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The Reaction between 5-(*p*-Toluenesulfonamido)tetrazole and *p*-Toluenesulfonyl Chloride

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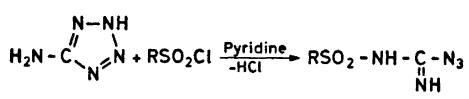
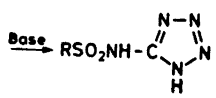
5-(*p*-Toluenesulfonamido)tetrazole reacts with *p*-toluenesulfonyl chloride and pyridine at room temperature with spontaneous evolution of nitrogen and formation of the dipolar compound II. This reacts with nucleophiles with elimination of pyridine. During the reactions with ethanol or ammonia an infrared absorption band near 2200 cm^{-1} appears for a short time, indicating that a carbodiimide, $RSO_2N=C=N-NHSO_2R$, is formed as an intermediate. Compound II also eliminates pyridine on heating or in solution, probably forming dimers (or polymers) of the carbodiimide.

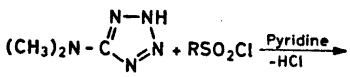
The primary product of the reaction between 5-aminotetrazole and *p*-toluenesulfonyl chloride in pyridine at temperatures below 70°C is known to be *p*-toluenesulfonylguanyl azide.[1] Under the influence of a base this is rearranged to the isomeric 5-(*p*-toluenesulfonamido)-tetrazole.

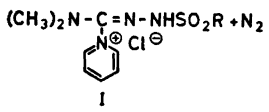
When, however, the temperature of the pyridine solution is raised above 90°C a violent reaction takes place with evolution of nitrogen.[2] Since we found [2] that 5-dimethylaminotetrazole reacted with *p*-toluenesulfonyl chloride in pyridine with evolution of nitrogen and formation of (1-*p*-toluenesulfonyl-4,4-dimethylcarbamohydrazonoyl)pyridinium chloride (I; semicarbazide numbering) it was anticipated





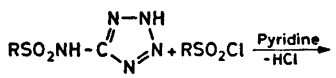
that a similar reaction had taken place between 5-(*p*-toluenesulfonamido)tetrazole and excess *p*-toluenesulfonyl chloride with the formation of compound II.

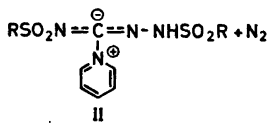




I

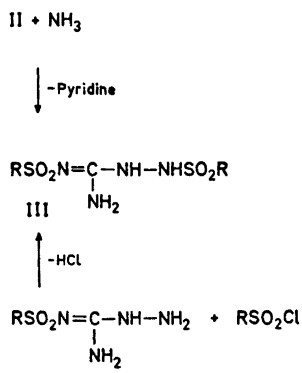
No compound corresponding to II could, however, be isolated from the solution. A possible reason for this negative result might be that the buffer solution pyridine/pyridinium chloride is too acid to induce a ring closure of the initially formed guanyl azide and that the nitrogen evolution originates from a thermal decomposition of the latter. However, the guanyl azide is not decomposed under the conditions of the experiment and it does not react with the sulfonyl chloride. It must therefore be concluded that some 5-(*p*-toluenesulfonamido)-tetrazole is actually formed and reacts with ex-





II

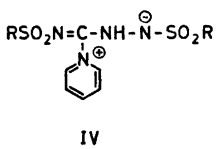
cess sulfonyl chloride under nitrogen evolution and formation of II, which, however, is unstable at the high temperatures used. We have now found that 5-(*p*-toluenesulfonamido)tetrazole in fact reacts with *p*-toluenesulfonyl chloride in pyridine solution with instantaneous release of nitrogen and the formation of a product which analytically corresponds to formula II. Chemical evidence in support of this structure is furnished by the reaction of II with ammonia. The product of this reaction was found to be in all respects identical with 1-(*p*-toluenesulfonamido-2-(*p*-toluenesulfonyl)guanidine (III), prepared from 2-(*p*-toluenesulfonyl)-1-aminoguanidine and *p*-toluenesulfonyl chloride.



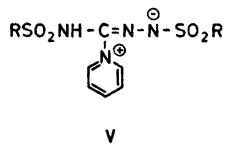
Analogously, the reaction of II with water gives rise to a product the elemental composition of which corresponds to that expected for 1,4-bis(*p*-toluenesulfonyl)semicarbazide. The corresponding thiosemicarbazide could not be obtained from the reaction of II with hydrogen sulfide (neither could it be prepared from *p*-toluenesulfonylhydrazine and *p*-toluenesulfonyl isothiocyanate).

The possibility that compound II should rather be represented by one of the tautomeric forms IV and V is rejected. These correspond to the conjugate base of I, which must be a very strong base since I is neutral. Compound II on the other hand is a very weak base (it does not form a hydrochloride). The structure depicted as II would also appear to be more plausible since it must be resonance-stabilized.

Compound I reacts with nucleophiles as a potential nitrilimine, $R_2N-C\equiv\overset{+}{N}-\overset{-}{N}-SO_2R'$, eliminating pyridinium chloride and forming a semicarbazide, a thiosemicarbazide, and an aminoguanidine with water, hydrogen sulfide, and ammonia, respectively.[2] Compound II, on the other hand, should be able to form a carbodiimide (VI) by elimination of pyridine. The product smells weakly of pyridine but could be recrystallized from acetone without change of composition, although with great loss. By evaporation of the filtrate, or by melting of compound II, a pyridine-free product was obtained the composition of which corresponds approximately to that of the carbodiimide. However, according to its infrared spectrum it cannot be a carbodiimide because there is no absorption near 2200 cm^{-1}. The spectrum exhibits an absorption band at 1725 cm^{-1}, which may originate from the grouping $RSO_2NHN=C$ since acetone *p*-toluenesulfonylhydrazone has a similar band. According to thin-layer chromatography the product contains at least ten compounds, which suggests the presence of four-membered rings since seven structurally isomeric diazetidines, each with several stereoisomers, could be formed by a dimerisation. Attempts at separation of the many compounds were not pursued further. However, the isolation of a small amount of crystalline *p*-tolyl *p*-toluenethiosulfonate, $CH_3C_6H_4SO_2-S-C_6H_4-CH_3$, from the melt shows that a possible dimerisation is accompanied by a more thorough decomposition. Incidentally, the molecular ion of this thiosulfonate and its fragmentation products account for most of the peaks in the mass spectrum of the melt. No ions corresponding to the carbodiimide or its polymers could be detected.

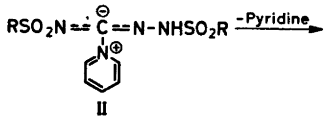


IV



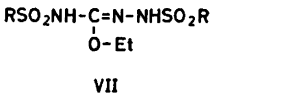
V

Evidence for the fleeting existence of the carbodiimide VI as an intermediate in the reactions of II with nucleophiles has been obtained from the reaction of II with ethanol. In this case products are obtained which initially show a strong infrared absorption in the range 2240–2260 cm^{-1}. This absorption disappears on drying, and the substance left is according to IR and NMR spectroscopic evidence *O*-ethyl 3-(*p*-toluenesulfonyl)-*p*-toluenesulfonimidocar-



II

VI



VII

bazate (VII). Accordingly the reaction of II with ethanol seems to proceed *via* the carbodiimide VI.

With *tert*-butyl alcohol II also gives a product with a strong infrared band at 2240 cm^{-1} which disappears on refluxing for a longer time. The reaction product is, however, not a carbazate but according to its infrared spectrum a similar mixture to that obtained by melting compound II.

During the reaction between compound II and ammonia in ethanol a transient absorption band is observed at 2180 cm^{-1}. The fact that this wavenumber differs somewhat from that observed during the reaction with ethanol may suggest that the carbodiimide forms a molecular complex with the nucleophile before reacting with it.

EXPERIMENTAL

^1H NMR spectra were recorded in $CDCl_3$ on a Varian A-60 instrument using TMS as internal standard. Chemical shifts are given in δ values (s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet). IR spectra were recorded on a Perkin-Elmer Model 337 grating spectrophotometer. Elemental analyses were performed by the analytical laboratory of this department (Mr. Preben Hansen and his staff).

p-*Toluenesulfonylguanyl azide* [2,3] could conveniently be prepared directly by the reaction of the commercially available 5-aminotetrazole hydrate and two equivalents of *p*-toluenesulfonyl chloride in pyridine. The crude azide, which is obtained in almost quantitative yield by pouring the pyridine solution into ice-cold water, still contains traces of pyridine and is specially suited for the following base-catalyzed isomerization. The ring closure to *5-(*p*-toluenesulfonamido)-tetrazole* could thus be achieved merely by refluxing the azide in ethanolic solution for *ca.* 6 h. The tetrazole was obtained by concentrating the ethanolic solution *in vacuo*. The best solvent for recrystallization is water + 15 – 20 % ethanol. Yield *ca.* 25 g of the recrystallized product (m.p. 187 – 189°C) from 20.6 g of 5-aminotetrazole monohydrate (52 %).

Pyridinio-p-*toluenesulfonylhydrazono*-p-*toluenesulfonimidoformate* II. A solution of *p*-toluenesulfonyl chloride (1.6 g) in acetone (10 ml) was added dropwise to a stirred solution of 5-(*p*-toluenesulfonamido)-tetrazole (2 g) and pyridine (2 ml) in acetone (10 ml). The reaction proceeds with evolution of nitrogen; the temperature was kept at 5°C by cooling with ice until all had been added (20 min) and the stirring was continued without cooling. A crystalline precipitate separated eventually or on scratching. The stirring was continued for ½ – 1 h or until the evolution of nitrogen had stopped (usually not more than 80 % of the calculated amount is evolved). The yield is very variable. In one case a yield as high as 70 % was obtained by carrying out the reaction in pyridine solution and adding acetone afterwards but this experiment could not be reproduced. In most cases only 20 – 30 % was obtained. The compound is unstable in contact with the solution and was isolated immediately by centrifugation. After a few washings with acetone, in which the residue is somewhat soluble, the analytically pure substance was obtained as a light yellow solid. (Found: C 54.00; H 4.55; N 12.67; S 14.37. Calc. for $C_{20}H_{20}N_4O_4S_2$: C 54.05; H 4.54; N 12.61; S 14.40). M.p. 121 – 122°C (decomp.). In the infrared spectrum ν(C=N) is found as a strong doublet at 1620 – 1630 cm^{-1}. In the ^1H NMR spectrum the protons of the two methyl groups give rise to a broad singlet at δ 2.40 and the other protons to complicated series of multiplets extending from δ 7 to 9.

On melting it gives off pyridine. According to chromatography ($CHCl_3$, silica gel) the amorphous product formed is a complicated mixture, from which, however, a few percent of *S*-*p*-tolyl *p*-toluenethiosulfonate (m.p. 78 – 79°C, infrared spectrum identical with that of an authentic sample) could be obtained.

The substance is only slightly soluble in benzene or ether, somewhat more soluble in acetone or chloroform. It dissolves in aqueous NaOH with an orange-red colour which disappears on heating. On acidification of the solution 1,4-bis(*p*-toluenesulfonyl)semicarbazide was precipitated; this could, however, be prepared in a purer state without the use of NaOH (see below). When II is dissolved in boiling acetone only a few percent crystallize out again on cooling and when the filtrate is evaporated a pyridine-free substance is obtained with essentially the same spectral properties as the melt. The IR spectrum has characteristic absorptions at 1725 (m), 1600 (s) and 1570 (s) cm^{-1} (difference from II). NMR-spectrum: broadened singlet at δ 2.38, multiplet at δ 7 – 9.

When the acetone solution from the prepara-

tion of II was diluted with water and extracted with chloroform a mixture was obtained which by chromatography (benzene-ethanol, silica gel) was separated into several fractions. One of these was identified as acetone *p*-toluenesulfonylhydrazone by comparison with an authentic sample. M.p. 158°C IR spectra superimposable, NMR spectrum: δ 1.82 (d, 6H); 2.38 (s, 3H); 7.5 (m, 5H). The other fractions were amorphous and apparently polymeric substances with varying N:S ratio (1.5 – 3) but similar infrared spectra except in the 1500 – 1600 cm^{-1} region. No well-defined compounds could be isolated. However, on boiling with water one fraction yielded *p*-toluenesulfonamide and another yielded 1,4-bis(*p*-toluenesulfonyl)-semicarbazide, in addition to insoluble resinous material.

1,4-Bis(p-toluenesulfonyl)semicarbazide. When II (100 mg) was boiled with water (8 ml) most of it went into solution. On cooling of the filtered solution and addition of a drop of dilute hydrochloric acid a white solid separated (45 mg). It was purified by crystallization from water-ethanol (10:1). M.p. 197 – 198°C. (Found: C 46.95; H 4.40; N 11.19; S 16.76. Calc. for $C_{15}H_{17}N_3O_5S_2$: C 46.99; H 4.47; N 10.96; S 16.72). Its infrared spectrum exhibits a strong C=O band at 1710 cm^{-1}.

1-(p-Toluenesulfonamido)-2-(p-toluenesulfonyl)guanidine (III). (a) A suspension of 2-(*p*-toluenesulfonyl)-1-aminoguanidine [1] (0.58 g) in pyridine (1 ml) was treated with *p*-toluenesulfonyl chloride (0.48 g) for 30 min at room temperature followed by a few min at 100°C until all had dissolved. On addition of water to the solution a white solid separated, which was filtered off, washed with water and dried over H_2SO_4. Yield 0.80 g. M.p. 240°C after recrystallization from ethanol. (Found: C 47.01; H 4.81; N 14.53; S 16.78. Calc. for $C_{15}H_{18}N_4O_4S_2$: C 47.12; H 4.75; N 14.66; S 16.74). As expected the substance is soluble in aqueous NaOH (to give a colourless solution). It is also soluble in ethanol saturated with ammonia but separates unchanged from the boiling solution as the ammonia escapes.

The infrared spectrum (KBr) exhibits three strong and sharp N – H stretching bands at 3310, 3375 and 3475 cm^{-1} and two strong bands at 1640 and 1560 cm^{-1} (amide I and amide II bands). The presence of some rather pronounced bands in the 2700 – 2800 cm^{-1} range indicates that the compound may in part exist in a dipolar form with an $=NH_2^+$ group.

(b) Compound II (0.30 g) was dissolved in ethanolic ammonia (5 ml). The solution initially had a deep red colour, which faded in 10 – 15 sec. After 1 h at room temperature a quantitative amount of III was obtained on dilution of the solution with ether. According to its m.p. (240°C), analysis (Found: C 46.90; H 4.83; N 14.53; S 16.61), and infrared spectrum this product was identical with that prepared by procedure (a).

O-Ethyl 3-(p-toluenesulfonyl)-p-toluenesulfonimidocarbazate (VII). II (400 mg) was boiled with ethanol (20 ml) until all had been dissolved and the yellow colour had disappeared. The solution was evaporated *in vacuo* and dried over H_2SO_4. Before the latter drying the infrared spectrum of the residue dissolved in $CHCl_3$ exhibited an absorption band at 2260 cm^{-1}, the intensity of which decreased slowly. The substance which had been dried over H_2SO_4 did not exhibit this band but had a strong band at 1602 cm^{-1}, presumably due to ν(C=N). The NMR spectrum was consistent with the expected constitution. The yield was almost quantitative (400 mg) but the product was glassy. It could only be purified with great loss by dissolving it in ethanol-benzene (1:4) and filtering the solution through silica gel; on addition of petroleum ether colourless crystals with m.p. 119 – 120°C were obtained. (Found: C 49.31; H 5.14; N 10.03. Calc. for $C_{17}H_{21}N_3S_2O_5$: C 49.62; H 5.14; N 10.21). NMR spectrum: δ 0.93 (t, 3H); 2.40 (d, 6H); 3.90 (q, 2H); 7.50 (m, 10H).

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2. Jensen, K. A., Holm, A. and Rachlin, S. *Acta Chem. Scand. 20* (1966) 2795.
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Crystal Structures of Synthetic Analgetics. II. *l*-Methadone

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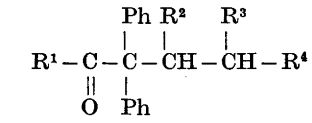
The molecular and crystal structure of *l*-methadone has been determined by X-ray methods. The crystals are orthorhombic, space group $P2_12_12_1$ with unit cell dimensions $a = 9.637$ Å; $b = 11.385$ Å; $c = 16.866$ Å. The phase problem was solved by direct methods and the model refined to an R-value of 0.038 for 1687 observed reflections. Estimated standard deviations are 0.003 – 0.004 Å in interatomic distances and 0.2 – 0.3° in angles.

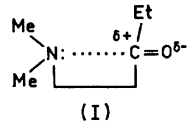
The dimethylamine group is (–)*syn clinal* relative to the quaternary carbon atom. There is a short intramolecular distance of 2.91 Å between the nitrogen atom and the carbonyl carbon atom and C3 is 0.06 Å out of the plane through C2, O and C4. The planes of the two phenyl rings make a dihedral angle of 80.6°.

The synthetic analgetic methadone may be characterized as the central compound among propylamines having morphine-like action. It belongs to a group of ketones with the general formula



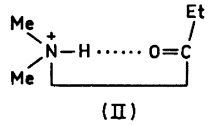
with $R^1 = Et$; $R^2 = H$; $R^3 = Me$; $R^4 = N(Me)_2$. The analgesic property of methadone and related compounds is believed to be dependent on a particular molecular conformation. Many investigations concerned with the preferred conformation of methadone have been reported.[1–4] Beckett *et al.*[1] and Beckett [2] proposed that the biologically important conformation of methadone resulted from an intramolecular interaction between the basic nitrogen atom and the carbonyl carbon atom (I).

This electrostatic attraction may bring the amine and the carbonyl group in close proximity.



(I)

Although an intramolecular hydrogen bond (II) is supposed to be present in solution,[1–4] no short distances were found between the nitrogen atom and the carbonyl group in the crystal structures of *d*-methadone hydrobromide [5] and dextropropoxyphene hydrochloride.[6]



(II)

The present structure determination was carried out to see if the particular conformation was preferred in the solid state of the free base.

EXPERIMENTAL

l-Methadone was prepared from commercially available *l*-methadone hydrochloride and single crystals were formed by recrystallization in diethyl ether by evaporation. The compound crystallizes as colourless, transparent parallelepipeds, and a crystal ground to a sphere ($r = 0.2$ mm) was used in the experiments.

Oscillation and Weissenberg photographs indicated orthorhombic symmetry. Systematically absent reflections were $h00$, $0k0$, $00l$ for odd indices; thus the space group is $P2_12_12_1$.

Unit cell dimensions were determined by least-squares methods from angular coordinates, measured on a Syntex P$\bar{1}$ diffractometer with graphite crystal monochromated Mo$K\alpha$-radiation. Three-dimensional intensity data were col-

Table 1. Observed and calculated structure factors. The columns are l, $10|F_o|$, and $10|F_c|$.

H=	0,K=	0																					
0	674	703	15	79	79	H=	0,K=	13	20	34	32	2	115	119	5	417	404	9	223	222	3	153	153
4	306	304	16	48	45	3	24	13	22	30	27	3	55	59	6	163	161	10	108	102	4	263	261
8	416	409	17	35	30	5	44	33	23	41	25	4	78	84	7	274	278	11	124	125	5	506	503
12	284	288	18	33	28	6	49	44	H=	1,K=	4	5	99	102	8	201	196	12	29	24	6	126	122
14	42	48	H=	0,K=	6	11	29	28	0	216	214	6	170	170	9	273	271	14	37	34	7	181	174
18	65	64	0	158	154	12	43	36	1	427	421	7	152	153	10	132	129	17	58	61	8	120	122
20	82	82	1	343	334	13	30	17	2	60	60	9	111	109	11	90	89	18	56	48	9	135	134
H=	0,K=	1	2	140	135	H=	0,K=	14	3	73	76	10	55	56	12	73	72	H=	2,K=	8	10	76	75
1	106	106	3	339	330	9	58	50	4	37	37	11	74	74	13	65	63	0	164	164	11	42	39
2	823	879	4	208	209	11	33	30	5	439	432	13	53	51	14	63	59	1	170	172	12	108	108
3	228	228	5	27	20	H=	0,K=	15	6	227	218	15	26	26	15	88	87	2	142	145	13	236	233
4	962	1026	7	82	84	4	41	40	7	252	251	16	40	38	17	88	90	3	44	36	14	113	110
5	458	454	9	89	89	6	39	32	8	205	210	19	29	15	18	31	33	4	136	139	15	81	79
6	201	195	10	52	47	9	42	37	9	196	193	H=	1,K=	10	20	35	37	5	43	39	17	33	27
7	136	138	11	124	123	10	35	35	10	77	76	0	126	128	H=	2,K=	3	6	69	74	H=	3,K=	2
8	364	360	12	138	133	11	35	34	11	87	86	1	33	38	0	88	86	7	167	174	0	217	215
9	91	91	13	101	103	H=	0,K=	16	12	57	59	2	112	118	1	330	323	8	128	132	1	56	58
10	176	172	14	62	66	3	37	29	13	89	87	3	107	108	2	249	237	9	136	138	2	177	170
11	142	143	15	56	58	7	42	39	14	36	44	4	63	68	3	341	339	10	83	84	3	201	202
14	87	87	16	32	28	8	39	39	15	66	64	5	113	108	4	582	577	11	77	79	4	138	138
15	28	21	18	32	24	H=	0,K=	17	17	50	51	6	88	85	5	437	424	12	77	79	5	465	451
17	30	24	H=	0,K=	7	1	32	20	18	28	33	7	97	99	6	330	321	13	61	52	6	454	452
18	132	132	1	198	198	H=	1,K=	0	20	28	21	8	45	45	7	51	48	15	52	52	7	206	201
19	32	36	2	92	91	1	666	677	21	39	37	9	60	55	8	161	160	H=	2,K=	9	8	186	186
20	82	79	3	70	76	2	193	192	H=	1,K=	5	11	31	30	9	64	64	0	27	22	9	48	45
H=	0,K=	2	4	128	127	3	302	304	0	191	185	12	28	36	10	66	65	1	125	129	10	102	106
0	814	839	6	55	51	4	94	93	1	87	86	14	59	55	11	58	58	2	31	28	11	68	65
1	887	964	7	94	89	5	288	293	2	77	79	H=	1,K=	11	12	75	75	3	98	96	12	61	57
2	561	565	8	247	254	7	282	279	3	241	233	1	88	84	13	27	21	4	91	90	13	84	84
3	62	61	9	68	76	8	78	74	4	22	44	2	48	44	14	65	64	5	81	85	14	62	63
4	455	448	10	148	148	9	156	158	5	346	339	3	86	89	15	141	139	6	56	57	15	140	136
5	632	626	12	53	53	10	20	24	6	216	216	4	137	137	16	57	61	7	35	43	16	56	57
6	388	384	13	23	6	12	71	71	7	81	85	5	27	33	18	47	43	8	54	53	18	57	59
7	228	219	14	71	72	14	79	79	8	105	104	6	31	33	H=	2,K=	4	9	46	45	19	42	39
8	86	82	16	51	56	16	42	38	9	157	160	8	38	39	0	434	419	16	53	49	H=	3,K=	3
9	25	26	17	34	40	17	97	99	10	60	60	9	36	35	1	41	42	H=	2,K=	10	0	102	101
11	81	74	18	32	22	18	54	49	11	114	113	10	71	66	2	44	46	0	65	67	1	243	239
12	75	73	20	59	53	20	53	46	12	115	118	11	72	69	3	254	247	1	64	69	2	121	118
13	63	61	H=	0,K=	8	H=	1,K=	1	13	72	78	12	32	41	4	211	210	2	45	38	3	132	137
14	61	53	0	89	91	0	733	756	14	47	51	14	39	34	5	315	318	3	156	161	4	479	469
15	24	20	2	100	104	1	315	306	16	41	43	15	29	23	6	418	407	4	64	66	5	291	279
16	32	27	4	28	21	2	556	566	18	30	26	16	33	31	7	67	74	5	71	67	6	198	195
17	31	41	5	53	49	3	796	818	19	30	29	H=	1,K=	12	8	174	172	8	38	31	7	56	51
18	35	32	6	354	358	4	567	567	H=	1,K=	6	0	97	95	9	134	136	11	44	32	8	50	46
20	46	41	7	181	183	5	207	207	0	139	142	1	48	48	10	65	67	15	36	32	9	100	101
H=	0,K=	3	9	41	47	6	239	233	1	160	160	2	67	69	11	70	72	H=	2,K=	11	10	165	168
1	265	267	10	53	55	7	157	159	2	130	132	3	48	46	12	128	130	0	154	154	11	30	24
2	254	260	11	65	64	8	259	260	3	99	106	4	66	65	13	40	36	1	76	78	12	109	115
3	79	76	12	65	65	9	32	31	4	147	146	5	37	40	14	119	121	3	59	59	14	39	44
4	337	334	13	38	42	10	98	102	5	43	47	8	41	41	15	30	38	4	53	54	15	64	65
5	221	221	14	72	71	11	118	118	6	188	186	9	59	55	16	35	29	5	28	30	17	78	76
7	85	84	15	43	38	12	91	94	7	92	93	12	34	41	17	65	64	6	39	34	18	54	55
8	89	89	16	66	58	13	96	93	8	23	37	H=	1,K=	13	18	25	26	8	59	64	19	27	30
9	32	27	H=	0,K=	9	14	60	63	9	145	148	1	38	43	21	43	36	9	43	38	H=	3,K=	4
10	70	67	1	28	20	15	105	105	10	21	23	5	42	41	H=	2,K=	5	11	40	40	0	216	211
11	60	62	2	32	32	16	41	38	11	71	74	7	29	35	0	144	147	12	31	28	1	180	181
12	99	100	3	176	173	17	101	102	12	60	59	11	43	34	1	125	128	13	38	34	2	143	137
13	32	26	4	107	107	19	66	67	13	32	33	H=	2,K=	0	2	222	221	H=	2,K=	12	3	114	117
14	60	56	5	87	92	20	52	48	14	73	71	0	41	42	3	164	159	1	112	109	4	94	98
15	41	50	6	84	81	H=	1,K=	2	15	37	30	1	27	29	4	227	222	2	45	45	5	108	110
16	45	48	8	114	114	0	214	219	16	39	37	2	399	399	5	142	138	3	63	68	6	45	39
17	45	39	9	55	58	1	785	823	20	27	24	3	1049	1091	6	111	114	4	29	30	7	163	166
18	41	38	11	35	34	2	599	594	H=	1,K=	7	4	210	205	7	104	107	5	38	36	8	81	82
19	35	31	12	43	41	3	544	548	1	85	81	5	266	265	8	153	158	11	59	59	10	127	129
22	28	19	13	57	52	4	292	292	2	66	67	6	159	156	9	141	146	12	44	41	11	82	84
H=	0,K=	4	14	99	94	5	96	95	3	82	84	7	156	155	10	104	104	H=	2,K=	13	12	108	110
0	286	289	15	45	45	6	276	272	4	191	191	8	224	222	11	162	164	2	55	50	13	71	72
2	90	92	17	51	45	7	253	252	6	45	50	9	63	62	12	119	121	5	26	26	14	83	90
3	56	56	18	36	30	8	98	102	7	94	96	10	44	37	13	36	36	6	48	49	15	72	71
4	151	156	19	28	16	9	104	104	8	41	46	11	37	34	14	41	34	8	43	39	16	36	38
5	63	63	H=	0,K=	10	10	189	191	9	66	68	12	23	30	15	67	69	10	47	40	18	31	30
6	114	111	0	114	114	11	120	120	10	77	73	13	73	76	17	36	32	13	32	28	20	35	44
7	214	208	1	97	100	12	132	132	11	67	66	17	25	29	19	41	40	H=	2,K=	14	H=	3,K=	5
8	142	144	2	89	90	13	45	43	12	36	42	18	46	48	22	37	36	4	44	41	0	144	143
9	231	230	4	174	176	14	103	106	13	63	63	H=	2,K=	1	H=	2,K=	6	5	36	35	1	114	114
10	41	41	6	186	190	15	104	102	14	75	76	0	640	647	0	176	181	6	40	32	2	297	283
11	37	38	7	115	117	16	38	43	16	42	43	1	385	380	1	168	164	10	32	25	3	163	162
12	24	28	8	96	102	17	46	42	17	41	35	2	675	673	2	263	256	H=	3,K=	0	4	93	97
13	24	26	10	91	87	18	49	48	19	28	25	3	842	851	3	153	151	1	140	141	5	101	95
14	61	64	12	60	56	19	37	28	21	37	17	4	247	243	4	117	113	2	205	203	6	141	141
15	56	48	13	28	22	21	36	37	H=	1,K=	8	5	212	211	5	47	48	3	793	782	7	64	61
16	23	18	15	40	34	H=	1,K=	3	0	23	23	6	235	230	6	84	83	4	99	98	8	99	100
17	30	28	H=	0,K=	11	0	161	163	1	50	56	7	167	166	7	123	126	5	950	958	9	162	166
18	47	43	1	34	31	1	516	516	2	79	79	8	69	68	8	98	95	6	214	215	10	95	97
21	33	31	2	29	21	2	351	343	3	49	49	9	37	35	9	94	90	7	456	461	11	110	109
H=	0,K=	5	3	84	86	3	247	250	4	54	58	10	124	125	10	211	214	8	92	100	12	61	67
1	24	15	5	63	59	4	311	314	5	149	149	11	87	90	11	192	198	9	34	28	14	39	42
2	32	29	7	29	17	5	261	256	6	111	109	12	121	120	13	26	17	10	27	30	15	49	55
3	218	223	10	35	40	6	232	234	7	150	149	13	36	39	14	53	60	11	102	99	16	67	68
4	146	153	11	61	60	7	256	253	8	53	47	14	81	80	15	66	67	12	43	45	H=	3,K=	6
5	68	66	12	43	40	8	217	221	9	123	122	15	112	115	16	28	18	13	48	47	0	54	48
6	66	66	15	33	37	9	55	52	10	84	80	16	35	34	H=	2,K=	7	14	32	27	1	106	107
7	19	9	19	32	27	10	80	83	11	93	89	17	50	44	0	173	175	15	54	50	2	196	199
8	75	70	H=	0,K=	12	11	93	97	12	58	58	18	37	33	1	109	105	16	56	59	3	89	86
9	29	20	0	37	29	12	52	53	13	83	81	H=	2,K=	2	2	82	83	17	43	40	4	185	183
10	40	37	1	76	81	13	56	61	15	33	28	0	102	103	4	127	127	19	63	60	5	124	129
11	52	54	4	42	42	14	27	14	17	34	38	1	147	140	5	32	35	H=	3,K=	1	6	56	57
12	75	79	8	50	50	15	38	43	H=	1,K=	9	2	298	300	6	114	116	0	434	415	7	182	186
13	43	47	12	40	27	17	95	92	0	33	33	3	583	576	7	96	98	1	179	177	8	234	236
14	46	41	14	41	41	18	48	48	1	73	74	4	516	514	8	65	64	2	162	158	9	57	58

Table 1. Continued.

10	103	100	1	263	265	5	41	42	6	59	58	2	116	113	7	147	151	7	92	95	8	28	36
11	35	39	2	142	137	6	107	110	6	85	85	3	25	15	9	44	44	8	34	31	9	62	59
12	129	134	3	308	295	7	80	81	7	86	83	4	89	89	10	36	36	9	30	30	11	63	55
13	56	65	4	168	164	8	56	56	8	197	202	5	54	59	11	94	96	10	38	39	Hz	7,Kz	10
14	37	39	5	210	206	9	23	18	9	190	194	6	96	102	13	43	32	11	47	47	4	45	46
15	26	30	6	43	55	10	52	54	10	287	296	7	38	39	16	45	42	12	86	83	7	55	60
20	40	34	7	318	319	11	69	70	11	70	72	8	37	41	Hz	6,Kz	5	13	46	47	9	69	61
21	48	41	8	207	207	12	62	62	12	60	66	9	56	55	0	143	141	14	38	34	Hz	7,Kz	11
Hz	3,Kz	7	9	155	151	13	36	28	13	29	26	10	39	38	1	116	122	15	45	41	0	39	35
0	165	168	10	173	177	Hz	4,Kz	8	14	61	63	11	48	49	2	69	72	16	35	34	6	32	34
1	241	239	11	160	162	1	47	49	15	81	80	14	74	68	3	105	108	17	33	29	Hz	8,Kz	0
2	112	116	12	212	216	2	93	90	16	29	24	Hz	5,Kz	10	4	59	62	Hz	7,Kz	2	1	22	24
3	152	151	13	92	93	3	47	45	Hz	5,Kz	3	0	27	24	5	66	70	0	51	49	2	40	40
4	58	64	14	82	83	4	67	67	0	86	85	1	61	56	6	75	78	1	93	91	3	81	82
5	22	22	16	88	90	5	37	34	1	72	69	2	42	45	7	104	102	2	55	50	4	77	73
6	135	142	17	37	34	6	85	84	2	57	54	4	83	82	10	67	71	3	30	27	5	239	246
7	49	47	Hz	4,Kz	2	8	69	67	3	154	150	5	58	54	12	48	45	4	68	74	7	57	63
8	133	135	0	60	61	9	39	40	4	70	67	6	45	45	14	48	45	5	147	151	8	79	82
9	65	69	1	108	109	10	42	46	5	77	79	7	34	32	15	40	30	6	33	33	9	60	57
10	114	120	2	198	202	11	32	27	6	100	101	8	37	45	Hz	6,Kz	6	7	172	175	13	60	53
11	60	55	3	300	293	12	31	31	7	95	92	9	48	52	0	46	45	8	74	71	15	113	106
12	30	31	4	142	141	Hz	4,Kz	9	8	59	61	10	27	21	1	90	94	9	52	56	16	37	33
13	40	38	5	139	134	0	93	88	9	165	167	12	28	28	2	87	82	10	59	59	Hz	8,Kz	1
Hz	3,Kz	8	6	190	189	1	141	142	10	93	96	Hz	5,Kz	11	3	105	110	11	33	39	0	63	62
0	48	50	7	162	158	2	74	75	11	34	42	0	52	48	4	154	153	12	30	32	1	58	60
1	185	185	8	111	114	3	94	94	12	76	75	2	46	46	5	33	26	13	70	71	2	45	43
3	48	51	9	130	133	4	130	132	15	54	58	5	48	45	6	40	46	14	50	44	3	150	153
4	92	93	10	170	176	5	98	94	16	31	32	7	71	61	7	59	56	16	51	42	4	70	66
5	92	90	11	54	50	6	91	88	Hz	5,Kz	4	9	43	41	8	51	48	Hz	7,Kz	3	5	132	134
6	128	128	12	79	78	7	81	80	0	24	15	Hz	5,Kz	12	10	53	53	0	82	80	6	48	53
7	61	61	13	68	73	9	26	28	1	41	44	0	36	42	11	31	31	1	140	144	7	23	28
8	95	97	15	103	103	10	48	44	2	154	156	2	64	62	12	67	72	2	102	107	9	56	57
9	72	74	16	60	53	11	62	59	3	50	52	3	28	37	15	56	56	3	72	72	11	50	50
11	42	47	19	46	48	13	60	59	4	50	52	4	49	49	16	59	48	4	94	95	13	67	65
12	86	86	Hz	4,Kz	3	15	36	34	5	147	149	5	28	31	Hz	6,Kz	7	5	79	81	14	46	42
13	42	43	0	19	16	16	45	33	6	32	35	Hz	5,Kz	13	0	40	44	6	154	153	Hz	8,Kz	2
16	41	36	1	23	22	17	39	34	7	23	22	1	57	50	1	147	143	7	159	161	0	95	98
19	46	39	2	209	205	18	53	44	8	109	113	3	29	32	2	60	60	8	51	58	2	54	54
Hz	3,Kz	9	3	381	368	Hz	4,Kz	10	9	98	102	5	30	20	3	187	186	9	31	27	3	86	84
1	95	90	4	145	140	0	83	79	10	34	38	Hz	6,Kz	0	4	125	130	10	54	53	4	156	159
2	139	139	5	330	329	1	54	56	11	66	68	0	314	311	6	64	63	11	76	74	5	196	203
3	98	98	6	108	109	2	82	84	12	50	49	2	55	58	7	76	79	13	52	51	6	61	69
4	47	48	7	97	92	3	115	111	13	32	37	3	73	75	8	51	58	14	25	34	7	72	77
6	109	108	8	121	118	4	69	67	14	24	28	4	36	35	9	50	50	15	48	43	8	45	41
7	104	98	9	75	77	5	43	47	15	36	37	5	56	53	10	50	54	Hz	7,Kz	4	10	42	41
9	32	40	10	84	85	6	41	45	Hz	5,Kz	5	6	26	27	11	31	30	0	244	243	15	39	40
10	32	29	12	137	134	7	70	64	0	166	167	7	63	64	15	67	58	1	98	101	16	46	41
11	53	42	13	122	123	9	55	53	1	21	13	8	28	35	Hz	6,Kz	8	2	155	153	Hz	8,Kz	3
15	40	39	14	42	38	14	46	48	2	119	119	9	98	97	1	97	96	3	121	122	0	151	152
Hz	3,Kz	10	15	141	142	Hz	4,Kz	11	3	58	51	10	56	55	2	51	49	4	138	142	1	63	59
0	36	40	16	24	22	0	30	25	4	45	47	11	38	48	3	111	107	5	178	176	5	34	29
1	153	157	17	35	34	1	114	113	6	106	112	12	82	82	4	34	29	6	107	108	7	35	35
2	99	100	Hz	4,Kz	4	3	59	60	7	29	32	13	125	126	5	53	62	7	132	133	8	47	55
3	101	106	0	164	160	4	64	61	8	130	129	17	41	41	6	74	78	8	52	55	9	38	35
4	49	47	1	141	138	6	54	50	9	25	27	Hz	6,Kz	1	7	60	63	9	28	16	10	27	16
5	76	74	2	289	286	7	34	45	10	26	30	0	264	270	10	53	40	10	24	33	11	24	22
6	25	20	3	42	39	9	50	47	11	49	47	1	158	158	11	45	39	11	51	56	12	42	43
7	78	73	4	178	176	11	38	34	12	38	46	2	117	118	12	42	38	12	28	26	13	27	23
8	48	50	5	209	200	Hz	4,Kz	12	13	40	41	4	178	178	13	53	52	14	47	50	Hz	8,Kz	4
10	43	40	6	88	92	1	95	95	Hz	5,Kz	6	5	77	80	14	59	51	16	37	35	0	135	135
11	69	69	7	238	235	3	37	27	0	76	77	6	68	70	Hz	6,Kz	9	17	40	28	1	55	56
13	67	62	8	112	117	8	60	47	1	154	152	7	170	177	0	33	30	Hz	7,Kz	5	2	89	91
Hz	3,Kz	11	9	98	101	9	34	38	2	174	177	8	50	53	1	61	61	0	309	314	3	55	55
1	52	58	10	79	79	Hz	4,Kz	13	3	145	150	9	222	222	2	55	49	1	134	134	4	91	93
2	59	57	11	130	128	1	51	44	4	23	31	10	110	109	3	93	96	2	82	86	5	40	33
6	27	27	12	52	54	3	48	53	5	81	83	11	80	81	4	79	83	3	83	82	6	49	59
7	54	52	13	76	82	4	53	45	6	90	91	12	48	50	5	81	77	4	70	66	7	77	80
11	72	64	14	57	58	5	48	43	7	56	53	13	68	64	7	25	26	5	45	48	8	36	40
12	37	38	15	30	44	Hz	5,Kz	0	8	48	53	14	44	45	Hz	6,Kz	10	6	103	107	10	38	36
Hz	3,Kz	12	16	44	42	1	71	68	9	39	40	Hz	6,Kz	2	0	46	45	8	67	71	11	30	32
0	28	29	17	47	46	2	370	369	10	87	87	0	251	249	2	35	39	10	29	35	14	32	27
1	56	52	19	38	33	3	40	38	11	47	48	1	108	109	3	24	23	11	31	34	Hz	8,Kz	5
2	57	53	Hz	4,Kz	5	4	137	135	13	48	41	2	171	171	5	41	37	12	39	39	0	115	120
6	45	53	0	21	19	5	118	122	17	44	35	3	49	51	6	46	46	13	32	37	1	37	31
9	30	33	1	92	89	6	55	53	18	34	26	4	102	101	8	63	59	Hz	7,Kz	6	2	37	37
Hz	3,Kz	13	2	219	211	7	119	120	Hz	5,Kz	7	5	82	79	11	43	35	0	215	218	3	101	98
0	50	48	3	112	103	8	213	216	0	30	38	6	73	78	12	46	39	1	185	185	4	70	68
4	60	55	4	45	45	10	392	399	1	87	90	7	105	111	Hz	6,Kz	11	2	68	68	5	80	79
8	53	50	5	140	140	11	199	202	2	99	98	8	86	84	0	50	53	3	63	64	6	56	53
Hz	3,Kz	14	6	86	85	12	144	150	3	92	96	9	136	135	1	49	39	4	102	105	7	44	42
0	41	43	7	101	104	14	113	111	4	117	117	10	82	88	4	56	53	5	27	24	10	65	66
1	40	36	8	135	136	16	81	77	5	54	50	11	37	37	6	66	62	7	59	62	13	34	32
2	52	54	9	106	110	17	30	37	6	174	175	12	29	34	7	32	38	12	31	30	14	57	52
5	44	36	11	161	163	19	38	32	7	89	97	13	35	29	8	52	52	14	68	65	Hz	8,Kz	6
Hz	3,Kz	15	12	57	56	Hz	5,Kz	1	8	57	60	14	69	69	10	30	22	Hz	7,Kz	7	0	85	88
5	44	41	13	64	62	0	31	25	10	55	57	18	64	52	Hz	6,Kz	12	0	93	95	2	134	132
Hz	4,Kz	0	14	25	21	1	123	119	11	29	34	Hz	6,Kz	3	2	46	35	1	107	103	3	81	79
0	152	151	15	39	43	2	308	303	12	47	50	0	163	166	7	42	31	2	45	42	4	88	88
1	44	43	16	33	38	4	40	37	14	45	48	1	181	184	Hz	7,Kz	0	3	73	73	8	52	58
2	427	425	Hz	4,Kz	6	5	90	91	16	47	40	2	135	133	1	143	147	4	62	58	10	42	42
3	327	335	1	104	105	6	194	193	Hz	5,Kz	8	3	91	94	3	141	141	5	65	62	11	40	30
4	233	227	2	147	144	7	79	78	1	41	42	4	75	73	4	26	32	6	47	46	Hz	8,Kz	7
5	106	104	3	162	158	8	241	249	2	56	55	5	74	70	6	27	37	7	39	30	0	46	44
6	178	172	4	104	107	10	291	301	3	149	151	6	92	98	8	81	80	8	59	60	1	104	101
7	31	35	5	60	63	11	72	76	4	241	245	7	228	235	9	99	102	11	50	48	2	36	38
9	24	16	6	110	111	12	58	59	5	38	41	8	119	116	10	37	37	12	60	49	4	60	57
10	117	128	7	60	61	13	26	27	6	77	78	9	84	83	11	46	53	Hz	7,Kz	8	12	37	27
11	62	63	9	93	94	14	86	84	7	70	67	10	53	59	13	25	28	1	55	60	Hz	8,Kz	8
12	46	52	11	45	48	15	45	47	8	59	63	11	67	68	15	46	42	2	28	26	1	59	54
13	98	98	12	24	23	16	48	49	9	30	33	13	52	55	16	38	34	3	35	37	2	56	56
14	30	36	13	57	57	18	53	52	10	69	72	16	42	41	Hz	7,Kz	1	4	35	36	9	39	37
15	62	56	14	38	39	Hz	5,Kz	2	11	60	61	Hz	6,Kz	4	0	142	144	8	39	36	11	68	57
16	28	33	Hz	4,Kz	7	0	220	213	12	64	68	0	45	42	1	70	57	Hz	7,Kz	9	Hz	8,Kz	9
17	36	39	0	62	68	1	105	106	15	53	48	1	109	114	2	115	116	0	38	37	1	34	32
21	52	54	1	95	94	2	188	187	16	75	66	2	24	25	4	49	51	2	59	56	3	42	47
Hz	4,Kz	1	2	51	52	3	98	98	Hz	5,Kz	9	3	87	93	5	79	77	4	37	44	7	45	39
0	55	56	4	50	52	4	112	115	0	39	43	5	181	185	6	60	59	6	71	73	8	39	32

Table 1. Continued.

9	55	49	7	63	65	6	27	24	H=	9,K=	6	5	31	31	1	62	56	4	50	46	1	53	48
10	44	37	8	56	54	7	29	21	0	29	28	6	41	36	2	69	73	5	67	54	3	44	44
H=	8,K=	10	9	45	44	8	61	70	1	96	94	8	49	45	3	44	45	6	36	46	4	43	40
6	45	38	11	33	35	H=	9,K=	4	2	58	53	12	86	73	4	50	42	7	51	44	H=	11,K=	4
8	33	37	12	64	60	0	47	43	3	52	59	H=	10,K=	1	6	68	67	H=	10,K=	7	0	41	29
H=	9,K=	0	14	39	32	1	52	50	4	59	58	0	70	68	H=	10,K=	4	4	72	71	1	53	51
1	25	31	H=	9,K=	2	2	54	53	H=	9,K=	7	1	58	56	0	52	47	6	58	44	3	49	47
2	46	45	0	78	79	3	134	134	0	44	34	2	47	42	1	94	92	H=	10,K=	8	4	31	32
3	36	39	1	55	54	4	72	72	1	76	74	4	43	40	2	44	39	0	61	51	H=	11,K=	5
4	67	70	2	50	46	5	27	12	2	40	45	5	53	56	4	39	41	4	36	39	5	43	36
5	31	26	3	72	74	6	54	54	4	57	48	7	59	59	5	51	41	H=	11,K=	0	H=	11,K=	6
6	117	116	4	57	62	8	60	58	6	53	50	8	46	45	6	36	31	1	51	60	3	36	32
7	30	23	5	106	107	9	28	36	9	46	36	10	59	49	H=	10,K=	5	10	98	87	H=	11,K=	7
8	50	46	6	44	42	10	30	28	10	35	25	H=	10,K=	2	0	55	52	H=	11,K=	1	2	35	33
9	94	89	8	64	64	H=	9,K=	5	H=	9,K=	8	0	146	140	1	50	48	1	38	36	H=	12,K=	0
10	38	31	9	54	57	0	80	76	3	37	31	1	46	43	2	48	42	2	40	44	4	33	31
13	64	61	12	32	30	1	72	71	6	35	29	2	104	104	3	38	38	4	34	22	6	43	32
H=	9,K=	1	13	49	47	2	75	71	H=	9,K=	9	3	61	59	5	55	47	7	37	39	H=	12,K=	2
1	99	102	H=	9,K=	3	3	65	62	4	42	33	4	45	48	H=	10,K=	6	8	75	72	1	53	46
2	83	80	0	81	74	4	43	43	H=	10,K=	0	8	58	60	0	42	30	H=	11,K=	2	2	35	30
3	39	34	2	92	90	5	50	49	0	93	97	12	80	70	1	41	39	10	59	51	5	43	43
4	107	106	3	91	93	8	42	39	2	58	61	H=	10,K=	3	2	31	34	H=	11,K=	3	H=	12,K=	3
5	108	109	4	96	94	10	36	25	4	45	40	0	99	96	3	51	45	0	41	43	4	28	30
6	39	37	5	102	104	12	38	35															

lected using the $2\theta-\theta$ autocollection program with variable scan rate and a cut-off for low intensities. The scan range was from 0.7° below $2\theta(\alpha_1)$ to 0.7° above $2\theta(\alpha_2)$, and the backgrounds were counted 0.7 times the intensity measuring time. The intensities of three standard reflections were measured periodically during the data collection. They showed no systematic variation. The e.s.d's in the intensities were taken as the square root of the total counts with a 2 % addition for instrumental instability.

A total of 1911 independent reflections were recorded within the limit of 0.66 for sin θ/λ, 1687 having a net count larger than 2 σ_I.

The data were corrected for Lorentz and polarization effects but not for absorption or secondary extinction.

All calculations were performed on a CDC 6600 computer using the programs described in Ref. 7, except for the phase determination which was done with the program MULTAN, written by Main *et al.*[8] Atomic form factors were those of Hanson *et al.*[9] for O, N, and C and of Stewart *et al.*[10] for H.

CRYSTAL DATA

(6:*R*)-6-Dimethylamino-4,4-diphenyl-3-heptanone, (*l*-methadone), $C_{21}H_{27}NO$, orthorhombic.

$a=9.637$ (1) Å, $b=11.385$ (2) Å, $c=16.866$ (2) Å.

$V=1850.4$ Å^3, M = 309.20, $Z=4$.

Melting point: 79 – 80°C.

$D_{obs}=1.10$ gcm^{-3} (flotation), $D_{calc}=1.11$ gcm^{-3}.

Systematic absences: $h00$, $0k0$, $00l$ for odd indices; space group $P2_12_12_1$.

STRUCTURE DETERMINATION

The structure was determined by direct methods. Preliminary scale and overall isotropic thermal vibration factor ($B=3.11$ Å^2) were derived by Wilson's statistical method and normalized structure factors were calculated. The phase determination was carried out by the program MULTAN, using the 344 highest E-values (≥ 1.25), and a total of 1400 relations of the $\sum_2$ formula.[11] The set of phases with the third highest figure of merit gave an E-map where 20 of the 23 non-hydrogen atoms could be located. Two successive Fourier refinement served to establish a trial structure of all the heavy atoms. Successive cycles of full matrix least-squares refinement, first with isotropic then with anisotropic thermal parameters gave an R-value of 0.09. Approximate positions of all the hydrogen atoms were calculated from stereochemical considerations. Giving these 27 atoms individual isotropic thermal parameters, the refinement converged at $R=0.038$ ($R_w=0.033$).

Inspection of the structure factor values of strong reflections, showed that nine probably were affected by secondary extinction. The differences $|F_o-F_c|$ for these reflections were less than 10 % of the F_c's, and the effect turned out to be negligible, both in the atomic parameters and the R-value.

Observed and calculated structure factors are listed in Table 1, and the atomic parameters in Tables 2 and 3. The anisotropic temperature factor is given by

$$\exp -(B_{11}h^2+B_{22}k^2+B_{33}l^2+B_{12}hk+B_{13}hl+B_{23}kl)$$

The e.s.d.'s in bond lengths and angles were calculated to be 0.003 – 0.004 Å and 0.2 – 0.3°, respectively.

Table 2. Fractional atomic coordinates and thermal parameters with standard deviations (10^5) for non-hydrogen atoms.

Atom	x	y	z	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
C1	64536	2111	33978	1256	1105	468	138	−549	−173
	33	32	21	40	33	13	60	42	36
C2	51829	7272	30097	1146	796	311	175	−113	−72
	29	24	15	32	23	9	48	32	26
C3	45648	−558	23784	773	629	299	−37	52	93
	22	20	12	24	19	8	39	26	23
C4	35029	5093	17984	729	486	299	35	−8	−14
	22	17	12	23	16	8	36	25	20
C5	41499	15653	13408	969	540	370	8	−136	116
	25	21	15	28	18	10	41	29	24
C6	53441	12585	7896	1276	731	316	−406	165	19
	26	22	14	34	22	9	47	32	24
C7	56281	22630	2047	1725	1727	578	−475	177	917
	47	43	26	57	50	19	95	60	54
C8	74416	836	8109	1885	1352	1120	773	1338	−35
	54	43	39	63	45	31	91	80	65
C9	73801	18503	15567	1133	1796	662	−676	141	−104
	39	44	27	41	52	20	78	52	56
C10	29192	−3389	11696	811	529	288	−46	25	96
	21	17	12	24	18	8	34	26	19
C11	16452	−814	8204	908	693	357	205	−81	−87
	24	22	14	29	21	9	45	29	25
C12	11306	−7336	1975	991	961	393	7	−316	−86
	27	24	16	31	26	11	50	32	29
C13	18638	−16688	−964	1459	786	344	−132	−156	−222
	30	22	15	37	23	10	52	34	26
C14	31222	−19488	2402	1390	704	396	306	46	−155
	29	22	15	38	22	11	51	36	26
C15	36428	−12987	8629	938	752	358	282	−111	−44
	26	21	14	30	23	10	43	31	25
C16	23099	9151	23492	776	728	324	73	−88	−113
	22	20	13	24	20	9	39	26	22
C17	19429	20728	24685	1066	778	565	127	81	−293
	28	25	18	31	24	14	50	37	31
C18	8614	23676	29718	1179	1251	680	517	46	−799
	31	27	20	34	32	17	60	45	38
C19	1306	15263	33618	981	1869	504	284	118	−831
	27	36	18	34	42	13	69	36	42
C20	4854	3662	32588	1140	1653	452	−279	341	−81
	28	30	17	35	41	12	62	38	36
C21	15631	631	27544	1033	1038	416	42	232	−51
	25	24	15	29	24	10	53	33	30
O	47886	−10987	23922	1614	536	508	179	−594	125
	18	13	10	25	13	8	31	24	16
N	65606	8915	12503	1022	873	512	299	321	89
	21	18	13	25	19	10	41	30	23

Table 3. Fractional coordinates (10^3) and isotropic thermal parameters ($Å^2$) for hydrogen atoms.

Atom	x	y	z	B
H1C1	682	75	380	5.3
H2C1	717	2	301	7.0
H3C1	631	−56	363	6.9
H1C2	534	152	279	5.0
H2C2	445	83	339	4.7
H1C5	448	218	172	2.3
H2C5	338	194	102	3.5
HC6	511	56	48	3.9
H1C7	577	299	50	7.9
H2C7	480	233	−12	7.9
H3C7	652	210	−9	7.7
H1C8	681	−61	65	11.2
H2C8	821	−17	117	11.8
H3C8	785	45	33	10.1
H1C9	684	239	184	7.7
H2C9	791	225	110	8.8
H3C9	805	153	194	13.0
HC11	111	55	103	4.0
HC12	32	−52	−3	4.5
HC13	155	−213	−53	4.4
HC14	370	−259	2	4.6
HC15	450	−151	108	3.9
HC17	244	267	222	5.5
HC18	59	319	303	7.2
HC19	−61	171	375	5.7
HC20	3	−26	352	6.4
HC21	184	−74	268	4.1

DISCUSSION

Interatomic distances and bond angles are given in Tables 4 and 5, and shown in Fig. 1. The absolute configuration (6:*R*) of *l*-methadone has been chosen according to earlier reports.[5,12]

The analysis of the thermal parameters showed that the two aromatic rings could be regarded as rigid bodies, and the positional parameters of these atoms, including C4, were corrected for librational effects. The corrected bond lengths are also listed in Table 4.

The crystal structure as seen down the *b*-axis is illustrated in Fig. 2. There are no particularly short intermolecular distances in the structure.

The distance C1 – C2 (1.508 Å) is found to be shorter than normal, 1.537.[13] This is probably due to the thermal motion of the methyl carbon atom, also reported for dextropropoxyphene hydrochloride.[7]

The mean C – N bond length is 1.455 Å, in agreement with the mean value for amines (1.456 Å) reported by Falkenberg.[14]

The expected lengthening of C – C single bonds in a compound having a quaternary carbon atom is shown by the bond lengths 1.555 Å (C3 – C4), 1.559 Å (C4 – C5), 1.544 Å (C4 – C10), and 1.552 Å (C4 – C16). Owing to the four large groups attached to C4, there is considerable deviations from tetrahedral values in the bond angles involving this atom. Similar distortions are reported in *d*-methadone. HBr [5] and dextropropoxyphene.HCl.[6]

Each of the two phenyl rings A (C10,C15) and B (C16,C21) is found to be planar. C4 is coplanar with ring B, whereas it is 0.16 Å out of plane A. This latter deviation from planarity is probably caused by the strain around C4. The two planes form an angle of 80.6°. The dihedral angles C5 – C4 – C16 – C17 and C3 – C4 – C10 – C15 are as small as −4.9° and −29.8°, respectively, and

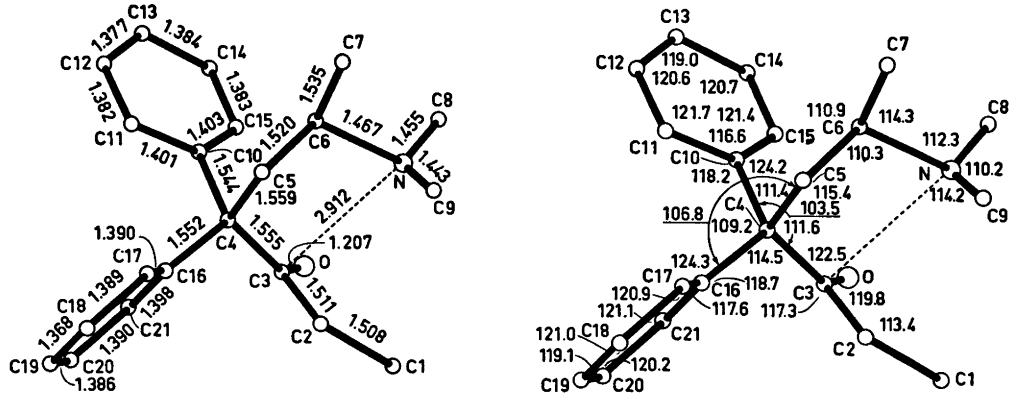


Fig. 1. (a) Bond lengths (Å). (b). Angles (°) in *l*-methadone.

Table 4. Bond lengths (Å) and bond angles (°) for the non-hydrogen atoms, with standard deviations.

Bond lengths		Corrected	Bond angles	
C1 – C2	1.508(4)		C1 – C2 – C3	113.3(.2)
C2 – C3	1.511(3)		C2 – C3 – C4	117.3(.2)
C3 – O	1.207(3)		O – C3 – C4	122.5(.2)
C3 – C4	1.555(3)		C3 – C4 – C5	111.6(.2)
C4 – C5	1.559(3)		C3 – C4 – C10	114.4(.2)
C4 – C10	1.541(3)	1.544	C3 – C4 – C16	103.6(.2)
C4 – C16	1.549(3)	1.552	C5 – C4 – C10	106.8(.2)
C5 – C6	1.520(3)		C5 – C4 – C16	111.3(.2)
C6 – C7	1.535(4)		C10 – C4 – C16	109.2(.2)
C6 – N	1.467(3)		C4 – C5 – C6	115.4(.2)
C8 – N	1.455(4)		C5 – C6 – C7	110.9(.3)
C9 – N	1.443(4)		C5 – C6 – N	110.3(.2)
C10 – C11	1.393(3)	1.401	C7 – C6 – N	114.2(.2)
C11 – C12	1.379(3)	1.382	C6 – N – C8	112.1(.3)
C12 – C13	1.371(3)	1.377	C6 – N – C9	114.3(.3)
C13 – C14	1.376(4)	1.384	C8 – N – C9	110.0(.3)
C14 – C15	1.379(3)	1.383	C4 – C10 – C11	118.7(.2)
C15 – C10	1.396(3)	1.403	C4 – C10 – C15	124.3(.2)
C16 – C17	1.379(3)	1.390	C10 – C11 – C12	121.7(.2)
C17 – C18	1.385(4)	1.389	C11 – C12 – C13	120.6(.3)
C18 – C19	1.359(4)	1.368	C12 – C13 – C14	119.0(.2)
C19 – C20	1.375(4)	1.386	C13 – C14 – C15	120.7(.3)
C20 – C21	1.386(4)	1.390	C14 – C15 – C10	121.4(.2)
C21 – C16	1.388(3)	1.398	C15 – C10 – C11	116.6(.2)
			C4 – C16 – C17	124.3(.2)
			C4 – C16 – C21	118.2(.2)
			C16 – C17 – C18	120.9(.3)
			C17 – C18 – C19	121.0(.3)
			C18 – C19 – C20	119.1(.2)
			C19 – C20 – C21	120.2(.3)
			C20 – C21 – C16	121.1(.3)
			C21 – C16 – C17	117.6(.2)
Intramolecular interaction			C2 – C3 – O	119.8(.2)
N···C3	2.912(3)			

Table 5. Bond lengths (Å) involving hydrogen atoms. Standard deviations are in the range 0.02 – 0.04 Å.

C1 – H1C1	0.98	C9 – H1C9	0.94
C1 – H2C1	0.97	C9 – H2C9	1.03
C1 – H3C1	0.97	C9 – H3C9	0.98
C2 – H1C2	0.99	C11 – HC11	0.95
C2 – H2C2	0.96	C12 – HC12	0.91
C5 – H1C5	0.99	C13 – HC13	0.95
C5 – H2C5	1.02	C14 – HC14	0.99
C6 – HC6	0.98	C15 – HC15	0.94
C7 – H1C7	0.98	C17 – HC17	0.93
C7 – H2C7	0.97	C18 – HC18	0.98
C7 – H3C7	1.01	C19 – HC19	0.99
C8 – H1C8	1.04	C20 – HC20	0.95
C8 – H2C8	1.01	C21 – HC21	0.96
C8 – H3C8	0.99		

the opening of the trigonal angles O – C3 – C4 (122.5°), C4 – C10 – C15 (124.3°), and C4 – C16 – C17 (124.3°) decreases the repulsions between C5 and C17, and between O and C15, respectively.

An interesting short intramolecular distance is that between N and C3 which are separated by 2.912 Å, approximately 0.1 Å shorter than the sum of the van der Waals radii. This close proximity of N and C_{co} may be explained by an interaction between the lone pair of the nitrogen atom and the electropositive carbonyl carbon atom, as discussed in the introduction (I). The carbonyl carbon atom is elevated 0.06

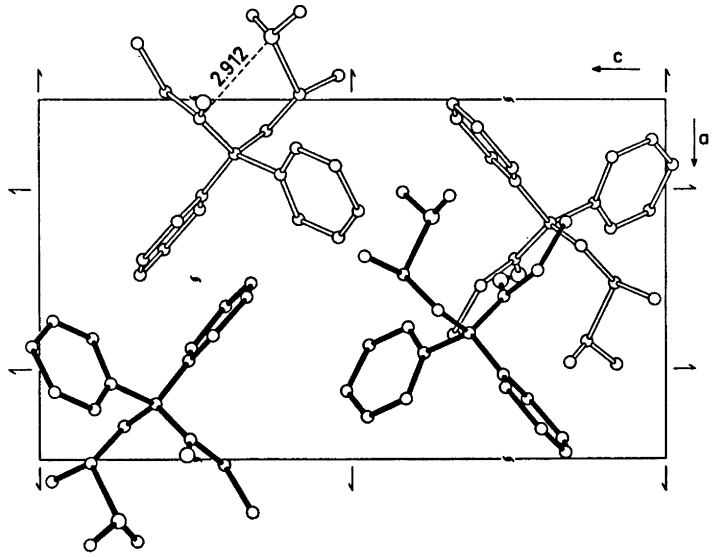


Fig. 2. The crystal structure of *l*-methadone as seen down the *b*-axis.

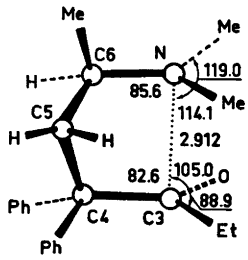


Fig. 3. Schematic drawing of the conformation of *l*-methadone.

Å out of the plane through the atoms to which it is bonded (towards the nitrogen atom.) The conformation of *l*-methadone, as seen perpendicular to a plane through C3,C4,C6 is shown in Fig. 3.

The conformation of methadone [1,2,4] proposed as necessary for analgesic activity is thus confirmed by this X-ray analysis. In the present structure the dimethylamine group is *gauche* relative to C4, the dihedral angle C4 – C5 – C6 – N being – 68.5°. It may be of great interest to see if other synthetic analgetics related to methadone are preferring a similar conformation in the solid state.

Note. The author has been made aware of the structure determination of the same compound carried out by H. B. Bürgi, E. Shefter and J. D. Dunitz, to be published in *Nature*. (The First European Crystallographic Meeting, Bordeaux, August 1973).

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The Conformations of Some *gem*-Dimethyl Substituted Cyclic Anhydrides

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Infrared spectroscopy shows that 3,3,6,6-tetramethyloctanedioic anhydride takes the same conformation in the crystal as in solution. Low-temperature ^{1}H NMR-spectroscopy of this 9-membered ring suggests C_2-symmetry, the two-fold axis passing through the ring oxygen in the anhydride group, and roughly D_3-symmetry for the ring skeleton. Two possible conformations are discussed for 3,3,7,7-tetramethylnonanedioic anhydride based on low-temperature NMR-spectroscopy, UV-absorptions, and dipole moment.

The acid anhydride group and two neighbouring carbon atoms should most likely tend to take the planar anti-anti form.[1] Also according to the determined crystal structure of monochloroacetic anhydride,[2a] electron diffraction of acetic anhydride [2b] and dipole moment investigations of benzoic and other anhydrides [3] the most stable conformation of the anhydride group seems to be the anti-anti form with varying deviations from planarity. In medium rings this conformation is not easily maintained and that is perhaps one of the reasons for the extreme instability of unsubstituted cyclic anhydrides.

We have earlier found that substitution with *gem*-dimethyl groups in certain positions facilitate the formation of 9- and 10-membered ring ketones.[4] Furthermore cyclic anhydrides of ring size 9 and 10, by substitution with *gem*-dimethyl groups were found to be easily formed and stable enough for conformational studies. In a previous paper [5] the syntheses of 3,3,6,6-tetramethyloctanedioic anhydride (a 9-membered ring), the corresponding dimer (an 18-membered ring), and 3,3,7,7-tetramethylnonanedioic anhydride (a 10-membered ring) have been described. A conformational analysis of these cyclic compounds is now reported.

In Table 1 the observed melting points, enthalpies and entropies of fusion and NMR coalescence temperatures at 100 MHz are listed. For comparison the anhydride of 3,3-dimethylpentanedioic acid (a 6-membered ring) has also been studied. (Pentane, octane, and nonanedioic anhydride = glutaric, suberic, and azelaic anhydride.)

In Table 2 the infrared absorption bands of the carbonyl groups, the ultraviolet absorp-

Table 1.

Compound	Ring-size	M.p. °C	Calorimetry ΔH_m	Calorimetry ΔS_m	NMR coalescense temp. at 100 MHc, °C
3,3,6,6-Tetramethyl-octanedioic anhydride	9	71	4.5	13.3	> 35
3,3,6,6-Tetramethyl-octanedioic anhydride dimer	18	liq.			
3,3,7,7-Tetramethyl-nonanedioic anhydride	10	59	4.9	14.9	*ca.* − 100
3,3-Dimethylpentane-dioic anhydride	6	123	4.3	11.9	> − 120

Table 2.

Compound	Infrared absorption cm^{-1}		Solvent	Ultraviolet absorption λ_{max}	ε	Solvent	Dipole moments [a] D
3,3,6,6-Tetramethyl-octanedioic anhydride							
Monomer	1758	1790	CCl_4	232	236	Hexane	4.1
Dimer	1735	1810	»				3.9
3,3,7,7-Tetramethyl-nonanedioic anhydride	1745	1790	»	230	215	»	3.7
3,3-Dimethylpentanedioic anhydride	1770	1815	»	223	< 100	»	5.0
Pentanedioic anhydride	1766	1811	CH_2Cl_2				4.14 [19]
Butanedioic anhydride	1797	1872	CCl_4	223	77	Ethyl ether	4.1
l-Methylbutanedioic anhydride				226	< 100	Heptane	
Acetic anhydride	1748	1824	None	217	56	None [18]	3.1 [3]
Propionic anhydride	1745	1810	»				3.3

[a] Solvent: Benzene.

tions and the dipole moments are shown, together with the corresponding values for some 5- and 6-membered ring anhydrides and the anhydrides of acetic and propionic acid.

No correlation was found between the splitting of the IR carbonyl bands and the conformation of the anhydride groups. Similarly the differences in the IR absorption intensities of the two carbonyl bands were not large enough to allow conclusions to be drawn concerning the angle between the two C=O bonds, as indicated by Fayat *et al.*[6] In the 6-membered cyclic anhydride the band at lowest frequency is the strongest, in the 9-membered ring the two bands are of the same intensity, in the 10- and 18-membered cyclic anhydrides the highest frequency bands are the strongest.

The shift of the UV-absorption of the carbonyl group is dependent on the resonance and inductive effect of the neighbouring groups. A positive resonance effect and a negative inductive effect will both shift the spectrum ($n-\pi^*$) towards shorter wavelengths.[7] The group

$$-O-\overset{\overset{O}{\|}}{C}-R$$

has a +R and −I effect. In our cyclic anhydrides the inductive effect is more or less the same for all possible conformations. The resonance effect through the free electron pairs of the "ether" oxygen and the electrons of the carbonyl group is dependent on the dihedral angle but this conjugation is possible both in the *cis-cis* conformations of rings and in the *trans-trans* conformation of acetic anhydride. This explains why the values for the 5- and 6-membered rings are almost the same as for acetic anhydride (Table 2). Conclusions about the conformations of the cyclic anhydrides are therefore difficult to draw from the UV absorption values.

3,3,6,6-TETRAMETHYLOCTANEDIOIC ANHYDRIDE

Studies of the conformations and barriers to inversion in 9-membered rings with *gem*-dimethyl groups in 4 and 7 positions have been reported earlier.[8,9] The compounds were all found to have C_2 symmetry, D_3 for the carbon skeleton, with the functional group and the *gem*-dimethyl substituted carbons on the three quasi symmetry axes of the D_3 ring skeleton.

The NMR-spectra of 3,3,6,6-tetramethyl-octanedioic anhydride in $CDCl_3$ at 35°, 20°, and 0° are shown in Fig. 1. There were no further changes in the spectrum below 0°. The coalescence temperature (above 35°C) is higher than found earlier for a correspondingly tetramethyl substituted cyclononanone.[8]

In the low temperature spectrum there are two lines for the *gem*-dimethyl protons, one for the methylenes between the *gem*-dimethyl

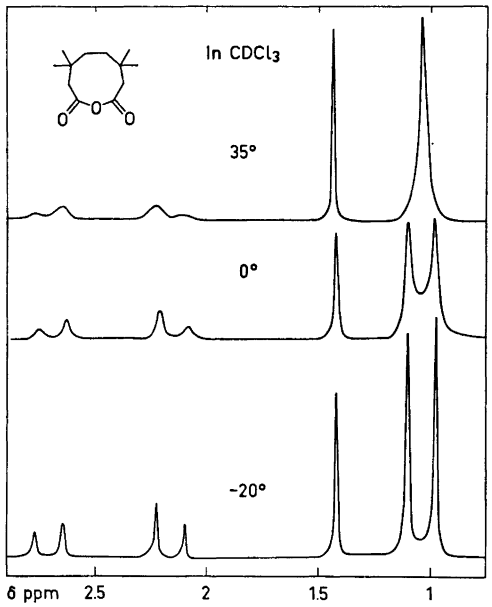

Fig. 1. The ^{1}H NMR-spectra of 3,3,6,6-tetramethyloctanedioic anhydride at different temperatures.

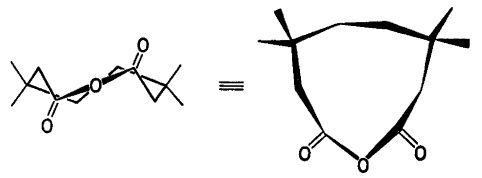

Fig. 2. Conformation of 3,3,6,6-tetramethyloctanedioic anhydride.

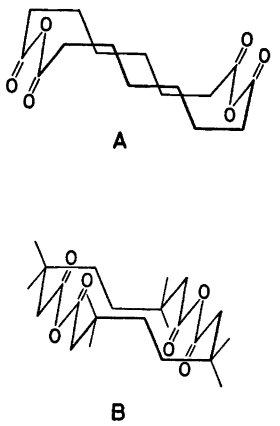

Fig. 3. A. Proposed ideal conformation for the unsubstituted dimer of octanedioic anhydride. B. Expected conformation for the dimer of 3,3,6,6-tetramethyloctanedioic anhydride.

groups and a quartet for the protons alpha to the anhydride groups. This means that there are only two types of methyl groups and two types of alpha protons. According to earlier considerations of possible conformations [8] this can only mean that the molecule has a C_2-symmetry, and probably D_3 symmetry for the ring skeleton, Fig. 2, as the earlier investigated tetramethyl substituted 9-membered rings.

The IR-spectra for the crystals and in CCl_4 solution were the same, indicating the same conformation in the crystals and in solution. The calorimetric values are in accordance with this observation.

DIMER OF 3,3,6,6-TETRAMETHYL-OCTANEDIOIC ANHYDRIDE

Hill and Carothers [10] found that the unsubstituted compound was stable up to the melting point 56 – 57°. Dale [1] has proposed that the ideal conformation for an unsubstituted cyclic dianhydride has the anhydride group and the two neighbouring carbon atoms coplanar to form the "bridges" as in Fig. 3 A; *gauche*-preferred bonds are in corner positions stabilizing the corners and the dipoles are anti-parallel.

In this conformation *gem*-dimethyl groups can, without serious interaction, only be placed in the corner positions, and not as they would in the tetramethyl substituted dimer, next to the corner positions.

Three other diamond lattice conformations are possible for the 18-membered cycloalkane.[11] Of these, the conformation, Fig. 3 B, would have planar anhydride groups, *gem*-dimethyl groups in the "corners", two-carbon bridges and dipoles opposed and would be the expected conformation for this compound, also because it most probably is the conformation for 1,1,4,4,10,10,-13,13-octamethylcyclo-octadecane.[12] However, the fact that the di-anhydride is a liquid and polymerizes by standing, may suggest that this conformation is not optimal, and makes it more probable that the dimeric anhydride exists as a mixture of conformations.

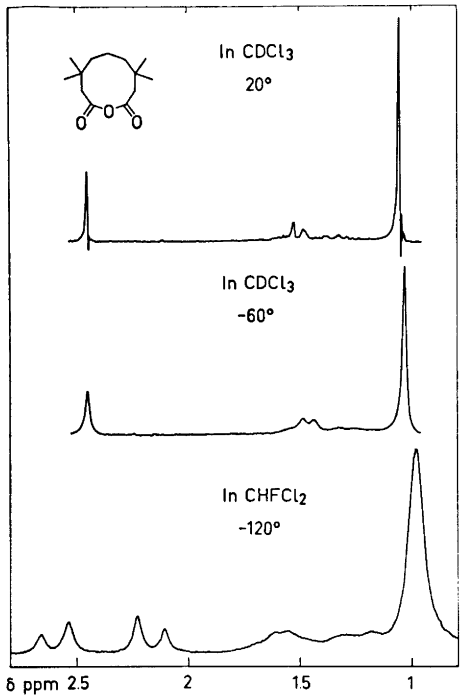

Fig. 4. The ^1H NMR-spectra of 3,3,7,7-tetramethylnonanedioic anhydride at different temperatures.

3,3,7,7-TETRAMETHYLNONANEDIOIC ANHYDRIDE

The NMR spectra of this 10-membered cyclic anhydride in $CDCl_3$ and $CHFCl_2$ at temperatures down to $-120°$ are shown in Fig. 4. The spectrum at the lowest temperature consists of a quartet for the alpha protons and the conclusion can be drawn that there are only two types of such protons and one type of α-CH_2 groups. The methyl signal has broadened but seems to require a lower temperature for full splitting. The spectrum at $-120°$ was the same in carbon disulphide. With only two types of alpha protons and the steric requirements of the two *gem*-dimethyl groups, the energetically possible conformations of this molecule are considerably reduced.

By semiquantitative calculations Dale [13] has found three probable conformations for 1,1,5,5-tetramethyl substituted cyclodecane. Two of these may be candidates for the conformation of the ring skeleton of 3,3,7,7-tetramethyl-

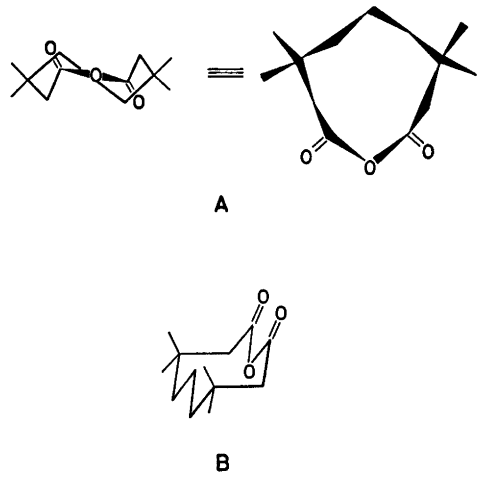

Fig. 5. Two possible conformations of 3,3,7,7-tetramethylnonanedioic anhydride.

nonanedioic anhydride. The third has no symmetry and is therefore not in accordance with the low temperature NMR-spectrum of the cyclic anhydride. The two actual conformations are shown in Fig. 5 A and B. Conformation A is the same as one of the two found by Dunitz in the crystal lattice of 4,4,8,8-tetramethylcyclodecane carboxylic acid.[14] Conformation B has already been proposed for cyclodecane-1,6-dione in solution.[15] The NMR-spectra, Fig. 4, shows a geminal coupling constant in the alpha protons of 12.5 cps. In conformation B one of the alpha protons is eclipsed with the C=O bond, in conformation A one alpha proton is almost eclipsed with the π-orbital in the C=O. In the first case the π-contribution to the coupling constant (dependent on the dihedral angle [16] should be below +0.5 cps, in the second case, −1.5 cps. As these contributions are small, and the observed coupling constant 12.5 cps is normal for such geminal protons (in the 9-membered ring anhydride, where the π-contribution should be zero, the corresponding J_{H-H}, was also 12.5) it is difficult to draw conclusions about the conformation from the geminal coupling constant of the alpha protons.

The similar degree of splitting of chemical shift of the two α-protons, as well as the similarity in the UV absorptions of the octane and nonane-dioic anhydrides indicates that the 10-membered cyclic anhydride may have the con-

formation A. The coplanarity in the anhydride group, preferred in open chain anhydrides may, however, be a reason for the molecule to chose conformation B. A surprising stability of this 10-membered cyclic anhydride, sligthly higher than the 9-membered ring anhydride is maybe explained by the coplanarity in conformation B and the stabilization of this conformation by the *gem*-dimethyl groups on the two "corners". As a cyclic anhydride the interactions are less and the deviations from the diamond lattice angles smaller than in the cycloalkane calculated by Dale and the conformation B therefore even more preferred.

The entropy and enthalpy of fusion of the tetramethylnonanedioic anhydride, Table 1, were on the same level as for the 9-membered ring anhydride. The IR-spectra were slightly different for the crystals in KBr and in CS_2 solution, however not sufficient to indicate different conformations in the two phases.

EXPERIMENTAL

Determination of dipole moments. Dielectric constants were measured at 20°C in a Weilheim Dipolmeter DM 01 in four different solutions of each compound. Refractive indices were measured on the same solutions in a Brice-Phoenix Differential Refractometer. Calculation of dipole moments was performed according to Hedestrand,[17] using no correction for atomic polarization.

Infrared spectra. These were recorded in a Perkin-Elmer Grating Infrared Spectrophotometer 457.

Ultraviolet spectra. The UV absorptions were determined in a Jasco Automatic Spectropolarimeter Model J-10.

Calorimetric measurements. A Perkin-Elmer Differential Scanning Calorimeter IB was used down to a temperature of −90°.

^1H NMR spectroscopy. The NMR spectra were recorded with a Varian HA 100 15 D instrument.

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Tobacco Chemistry. 22. Structures and Syntheses of a Nor- and a Seco-terpenoid of the Drimane Series Isolated from Tobacco

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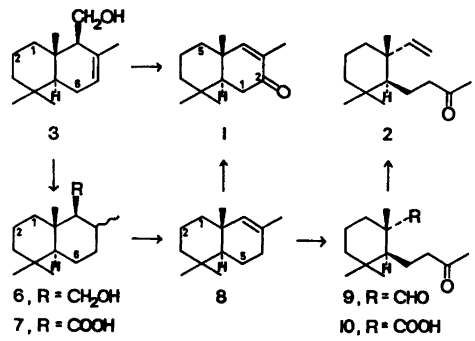
The structures of two new tobacco compounds, (4a*R*,8a*S*)-4a,5,6,7,8,8a-hexahydro-3,4a,8,8-tetramethylnaphthalen-2(1H)-one (*1*) (isonordrimenone) and (1′*S*,6′*S*)-4-(2′,2′,6′-trimethyl-6′-vinylcyclohexyl)-2-butanone (*2*) were assigned on the basis of the spectral data and confirmed by synthesis. Both compounds are likely products of degradation of cyclic terpenoid precursors.

A neutral fraction of the volatile material from an extract of sun-cured Greek tobacco, *Nicotiana tabacum* L., yielded two minor components which were identified as the α,β-unsaturated ketone (*1*) and the vinyl ketone (*2*).[1] We now wish to report the structure elucidations, limited to a consideration of the spectral data because of the small quantities isolated, and the syntheses of these compounds from a common bicyclic intermediate (*8*) obtained by degradation of the sesquiterpene alcohol drimenol (*3*).

(4aR,8aS)-4a,5,6,7,8,8a-Hexahydro-3,4a,8,8-tetramethylnaphthalen-2(1H)-one (*1*). Accurate mass measurement of the molecular ion (*m/e* 206) in the mass spectrum of this tobacco compound gave the molecular formula $C_{14}H_{22}O$. The carbonyl absorption (ν_{max} 1673 cm^{-1}) and ultra-violet absorption (λ_{max} (EtOH) 237 nm, ε 6200) indicated a disubstituted α,β-unsaturated ketone. The NMR spectrum displayed singlets for three methyl groups (δ 0.88, 0.91 and 1.07) and a doublet for a methyl group on a double bond (δ 1.72, *J* 1 Hz). Irradiation at the frequency of the vinylic methyl group showed that the single olefinic proton (δ 6.37, q, *J* 1 Hz) was coupled to this methyl group. With no evidence for further unsaturation in



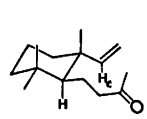
the molecule the molecular formula indicated a bicyclic compound, and the IR and UV spectral data supported a substituted α,β-unsaturated cyclohexanone structure. The downfield position of the quartet in the NMR spectrum for the olefinic proton indicated that it was β to the carbonyl group, and the collapse of the signal to a singlet in the above double irradiation experiment showed it to be coupled only to the methyl group and, hence, adjacent to a quaternary carbon atom. On addition of $Eu(fod)_3$-d_{27} a two proton multiplet at δ 2.12 – 2.62, assigned to the methylene group adjacent (α′) to the carbonyl function, was resolved into the strongly downfield-shifted AB part of an ABX system; the less strongly downfield-shifted X part was ascribed to a single proton, β′ to the carbonyl group, which lacked further spin-spin couplings and had to be adjacent to two fully substituted carbon atoms. These spectral data require the presence of a 2-methylcyclohex-2-en-1-one moiety and demonstrate how six of the remaining seven carbon atoms are linked to the ring. Since

three of these carbon atoms are present as tertiary methyl groups and another is quaternary it follows that the three remaining carbon atoms (C_3H_6) are present as methylene groups and the tobacco compound has structure *1*. The strong lanthanide induced shifts (LIS) observed in the NMR spectrum for C(3)CH_3 and C(1)H_2 and the less strongly induced shifts for the olefinic proton and C(8a)H are as expected. The relatively strong induced shift observed for C(4a)CH_3 and the weak induced shifts for C(8)(CH_3)$_2$ support the *trans*-fused structure *1*.

This tobacco compound (*1*) has not been isolated previously from a natural source but it has been reported as an oxidation product of the sesquiterpene alcohol drimenol (*3*).[2] Oxidation of the bicyclic olefin *8*, prepared from the sesquiterpene alcohol drimenol (*3*) as described below, with chromium trioxide in acetic acid solution provided an unambiguous synthesis of *1*. This product is identical in all respects to *1* isolated from tobacco. We have also confirmed that oxidation of drimenol (*3*) with chromium trioxide in pyridine gives isonordrimenone (*1*).[2]

(1'S,6'S)-4-(2',2',6'-Trimethyl-6'-vinylcyclohexyl)-2-butanone (2). The IR spectrum of this tobacco compound (*2*) with molecular formula $C_{15}H_{26}O$ (accurate mass measurement of molecular ion at *m/e* 222) showed absorptions characteristic of a carbonyl (1718 cm^{-1}) and a vinyl group (1637, 1008, 917 cm^{-1}). The three protons of the vinyl group appeared as an ABC system in the NMR spectrum (δ 4.89, H_A; 4.92, H_B; 5.64, H_C; $J_{AB} \sim 1$, J_{BC} 10, J_{AC} 18 Hz) and the absence of any coupling to other protons indicated that it was attached to a quaternary carbon atom. The presence of the $>CHCH_2CH_2COCH_3$ residue was established from the NMR spectrum (δ 2.06, CH_3CO) and the lanthanide induced shifts observed on the addition of Eu(fod)$_3$-d_{27}. The two proton multiplet at δ 2.15–2.5 for the methylene protons α to the carbonyl group was resolved into a strongly downfield-shifted triplet; spin-decoupling experiments confirmed the coupling of these protons to the two protons at C-4 which appeared as a less strongly downfield-shifted multiplet and, in turn, were coupled to the single proton γ to the carbonyl group which gave rise to a weakly downfield-shifted triplet. The absence of any further couplings to this last proton indicated that the $>CHCH_2CH_2COCH_3$ residue was attached to two fully substituted carbon atoms. The presence of the butanone sidechain was also supported by a prominent peak in the mass spectrum at *m/e* 164 ($M-C_3H_6O$) evidently arising from a McLafferty-type cleavage. With the vinyl group, the two quaternary carbon atoms carrying the 2-oxo-5-pentylidene moiety and the three methyl groups that appear as singlets in the NMR spectrum (δ 0.90, 0.92 and 1.02) there remains three methylene groups (C_3H_6) to construct the one ring required to satisfy the molecular formula. Since the two carbon atoms of the six-membered ring adjacent to the carbon atom bearing the butanone sidechain must be tetrasubstituted the structure *2* (stereochemistry undetermined) follows for this tobacco compound.

The relative stereochemistry may be deduced by distinguishing between the two configurations *4* and *5* possible when only chair conformations with the butanone sidechain in an equatorial position are considered. This was possible from an analysis of the LIS observed in the NMR spectrum on the addition of Eu(fod)$_3$-d_{27}. The preferred position of the europium complex about the carbonyl group



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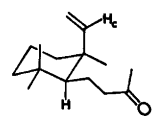

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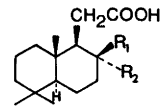
would be on the sterically less hindered side, α-side in *4* and *5*, and this is supported by the relatively strong LIS observed for C(1')H. The observed relative LIS for the three methyl groups and the vinyl proton H_c are in the order $Me \geq H_c \gg 2Me$ which is as expected for *4* but does not support the alternative configuration (*5*) for which the signals for the two α-methyl groups would be expected to display similar LIS which, in turn, would be greater than those for the β-methyl and the vinyl proton, H_c.

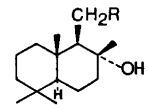
The structure *2* for this tobacco compound with stereochemistry as shown in *4* was confirmed by synthesis from the sesquiterpene alcohol drimenol (*3*) as outlined in the scheme.

The crude mixture of epimeric alcohols (*6*) obtained by the catalytic hydrogenation of drimenol (*3*)[3] was oxidised with chromium trioxide in 80 % acetic acid at room temperature in the presence of potassium hydrogen sulphate to give a 70 % yield of the epimeric acids *7*. Smooth decarboxylation of *7* was effected with lead tetraacetate in refluxing benzene in the presence of cupric acetate and pyridine[4] for an 80 % yield of the desired octahydronaphthalene intermediate *8*.

Ozonolysis of *8* at −78° in methylene chloride solution containing pyridine (1 equiv.) followed by a reductive work-up with zinc and acetic acid gave, in high yield, the unstable keto-aldehyde *9*. This essentially pure product was used in the next step without further purification. Only small amounts of the keto-aldehyde *9* were detected in the reaction product when ozonolysis of *8* in methanol suspension at −30° was followed by reduction of the hydroperoxides with dimethyl sulphide.[5] The main product of this reaction appeared to be the keto-acid (*10*). The attempted selective Wittig condensation of methylene triphenylphosphorane with the more reactive carbonyl group of *9* to give the desired vinyl ketone *2* gave a complex mixture of products. Eventually, inverse addition of a solution of methylenetriphenylphosphorane (1 equiv.) in dry dimethyl sulphoxide to a solution of the keto-aldehyde *9* in dry dimethyl sulphoxide at room temperature gave a low yield of the vinyl ketone *2*. The product was isolated by preparative gas-chromatography and displayed NMR, IR, and mass spectra identical to those of the tobacco compound *2*. The natural and synthetic products did not separate when co-injected on a capillary GC-column. The low specific rotation ($[\alpha]_D$ −0.5°) measured for the tobacco compound *2* in comparison to that for the synthetic material ($[\alpha]_D$ −10°) could not be used to establish the stereochemistry of the natural product. However, the very similar partial ORD curves of the natural and synthetic products obtained by measurement of the specific rotation at five different wavelengths (365 − 589 nm) confirmed that the synthetic product *2* and the tobacco compound *2* have identical absolute configurations. This is the first isolation of the C_{15} compound *2* from a natural source although it had been identified previously[6] as a cleavage product in the electrolytic decarboxylation of salts of the acids *11* and *12*.



11, $R_1 = CH_3$, $R_2 = OH$
12, $R_1 = OH$, $R_2 = CH_3$



13

The formation of isonordrimenone (*1*) and the vinyl ketone *2* by oxidation[2] of drimenol (*3*) and electrolysis of the salts of *11* and *12*, respectively, makes it attractive to suggest that bicyclic sesqui- or diterpenoids of the drimane (*e.g. 3*) or labdane type (relevant part of the labdane skeleton as in *13*) might act as precursors for the new nor- and seco-sesquiterpenoids *1* and *2*. Several natural products in the series *13* have recently been identified in Greek tobacco,[7] and related compounds such as 12-norambreinolide,[8] 8,13- and 8,13β-epoxylabd-14-en-12-one,[9] 12α-hydroxy-13-epimanoyl oxide,[10] and epimeric levantenolides,[11] are known tobacco leaf or smoke constituents.

EXPERIMENTAL

NMR, IR, UV, and mass spectra were recorded on Varian HA 100D and A60-A, Digilab FTS-14 and Perkin-Elmer 257, Beckmann DK-2A and LKB 9000 (70 eV) instruments, respectively. Optical rotations were measured on a Perkin-Elmer 141 instrument. Accurate mass measurements were carried out at the Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm. The mass spectra were obtained by gas liquid chromatography in combination with mass spectrometry (GLC-MS). A steel capillary column (0.5 mm × 50 m, Handy and Harman grade 316-S) coated with Emulphor by the dynamic packing method[12] was used for GLC-MS and analytical gas chromatography. A Varian 1700 gas chromatograph equipped with a glass column (3.2 mm × 3 m) packed with 5 % Carbowax 20M on Chromosorb G was used for preparative GLC.

Isolation. Compounds *1* and *2* were isolated from fraction B3 of an extract of sun-cured *Nicotiana tabacum* L. by a combination of liquid and preparative gas chromatography.[1]

(*4a*R,*8a*S)-*4a,5,6,7,8,8a-Hexahydro-3,4a,8-tetramethylnaphthalen-2-(1H)-one* (*1*) MS: *m/e* 206 (M^+, 42 %), 83 (100), 109 (48), 55 (34), 108 (33), 41 (33), 121 (27), 69 (26), 163 (24), 123 (23);

accurate mass measurement: $C_{14}H_{22}O$, found 206.1672, calc. 206.1671; λ_{max}(EtOH) 237 nm (ε 6200); λ_{max}(film) 1673 (s), 1640 (w), 1017 (m) cm^{-1}; δ ($CDCl_3$) 0.88 (3 H, s), 0.91 (3 H, s), 1.07 (3 H, s), 1.72 (3 H, d, J 1 Hz), 2.12–2.62 (2 H, m), 6.37 (1 H, q, J ~1 Hz). On addition of Eu(fod)$_3$-d_{27}: C(1)H_2 and C(8a)H appear as an ABX, r (relative induced shift) = 3.88 [C(5)H_2, 8 lines, J 4, 14, 18 Hz], 2.56 [C(3)CH_3, broad s], 1.9 [C(8a)H, dd, J 4, 14 Hz], 1.12 [C(4)H, broad s], 1.0 [C(4a)CH_3, s], 0.28 and 0.36 [C(8) (CH_3)$_2$, 2 s]. $[\alpha]_D^{20}$ +17.6 (c 0.21 in benzene).

(1'S,6'S)-4-(2',2',6'-Trimethyl-6'-vinylcyclohexyl)-2-butanone (*2*) MS: *m/e* 222 (M$^+$, 4.5 %), 43 (100), 41 (42), 109 (42), 81 (40), 82 (40), 95 (38), 67 (37), 55 (35), 69 (34), 123 (34); accurate mass measurement: $C_{15}H_{26}O$, found 222.1990, calc. 222.1984; $C_{12}H_{20}$, found 164.1558, calc. 164.1565; ν_{max}(film) 1718 (s), 1637 (w), 1162 (m), 1008 (w), 917 (m) cm^{-1}; δ ($CDCl_3$) 0.90 (3 H, s), 0.97 (3 H, s), 1.02 (3 H, s), 2.06 (3 H, s), 2.15–2.5 (2 H, m), $-CH=CH_2$ appears as an ABC: 4.89, H_A; 4.92, H_B; 5.64, H_C; J_{AB} 1, J_{BC} 10, J_{AC} 18 Hz. On addition of Eu(fod)$_3$-d_{27} r = 3.2 [C(3)H_2, t, J ~8 Hz], 3.2 [C(1)H_3, s], 2.4 [C(4)H_2, m], 1.0 [C(1')H, broad t, J ~5 Hz], 0.86 ($-CH=CH_2$,m), 0.5 and 0.58 ($-CH=CH_2$, 2 m), 0.66 (3 H, s), 0.44 (3 H, s), 0.34 (3 H, s). $[\alpha]^{20}$ −0.5° (589 nm), −12.7° (578), −15.2° (546), −33.3° (436), −74.2° (365) (c 0.2 in chloroform).

8ξ-Drimanol (*6*). Drimenol (*3*) (1 g) was hydrogenated in ethyl acetate solution (50 ml) with Adams catalyst (150 mg) at 20°/1 atm.[3] Filtration and removal of solvent afforded a colourless oil (1 g) that crystallised slowly on standing. No attempt was made to separate the minor product of hydrogenation, 8α-drimanol, from the major product, 8β-drimanol.

8ξ-Driman-11-oic acid (*7*). Oxidation of the crude product *6* from the hydrogenation of drimenol (*3*) by the method of Appel, Brooks and Overton [3] gave a poor yield of 8ξ-driman-11-oic acid *(7)*. The following procedure gave a 70 % yield of crystalline acid: A solution of chromium trioxide (345 mg) in 80 % acetic acid (12 ml) containing potassium hydrogen sulphate (340 mg), was added dropwise at room temperature to a stirred solution of the crude hydrogenation product *6* (1 g) in 10 % acetic acid (12 ml). After stirring at room temperature for a further 2 h the reaction mixture was diluted with water and extracted with ether. The combined ether extracts were washed with saturated sodium bicarbonate solution (to remove only 39 mg of acidic material that contained no *7* on acidification and extraction into ether) followed by 2 M sodium hydroxide solution. The combined alkaline extracts were acidified and extracted with ether, the ether fractions washed with water and the solvent evaporated to yield crystalline 8ξ-driman-11-oic acid (*7*) (0.55 g). The neutral residue (0.35 g) from this oxidation contained unreacted drimanol (6) and related aldehydes which, on further oxidation in 80 % acetic acid solution (5 ml) with chromium trioxide (230 mg) in 80 % acetic acid (5 ml) containing potassium hydrogen sulphate (230 mg) and work-up as described above, gave a further 0.2 g of crystalline 8ξ-driman-11-oic acid (*7*) ν_{max}(KBr) 2500–3600 (broad), 1700 cm^{-1} (s).

trans-1,2,3,4,4aα,5,6,8a-Octahydro-4α,4β,7,8aβ-tetramethylnaphthalene (8). A mixture of 8ξ-driman-11-oic acid (0.5 g), lead tetraacetate (2.5 g, freed of excess acetic acid under high vacuum), copper(II) acetate (0.4 g) and pyridine (0.25 ml) in dry benzene (60 ml) was heated under reflux for 2 h in a nitrogen atmosphere.[4] The insoluble lead salts were filtered off at room temperature and the filtrate was washed with water, dried (Na_2SO_4) and the benzene removed under reduced pressure. The crude product was filtered through silica (25 g) in 1 % ether/pentane to yield pure *8* (320 mg, 80 %). Accurate mass measurement: $C_{14}H_{24}$, found 192.1886, calc. 192.1878; δ ($CDCl_3$) 0.85 (3 H, s), 0.90 (3 H, s), 0.94 (3 H, s), 1.58 (3 H, broad s), 5.06 (1 H, q, J ~1.5 Hz); ν_{max}(film) 3000 (m), 1390 (m), 1379 (m), 1370 (m), 1030 (w), 848 (m) cm^{-1}; $[\alpha]_D^{20}$ +56.8° (c 0.95 in pentane).

Keto-aldehyde 9. Ozone was bubbled through a solution of *8* (320 mg) in methylene chloride solution (10 ml) containing pyridine (140 mg) at −78° until there was an excess of ozone at the outlet from the reaction vessel. Zinc (600 mg) and acetic acid (1.5 ml) were added and the reaction mixture was brought quickly to room temperature. After 2 h stirring at room temperature the reaction mixture was diluted with water and extracted with ether. The combined ether extracts were washed with water, saturated sodium chloride solution and dried (Na_2SO_4). Evaporation of the solvent under reduced pressure gave the keto-aldehyde *9* as a pale yellow liquid (368 mg). This unstable but essentially pure reaction product darkened quickly on standing and could not be purified successfully by chromatography on silica. It was reacted in the next step without further purification. MS: *m/e* 224 (M$^+$, 1 %), 43 (100), 69 (65), 41 (38), 95 (33), 81 (30), 109 (28), 123 (25), 55 (24); δ ($CDCl_3$) 0.92 (3 H, s), 0.95 (3 H, s), 1.10 (3 H, s), 2.07 (3 H, s), 2.2–2.45 (2 H, m), 9.28 (1 H, s). ν_{max} (film) 2690 (w), 1720 (s).

Vinyl ketone 2. A solution of methylene triphenylphosphorane (1 mM) in anhydrous dimethyl sulphoxide (10 ml), from the *in situ* reaction of methylsulphinyl carbanion (generated with 24 mg of sodium hydride) and methyl triphenylphosphonium bromide (360 mg),[14] was added under nitrogen pressure to a stirred solution of the crude keto-aldehyde *9* (224 mg, 1 mM) in anhydrous dimethyl sulphoxide (20 ml) over 1 h at room temperature. After a further hour at room temperature the reaction

mixture was diluted with water, acidified, and extracted with ether. The combined ether extracts were washed with water, saturated sodium chloride solution and dried (Na_2SO_4). Evaporation of the solvent gave a yellow oil (240 mg) which TLC showed to be a complex mixture of compounds. Pure vinyl ketone *2* was isolated in low yield by preparative gas chromatography. It displayed NMR and IR spectra indistinguishable from the spectra of *2* isolated from the tobacco. The synthetic and natural products did not separate when co-injected on a capillary GC column. $[\alpha]^{20}$ −10.0° (589 nm), −15.2 (578), −17.7 (546), −36.5 (436), −78.6 (365) (*c* 0.52 in chloroform).

Isonordrimenone (*1*). (i) The octahydronaphthalene *8* (20 mg) in acetic acid (2 ml) at 70° was treated dropwise (20 min) with a solution of chromium trioxide (20 mg) in acetic acid (1 ml). After a further 30 min at 70° the cooled reaction mixture was diluted with water and extracted with ether. The combined ether extracts were washed with saturated sodium bicarbonate solution, water and dried (Na_2SO_4). Chromatography on silica of the residue obtained on evaporation of the solvent gave pure isonordrimenone (*1*) (8 mg). It displayed NMR, IR, and mass spectra identical to those obtained for *1* isolated from the tobacco, and the synthetic and natural products did not separate when co-injected on a capillary GC column. $[\alpha]_D^{20}$ + 21.8° (*c* 0.22 in benzene).

(ii) Oxidation of drimenol (*3*) (30 mg) with chromium trioxide (75 mg) in dry pyridine (0.75 ml) as described by Appel, Brooks and Overton [3] gave a mixture of compounds from which isonordrimenone (*1*) (3 mg) was isolated by column chromatography on silica. This material was identical in all respects to *1* isolated from the tobacco and prepared by oxidation of *8* as described above.

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Transformations of Steroids by Cell-free Preparations of *Penicillium lilacinum* NRRL 895. IV. Enzyme Catalyzed Acyl Transfer

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The cleavage of testosterone acetate by cell-free preparations from *P. lilacinum* was stimulated up to 2.3-fold by the addition of equimolar amounts of testosterone. Testosterone was found to be an acceptor of acyl groups derived from the acetyl esters of testosterone, epitestosterone, oestrone, epiandrosterone and desoxycorticosterone and from testosterone propionate. Epitestosterone inhibited the cleavage of testosterone acetate and did not act as an acyl acceptor to any significant degree. 4-Androstene-3,17-dione slightly stimulated the cleavage of testosterone acetate, probably acting as a modifier molecule.

Whereas the hydrolysis of steroid esters by microorganisms is well documented, reports on microbial esterification of hydroxysteroids seem to be less frequent.[1] Transformation of 4-androstene-3,17-dione into testosterone and testosterone acetate by *Saccaromyces fragilis* was demonstrated by McGuire *et al.*[2] Capek and co-workers showed that of 45 *Endomycetes* only the lactose fermenting species *S. fragilis*, *S. lactis*, *Candida pseudotropicalis*, and *Torulopsis sphaericus* were capable to acetylate testosterone. Steroids with 11α, 11β, 17α (sec), 20β, and 21-hydroxyls were not acetylated.[3] 3β-Hydroxy-C_{19}-steroids have been converted into the corresponding 3β-acetoxy compounds by *Penicillium oxalicum*.[17] 21-Acetylation of the acetonide of 9α-fluoro 16α-hydroxyhydrocortisone was demonstrated in *Trichoderma glaucum* by Holmlund *et al.*[4] Esterification of sterols with fatty acids and with succinic acid has been demonstrated in *Mycoplasma*, intestinal bacteria and *Mycobacteria*[5–8] *Aspergillus flavus* has been shown to convert oestrone into its sulphate.[9]

In the cases mentioned above, the acyl moiety was appearantly not derived from the steroid structure. The microbial formation of 17β acetates from 20-oxo-C_{21}-steroids is a result of a Baeyer-Villiger type of oxidation and must not be considered as an esterification.[1]

The steroid inducible esterase activity in cell-free preparations from *P. lilacinum* readily cleaves the ester bond of testosterone acetate.[10] When this reaction was studied, the product testosterone was found not to inhibit but instead to stimulate the reaction and to act as an acceptor of the acetyl group. The present communication describes transacylations carried out by esterase-active cell free preparations from *P. lilacinum* and the influence of some Δ^4-3-oxo-C_{19} steroids upon the esterase activity in such preparations.

MATERIALS AND METHODS

Abbreviations and trivial names. GLC: gas liquid chromatography; GC-MS: gas chromatography-mass spectrometry; TLC: thin layer chromatography; Desoxycorticosterone: 21-hydroxy-4-pregnene-3,20-dione; Epiandrosterone: 3β-hydroxy-5α-androstan-17-one; Epitestosterone: 17α-hydroxy-4-androsten-3-one; Oestrone: 3-hydroxy-1,3,5,(10)-oestratrien-17-one; Progesterone: 4-pregnene-3,20-dione; Testosterone: 17β-hydroxy-4-androsten-3-one.

Radioactive chemicals. [^{3}H]-Acetic anhydride (specific activity 0.050 Ci/mmol), [4-^{14}C]-4-

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androstene-3,17-dione (specific activity 0.0588 Ci/mmol), [7-^{3}H]-testosterone (specific activity 25 Ci/mmol) and [1,2-^{3}H]-epitestosterone (specific activity 49 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. The steroids were purified by TLC before use. Acetates of radioactive steroids or with ^{3}H-labelled acetyl groups were prepared by acetylation with acetic anhydride in pyridine, followed by purification by TLC. In the enzyme experiments the solutions of radioactive steroids were diluted with unlabelled steroids to give a final radioactivity corresponding to approx. 300 cpm per 0.3 μmol steroid in 10 μl of ethanol for desoxycorticosterone-[^{3}H] acetate and 10 000 – 50 000 cpm for the other steroids.

Unlabelled steroids. 4-Androstene-3,17-dione, desoxycorticosterone acetate, epiandrosterone acetate, progesterone, testosterone and its acetate and propionate were obtained from Sigma Chemical Co., St Louis, Mo. Epitestosterone and its acetate were obtained from Steraloids Inc., Pawling, N.Y. and desoxycorticosterone from Ikapharm, Ramat-Gan, Israel. They were checked for purity by TLC and/or GLC.

Other chemicals. All other chemicals were of reagent grade and treated as described previously.[10]

Chromatographic systems. TLC was carried out on Silica gel GF_{254} (system 1)[11] and on Al_2O_3 GF_{254} (0.5 % ethanol in benzene as solvent). GLC on OV-17 and GC-MS were performed as previously described.[11]

Growth of organism and preparation of cell free extracts. P. lilacinum NRRL 895 was grown on Czapek-Dox medium, induced, washed and frozen as described previously.[11] Progesterone was used as induced (70 mg in 2 ml of dimethylformamide per 300 ml of culture). 0.06 M Tris-HCl pH 7.2 was used as buffer in the washing of the cells and in the preparation of cell free extracts.

Cell free extracts were prepared using high speed grinding with glass beads according to Hedenskog *et al.*[12] All procedures were carried out at +4°C. 7 – 9 g of frozen cells were thawed and suspended in buffer to a final volume of 21 ml. The suspension was ground for 30 min with an equal volume of glass beads (diameter 0.45 – 0.50 mm) in a 28 mm i.d. glass tube with a loop stirrer of 20 mm diameter, rotating at approx. 1500 – 2000 rpm. The supernatant was decanted and the beads were washed with two small portions of buffer which were combined with the first supernatant. Cell debris was removed by centrifugation at 6000 rpm for 15 min, yielding about 30 ml of crude supernatant. After centrifugation at 100 000 *g* for 60 min, the resulting supernatant was frozen in suitable aliquots at −22°C. After thawing at room temperature the extract was centrifuged at 6000 rpm for 15 min in order to remove inactive protein precipitated during the freezing and thawing procedure. The clear extract was diluted with 0.06 M Tris-HCl pH 7.2 (usually to a fourfold dilution) and used in the enzyme experiments.

Incubation of steroids with cell-free extracts. The procedures for incubation and for extraction of the reaction mixture were the same as those previously described.[13] Due to short reaction times (2 min) the incubations were carried out on a vortex mixer. Each steroid was added in 10 μl of ethanol and in each series the ethanol concentration was equalized. The enzyme solution was preincubated with the steroid additive, usually for 45 s (for technical reasons), and the steroid ester substrate was then added. All incubations were carried out in duplicates.

The esterase activity in these preparations is unstable at room temperature, loosing approx. 10 – 20 % of its original activity in 40 – 60 min.[10] Therefore reference incubations with enzyme and steroid ester were run at the beginning and at the end of the series. The decrease in esterase activity was assumed to be linear and was corrected for in the calculations.

Steroid analysis. The relative amount of steroid alcohol and steroid ester was determined by TLC and liquid scintillation counting as described previously.[13] It was checked that side reactions (*e.g.* alcohol dehydrogenation) did not take place to any detectable degree. The variation between individual samples in duplicate incubations was calculated as $S.D. = (\sum d^2/2N)^{\frac{1}{2}}$. In two series, one with a mean testosterone concentration of 31.6 mol % (4.2 – 49.3 %, N = 61) and another with a mean concentration of 80.3 mol % (51.9 – 95.8 %), N = 20) the S.D. was ± 1.5 mol %.

Identification of steroids. The fractions containing [7-^{3}H] testosterone acetate formed by acetyl transfer from unlabelled steroid acetates (with exception of epitestosterone acetate) were isolated individually by TLC on Silica gel GF_{254}, rechromatographed on Al_2O_3 GF_{254} and analyzed by GC-MS and GLC using OV-17 as stationary phase. Crystallization to constant specific activity was also carried out after addition of 30 mg of carrier testosterone acetate. The [7-^{3}H]testosterone acetate fractions obtained from the acetates of desoxycorticosterone, epiandrosterone and oestrone were pooled and crystallized. The [7-^{3}H]testosterone acetate obtained from [7-^{3}H]testosterone and unlabelled testosterone acetate was crystallized separately.

Due to difficulties in the separations of the 17-epimeric acetyl esters, the [7-^{3}H]testosterone acetate obtained by transacetylation from unlabelled epitestosterone acetate was only tentatively identified by its TLC R_F-value.

[7-^{3}H] testosterone propionate obtained by transacylation from non radioactive testosterone propionate was isolated by TLC on Silica gel GF_{254} and by rechromatography on Al_2O_3 GF_{254}. In the latter system the acetates and propionates of testosterone separated fairly

Table 1. Transacylation of [7-^{3}H]testosterone by cell-free preparations from *P. lilacinum*.

Experiment	Acyl donor	% of [7-^{3}H]testosterone transacylated
1[a]	Testosterone acetate	19.8
1[a]	Testosterone propionate	4.9
1[a]	Desoxycorticosterone acetate	6.3
1[a]	Epiandrosterone acetate	10.4
1[a]	Oestrone acetate	21.0
2[a]	Testosterone acetate	20.5
2[b]	Epitestosterone acetate	4.2

[a] Initial concentration of [7^{3}H]testosterone 3.47×10^{-4} M, of steroid ester 3.04×10^{-4} M, preincubation time 45 s, incubation time 2.0 min. Temp. 30°C. Hydrolysis of [7-^{3}H] testosterone acetate without additive 31.2 %. [b] Conditions as in Note *a*, hydrolysis of [7-^{3}H] testosterone acetate and [1,2-^{3}H]-epitestosterone acetate without additive 36.2 and 30.8 %, respectively.

well (R_F-value for testosterone acetate 0.45, for testosterone propionate 0.53). The propionate was identified by crystallization to constant specific activity.

RESULTS

Acylation of testosterone. When [7-^{3}H]testosterone was incubated together with steroid acetates and with testosterone propionate, transfer of the acyl group from the ester substrate to testosterone could be demonstrated (Table 1). All the esters used are hydrolyzed by the esterase preparation.[10] TLC, GLC, and GC-MS properties for the testosterone acetate and propionate formed by transacylation were identical to those of authentic reference compounds. The results from the crystallizations to constant specific radioactivity are shown in Table 2.

When desoxycorticosterone-[^{3}H]acetate was incubated together with unlabelled testosterone, significant amounts of radioactivity could be recovered only in the testosterone acetate and desoxycorticosterone acetate fractions.

Ethyl acetate and sodium acetate did not serve as acetyl donors, even after prolonged incubation (20 min). Boiled extracts did not catalyze transacylations.

When [1,2-^{3}H]epitestosterone was incubated with enzyme preparation and testosterone acetate or epitestosterone acetate, significant amounts of [1,2-^{3}H]epitestosterone acetate could not be detected.

Effect of Δ^4-3-oxo-C_{19} steroids on the cleavage of [7-^{3}H]testosterone acetate and [1,2-^{3}H]epitestosterone acetate. The cleavage of testosterone acetate was clearly stimulated by testosterone and to a less degree also by 4-androstene-3, 17-dione (Tables 3–5). It was inhibited by epitestosterone (Tables 3 and 5). The hydrolysis of epitestosterone acetate was inhibited by epitestosterone and to a smaller degree also by testosterone. It was slightly stimulated by 4-androstene-3,17-dione (Table 3).

The increase in testosterone acetate cleavage depended on the amount of initially added free testosterone (Table 4). The figures clearly indicate that the effect was due to alcoholysis (transacylation) and thus the net amount of tritiated + unlabelled testosterone acetate in the mixture was not significantly affected. In

Table 2. Results from crystallizations to constant specific activity of [7-^{3}H]testosterone acetate and [7-^{3}H]testosterone propionate obtained by transacylation from unlabelled steroid esters.

Crystallization	Specific activity, cpm/mg		
	[7^{3}H]Testosterone acetate from unlabelled testosterone acetate	[7^{3}H]Testosterone acetate from other unlabelled steroid acetates (pool)	[7^{3}H]Testosterone propionate
First	220	194	33.5
Second	226	191	30.5
Third	214	191	31.2

Table 3. Effect of Δ^4-3-oxo-C_{19} steroids on the cleavage of [7-^{3}H]testosterone acetate and [1,2-^{3}H]epitestosterone acetate by cell-free preparations from *P. lilacinum*.

Experiment [a]	Additive [b]	% relative ester cleavage
3 T	none	100 (14.6 % hydrolysis)
3 T	testosterone	226
3 T	epitestosterone	56
3 T	4-androstene-3,17--dione	170
3 E	none	100 (13.6 % hydrolysis)
3 E	testosterone	86
3 E	epitestosterone	66
3 E	4-androstene-3,17--dione	115

[a] Initial concentration of [7-^{3}H]testosterone acetate (3T) or [1,2-^{3}H]epitestosterone acetate (3E) 3.04×10^{-4} M, incubation time 2.0 min, temp. 26°C. [b] Initial concentration 3.47×10^{-4} M. Preincubation time 45 s.

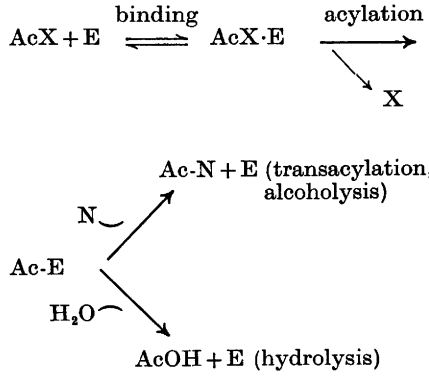
separate experiments the effect was found to be independent upon preincubation time (0 – 600 s).

Addition of equimolar amounts of 4-androstene-3,17-dione together with the 17-hydroxysteroids did not significantly influence the effects of testosterone (transacylation or stimulation of [7-^{3}H]testosterone acetate cleavage). The inhibitory effect of epitestosterone was slightly diminished by 4-androstene-3,17-dione (Table 5). When [4-^{14}C]4-androstene-3,17-dione was incubated with unlabelled testosterone acetate, no radioactivity could be detected in the testosterone acetate fraction. It was also found that the 17-oxo-group of [4-^{14}C]4-androstene-3,17-dione was not reduced in this system.

DISCUSSION

Acyl group transfer catalyzed by hydrolytic enzymes is well known.[14] However, transacylation between steroid esters and alcohols does not seem to have been reported to occur in microbial steroid metabolism.

The increase in testosterone acetate cleavage caused by testosterone is appearently due to "alcoholysis" since it corresponds to the amount of transacetylated steroid alcohol (Table 4). It is generally assumed that the enzyme catalyzed ester cleavage starts with a rapid binding step and proceeds *via* an acyl enzyme intermediate according to the following equation:[14]



where AcX is the ester, E the enzyme, AcX·E the enzyme substrate complex, X the alcohol, Ac – E the acyl-enzyme intermediate and N a nucleophile such as testosterone. If the acylations step is fast, the acyl-enzyme can be supplied as fast as it reacts further. Thus the rate of ester cleavage will be determined by the deacylation steps, *i.e.* hydrolysis or alcoholysis. The total rate of ester cleavage will increase linearly with increasing concentration of nucleophile.[15] The results presented in Table 4 clearly indicate that this is the case for the testosterone acetate cleavage.

The activation caused by 4-androstene-3,17-dione is probably analogous to the modifier action of acetone and other non hydroxylic compounds on esterases from pig and beef liver.[14,15] Greenzaid and Jencks found that nucleophiles such as methanol as well as modifier molecules (acetone, dioxane) increased the activity of pig liver esterase.[15] However, the modifiers were found to specificially suppress the degree of methanolysis. From this the authors postulated an alcohol binding site which might be occupied by the alcohol as well as by the modifier. The lack of effect of 4-androstene-3,17-dione on the transacylation

Table 4. Relation between increased cleavage of [7-^{3}H]testosterone acetate and transacylation of [7-^{3}H]testosterone by cell-free preparations from *P. lilacinum*.

Experiment	Substrate	Added testosterone or [7-^{3}H]testosterone, μmol	Increased in liberated [7-^{3}H]testosterone, μmol	Transacylated[7-^{3}H] testosterone, μmol
4[a]	[7-^{3}H]testosterone acetate	0.087	0.025	
»	»	0.174	0.033	
»	»	0.347	0.068	
»	testosterone acetate	0.087		0.020
»	»	0.174		0.034
»	»	0.347		0.073

[a] Initial steroid ester concentration 3.04×10^{-4} M, preincubation time 45 s, incubation time 2.0 min. Temp. 26°C. Hydrolysis of [7-^{3}H]testosterone acetate without additive 21.0 %.

of testosterone does not necessarily speak against the existence of such an alcohol binding site in the preparation from *P. lilacinum*. It might be due to differences in affinity towards the 17 β-alcohol and the 17-ketone.

Epitestosterone was found to suppress the hydrolysis of testosterone acetate as well as of epitestosterone acetate. It was also not possible to demonstrate transacylation of epitestosterone. This latter finding can be easily explained by the steric differences between the epimeric 17-alcohols. Wynne and Shalitin have studied the influence of the structure of alcohols upon their action of beef liver esterase activity.[16] They assumed the alcohol binding site to be a narrow cleft "padded" with hydrophobic groups. This will fit well with the different behaviour of the two epimeric 17-hydroxysteroids: see Fig. 1. The less favourable configuration of the 17α-hydroxyl might also

Table 5. Influence of the addition of 4-androstene-3,17-dione together with testosterone or epitestosterone.

Experiment[a]	Substrate	Additive	% relative ester cleavage	% of [7-^{3}H]testosterone transacylated
5	[7-^{3}H]testosterone acetate	testosterone	183	
»		4-androstene-3,17-dione	126	
»		testosterone + 4-androstene-3,17-dione	195	
»		epitestosterone	45	
»		epitestosterone + 4--androstene-3,17-dione	70	
»	testosterone acetate	[7-^{3}H]testosterone		11,2
»		[7-^{3}H]testosterone + 4-androstene-3,17-dione		9,2

[a] Initial concentration of additive 3.47×10^{-4} M, of steroid ester 3.04×10^{-4} M. Preincubation time 45 s, incubation time 2.0 min. Temp. 24°C. Hydrolysis of [7-^{3}H]testosterone acetate without additive 9.4 % (= 100 % relative ester cleavage).

explain its resistence towards enzymatic acetylation by yeasts as shown by Capek *et al.*[3]

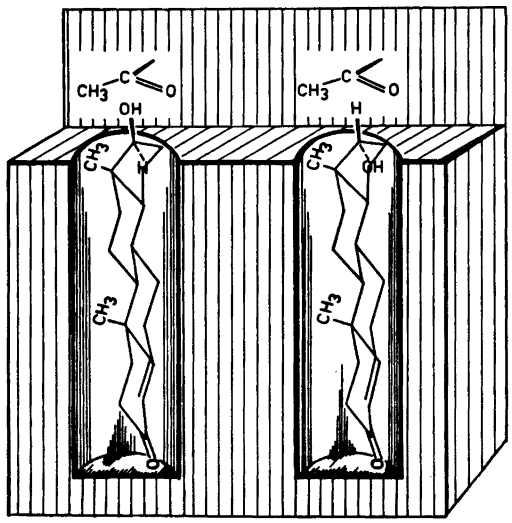


Fig. 1. Differences between testosterone and epitestosterone in reactivity: The 17β-hydroxyl group has a far more favourable position towards the acetyl group in the acetyl-enzyme complex.

While the nucleophile testosterone does not affect the hydrolysis of testosterone acetone to any considerable degree but stimulates the total disappearance rate by alcoholysis, its action upon the cleavage epitestosterone acetate is quite different. Transacylation of testosterone from epitestosterone acetate occurs to only about one fifth of that observed in the testosterone-testosterone acetate system. The hydrolysis is partly inhibited and this results in a slight suppression of the total cleavage rate. Similar observations have been made previously by Greenzaid and Jencks, using highly purified preparations of pig liver esterase.[15] While the addition of methanol causes a slight suppression of the hydrolysis of phenyl acetate but an increase on total cleavage due to alcoholysis, the hydrolysis of smaller acetates such as methyl acetate was greatly suppressed which in some cases resulted in a decrease of the total cleavage rate. These findings together with differences in response to organophosphorus inhibitors led to the conclusion that at least two different types of active sites with differences in substrate specificity must exist. This might also be true for the esterase active preparations from *P. lilacinum*, especially when its adaptive nature is considered. It must also be kept in mind that this preparation is rather crude and might contain several esterase active proteins with different substrate specificity.

Acknowledgement. The GC-MS analysis was carried out at the Department of Chemistry 1, Karolinska Institutet, Stockholm, Sweden.

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The Oxidation of Glycosides. XVII. Preparation of Some Methyl-hexodialdopyranoside-(1,5)-s

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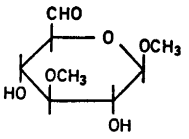
The 6-aldehydo derivatives of methyl 3-*O*-methyl-β-D-glucopyranoside and of the anomeric methyl glycopyranosides from L-glucose, L-mannose, D-galactose, and D-gulose have been prepared. During the oxidation of methyl 3-*O*-methyl-β-D-glucopyranoside small amounts of two keto-compounds were obtained: methyl 3-*O*-methyl-β-D-*arabino*-hexopyranosidulose and another one, which most likely is a diketo derivative of the parent glucoside.

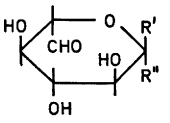
The previously unreported methyl α- and β-D-*glycero*-D-*talo*-heptopyranosides were also prepared.

It was observed that 6-aldehydo derivatives of methyl β-D-glucopyranoside and its 4-*O*-methyl derivative were hydrolysed much faster than the unoxidised glucosides.[1] In order to study this effect in some detail, a number of 6-aldehydo-hexosides have been prepared, and their acid hydrolysis will be investigated. In the present paper we report the synthesis of methyl 3-*O*-methyl-β-D-gluco-hexodialdopyranoside-(1,5) (I), the 6-aldehydo derivatives of the anomeric methyl glycopyranosides of L-glucose (II, III), L-mannose (IV, V), D-galactose (VI, VII), and D-gulose (VIII, IX).

Methyl 3-*O*-methyl-β-D-gluco-hexodialdopyranoside-(1,5) (I) was prepared by a route similar to that followed for the corresponding 4-*O*-methyl derivative,[2] that is, by oxidation of methyl 3-*O*-methyl-β-D-glucopyranoside with chromium trioxide in acetone and subsequent fractionation of the products on carbon and cellulose columns. Compound I was obtained as an amorphous ($[\alpha]_D^{20}$ −13°) material and showed the chromatographic and electrophoretic properties of an aldehydo-glycoside.[3] The structure was demonstrated through the following reactions: Borohydride reduction gave only one product, methyl 3-*O*-methyl-β-D-glucopyranoside which, when hydrolysed, released only one sugar, 3-*O*-methyl-D-glucose. After acid hydrolysis of I to the free dialdose, partial reduction and demethylation with



I



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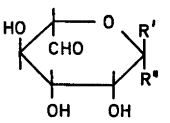
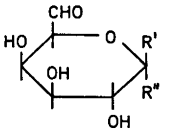
III R' = H; R" = OCH_3



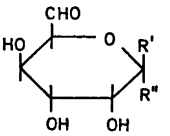
IV R' = OCH_3; R" = H

V R' = H; R" = OCH_3



VI R' = H; R" = OCH_3

VII R' = OCH_3; R" = H



VIII R' = H R" = OCH_3

IX R = OCH_3 R' = H

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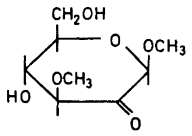
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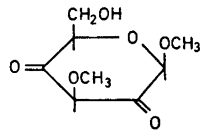
XI

boron trichloride,[4] glucose and gulose were the only monosaccharides detectable by paper chromatography and electrophoresis. Besides, supporting the structures of I, this result also indicated that no isomerisation of I or its hydrolysis product had occurred during the acid treatment.

Preparation of compound I was the primary object of this experiment, but two keto-compounds, formed in small amounts were also characterised. One of these compounds was amorphous ($[\alpha]_D^{20}$ −32°) and according to its chromatographic properties and migration in hydrogen sulphite electrophoresis [3,5] ($M_{vanillin}$ = 0.39, *cf.* 1.19 for compound I), appeared to be a mono-keto derivative. After borohydride reduction and demethylation this compound gave glucose and mannose as the only detectable monosaccharides, indicating that the compound is methyl 3-*O*-methyl-β-D-*arabino*-hexopyranosidulose (X).

The other compound was obtained crystalline (m.p. 141 – 142°; $[\alpha]_D^{20}$ −45.2°) and its chromatographic properties, migration in hydrogen sulphite electrophoresis ($M_{vanillin}$ = 1.37)[3,5] and elemental analysis indicated that it was a diketo compound, which make structure XI most likely. Borohydride reduction and demethylation yielded glucose, galactose, and small amounts of mannose as the main products in agreement with the proposed structure, XI.

D-Galactose oxidase has been widely used to oxidise the hydroxyl group at C-6 in D-galacto-pyranose residues to an aldehyde group.[6,7] The reaction is used when analysing such residues or when making tritium labelled compounds *via* reduction.[8] A discussion of previous work and substrate specificity of this oxidase has been published.[9] In the present work this method was used for the preparation of methyl β-D-*galacto*-hexodialdopyranoside-(1,5) from the corresponding galactoside. The reaction was not at all complete which is in accordance with the

Table 1. Preparation of methyl heptopyranosides.

Substance	Yield %	Melting point obs.	Melting point lit.	$[\alpha]_D^{20}$ (H_2O) obs.	$[\alpha]_D^{20}$ (H_2O) lit.
Methyl α-D-*glycero*-D-*gulo*-hepto-pyranoside	22	–	104 – 107°[15]	+111.5°	+112°[15]
Methyl β-D-*glycero*-D-*gulo*-hepto-pyranoside	41	171 – 172°	168 – 169°[15]	−70°	−75°[15]
Methyl α-D-*glycero*-D-*ido*-hepto-pyranoside	13	–	–	+80°	–
Methyl β-D-*glycero*-D-*ido*-hepto-pyranoside	7	–	–	−38°	–
Methyl α-D-*glycero*-D-*talo*-hepto-pyranoside	13	196 – 197°	–	+83.6°	–
Methyl β-D-*glycero*-D-*talo*-hepto-pyranoside	7	136.5 – 138°	–	−48.0°	–
Methyl α-D-*glycero*-D-*galacto*-hepto-pyranoside	29	141 – 143°	141°[16]	+179°	+178°[16]
Methyl β-D-*glycero*-D-*galacto*-hepto-pyranoside	2	166 – 169°	168°[16]	+16°	−5.1°[16]
Methyl α-D-*glycero*-L-*gluco*-hepto-pyranoside	13	167,5 – 169°	154 – 155°[16]	−142°	−108°[15]
Methyl β-D-*glycero*-L-*gluco*-hepto-pyranoside	4	185 – 187°	182 – 183°[15,17]	+36.8°	+36°[15,17]
Methyl α-D-*glycero*-L-*manno*-hepto-pyranoside	55	132 – 133°	132°[15,18]	−70°	−70.2°[15,18]
Methyl β-D-*glycero*-L-*manno*-hepto-pyranoside	5	–	–	+74°	+74°[16]

Table 2. Preparation of methyl hexodialdopyranoside-(1,5)-s.

Substance	Yield %	$[\alpha]_D^{20}$ (H_2O)
Methyl 3-*O*-methyl β-D-*gluco*-hexodialdopyranoside-(1,5) (I)	10	−13°
Methyl α-L-*gluco*-hexodialdopyranoside-(1,5) (II)	44	−120°
Methyl β-L-*gluco*-hexodialdopyranoside-(1,5) (III)	70	+40°
Methyl α-L-*manno*-hexodialdopyranoside-(1,5) (IV)	60	−60°
Methyl β-L-*manno*-hexodialdopyranoside-(1,5) (V)	28	+69°
Methyl α-D-*galacto*-hexodialdopyranoside-(1,5) (VI)	34	+132°
Methyl β-D-*galacto*-hexodialdopyranoside-(1,5) (VII)	93[a] 8[b]	+8°
Methyl α-D-*gulo*-hexodialdopyranoside-(1,5) (VIII)	31	+116°
Methyl β-D-*gulo*-hexodialdopyranoside(-1,5) (IX)	47	−71°

[a] Prepared from the methyl heptoside with periodate oxidation. [b] Prepared from the methyl galactoside with enzymatic oxidation.

results obtained from oxidation of *o*-nitrophenyl-β-D-galactopyranoside.[8]

The aldehydo compound obtained from enzyme action was shown to be identical with a sample prepared by the route discussed below. Further verification of the structure was obtained by hydrolysis to *galacto*-hexodialdose and by conversion to galacturonic acid by bromine oxidation and hydrolysis.

Antia and Perry [10] prepared methyl β-D-gulopyranoside by oxidation of methyl β-D-*glycero*-D-*gulo*-heptopyranoside with 1 mol of periodate to the 6-aldehydo-glycoside, followed by borohydride reduction. We have used this synthetic route, but stopping at the 6-aldehydo stage, in the preparation of a series of methyl hexodialdopyranoside-(1,5)-s. The heptoses prepared from D-glucose by the cyanohydrin synthesis and from D-mannose and D-galactose by the nitromethane synthesis, were glycosidated with methanol-hydrogen chloride. The products after glycosidation were fractionated on a basic ion-exchange resin column.[11] Yields and properties of the pure anomeric methyl heptosides obtained are summarised in Table 1. Methyl α- and methyl β-D-*glycero*-D-*talo*-hepto-

Table 3. Electrophoretic mobilities of the aldehydo-glycosides and corresponding dialdoses.

Substance	Hydrogen sulphite pH 4,7 $M_{vanillin}$	Borate pH 10 $M_{glucose}$
Methyl β-D-*gluco*-hexodialdopyranoside-(1,5)	1.25	0.62
Methyl 4-*O*-methyl β-D-*gluco*-hexodialdopyranoside-(1,5)	1.22	0
Methyl 3-*O*-methyl β-D-*gluco*-hexodialdopyranoside-(1,5)	1.19	0.57
Methyl α-L-*gluco*-hexodialdopyranoside-(1,5)	1.07	0.47
Methyl β-L-*gluco*-hexodialdopyranoside-(1,5)	1.25	0.60
Methyl α-L-*manno*-hexodialdopyranoside-(1,5)	1.17	0.66
Methyl β-L-*manno*-hexodialdopyranoside-(1,5)	1.29	0.62
Methyl α-D-*galacto*-hexodialdopyranoside-(1,5)	1.29	0.30
Methyl β-D-*galacto*-hexodialdopyranoside-(1,5)	1.45	0.43
Methyl α-D-*gulo*-hexodialdopyranoside-(1,5)	1.25	0.52
Methyl β-D-*gulo*-hexodialdopyranoside-(1,5)	1.33	0.73
D-*Gluco*-hexodialdose	1.36	1.15
4-*O*-Methyl D-*gluco*-hexodialdose	1.29	0.78
3-*O*-Methyl-D-*gluco*-hexodialdose	1.26	0.98
L-*gluco*-Hexodialdose	1.36	1.15
L-manno-Hexodialdose	1.57	0.79
D-*galacto*-Hexodialdose	1.43	0.93
D-*gulo*-Hexodialdose	1.42	1.09

pyranosides have, to our knowledge, not been previously reported.

The heptosides (1 mol) were oxidised at low temperature with periodate (1.05 – 1.15 mol). The products were fractionated by cellulose column chromatography and amorphous, but, according to paper chromatography and electrophoresis, pure fractions of the 6-aldehydo-glycopyranosides II – IX were obtained. Yields, based on the heptosides used as starting materials, and optical properties of the pure top-fractions are given in Table 2. The corresponding derivatives of D-idose and D-talose were obtained in small yields only and were impure.

Characteristic for aldehydo-glycopyranosides as well as for dialdoses and osuloses is the appearance on the paper chromatograms of elongated spots, which are often multiple with tailing between, which indicates equilibria between various isomeric forms.[3] This, of course, complicates the chromatographic purification of the aldehydo-glycosides. On paper electrophoresis, either in hydrogen sulphite [5] or borate buffer, the aldehydo-glycosides show characteristic mobilities and give distinct spots (Table 3). Studies on the acid hydrolysis of the aldehydo-glycosides will be reported separately.

EXPERIMENTAL

Concentrations were carried out under reduced pressure below 40°. The following solvent systems were used for paper chromatography:

A. Butan-1-ol – ethanol – water (10:3:5).
B. Ethyl acetate – acetic acid – water (3:1:1).
C. Ethyl acetate – pyridine – water (8:2:1).

In paper electrophoresis the following buffers were used:
D. 0.1 M Sodium hydrogen sulphite,[5] pH 4,7 at 40°.
E. 0.1 M Borate, pH 10.
F. 0.4 M Boric acid, 1.0 M glycerol, pH 6.8 at 40°.[12]

Whatman No. 1 papers were used for the chromatographic separations. Visualization of the spots was through the following conventional carbohydrate spray reagents: *p*-anisidine hydrochloride, silver nitrate-sodium hydroxide and resorcinol-hydrochloric acid.

Preparation and characterisation of methyl 3-O-methyl-β-D-gluco-hexodialdopyranoside-(1,5) (I). Methyl 3-*O*-methyl-β-D-glucopyranoside was prepared as previously reported.[13,14] The glucoside was oxidised in a way similar to that described for the corresponding 4-*O*-methyl ether.[2]

Chromium trioxide (8.3 g) in small portions was added with agitation to cold, freshly distilled acetone (835 ml). A solution of the glucoside (12.5 g) in cold acetone (250 ml) was poured in a slow stream to the agitated chromium trioxide solution. The temperature was kept below +15°. The reaction mixture was then kept at room temperature for half an hour and after that refluxed for 1 h. After cooling, the precipitate was filtered off and heated under reflux with acetone (4 × 175 ml) with intermediate filtrations. The combined filtrates were concentrated to a small volume and diluted with water to a final volume of 200 ml. The aqueous solution was extracted with chloroform (4 × 100 ml) and then treated with cation exchanger (Dowex 50, H^+) and anion exchanger (Dowex 3, free base). After concentration the oxidation product was separated on a carbon-Celite column (6.5 × 70 cm) and eluted with aqueous ethanol 0 – 25 % (20 l) and, subsequently, with 75 % ethanol (4 l). The fraction (0.65 g) which contained the aldehydo-compound, also contained some starting material and probably some keto-compounds and was further fractionated on a silicic acid column (3.5 × 50 cm) with chloroform-ethanol (3:1) as eluent.

A chromatographically and electrophoretically pure top-fraction of methyl 3-*O*-methyl-β-D-*gluco*-hexodialdopyranoside-(1,5) I (0.16 g) was obtained (but the total amount of the aldehyde was considerably higher). The compound I was identified as follows. I (0.037 g) was reduced with excess potassium borohydride and after reaction the solution was treated with cation exchanger (Dowex 50, H^+) and evaporated. Remaining boric acid was removed by evaporation with methanol. The reduction product, according to paper chromatography (solvent A and B) and thin layer chromatography in chloroform-ethanol (3:1), was identical with authentic methyl 3-*O*-methyl-β-D-glucopyranoside. No further products were detected. The reduction product (0.022 g) was hydrolysed and the crystalline product obtained (m.p. 164.5 – 166.5°) was identified as 3-*O*-methyl-D-glucose by IR, mixed m.p., and its chromatographic properties compared to authentic material. No other sugars were detected in the mother liquor.

Part of compound I was treated with 0.5 M sulphuric acid at 80° for 4 h. After neutralisation with barium carbonate, the product was reduced with an amount of potassium borohydride sufficient to convert one aldehyde group only. After demethylation [4] with boron trichloride in methylene chloride at −80° the mixture of monosaccharides was investigated by paper chromatography (solvent A, B, and C) and electrophoresis (buffer E and F). These separations revealed the presence of the ex-

pected sugars glucose and gulose, but the absence of the other hexoses.

During the separation of the oxidation products, two other compounds, giving yellow colour with the anisidine spray, were collected (a: 0.010 g and b: 0.018 g).

Substance a was crystalline and after recrystallisation from ethanol exhibited a m.p. of 141 – 142°, $[\alpha]_D^{20} - 45.2°$ (*c* 0.6 water), and no mutarotation. Elemental analysis gave: C 47,1; H 6.44; O 47.0. Diketo compound $C_8H_{12}O_6$ requires: C 47.1; H 5.92; O 47.0; and monoketo compound $C_8H_{14}O_6$ requires: C 46.6; H 6.85; O 46.7. $M_{vanillin} = 1.37$ in buffer D and the elemental analysis indicated that the compound was a diketo compound. It was reduced with excess potassium borohydride and the reduction product was demethylated with boron trichloride in methylene chloride at −80°. Paper chromatography (solvents A, B, and C) and paper electrophoresis (buffer E and F) revealed the presence of glucose, galactose, and small amounts of mannose as the only sugars. This indicates that the positions of the keto groups are at C-2 and C-4 in methyl 3-*O*-methyl glucoside.

Mass spectrometry gave M^+ 204, which correspond to structure XI. No more material was available for further elucidation of the structure.

Compound b was a chromatographically pure, amorphous compound, $[\alpha]_D^{20} - 32°$ (*c* 0.3 water). After reduction and demethylation as above, glucose and mannose were the only sugars detected by paper chromatography and electrophoresis. This indicates that the compound has one keto group at C-2 and thus is *methyl 3-O-methyl-β-*D-arabino-*hexopyranosidulose* (*X*). The mobility in hydrogen sulphite electrophoresis ($M_v = 0.39$) is characteristic of a monoketo-glycoside.

*Preparation and characterisation of methyl β-*D-galacto-*hexodialdopyranoside-(1,5) (VI).* In small scale experiments the extent of oxidation of methyl β-D-galactopyranoside with D-galactose oxidase was estimated by paper chromatography. When amounts of enzyme reasonable for preparative scale work were used, the oxidation was far from complete.

In a typical larger scale experiment methyl β-D-galactopyranoside (3.30 g) in 0.01 M, pH 7.0, phosphate buffer (330 ml) containing catalase (0.16 g) and galactose oxidase (0.066 g); was shaken for 1.5 h at 37°. Both enzymes were obtained from Worthington Biochemical Company. Ethanol (80 ml), active carbon (10 g) and some Celite was added and the stirred mixture filtered and washed with 25 % ethanol. After standing in the cold one day some inorganic salts could be filtered off.

The filtrate was evaporated to a small volume, passed through ion exchange columns of Dowex 50 (H^+) and Dowex 3 (free base) and evaporated to dryness. Some methyl β-D-galactopyranoside (0.90 g) was removed after crystallisation and the mother liquor was fractionated on a cellulose column (Whatman CF 11 powder, 4.5 × 85 cm) using solvent A. A top-fraction of compound VI (0.27 g) was collected and was pure according to paper chromatography and electrophoresis.

Part of compound VI was hydrolysed with 0.5 M sulphuric acid at 100° for 4 h and the product obtained shown to be identical (by paper chromatography and electrophoresis) with a sample of *galacto*-hexodialdose prepared by a Rosenmund reduction of tetra-*O*-acetyl-galactaroyl dichloride.[19] Another part of VI was transformed to a product shown to be identical with galacturonic acid by bromine oxidation[20] and acid hydrolysis with 0.5 M sulphuric acid at 100° for 4 h.

Preparation and characterisation of the methyl hexodialdopyranoside-(1,5)-s (II – IX). The general route for the preparation of compounds II – IX was preparation of heptoses, glycosidation of the latter with methanol/hydrogen chloride and separation of the resulting anomeric methyl heptopyranosides. Controlled periodate oxidation of the pure heptosides and chromatographic purification of the corresponding methyl hexodialdopyranoside-(1,5)-s was then performed. Yields and physical properties of the methyl heptopyranosides and of the methyl hexodialdopyranoside(1,5)-s are given in Tables 1 and 2, respectively. Paper electrophoretic properties of the latter and the corresponding hexodialdoses are given in Table 3. The yields in Table 1 are based on the heptoses and in Table 2 on the methyl heptopyranosides.

D-*glycero*-D-*gulo*-Heptopyranose and D-*glycero*-D-*ido*-heptopyranose were prepared by cyanohydrin synthesis from D-glucose,[21] but the reductions of the intermediate heptono-1,4-lactones were made with sodium borohydride[22] instead of sodium amalgam.[21] D-*glycero*-D-*galacto*-Heptopyranose and D-*glycero*-D-*talo*-heptopyranose were prepared as previously reported by nitromethane condensation and the Nef reaction starting from D-mannose.[23–25] D-*glycero*-L-*manno*-Heptopyranose and D-*glycero*-L-*gluco*-heptopyranose were similarly prepared but starting from D-galactose[26] instead of the D-mannose.

The same conditions for the glycosidation were used for all heptoses and were essentially as previously described.[15] To a solution of the heptose in methanol, was added a methanol/hydrogen chloride mixture to give a final concentration of 10 % sugar and 1.5 % hydrogen chloride. After refluxing the solution for 6 h, most of the solvent was removed under reduced pressure at 30°. Water was then added and the solution neutralised with an anion exchange resin (Dowex 3, free base). The resulting mixture of methyl glycosides was fractionated on an anion exchange column (Dowex 1–X8, OH^-, 200 – 400 mesh).[11] Pyranosides were eluted faster than furanosides (the latter were not collected) and α-pyranosides

Table 4. Characterisation of the aldehydo-glycosides by (A) reduction and (B) hydrolysis and subsequent reduction.

Compound	Reaction products A	B[a]
Methyl α-L-*gluco*-hexodialdopyranoside-(1,5) (II)	Methyl α-L-glucopyranoside (identified by paper chromatography and electrophoresis)	Glucitol
Methyl β-L-*gluco*-hexodialdopyranoside-(1,5) (III)	Methyl β-L-glucopyranoside (identified as above)	Glucitol
Methyl α-L-*manno*-hexodialdopyranoside-(1,5) (IV)	Methyl α-L-mannopyranoside (identified as above)	Mannitol
Methyl β-L-*manno*-hexodialdopyranoside-(1,5) (V)	Methyl β-L-mannopyranoside (identified as above)	Mannitol
Methyl α-D-*galacto*-hexodialdopyranoside-(1,5) (VI)	Methyl α-D-galactopyranoside (identified as above)	Galacticol
Methyl β-D-*galacto*-hexodialdopyranoside-(1,5) (VII)	Methyl β-D-galactopyranoside (identified as above)	Galactitol
Methyl α-D-*gulo*-hexodialdopyranoside-(1,5) (VIII)	$[\alpha]_D^{20}$ +110° (H_2O) found $[\alpha]_D^{20}$ +109.4° (H_2O) lit. Me α-D-gulopyranoside	Glucitol
Methyl β-D-*gulo*-hexodialdopyranoside-(1,5) (IX)	$[\alpha]_D^{20}$ −80.5° (H_2O) found $[\alpha]_D^{20}$ −83° (H_2O) lit. Me β-D-gulopyranoside	Glucitol

[a] The retention times of the hexaacetates on GLC were: mannitol 0.78, galactitol 0.89, glucitol 1.00 and, as comparison, iditol 1.22.

well separated from β-pyranosides. The separations were followed polarimetrically and by paper-chromatography and pure fractions of anomeric methyl heptopyranosides were obtained. The physical data of the crystalline compounds were in good agreement with previously reported values (Table 1). To our knowledge the methyl α- and β-D-*glycero*-D-*talo*-heptopyranosides have not been previously reported. Elemental analysis: required for the methyl heptosides C 42.9; H 7.20; O 50.0. Obtained for methyl α-D-*glycero*-D-*talo*-heptoside, C 43.2; H 7.10; O 49.6. Obtained for methyl β-D-*glycero*-D-*talo*-heptoside, C 43.1; H 7.30; O 49.4.

The periodate oxidation of the various methyl heptopyranosides was made essentially as previously described for the oxidation of methyl β-D-*glycero*-D-*gulo*-heptopyranoside,[10] but with somewhat more than the theoretical amount of periodate. Based upon smallscale experiments the following excess was used in the preparations (otherwise made under similar conditions): 5 % and 15 % excess of sodium metaperiodate for the methyl α-D-*glycero*-D-*gulo*-heptoside and its β-anomer, respectively, and otherwise 10 % excess. The preparation procedure (10 % excess of oxidant) was as follows:

To a solution of the methyl heptoside (1.00 g in 50 ml water) at 0° was added with stirring 0.09 M sodium metaperiodate (55 ml) while maintaining the temperature below +3°. After one hour stirring at room temperature the solution was kept at +4° for 20 h. Barium carbonate equivalent to the original periodate was then added and the mixture again stirred. After filtering, ion exchange (Dowex 50, H^+ and Dowex 3, free base) and evaporation to a syrup, the product, obtained by extraction with warm 50 % ethanol, was (after evaporation) fractionated on a cellulose column (90 × 4 cm) using butan-1-ol saturated with water as eluent. Top-fractions (both fast- and slow-moving with similar equilibrium patterns) of pure methyl hexodialdopyranoside-(1,5)-s were collected (Table 2).

The aldehydo-glycosides were amorphous but behaved as pure components on paper chromatography and paper electrophoresis[3] (Table 3).

Each of them yielded essentially one component, the dialdose, on acid hydrolysis. Borohydride reduction of the aldehydo-glycosides and the dialdoses yielded the expected glycosides and alditols, respectively (Table 4).

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The Use of Optically Active Half-esters of Methoxy-substituted Succinic Acids in the Stereospecific Synthesis of Long Chain Oxygenated Compounds

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The isomeric half-esters of (*S*)-methoxysuccinic acid have been prepared in the search for suitable intermediates for the synthesis of the β-glycol group of phthiocerol. The half-esters have very similar properties and differ mainly in the mass spectra and IR-spectra. Chain-lengthening of (*S*)-methyl 2-methoxy-3-carboxypropanoate by mixed electrolysis with dodecanoic acid in dimethylformamide gave a 21 % yield of (*S*)-methyl 2-methoxytetradecanoate.

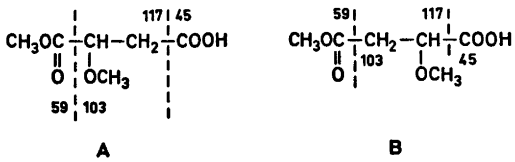
In the further search for routes for the stereospecific synthesis of phthiocerol and other optically active complex glycols we have investigated the possibilities of using the monoesters of methoxy succinic acids as suitable initial materials. These compounds were considered attractive as they are readily prepared from the commercially available D- and L-malic acids. The half-esters should be suitable for chain-lengthening by the use of the Kolbe reaction. There appears to be only one report in the literature concerning the synthesis of such a half-ester.[1] In this work the half-ester was prepared by boiling a solution of the anhydride of methylated L-malic acid in methanol. The optical rotation of the resulting product is reported as $[\alpha]_D^{20}$ −57.64° (*c*, 3.4874, acetone). No indication of the structure of this material is given.

Present work. It is well known that introduction of electron attracting groups (*e.g.* $-OCH_3$) next to the carbonyl carbon of the ester group markedly increases its positive character and hence will increase the velocity of base-catalyzed hydrolysis. Partial hydrolysis of dimethyl methoxysuccinate would thus predominantly result in the half-ester with the methoxyl substituent next to the free carboxyl group.

In contrast, formation of half-esters from unsymmetric anhydrides by reaction with hydroxyl compounds leads to predominance of ester on the more acidic carboxyl group. Accordingly, the material obtained by Purdie *et al.*[1] should have the structure (*S*)-methyl 2-methoxy-3-carboxypropanoate. We have repeated this work and obtained a product (A) with $[\alpha]_D^{22}$ −55.9° (*c*, 3.775, acetone), which is in good agreement with the value reported previously. An attempt by us to prepare the isomeric monoester (*S*)-methyl 3-methoxy-3-carboxypropanoate (B) *via* partial alkaline hydrolysis of the diester resulted in a compound with $[\alpha]_D^{24}$ −45.8° (*c*, 3.290, acetone).

The two monoesters (A and B) had practically identical gas chromatographic retention times on polar and non-polar stationary phases. This means unfortunately that it is at present impossible to state the isomeric purity of the two half-esters. The NMR spectra were also virtually identical whereas the infra-red spectra showed minor differences in the 1400 and 1050 cm^{-1} regions.

The mass spectra of the two esters show differences, however, which are consistent with the structures expected on theoretical grounds. None of the esters shows a peak due to the molecular ion (M = 162). The peaks of highest mass



number in both spectra are seen at $m/e = M - 18$ (loss of water). Significant peaks appear at the following mass numbers. Half ester A: 61(100), 103 (67), 61(27), 75(18), 132(16), 117(6), 114(5), 144(2); half ester B: 117(100), 59(64), 61(60), 103(60), 75(46), 71(30), 89(20), 99(20), 131(17), 144(5).

Attempts to lengthen the chain of the two half-esters by mixed anodic coupling with dodecanoic acid in dimethylformamide [2] gave a 21 % yield of (*S*)-methyl 2-methoxytetradecanoate when halfester (A) was used. Practically no cross-coupling was observed for half-ester (B). Similar experiments with (*S*)-methyl 2-acetoxy-3-carboxypropanoate as optically active unit as used by Horn and Pretorius [3] gave a yield of 14 % of the corresponding acetoxy-substituted compound.

We feel that optically active 2-methoxy-substituted esters prepared in the manner described are suitable as intermediates in the syntheses of more complex lipids containing methoxyl or hydroxyl groups. It has been shown previously [4] that the methoxyl compounds may be demethylated to the corresponding hydroxyl compounds without loss of optical activity. The methoxyl group can be retained as protecting group during chain lengthening operations to give for example polymethoxy or, after demethylation, polyhydroxy compounds.

EXPERIMENTAL

L-*Malic acid* (Fluka AG, Buchs, Switzerland) was used as initial material.

(S)-*Dimethyl 2-hydroxysuccinate* (dimethyl malate) was prepared from 50.0 g (0.373 mol) of L-malic acid by refluxing overnight with 500 ml of methanol containing 1 % of dry hydrogen chloride. After removal of the excess of methanol the crude ester was distilled. Yield 42.2 g (70.0 %) of b.p. 134–136°, 11 mm.

Dimethyl (S)-*2-methoxysuccinate*. To a mixture of 30.0 g (0.185 mol) of dimethyl malate and 105 g (0.740 mol) of methyl iodide was added during a period of 6 h 86 g (0.370 mol) of silver oxide (Fluka AG, Buchs, Switzerland). A spontaneous reaction occurred after initial heating. A further portion (25 g) of methyl iodide was added when about 2/3 of the silver oxide had been used. The reaction mixture was finally heated for 2 h and, after cooling, triturated with ether. After filtration and removal of volatile material the product was distilled yielding 24.1 g (74.0 %) of the methoxy ester. B.p. 116°, 11 mm. Purity 98.5 % as indicated by GC with HI-EFF 4 B as stationary phase. $[\alpha]_D^{25}$ −50.7° (acetone; *l*, 0.2; *c*, 3.24); $[\alpha]_D^{25}$ −42.2° (chloroform; *l*, 0.2; *c*, 1.60). Lardon and Reichstein [5] reported $[\alpha]_D^{16}$ −47.8° (*c*, 3.047 in acetone) and Fodor and Sóti [6] $[\alpha]_D^{28}$ −50.09° (*c*, 3.27 in acetone).

(S)-*2-Methoxysuccinic acid*. 17.3 g (0.0550 mol) of $Ba(OH)_2.8H_2O$ in 125 ml of distilled water was added to 8.7 g (0.0455 mol) of the above diester. The mixture was left for 1 h at room temperature and then for 2 h at 50°. After cooling the product was run through 70 g of Dowex 50 WX8 50/100 mesh ion exchange (H^+ form). Water was removed in a rotatory evaporator and the residue dried under vacuum over P_2O_5. The acid had m.p. 78.8–80.2°. Yield 5.6 g (77.0 %).

(S)-*Methyl 2-methoxy-3-carboxypropanoate (half-ester A)*. The free acid (5.5 g) was refluxed by heating in an oil bath at 60° for 4.5 h with 30 ml of acetyl chloride. The crude anhydride formed weighed 4.6 g after evaporation of volatile material. A mixture of 4.58 g (0.0353 mol) of the anhydride and 1.13 g (0.0353 mol) of methanol was heated at 50° for 4.5 h. 2.0 g of the reaction product which contained in addition to half-ester a small amount of diester (5 %) as indicated by GLC was chromatographed on silicic acid (50 g) (Mallinckrodt, 100–200 mesh, activated at 110° overnight) with ether:light petroleum (b.p. 40–60°) (35:65 v/v). After a small forerum (diester) 1.32 g of half-ester was eluted. It was a colorless viscous oil and had a purity of 98.5 % as indicated by GLC. $[\alpha]_D^{26}$ −55.9° (acetone; *l*, 0.2; *c*, 3.775). Purdie *et al.*[1] reported $[\alpha]_D^{20}$ −57.64° (*c*, 3.4874 in acetone).

(S)-*Methyl 3-methoxy-3-carboxypropanoate (half-ester B)*. A solution of 5.68 mmol of potassium hydroxide dissolved in 5 ml of methanol was added drop-wise at +4° to 1.00 g (5.68 mmol) of the diester dissolved in 5 ml of methanol. The reaction mixture was stirred overnight at room temperature and then evaporated to dryness. 2 ml of distilled water was added to the residue and the solution extracted twice with light petroleum (b.p. 40–60°). The aqueous phase was acidified with diluted hydrochloric acid (pH 1), saturated with sodium chloride and extracted five times with ethyl acetate. The combined organic extracts were dried ($MgSO_4$) and evaporated to dryness. The crude half-ester was purified by chromatography on silicic acid as described for (A). Yield 0.82 g of half-ester (B), being a colourless viscous oil. $[\alpha]_D^{24}$ −45.8° (acetone, *l*, 0.2; *c*, 3.290).

Chain-lengthening of half-ester (A). A mixture of (A) (1.083 g, 6.70 mmol) and 2.68 g (13.40 mmol) of dodecanoic acid in 35 ml of dimethylformamide containing 203 mg (2.01 mmol) of triethylamine was electrolyzed at 0.4 A (100 V) between platinum electrodes for 6 h. The methoxyester obtained was worked up by addition of light petroleum (b.p. 40 – 60°) and water to the reaction product. The aqueous phase was extracted once more with light petroleum and the combined organic phases washed with sodium carbonate, (5 % solution in water) and with water until neutral. Yield after drying ($MgSO_4$) and evaporation of solvent 1.40 g. The product was chromatographed on 30 g of the silicic acid described with ether:light petroleum (b.p. 40 – 60°) mixtures. 458 mg of docosane was eluted with 1:99 (v/v) and 389 mg (21.4 %) of (*S*)-methyl 2-methoxytetradecanoate with 1:1 (v/v). GLC indicated satisfactory purity (98 %) and the mass spectrum was consistent with the structure assumed. α_D^{30} −0.22° (*l*, 0.2; *c*, 4.20, chloroform), $[\alpha]_D^{30}$ −26.4° and $[M]_D^{30}$ −72. A similar experiment using half-ester (B) was made. GLC of the reaction product indicated only 2 % of methoxyester.

Chain-lengthening of (S)*-ethyl hydrogen 2-acetoxysuccinate.* A mixture of the acetoxy half-ester (4) 1.37 g (6.70 mmol) and 2.68 g (13.40 mmol) of dodecanoic acid was electrolyzed for 10 h in 40 ml of dimethylformamide containing 203 mg (2.09 mmol) of triethylamine (0.4 A, 120 V). The reaction product was worked up and purified as described. 303 mg (14 %) of (S)-ethyl 2-acetoxytetradecanoate was obtained.

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Formation of a Non-planar Dienamine from 1-(2-Indanylidene)-indan-2-one and Pyrrolidine

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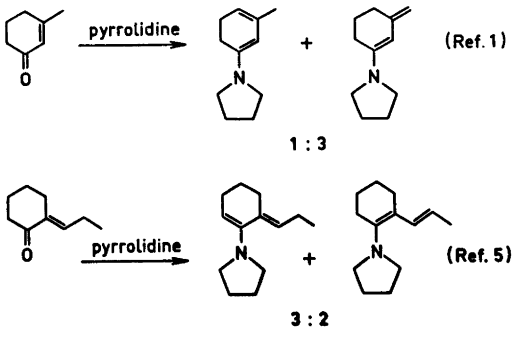
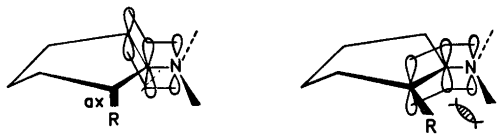
1-(2-Indanylidene)indan-2-one (I) may be converted into a mixture of the linear dienamine, 3-(2-indenyl)-2-(*N*-pyrrolidyl)-indene (II), and isomeric enamine, 1-(2-indenyl)-2-(*N*-pyrrolidyl)indene (III). In the main product (II), isolated by recrystallization, the indenyl substituent is proposed to be more or less twisted out of conjugation as shown by protium-deuterium exchange and PMR data. Factors affecting the formation and the relative stability of the isomeric forms are briefly discussed.

Several investigations concerning the preparation and the structure of dienamines from cyclic and acyclic ketones have been published.[1-4] In the case of ketones with an endocyclic double bond the products generally appear as mixtures of dienamines among which linear forms seem to be predominant, or exclusive. A different behaviour has been reported for cisoid, unsaturated cyclohexanones where the cross-conjugated isomer is the major component in the dienamine mixture[5,6] (Scheme 1). In the case of enamines derived from 2-substituted ketones the isomeric distribution is controlled

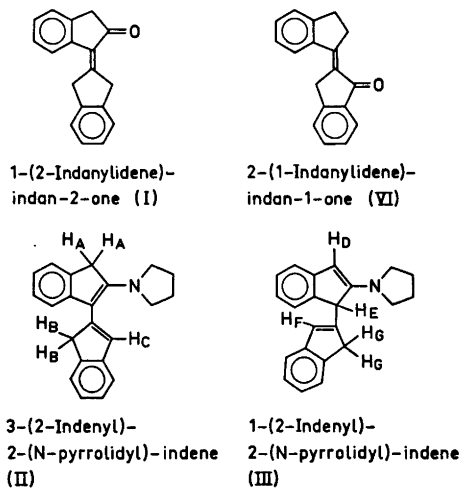


Scheme 1.



Scheme 2.

by various steric and electronic factors which affect the overlap between the nitrogen lone pair and the double bond of the enamine. Gurowitz and Joseph[7] have proposed that, within a given series, the greater the overlap the greater is the amount of the less substituted enamine (Scheme 2). In connection with the study of enamines from 1-methylindan-2-one we observed an isomeric ratio which deviated



Scheme 3.

from the general formulation mentioned above.[8] In the pyrrolidine enamine case, where overlap between the nitrogen lone pair and the double bond is strongest, the proportion of the most substituted enamine was greater than in the cases with other amine components. Since the controlling factors may or may not act in the same direction or will be differently weighted in our system, we have tried to obtain more information by studying the condensation between the reactive, secondary amine, pyrrolidine, and the planar, cisoid ketone 1-(2-indanylidene)indan-2-one (I)[9] (Scheme 3).

RESULTS AND DISCUSSION

Treatment of (I) with pyrrolidine in chloroform affords at room temperature two isomeric amines, (II) and (III), roughly in the ratio 9:1. Crystallization of the crude mixture from tetrachloroethylene gave only the pure isomer (II). The isomer (III) could not be obtained in a pure state. The ease of formation and the stability against hydrolysis are the same properties as ascribed to enamines of simpler 2-indanones.[8,10] In a comparative study, a similar compound, 2-(1-indanylidene)indan-1-one, ((VI),

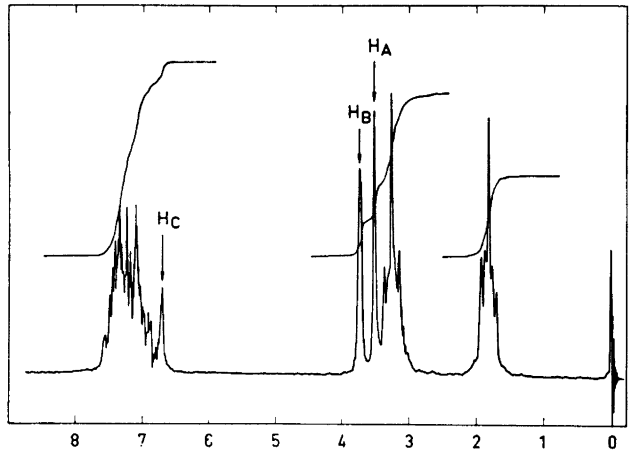


Fig. 1. PMR-spectrum of the pure isomer (II) (Concentration: 0.5 M) in chloroform-D. A small amount of 1,8-(*N*,*N*,*N'*,*N'*-tetramethyl)diaminonaphthalene is added to avoid equilibration due to acidic impurities in the solvent.

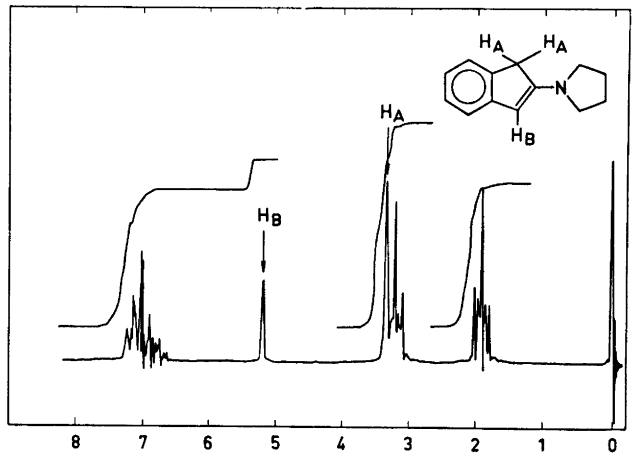


Fig. 2. PMR-spectrum of 2-(*N*-pyrrolidyl)indene (Concentration: 0.5 M) in chloroform-D.

Scheme 3) prepared according to Kipping,[11] was shown to be completely unreactive against condensation with pyrrolidine even after a prolonged reaction period. This demonstrates that the unusual reactivity observed for the simpler 2-indanones seems to be valid also in this unsaturated case (I). The PMR spectrum of the pure dienamine (II) is given in Fig. 1. This spectrum could be obtained only if the solvent chloroform-D had been carefully dried and with a trace of 1,8-(*N*,*N*,*N'*,*N'*-tetramethyl)diaminonaphthalene added in order to inhibit acid-catalyzed isomerization. The signals at $\delta \approx 1.6-2.0$ (ppm) and $\delta \approx 3.0-3.4$ (ppm) are ascribed to the protons of the pyrrolidine ring and the singlet at $\delta = 3.5$ (ppm) to the protons at the 1-position (H_A) in the enamine part. This assumption is based on the similarities with the spectrum of 2-(*N*-pyrrolidyl)indene shown in Fig. 2. The peaks from the protons at the 1- and 3-position in the indenyl part appear at $\delta = 6.7$ (ppm) (H_C) and $\delta = 3.7$ (ppm) (H_B), respectively, and the complex signals at $\delta \approx 6.7-7.7$ (ppm) are assigned to the eight aromatic protons. If the solution of (II) contains traces of acidic impurities, the PMR spectrum rapidly changes to that given in Fig. 3. In this spectrum the peaks at $\delta = 4.6$ and 5.3 (ppm) are interpreted as resulting from the protons H_E and H_D, respectively, in the isomeric form (III). The signals from the remaining protons in (III) are as expected overlapped by the signals from (II). As no changes in the spectrum (Fig. 3) was observed after addition of *p*-toluenesulphonic acid, the equilibrium ratio between (II) and (III), found to be 9:1, could be determined by integration. The area under the peak at $\delta = 3.7$ (ppm) due to H_B in (II) and H_G in (III) was compared with the area of the H_D and H_E peaks in (III) using the pyrrolidine proton signals at $\delta \approx 1.6-2.0$ (ppm) as internal standard. The equilibrium ratio is equal to that reported for pyrrolidyl enamines of 1-phenylindan-2-one.[12]

An interesting observation from this spectrum of (II) is the lowfield signal assigned to the terminal vinylic proton (H_C) with a shift value ($\delta = 6.7$ (ppm)) close to that ascribed to the olefinic proton in the 3-position of indenes ($\delta \approx 6.4-6.7$ (ppm)).[13,16,17] This fact cannot be in accordance with any significant increase in electron density at the carbon atom bearing the vinylic proton in our dienamine. This proposal is based on earlier reports [1,3] which clearly show that an increased conjugation caused a marked upfield shift of the signal from the terminal vinylic proton of the dienamine. Thus the PMR spectrum shows much greater likeness to a superposition of an enamine and an indene than a dienamine. This together with above-mentioned isomeric ratio and the non-coplanarity proposed for enamines of 1-phenylindan-2-one [12] suggests that the indene systems are twisted out of conjugation.

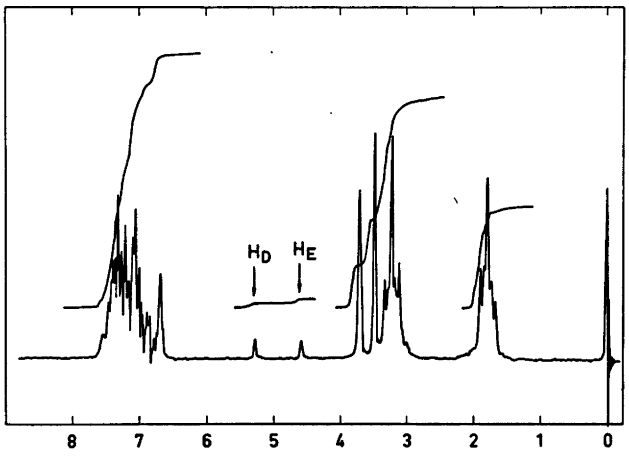
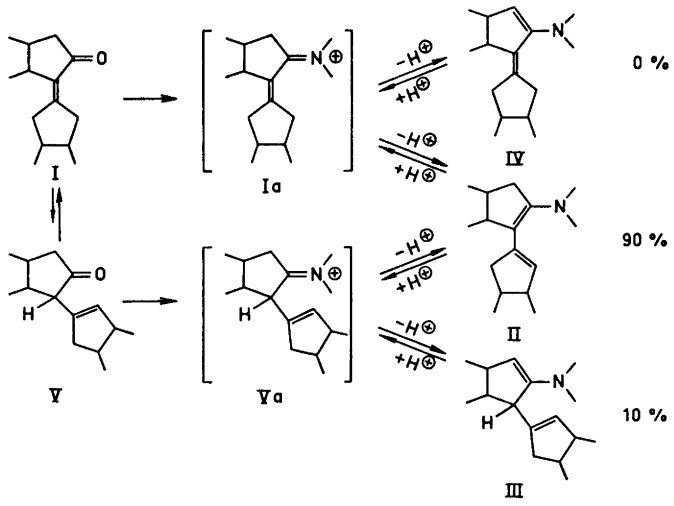


Fig. 3. PMR-spectrum of a mixture of (II) and (III) (9:1) (Concentration: 0.5 M) in chloroform-D. A catalytic amount of *p*-toluenesulphonic acid is added to confirm the equilibrium proportions (II/III = 9:1).



Scheme 4.

In order to confirm the assumption that the enamine part remains isolated from the indenyl substituent we have studied isotopic exchange between the equilibrium mixture (II + III) and methanol-OD, since deuterium incorporation rates have been shown to correlate well with estimated electron densities at different sites.[14] These results are summarized in Table 1, which shows that there is a fast exchange in the enamine part as compared to the indenyl part when chloroform was used as solvent. The decreased rate observed in pyridine could possibly be explained by hydrogen bonding between methanol-OD and pyridine. Even a small but significant deuterium content is observed in the indenyl substituent. This could be caused by involvement of an enimmonium structure like (I a, Scheme 4) but it can more likely be explained in terms of a simple base-catalyzed isotopic exchange as earlier reported.[15] To summarize, these results strongly indicate a decreased coplanarity and a decreased orbital interaction between the nitrogen lone pair and the indenyl substituent in agreement with our proposal.

Table 1. Protium-deuterium exchange between 3-(2-indenyl)-2-(*N*-pyrrolidyl)-indene (II) and 1-(2-indenyl)-2-(*N*-pyrrolidyl)indene (III) and methanol-OD at 27°C. Molar ratio: Amines: methanol-OD (1:4) Concentration of amines: 0.32 M.

Solvent	Time (h)	Percentage deuterium [a] in the enamine part (A,D,E)	Percentage deuterium [a] in the indenyl part (B,C,G,F)
Chloroform	0.5	18	0
	1.0	30	0
	2.0	50	0
	4.5	63	0
Pyridine	4.0	10	0
	27.0	33	5
	45.0	48	10
	64.0	50	13

[a] Estimated error ±4 %.

Consideration of the route of formation of the isomeric mixture (II + III) indicates that at least two different pathways are plausible (Scheme 4). One possibility is a direct formation of the highly sterically strained enimmonium structure (I a) followed by proton abstraction theoretically yielding two different structures (II + IV). The linear form (II) is thermodynamically much more stable than (IV). The observed equilibrium between (II) and (III) is then established through structure (V a) in accordance with the above-mentioned results from hydrogen-deuterium exchange. Another plausible pathway is a base-catalyzed inter-

conversion of the cisoid unsaturated ketone (I) to an indenyl-substituted 2-indanone (V). Subsequent reaction with pyrrolidine, in analogy with the preparation of alkyl-substituted enamines of 2-indanones, gives a less sterically restricted intermediate (V a) compared with (I a). Whether one or both of these mechanisms is operative cannot be clarified. No trace of formation of the isomer (IV) could be detected during the course of the reaction. This fact can be explained either by assuming that the mechanism proceeding *via* (I a) does not operate, or that (IV) possibly formed too rapidly isomerizes to structure (II) in order to be detected. Referring to the very high reactivity of 2-indanone and the alkylsubstituted 2-indanones it seems plausible to exclude the path *via* (I a).

The ratio between the obtained isomeric compounds (II + III) is quite different from that expected from earlier investigations of cisoid unsaturated ketones. In these studies the cross-conjugated isomer was, as mentioned, the predominating product. The factors, which affect the isomeric proportions in our case, seem to be the same as in the case of enamines of substituted 2-indanones. The unusually large proportion of the most substituted isomer, that was obtained when pyrrolidine was used as amine component, could possibly be caused by differently weighted steric effects in our enamine systems compared to the conditions in enamines of other substituted ketones. An extension of conjugation by the aromatic part and an increased difference in hyperconjugative stabilization between the 1-substituted and 3-substituted forms in our systems are factors that also could be responsible for this unexpected effect. However, these differences in isomeric proportions from those expected, on the basis of the now widely accepted rule of Gurowitz and Joseph,[7] correspond to very small differences in free energy of the different structures. This means, that any closer treatment of these factors is very difficult.

EXPERIMENTAL

A JEOL C-60 HL instrument was used to obtain the PMR spectra. The mass spectrum was recorded on a LKB 9000 mass spectrometer, and the IR spectrum was determined on a Perkin-Elmer 257 Spectrophotometer. Melting points are uncorrected.

1-(2-Indanylidene)indan-2-one (I) was prepared from 2-indanone according to a method published by Treibs and Schroth.[9] m.p. 176.5 – 178°C (lit.[9] 178°).

3-(2-Indenyl)-2-(N-pyrrolidyl)indene (II). A mixture of 2.0 g 1-(2-indanylidene)indan-2-one (0.007 mol) and 1.07 g pyrrolidine *p.a.* (0.015 mol) dissolved in 20 ml chloroform was allowed to stand for two days at room temperature over anhydrous calcium sulphate. Evaporation in vacuum affords a crude mixture mainly consisting of (II) and (III) approximately in a ratio 9:1. Recrystallization from tetrachloroethylene yields pure (II). The yield was 1.28 g. (52 %) m.p. 140 – 142°C. (Found: C 88.2; H 7.0; N 4.7. Calc. for $C_{22}H_{21}N$ (299): C 88.3; H 7.1; N 4.7.) $M^{+\cdot}$ (*m/e*): 299. $\nu_{C=C}$ (KBr): 1600, 1580 sh, 1550 (cm^{-1}).

PMR spectrum (Fig. 1, Fig. 3). Isomer (II): 1.6 – 2.0 (4 H complex), 3.0 – 3.4 (4 H complex), 3.5 (2 H_A singlet), 3.7 (2 H_B singlet), 5.7 (1 H_C singlet), 6.7 – 7.7 (8 H complex).

Isomer (III): 4.6 (1 H_E singlet), 5.3 (1 H_D singlet), (δ (ppm) in chloroform-D (TMS). Conc. 0.5 M).

Protium-deuterium exchange. Pyridine p.a. (Mallinckrodt) was freshly distilled and dried over calcium hydride. Chloroform *p.a.* was purified from alcohol. Methanol-OD (CIBA) was > 99 % D. The isotopic incorporation was followed by PMR-integration with sealed NMR tubes. Further data is given in Table 1.

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The Binding of Quinacrine Mustard to Nucleic Acids

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Quinacrine mustard forms complexes with nucleic acids in solution, as indicated by the yellow precipitation forming when native DNA and high concentrations of quinacrine mustard are mixed. This complex shows different biophysical properties as compared to the nucleic acid alone in solution. The viscosity is elevated, T_m is shifted towards higher temperatures, and the sedimentation constant is reduced. The polymer changes the fluorescence intensity of the dye: a net quench is obtained with all natural DNAs. The bonds between the dye and DNA depend on the pH and ionic strength in the solution. The binding between the dye and nucleic acids is also affected by the strandedness of the polymer. A greater quenching of the fluorescence intensity of quinacrine mustard is obtained with single stranded molecules than with double stranded ones. In a methyl green-DNA complex the methyl green is displaced by quinacrine mustard. This probably reflects competition between the two dyes.

The basic acridine dyes are able to bind nucleic acids in different ways. They are all capable of ionic binding to the phosphate groups in the nucleic acids with or without intercalation.[1-4]

The mode of interaction between acridine dyes and nucleic acid polymers has been studied by Dourlent and Hélène [5] with the dye proflavine. They found two bond species: a cooperative binding along the phosphate backbone and an interaction between the aromatic rings of the dye and nucleic acid bases, *i.e.* intercalation. These two processes are linked together. Further, the intercalation process is closely correlated to the secondary structure of the polymer. At low ionic strength and low concentration ratios of nucleic acid to proflavine the binding is an electrostatic interaction between the positively charged dye and the negatively charged phosphate groups. At high concentration ratios of nucleic acid to dye, a new complex is formed which suggests a van der Waals type of interaction between proflavine and the bases of the nucleic acid. Two models have been proposed for this second type of binding: the complete model of Lerman [6] and the partial intercalation model of Pritchard.[7] In the first model the dye is spaced between two bases in the opposite strands of the DNA helix. In the partial intercalation model the dye is spaced between two bases in the same strand of the nucleic acid. A complex formation between acridine dyes and single stranded nucleic acids provides evidence in support of the latter model.

Caspersson *et al.*[4] introduced the use of quinacrine and quinacrine mustard for staining cytological preparations. They chose quinacrine mustard because of the assumed guanine specificity of the dye. However, Ellison and Barr,[8] Weisblum and de Haseth,[9,10] Pachmann and Rigler,[11] and Michelson *et al.*[12] all showed that quinacrine and quinacrine mustard preferably stain chromosome regions rich in AT base pairs. Alternatively, proteins associated with (A+T) rich DNA might contribute to strong fluorescence, although this is hardly the case when purified DNAs or nucleotides in solution are tested.

The aim of the present investigation was to study the characteristics of the binding between quinacrine mustard and natural nucleic acids. Changes caused by the dye in some of the biophysical properties of DNA are reported. The results are discussed with reference to findings reported by other investigators using related dyes which also interact with nucleic acids.

EXPERIMENTAL

DNAs. DNA from mouse liver, calf thymus and *Saccharomyces cerevisiae* was isolated according to the method described by Marmur.[13]

The absorbance of 1 mg/ml of DNA from mouse liver and calf thymus was 21 at 257.5 nm in 0.1 × SSC * solution and of DNA isolated from *S. cerevisiae* 25.4 at 260.0 nm. This DNA was denatured, *i.e.* in single stranded form. The (G+C) content was 40 % for the DNA from mouse liver and calf thymus, and 43 % for the DNA isolated from *S. cerevisiae*. Phosphorus analysis gave 11.7 % phosphorus in mouse liver, 10.0 % in calf thymus, and 11.2 % in *S. cerevisiae* DNA. The absorbance of the sodium salt of highly polymerized calf thymus DNA (G+C, 45 %), purchased from the Sigma Chemical, U.S.A, was 21 at 260.0 nm, and the phosphorus content was 11.9 % (0.1 × SSC solution).

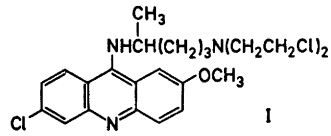
Deoxyribonucleic acid from *Escherichia coli*, (G+C, 50 %) and from *Clostridium perfringens* (G+C, 31 %) were purchased from the Sigma Chemical, U.S.A.

Denaturation was achieved by heating DNA (approx. 100 μg/ml in 0.1 × SSC solution) at 100°C for 10 min and then rapidly cooling it to 0°C. The change in absorption was measured spectrophotometrically and the temperature which produced 50 % of the total hyperchromicity resulting from the denaturation was taken to be T_m.

Native and denatured DNA (approx. 1 mg/ml) could be stored for at least 6 months at −70°C without degradation. The DNA concentration was determined either at the absorption peak at 260.0 nm or with the diphenylamine reaction.[14] The phosphorus analysis was as described by Tuan and Bonner.[15] The phosphorus content for the DNAs as well as the RNAs (see below) was rather high, indicating some contamination during the preparation of the nucleic acids. Deoxyribonuclease I from bovine pancreas was purchased from the Sigma Chemical, U.S.A.

RNAs. RNA from mouse liver and *S. cerevisiae* was isolated according to the method described by Kirby.[16] Stock solutions (approx. 1 mg/ml) were prepared in 0.1 × SSC solution and could be stored for 6 months at −25°C without degradation. The absorbance of mouse liver RNA at 260.0 nm was 20 and that of *S. cerevisiae* RNA 24.9. The (G+C) content of mouse liver RNA was 40 %. Phosphorus analysis gave 15.2 % phosphorus in mouse liver and 14.2 % in *S. cerevisiae* RNA. The RNA concentration was determined either at the absorption peak at 260.0 nm or with the orcinol reaction.[17]

Quinacrine mustard. Quinacrine mustard * (I) was a gift from the Sterling-Winthrop Research Institute, U.S.A. The fluorescence and absorption spectra and some other characteristics of the dye (anion and pH dependence) have been previously reported.[18] Stock solution of QM (2 mg/ml) in distilled water was stable for two weeks at 4°C in the dark. The solution remained clear the whole time without any changes in the absorption or fluorescence properties.



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Fluorescence intensity. A fluorescence attachment ZFM4 to a Zeiss (PMQ II) spectrophotometer equipped with an excitation filter at 436 nm was used for determining the fluorescence intensity of the solution. The spectrophotometer was standardized with a fluorescence standard cuvette F 53 with excitation at 436 nm and with a fluorescence peak at 530 nm and a No. 10 slit arrangement.

The nucleic acids did not interfere with the QM fluorescence at 514 nm.[19] The theory and method of fluorimetric titration have been described by LePecq and Paoletti.[20] The binding parameters could be calculated by measuring the alteration of the fluorescence intensity of the dye under constant conditions of excitation, temperature, solvent composition and concentration. It was established that the fluorescence intensity alterations which occurred after mixing the polymer and the dye were complete within a few seconds and showed no further changes with time or additional mixing.

Absorption studies. Absorption spectra were recorded with a Zeiss (PMQ II) spectrophotometer. The absorption of the polymer did not disturb the determination of QM at 424.0 nm. The theory which was applied to the analysis of QM binding experiments has been described by Peacocke and Skerrett.[21] The association constants and the number of binding sites of the dye can be directly determined from the titration data with the Scatchard equation.[22] Although the curves obtained from the titration experiments with QM and polymers of unknown length deviate from the theoretical curves of Scatchard, his equation has been used in many investigations to determine the number of dye molecules bound to polymers.[5,23–26]

RESULTS

Effect of pH on QM. The effect of pH over the range 2 to 12 (in 0.015 M NaCl) on the ratio of the absorption at 424 nm and 365 nm of unbound QM is shown in Fig. 1. At pH ≥ 9.7 only the nitrogen atom of the diethylamine group of the side chain is protonated, but at pH ≤ 7 the dye is doubly protonated. The increase in the 424 nm to 365 nm ratio between the pH values 9.7 and 7 corresponds to a decrease in the fluorescence intensity of the dye.[18] The corre-

* Abbreviations: 0.1 × SSC, 0.015 M NaCl-0.0015 M sodium citrate, pH 7. QM, quinacrine mustard.

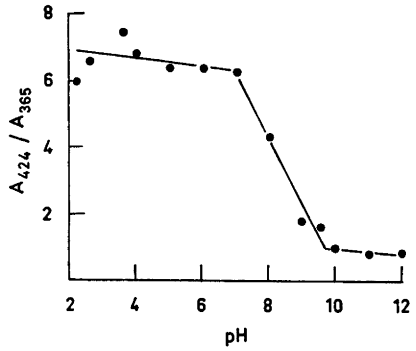

Fig. 1. Effect of pH on QM. Determinations were made in 0.015 M NaCl and the concentration of QM was 156 μM. pH was plotted against the changes in the ratio of absorption at 424 nm and 365 nm.

sponding pK_a values for quinacrine are 10.3 and 7.7, respectively.[27]

Interaction between QM and DNA. The reaction between QM and native DNA was indicated by the precipitation of yellow fibres brought about by the mixing of a solution containing a high concentration of the dye. At lower concentrations of the dye no precipitation occurred. In all the experiments reported below, concentration ranges of polymer and dye were chosen so that precipitation of the complex did not occur.

Effect of DNA on the fluorescence and absorption spectra of QM. In a QM-DNA mixture, quenching of the fluorescence intensity of the dye was noted at 514 nm, but no shift was found in the fluorescence spectrum of QM. However, DNA alters the absorption spectrum of QM as shown in Fig. 2. Increasing amounts of native mouse liver DNA were added to a solution with a constant amount of QM. The shift of the absorption maximum from 424 nm to higher wavelengths and the presence of an isosbestic point at 455 nm indicate the formation of specific complexes of the dye with DNA.[5] The ratio of the absorption at 424 nm and 365 nm decreases as the concentration of DNA increases. At very high concentration ratios of polymer to dye, there is a marked change in the absorption spectrum of QM (Fig. 2).

Fig. 3 depicts the difference spectra of QM in mixtures with native mouse liver DNA and with mouse liver RNA in 0.1 × SSC solution.

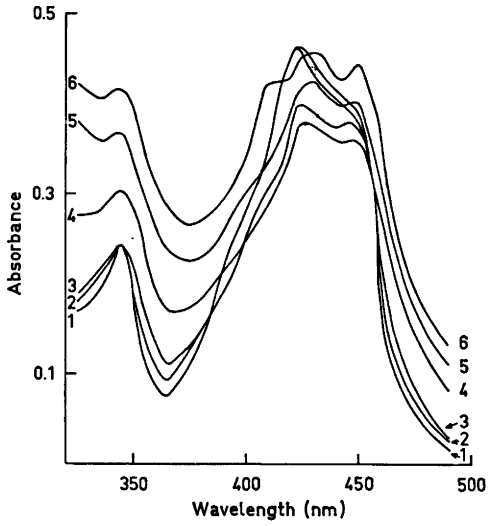

Fig. 2. Absorption spectra of QM partially and completely bound to native DNA. Determinations were made in McIlvaine's buffer, pH 7,[28] and the concentration of QM was (1) 50 μM. Concentrations of native mouse liver DNA phosphorus were: (2) 0.012 mM; (3) 0.129 mM; (4) 0.523 mM; (5) 0.928 mM and (6) 1.230 mM.

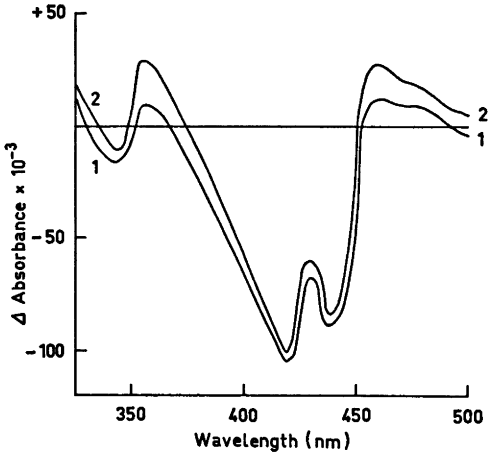

Fig. 3. Difference spectra of QM produced by nucleic acids. Determinations were made in 0.1 × SSC solution, pH 7, with 12.6 μM QM. (1) 0.535 mM RNA phosphorus, and (2) 0.464 mM double stranded DNA phosphorus. Both RNA and DNA were isolated from mouse liver (*cf.* Experimental).

Both nucleic acids depress the absorbance at 424 nm, although there were differences between double and single stranded nucleic acids.

Non-equilibrium dialysis. Since this was not an equilibrium dialysis, the possible dissociation of bound QM as the free QM concentration approached 0 would cause the fluorescence estimation at 514 nm to be too low. Mixtures of 0.5 mg/ml native calf thymus DNA with 0.05 mg/ml QM in 0.05 M Tris solution, pH 7.5, were dialyzed for 50 h at 0°C in viscose cellophane bags. The dialysis solution was changed frequently. After dialysis, the concentration of calf thymus DNA in the dialysis bag was 0.46 mg/ml and that of QM was 0.018 mg/ml which gave n 0.44. Native mouse liver DNA and QM, dialyzed in McIlvaine's buffer at pH 7.0, showed that 0.322 mg/ml DNA bound 0.014 mg/ml QM, *i.e.* n 0.45. The absorption of QM by the dialysis bag was slight as was the loss of nucleic acid to the external solution.

Effect of urea. Native mouse liver DNA (0.032 mg) was mixed with 0.02 mg QM in 0.1 × SSC solution with 3 M urea and 0.025 M sodium sulphate. In a solution without urea the quenching with excess DNA was $-22.8\ \Delta$ %, and with 3 M urea $-10.6\ \Delta$ %, *i.e.* a 50 % enhancement of the fluorescence intensity of QM in the presence of urea. [Δ % is the change of the fluorescence intensity of the mixture (in per cent) relative to the fluorescence intensity of QM alone.] The QM-DNA complex was unstable in urea, *i.e.* also some hydrogen bonds might be formed when QM reacts with DNA. The interaction between chloroquine as well as quinacrine and nucleic acids is stable in urea [29] while actinomycin D forms hydrogen bonds with DNA.[30]

Effect of pH on QM-DNA mixtures. The spectrum of bound QM varies considerably within the pH range examined (Fig. 4). Since the protonation of QM is largely unaffected at pH values below 7.0 (*cf.* Fig. 1) the pH-induced changes seen in acidic solutions must result from protonation in the nucleic acid. The ratio of the absorption at 424 nm and 365 nm of QM-calf thymus DNA (double stranded) shows a rapid decline at pH ≦ 3 and at pH ≧ 6 (Fig. 4). The single stranded molecules show a more irregular dependence on pH. The protonation of DNA begins at pH 3.5 – 4.[31] Adenine and cytosine protonate first (pK_a values between 5

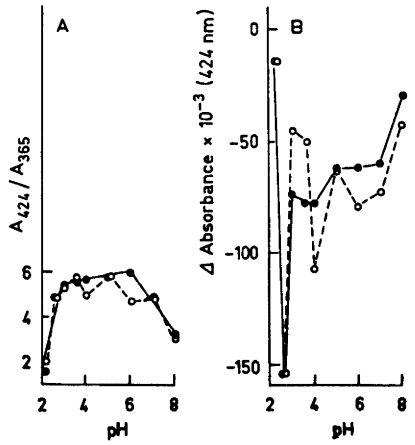

Fig. 4. Effect of pH on QM bound to nucleic acids. The determinations were made with 50 μM QM in McIlvaine's buffer. A: pH was plotted against the changes in the ratio of the absorption at 424 nm and 365 nm; B: pH was plotted against the difference in the absorption at 424 nm. (●) double stranded and (○) single stranded calf thymus NaDNA (Sigma). Concentration of DNA phosphorus was 0.129 mM.

and 4) followed by guanine ($pK_a < 3$), which is almost fully protonated at pH ≦ 2.4. The decline in the 424 nm to 365 nm ratio at very acid pH is followed by an enhancement of the fluores-

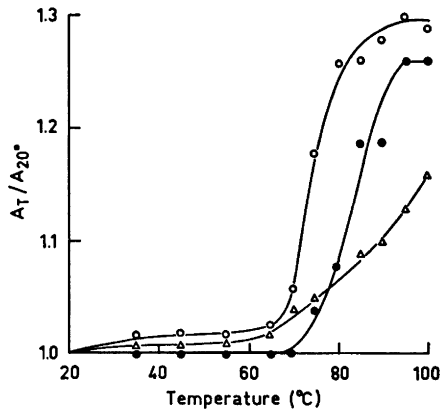

Fig. 5. Effect of QM on thermal denaturation of calf thymus DNA. Determinations were made in 0.05 M Tris solution, pH 7.5. Concentration of calf thymus NaDNA (Sigma) alone was, (○) 0.935 mM DNA phosphorus. (●) was dialyzed complex of 35 μM QM and 3.44 mM DNA phosphorus and, (△) was a mixture of 15 μM QM and 0.925 mM calf thymus DNA phosphorus.

cence at 514 nm and an enhancement of the absorption at 424 nm (Fig. 4). The absorption measurements at low pH were made within 10 min, so that possible depurination due to the acidic solution was negligible.[31] The denaturation of DNA at pH below 3 leads to a change in the binding of the dye through intercalation which leads to an increased binding of QM to the phosphate groups.[5] Since the spectrum of unbound QM varies within the pH range 7 to 8 (Fig. 1) it can be inferred that the changes found at pH above 6 for the bound ligand only depict the pH dependence of the unbound dye. Furthermore the denaturation of DNA at high pH occurs at pH above 11.[20]

Thermal denaturation of QM-DNA mixtures. The interaction between QM and native DNA is further demonstrated by the stabilizing effect of QM on DNA against denaturation by heat, as shown in Fig. 5. The T_m for (Sigma) calf thymus DNA was 73°C and the hyperchromicity 30 %. For a QM-DNA mixture (DNA-P/QM = 100) the T_m value was 83°C and the hyperchromicity 26 %, *i.e.* an increase in T_m of 10 degrees. When the proportion of QM in the mixture was raised (DNA-P/QM = 62), the T_m was increased to 90°C and the hyperchromicity reduced to 16 %. From these data it is evident that an increased stability of DNA was obtained with increased concentration ratios of QM to DNA. According to Cohen and Yielding[24] the T_m raises 9 degrees at a concentration ratio of chloroquine to DNA equal to 1:10. When the concentration ratios of the dye to DNA approaches 0, the T_m for the dye-DNA complex approaches the T_m for native DNA alone.

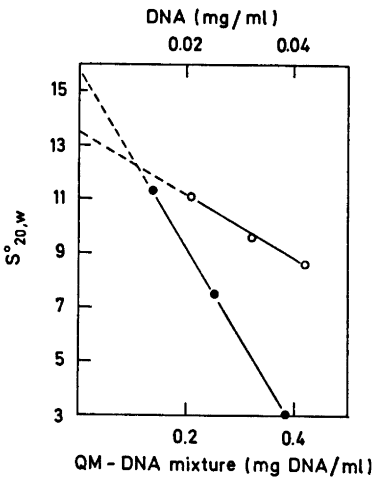

Fig. 6. Sedimentation constant of DNA and of QM-DNA mixtures. (●) DNA alone expressed in mg/ml, and (○) QM-DNA mixtures expressed in mg DNA/ml with 26 μM QM.

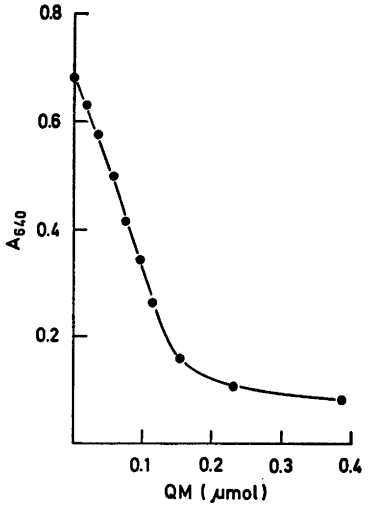

Fig. 7. Effect of QM on the methyl green-DNA complex. Increasing amounts of QM were added to 0.05 M Tris solution, pH 7.5, containing 33 mmol native mouse liver DNA phosphorus and 25 μmol methyl green. The mixtures were then allowed to stand for 18 h at room temperature (in the dark) before the absorption at 640 nm was estimated.

Analytical centrifugation. Analytical ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge equipped with an RTIC unit, a monochromator and a split beam photoelectric scanner as an accessory. The sedimentation of native mouse liver DNA was observed at 260 nm at 20°C and that of QM-DNA mixtures at 424 nm in 0.1 × SSC solution, pH 7. A 12 mm double sector cell with sapphire windows was used in the An-D-rotor at 42040 rpm. The sedimentation coefficients ($S_{20,w}^\circ$) were calculated from a plot of log *r versus* time and was corrected to the values in a solvent with the viscosity of water at 20°C. In Fig. 6 the sedimentation constant is plotted against the concentration of DNA. This extrapolates to a sedimentation constant of 13.5 S for the QM-DNA complex at zero concentration and to 15.7 S for native mouse liver DNA

alone, *i.e.* a decrease of approximately 14 % in the sedimentation constant. These results agree with those of Lerman [32] and Kurnick and Radcliffe [33] for the quinacrine-DNA complex. The sedimentation constants obtained were 12.4 S for DNA alone and 9.3 S for the quinacrine-DNA complex at zero concentration.[33] The decrease in the sedimentation constant suggests that the QM-DNA complex is more asymmetric than DNA alone.[32]

Displacement of methyl green. The effect of the addition of QM to the methyl green-DNA substrate [34] is presented in Fig. 7. At pH 7.5 free methyl green was converted to leukobase, with a resultant fall in the absorbance at absorption maximum of the methyl green-DNA complex (640 nm).[35] As the QM concentration was increased there was a progressive displacement of methyl green, although complete displacement did not occur. It is possible that the dye methyl green interferes with the approach of QM to the DNA molecule by preventing the intercalation of QM.

Inhibition of the DNase activity by QM. The methyl green-DNA substrate [34] could not be used in this study because of the progressive displacement of the dye from the complex when the concentration of QM was increased (cf. above) DNase assays were therefore performed by viscosimetry. Ostwald Fenske viscosity pipettes were used at 25°C. The pipettes were calibrated with water and gave an outflow time (with water) of 35 s at 25°C.

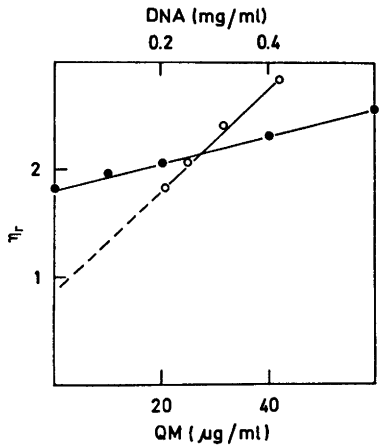

Fig. 8. Effect of QM on the viscosity of DNA. The results are presented as the relative viscosity (η_r), *i.e.* the outflow time of the solution divided by the outflow time of water. (O) increasing amounts of native mouse liver DNA (mg/ml), (●) 0.2 mg/ml native mouse liver DNA with increasing amounts of QM (μg/ml). The solution was 0.1 × SSC, pH 7, and each point represents the mean of three determinations.

In Fig. 8 the relative viscosities are plotted against the concentration of QM for the QM-DNA mixtures. The relative viscosities of DNA solutions are also shown in the figure. It was found that as the amount of QM was increased, the relative viscosity also increased up to 60 μg QM/ml, *i.e.* 0.3 mg dye per mg DNA. Kurnick and Radcliffe [33] reported a similar rise in the relative viscosity of a quinacrine-DNA mixture, with the maximum at 0.4 mg quinacrine per mg DNA.

In a series of assays with a DNase concentration of 20 μg/ml, 0.2 mg/ml native mouse liver DNA and varying amounts of QM in 0.1 × SSC solution, progressive reduction in the enzyme activity was observed as the relative concentration of QM increased. After a solution with 0.2 mg QM per mg DNA had been incubated at 37°C for 3 h the relative viscosity of DNA alone decreased by 17 %, while that of the QM-DNA mixture decreased by 12 %. The corresponding values for a solution with 0.1 mg QM per mg DNA was 40 % for DNA alone, and 37 % for the QM-DNA mixture (incubated for 6 h). The inhibition is obviously dependent on the concentration ratios of dye to DNA. A similar inhibition of DNase activity with quinacrine and a complete inhibition of the enzyme activity with chloroquine has been reported.[29,33]

Calculation of binding parameters. When increasing amounts of QM were added to a constant amount of polymer, the fluorescence intensity of the bound dye was quenched (Fig. 9). It is evident that those binding sites resulting in an increase of the quantum yield are occupied first, and that further additions of the dye result in reduction of the fluorescence enhancement. The reverse is illustrated in Fig. 10, where the effect of increasing amounts of native DNA and constant amounts of dye is shown. In Fig. 9 the greatest enhancement of the fluorescence intensity of the dye was obtained with native mouse liver DNA, while the curve of denatured (single stranded) DNA is about the same as

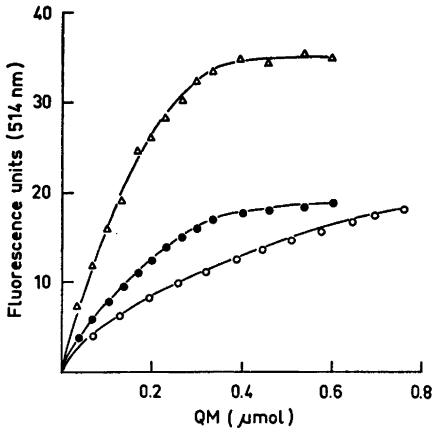

Fig. 9. Fluorescence enhancement of QM bound to nucleic acids. (△) to constant amount of native mouse liver DNA (3.77 mmol DNA phosphorus), (●) constant amount of denatured (single stranded) mouse liver DNA (3.77 mmol DNA phosphorus) and (○) constant amount of mouse liver RNA (4.91 mmol RNA phosphorus) was added increasing amounts of QM (μmol) in McIlvaine's buffer, pH 7.

that of mouse liver RNA. This quenching agrees with that of the DNAs of quinacrine and QM fluorescence intensity reported by others.[9-12]

The titration of DNAs by the fluorimetric method leads to anomalous binding curves which deviate from linearity in the Scatchard plot. Accordingly, the correct Scatchard plot is a line in which the slope of the first part of the

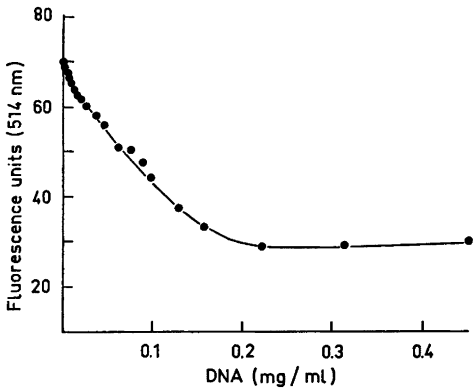

Fig. 10. Effect of DNA on the fluorescence intensity of QM. To a constant amount of QM (71.5 μmol) was added increasing amounts of native mouse liver DNA (mg/ml) in 0.1 × SSC solution, pH 7.

Table 1. Binding of QM to nucleic acids.[a]

Nucleic acid	Buffer system	Binding parameters n_1	n_2
DNA			
native mouse liver	0.1 × SSC, pH 7	0.17	1.11
denatured mouse liver	0.1 × SSC, pH 7	0.13	0.53
denatured *S. cerevisiae*	McIlvaine, pH 7	0.25	1.10
RNA			
mouse liver	0.1 × SSC, pH 7	0.09	0.19
mouse liver	McIlvaine, pH 7	0.31	0.50
S. cerevisiae	McIlvaine, pH 7	0.17	0.37

[a] Fluorimetric titration was used to estimate the parameter n, which was calculated according to the equation of Scatchard.[22] Each n value represents the mean of three determinations.

curve is negative. The line for titration of DNAs with QM does not conform with the hypothesis of independent binding sites because it is not a straight line. However, the intercept with the r axis (r is the molar ratio of bound dye to DNA phosphorus) can be determined, *i.e.* the value of n (the apparent number of binding sites). These values are given in Table 1. The value of n_1 for native DNA was 0.17 and that of n_2 was 1.11. The high value of n_2 might depend on the high phosphorus content in mouse liver DNA (*cf.* Experimental). For chloroquine-nucleic acid mixtures n values were between 0.15 and 0.75.[24] The n values for native DNA determined by fluorimetric titration agree well with those obtained by dialysis (*cf.* above) and by analytical ultracentrifugation (0.75).

Precipitation of QM-DNA complexes. When a QM-nucleic acid complex is precipitated from a mixture containing an excess of both QM and nucleic acid the value of n increases. After the precipitation, n for native calf thymus DNA was 0.81 and for denatured calf thymus DNA, 0.66. These high n values were partly caused by the high concentration of salt in the solution during precipitation of the yellow QM-nucleic acid complex with alcohol. This high salt concentration may cause aggregation of the dye molecules.[36]

Comparison of different nucleic acids. The

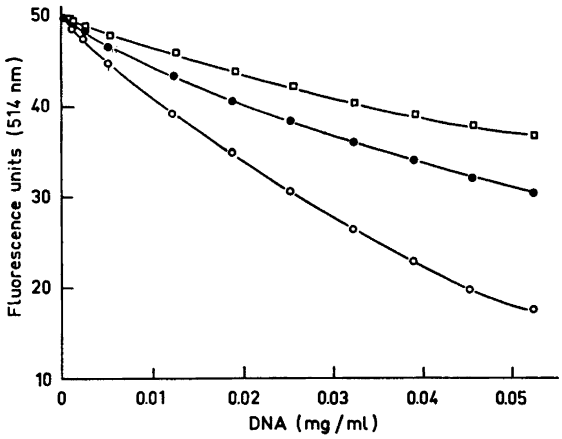

Fig. 11. Effect of different DNAs on the fluorescence intensity of QM. To a constant amount of QM (26 μmol) was added increasing amounts of native DNAs (mg/ml) in 0.1 × SSC solution, pH 7. DNAs: (□) *C. perfringens* (G+C, 31 %); (●) mouse liver (G+C, 40 %), and (○) *E. coli* (G+C, 50 %).

fluorimetric titration curves for different DNAs are shown in Fig. 11. The figure depicts the correlation between the (G+C) content of the DNA tested and the quenching effect on the fluorescence intensity of QM. It can be seen that the higher is the (G+C) content, the more readily the DNA quenches the fluorescence intensity of the dye. This finding is in line with the results reported by Weisblum and de Haseth [9,10] and Pachmann and Rigler [11] for the effect of DNAs on the fluorescence intensity of quinacrine. Polynucleotides behave in a similar way.[9,10,12,18]

The same relation between (G+C) content and quenching is also shown with denatured DNAs. The quenching of the fluorescence intensity of QM with excess denatured calf thymus or mouse liver DNA (G+C, 40 %) was approx. $-50\ \Delta$ % and for en equal amount of denatured *E. coli* DNA (G+C, 50 %), $-62\ \Delta$ %.

Interaction between QM and single stranded nucleic acids. Mixtures of either single stranded DNA or RNA with QM do not give rise to yellow precipitates at any of the concentration ranges of polymer and dye. However, there is an interaction between QM and RNA as is shown by changes in the absorption spectrum of QM when RNA is added to the solution (Fig. 3). This leads to quenching of the fluorescence intensity of QM. The fluorimetric titration with a constant amount of mouse liver RNA and increasing amounts of QM is shown in Fig. 9. The enhancement of the fluorescence intensity of the dye with RNA was about the same as with denatured DNA.

The n values for QM-RNA complexes are summarized in Table 1. They lie between 0.09 and 0.31 for n_1 and between 0.19 and 0.50 for n_2 depending on the ionic strength and pH of the solution. The different n values obtained for RNAs might also be due to the extremely high phosphorus content in the RNAs (*cf.* Experimental). Non-equilibrium dialysis of a mixture containing 0.5 mg/ml mouse liver RNA and 0.05 mg/ml QM in McIlvaine's buffer, gave n 0.27.

The effect of increasing amounts of the dye and a constant amount of denatured mouse liver DNA is also shown in Fig. 9. The enhancement of the fluorescence intensity of the dye was slighter with denatured DNA than with native DNA. A similar effect of the strandedness of the polymer on the binding of acridines has also been reported earlier.[6,24,33,37,38] Fluorimetric titration of denatured DNA (from mouse liver and from *S. cerevisiae*) with QM gave n_1 between 0.13 and 0.25, and n_2 between 0.53 and 1.10 (Table 1).

DISCUSSION

Binding mechanisms. Two different processes are involved in the interaction between dyes and polymers. The first binding (process I) is a weak ionic interaction between positively labelled dye molecules and negatively labelled groups of the polymer. Being an electrostatic interaction, the binding depends on the ionic strength in the solution, *i.e.* increased ionic strength leads to a decrease in process I. At high dye concentrations the absorption maximum of the dye is shifted to higher wavelengths. The isosbestic point suggests the existence of only one kind of binding (process I), *i.e.* the dye molecules are stacked along the phosphate backbone outside the double helix.[5]

The apparent number of binding sites (n) for interaction between QM and DNA in process I are higher than those reported for the interaction between chloroquine and DNA.[24] However, the QM-DNA interaction also shows a dependence on the ionic environment. Thus electrostatic factors are also important in the QM-DNA complex formation.

The second interaction (process II) is a strong binding, where the dye slips into the space between base residues in the helix of the polymer. According to Cohen and Yielding [24] this second type of binding comprises strongly reacting sites associated with bases, *i.e.* intercalation. Although the molecular structure of QM differs from that of chloroquine, the n values for process II were of the same order.[24] Pachmann and Rigler [11] found similar n values for the binding of proflavine and quinacrine with nucleic acids. Thus the side chain at C-9 of the dye does not influence the intercalation (process II) between the dye and the polymer.

Since the interaction between QM and native DNA was affected by urea as shown in this paper, some bindings between QM and DNA are hydrogen bonds. This idea is supported by the observations by Adkisson *et al.*[39] They showed that the bands which are brightly fluorescent after staining of *Drosophila* chromosomes with QM could be removed with acid.

Base-specificity. Several authors have reported base-specificity in the interaction between acridine dyes and nucleic acids.[9–12,40] However, acridines such as acridine orange [9] and 9-aminoacridine [10] show no base-specificity in the interaction with DNA in solution. The base-specificity can be demonstrated in solution with either mono- or polynucleotides containing the base guanine, or with DNAs that differ in the (G+C) content. In two previous papers I presented evidences for base-specificity of QM.[18,41] Quenching of the fluorescence intensity of QM was obtained with polyG, while polyA enhanced the fluorescence intensity of the dye. The relationship between the base content of the DNA and the degree of quenching of the fluorescence intensity of QM is further demonstrated in this paper. The higher the (G+C) content of the DNA the more the nucleic acid quenches the fluorescence intensity of QM in solution. A similar enhancement and quenching of fluorescence is obtained with acridines without a long side chain at C-9 (*e.g.* proflavine.)[9] Thus the side chain at C-9 of QM is not responsible for the base-specific reaction.

Conformational changes of the polymer. When a polymer is mixed with a dye in solution, the polymer undergoes molecular changes. These changes can be seen as altered hydrodynamic properties of the polymer. In the quinacrine-DNA complex, Kurnick and Radcliffe [33] reported an increased viscosity and a reduced sedimentation constant compared to those of native DNA alone. At low dye concentrations, *i.e.* at intercalation, the length of the helix is increased by the dye. At high concentration ratios of dye to polymer, the dye is lined up opposite the phosphate groups, thus increasing the diameter of the helix.

The stabilizing effect of QM on the polymer was shown by increased T_m and increased resistance to DNase depolymerization. The effect on T_m as well as on depolymerization by DNase depends on the concentration ratio of the dye to the polymer. An increased concentration ratio of the dye to polymer increases the protective effect of the dye on the polymer. The protective effect also depends on the salt concentration in the solution. When the salt concentration is increased the protective effect of the dye is reduced.

To stabilize a native nucleic acid against thermal strand separation it is not necessary for the acridine ring to have a side chain at C-9. Ramstein and Leng [40] reported a rise of the T_m for *Micrococcus lysodeikticus* DNA when mixed with proflavine, which does not have any side

chain at C-9. The agent can also be a simple aliphatic compound without any ring structure. Spermine, an aliphatic diaminopentane with two amino groups separated by four carbon atoms, is also active in raising the T_m of nucleic acids.[42] The two exocyclic amino groups in QM are separated by four carbon atoms, hence the dye can be considered a substituted 1,4-diaminopentane (see formula given above). Thus, it is not surprising that QM was as active as spermine in producing an effect on T_m.

Cytological implications. It is not easy to evaluate the relationship between results obtained with QM and nucleic acids in solution on the one hand and cytological fluorescence investigations on the other. There are several fundamental differences between the two systems. Firstly, metaphase chromosomes contain 50–80 % protein.[43] It is already known that proteins modify several of the fluorescent characteristics of QM in solution.[44] Secondly, the concentration of DNA in the interphase nucleus is greater than that normally used in solution.[10] This is apparent *e.g.* from the extremely rapid reassociation of denatured chromosomal DNA *in situ*.[45–47] Thirdly, the procedures used to obtain metaphase chromosome spreads certainly affect the structure and properties of both DNA and proteins.[48,49] Nevertheless, the base-specificity of the fluorescence intensity of QM with natural DNAs and nucleotides in solution is a striking phenomenon, which may well be related to the occurrence of differential brightness of fluorescence along metaphase chromosomes.

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Numerical Resolution of CCD-Curves

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A Fortran program using correct statistical methods for resolution of counter-current distribution (CCD) curves is described. The program has been tested on CCD-curves of the isoenzymes of enolase and lactic dehydrogenase. The results show excellent reproducibility and that CCD on proteins can well be used for analytical purpose.

Partition in aqueous polymer two-phase systems is a method of great usefulness in the study of biological materials.[1] The partition behaviour depends mainly on such properties as charge and size. It is best expressed by the partition coefficient K defined as the concentration of the material in the upper phase/its concentration in the lower phase. If the K-values of two components in a sample differ sufficiently a couple of extractions are enough to achieve a satisfactory separation. However, for complex mixtures consisting of many components a multistage procedure (consisting of a large number of extractions) such as counter-current distribution (CCD) must be used.[1-3] CCD has been successfully used to study complex biological systems such as isoenzymes.[4] The resolution of the distribution curves obtained can be accomplished by calculation of the theoretical curves for the various components and by subsequently fitting a weighted sum of these theoretical curves to the observed data. The use of a computer will considerably simplify this tedious task and, in addition, allow the use of correct statistical methods. Moreover several different curves can be computed for the same case at the same time. By comparing the different theoretical curves with the experimental curve the most likely curve can easily be selected. In this paper we describe a Fortran computer program for resolution of CCD-curves and its application to some CCD-experiments with isoenzyme systems.

MATERIALS AND METHODS

The polymers used were Dextran T 500, batch No. 5996, Pharmacia Fine Chemicals, Uppsala, Sweden, and trimethyl aminopoly(ethylene glycol) (TMA PEG). The TMA PEG [5] was a generous gift from Dr. Göte Johansson. Dextran T 500 had a weight average molecular weight of 518 000 and the molecular weight of TMA PEG was about 6000. Enolase was prepared from baker's yeast by the method of Malmström [6] and was bought under the name Specialjäst from Jästbolaget, Sollentuna, Sweden. Lactic dehydrogenase (LDH) was prepared from pig liver. 500 g fresh pig liver was homogenized in 1 liter 10 mM potassium phosphate buffer, pH 7.3, in a Turmix mixer. The homogenate was centrifuged at 9000 g for 45 min and the supernatant was used as liver extract.

All chemicals used were of analytical grade. The water used was distilled twice in a quartz apparatus.

Enzyme assays. Enzyme activity was measured as change in absorbance at a suitable wavelength. All measurements were carried out at 20°C using a Unicam SP-800 spectrophotometer with tempered 1 cm quartz cuvettes.

Enolase was measured according to the method of Warburg and Christian,[7] and lactic dehydrogenase according to the method of Neilands.[8] No absolute activities were calculated.

Phase system. The phase system used contained 6.6 % dextran, 6.4 % TMA PEG and 5 mM potassium phosphate buffer, pH 6.5. In the experiments with lactate dehydrogenase 50 mM glycine–NaOH buffer pH 8.5 was used.

Counter-current distribution. The CCD experiments were carried out in the thin-layer counter-current distribution apparatus described by Albertsson.[1] Each chamber contained

0.7 ml upper phase and 0.7 ml lower phase. The enzyme mixture to be analysed was dissolved in 1.4 ml phase systems and loaded in chamber 0. Settling time used was 8 min and shaking time 30 s. 60 transfers were completed and the temperature was 22 ± 1°C.

After the CCD, 0.7 ml water was added to each chamber to obtain one phase and each chamber was analysed for enzyme activity.

COMPUTATION

1. Equations. The theoretical concentration in the i:th tube of a component with its maximum in the R:th tube is[2]

$$\eta_i = [60!/i!(60-i)!] \frac{K^i}{(K+1)^{60}} \varepsilon_R = T_{iR}\varepsilon_R \qquad (1)$$

with the partition coefficient K measured by

$$K = (R+0.5)/(60-R+0.5) \qquad (2)$$

Eqns. 1 and 2 are formulated for 60 transfers; in other cases the number 60 is changed appropriately. It must be noted that the model specified by eqns. 1 and 2 applies only to tubes 8 – 53.[2]

In order to completely specify the concentrations (η) of one component in the different tubes (60 in number), it is sufficient to specify the value of the parameter R (the tube number with maximal concentration of the component) and the relative concentration ε_R. Hence the resolution of a CCD curve involves (a) the selection of the number of peaks (N) and thereafter (b) estimation of the 2N parameters (ε_k, R_k, k = 1,2, . . . ,N) in the following eqn. (3) so that the curve fit is optimized.

$$y_i = \sum_{k=1}^{N} T_{iR_k}\varepsilon_k + e_i \qquad (3)$$

The residuals (e_i) describe the deviations between theoretical (η) and observed concentration (y) values. These residuals contain both errors of measurement and "model errors" due to simplifications inherent in the model (eqns. 1 and 2).

2. Criteria of goodness of fit. In the investigation of several CCD curves, it was found that the ordinary least squares criterion (eqn. 4 with all weights $w_i = 1$) was inadequate.

$$U = \sum_{i=1}^{60} e_i^2 w_i \qquad U = \min \qquad (4)$$

This inadequacy was interpreted as due to imperfections in the model for the peak shape (eqns. 1 and 2) in areas far from the peak maximum (R in eqn. 2). To compensate for this nonideal behaviour and, in addition, to decouple the peaks, we have instead used a weighted least squares criterion (eqn. 4) with the weights defined by

$$w_i = 0.1 + \sum_{k=1}^{N} e^{-0.5\Delta_{ki}} \qquad (5)$$

In eqn. 5 the summation is made over the N peaks (with index k) and Δ_{ki} denotes the distance between the i:th tube and the maximum of the k:th peak (R_k). Hence, the weight value for the i:th tube changes as different combinations of the peak maxima are tried in the iterative computational procedure. The weights have the values from 0.1 to *ca.* 1.5 at a peak maximum with two other maxima at the closest allowed distance (two points below and above, respectively).

In the computer program, however, options are provided also for the use of standard criteria, eqn. 4 with $w_i = y_i$ if the user feels that his data are behaving ideally.

3. Optimization strategy. The estimation of the parameters R_k and ε_k, $k = 1, 2, \ldots, N$; is a nonlinear problem corresponding to the minimization of the criterion U in eqn. 4. The problem can be linearized by the specification of the R_k parameters since this makes the weights in eqn. 5 defined as well as the T-values in eqn. 3. The strategy employed in the present computer program is based on this linearization and consists of a systematic variation of the set of R_k-parameters until the minimum of U in eqn. 4 is reached. For each new set U and the corresponding relative activity parameters (ε_k, $k = 1, 2, \ldots, N$) are computed by linear least squares. The following scheme of variation of the R_k-values has been used and found efficient.

1. Specify the number of peaks (N) and the starting values of the peak maxima (R_k, k = 1, 2, . . . , N). This is made at the input.

2. Compute U (eqn. 4) for this set of R_k values.

3. Set j = 1. This is an index which specifies the particular peak location which is currently sub-optimized.

4. Increase R_j with 1. Compute U.

5. Test if this new U is smaller than the last U (in step 2, 5, or 6). If "yes" save the new U-value and go to step 8.

6. Decrease R_j with 2, compute U.

7. Test if this new U is smaller than the last U (in step 2, 5, or 6). If "yes" save the new U-value and go to step 8. If "no" reset R_j to original value (add 1).

8. Increase j with 1. If j larger than N, go to step 9, otherwise to step 4.

9. If any change has been made, that is R_j+1 or R_j-1 has given better fit for at least one j, go back to step 3 and start a new cycle. (If the number of iterations already made is larger than a limit, usually 25, the procedure is terminated).

10. Output. (Number of iterations, parameter values, observed and calculated activity values for i=1 to 60, residuals and corresponding plot).

Some additional restrictions have been found practical. First, the peak maxima are not allowed to get closer than two tubes apart. Secondly, the peaks are not permitted to be closer than two tubes from the edges of the measured data.

The procedures has been programmed in Fortran for the CD3300 computer at the University of Umeå, Sweden. Program listings and a manual are available on request.

RESULTS AND DISCUSSIONS

As an example a comparison between experimental and theoretical curves of a CCD-experiment with enolase from baker's yeast is shown in Fig. 1. Fig. 1a shows the theoretical curve (○), and the experimental curve (□) when two components are assumed in the sample. In Fig. 1b three components are assumed. In Fig. 1a there is a gap between the sum of the two theoretical curves and the experimental curve around tube 32. However, when three components are assumed the two curves fit well except for tubes 49–55 which indicate a fourth component. It has previously been shown that enolase from baker's yeast can be separated into three components when subjected to CCD or electrophoresis.[5,6]

A more complex case to analyse is a CCD-experiment of the LDH isoenzyme system. As is well known LDH from different sources is composed of up to five isoenzymes which can be separated on gel electrophoresis. In a CCD-experiment with 60 transfers the different LDH isoenzymes overlap and make the numerical resolution of the experimental curve very time consuming. By use of the present computor program the calculation of the theoretical curves is simple and the comparison between theoretical curves and experimental curves (assuming different numbers of isoenzyme components) can easily be undertaken. In Fig. 2a a comparison of the experimental curve (□) and the theoretical curves (○) of a CCD-experiment assuming 4 components of LDH in the liver extract shows a good agreement. However, when 5 components of LDH are assumed (Fig. 2b) there is an excellent agreement between the experimental curve (□) and the sum of the theoretical curves (–).

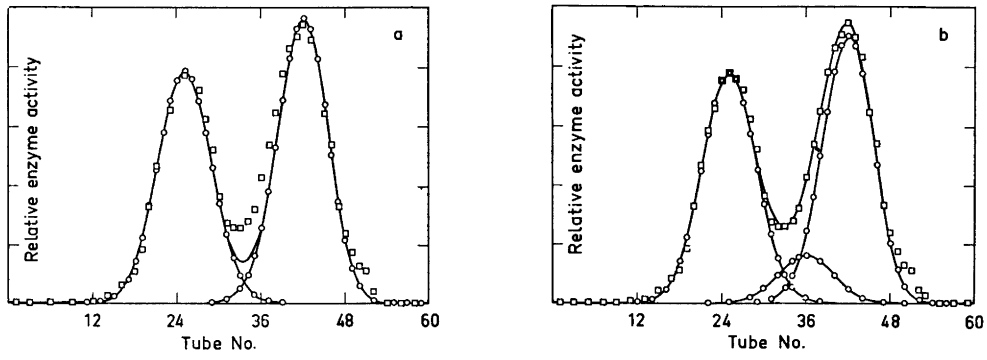

Fig. 1. Counter-current distribution of enolase from baker's yeast (□) represents experimental curve (○) represents theoretical curves and full line the sum of theoretical curves. In Fig. 1a two components are assumed while in Fig. 1b three components are assumed in the isoenzyme sample.

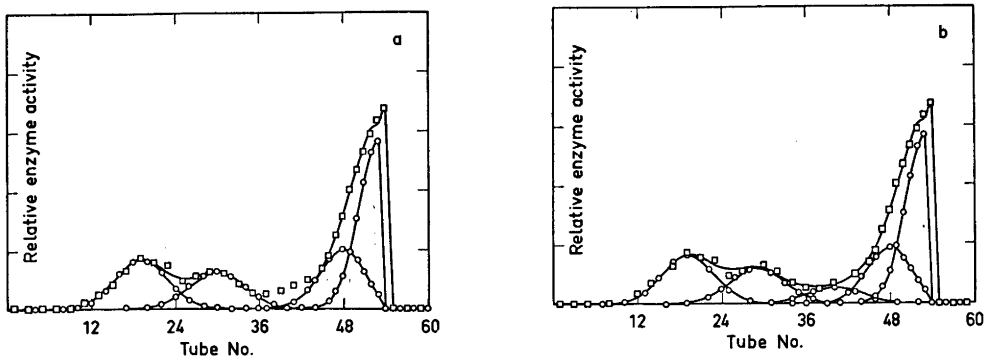

Fig. 2. Counter-current distribution of lactic dehydrogenase from pig liver. (□) represents experimental curve (○) represents theoretical curves and full line the sum of theoretical curves. In Fig. 2a 4 components are assumed while in Fig. 2b 5 components are assumed in the isoenzyme sample.

To study the reproducibility of CCD experiments and of the plotting of theoretical curves three CCD-experiments were performed at different times with the same enzyme preparation. The enzyme was stored at −25°C between the experiments which were carried out at 6 month intervals. Table 1 shows the position and amount of the different components of yeast enolase when subjected to CCD in the three experiments. The table indicates a very small variation in position of the three components from experiment to experiment. The variations are only ±1 tube which is within experimental error. The small

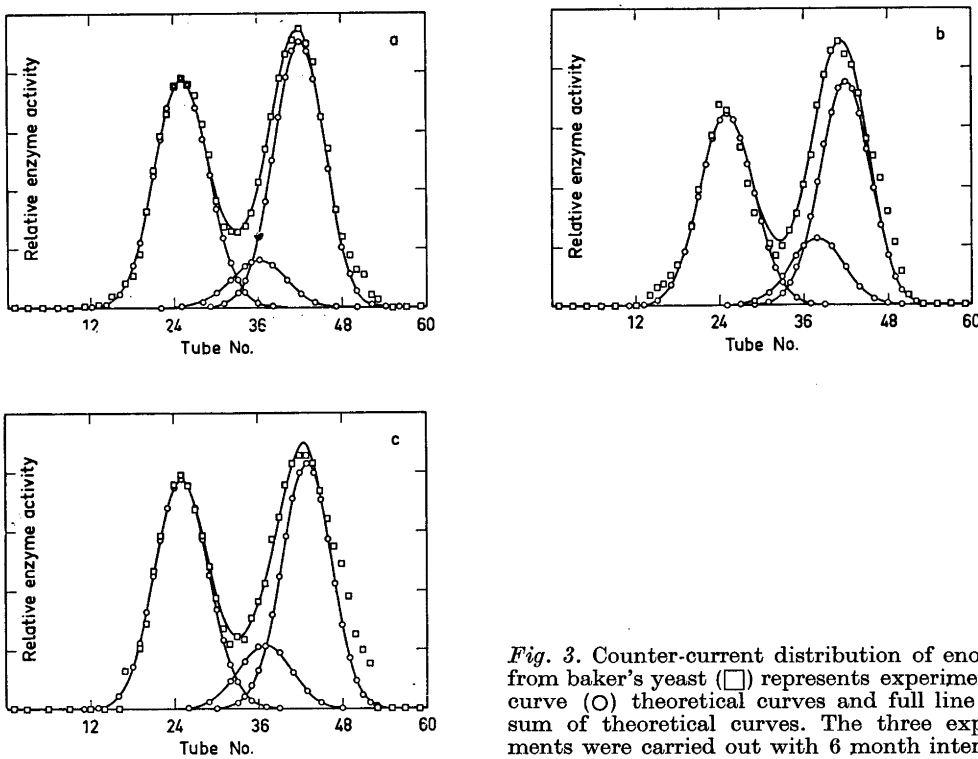

Fig. 3. Counter-current distribution of enolase from baker's yeast (□) represents experimental curve (○) theoretical curves and full line the sum of theoretical curves. The three experiments were carried out with 6 month interval.

Table 1.

		CCD-experiment 1	2	3
Position of peak No.	1	25	25	25
	2	36	38	37
	3	44	42	43
Percentage of total enzyme activity under peak No.	1	43	43	41
	2	9	12	14
	3	48	45	45

differences in the precentage of total enzyme activity under each peak in the three experiments may be explained by changes in the isoenzyme ratios due to storage.

In Fig. 3 (a, b and c) the three experiments are plotted. The reproducibility is very good and shows the value of the method for analytical purposes. Fig. 3 also shows that a fourth component may be to the right of the third peak. From gel electrophoresis enolase from baker's yeast is known to consist of three components.[9] It is never the less possible that a fourth component may be present in the enzyme preparation. To investigate this, further experiments with different enzyme preparations are necessary.

The computer program has been found to be excellent in the cases tested. It is not valid for calculations at the ends of the plot as can be seen in Fig. 2. To do further studies on the peak to the far right, one can change pH to make the K-value lower. The peak is then transferred to an area in which the computer program is valid and a theoretical resolution of the peak can be obtained.

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^{1}H- and ^{13}C-NMR Spectra of Phenyl-substituted Azole Derivatives. II. A Conformational Study

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^{13}C-NMR spectra of a number of phenyl-substituted pyrazolium bromides *1*, imidazolium bromides *2*, 1,2,3-triazolium bromides *4* and *5*, pyrazol-4-in-3-ones and -thiones *13* Z = O or S, imidazol-4-in-2-ones and -thiones *7* Z = O or S, 4-(1,2,3-triazolio)oxides and -sulfides *16*; Z = O or S, and 1,2,3-triazol-3-in-5-ones and -thiones *9*; Z = O or S have been recorded. Certain effects of substitution with methyl or chlorine have been measured. The magnitude of the chemical shifts of the benzene carbon atoms, particularly the *ortho*-carbon atoms, depends on the extent of interannular conjugation, and hence may provide information about the preferred conformation. Values for δ_{ortho}-C and the difference δ_{meta}-C − δ_{ortho}-C have been determined and their usefulness for assessing the extent of interannular conjugation in phenyl-substituted azoles with charged, zwitterionic, or partly aromatic heterocyclic rings has been demonstrated. ^{13}C-NMR data reveal that the heterocyclic ring of pyrazol-4-in-3-ones *13*; Z = O and triazol-3-in-5-ones *9*; Z = O, presumably takes up a twisted conformation, whereas the corresponding thiones are planar or nearly so. The ^{13}C-NMR-spectra render reliable information even in cases where ^{1}H-NMR-spectra fail to give correct results.

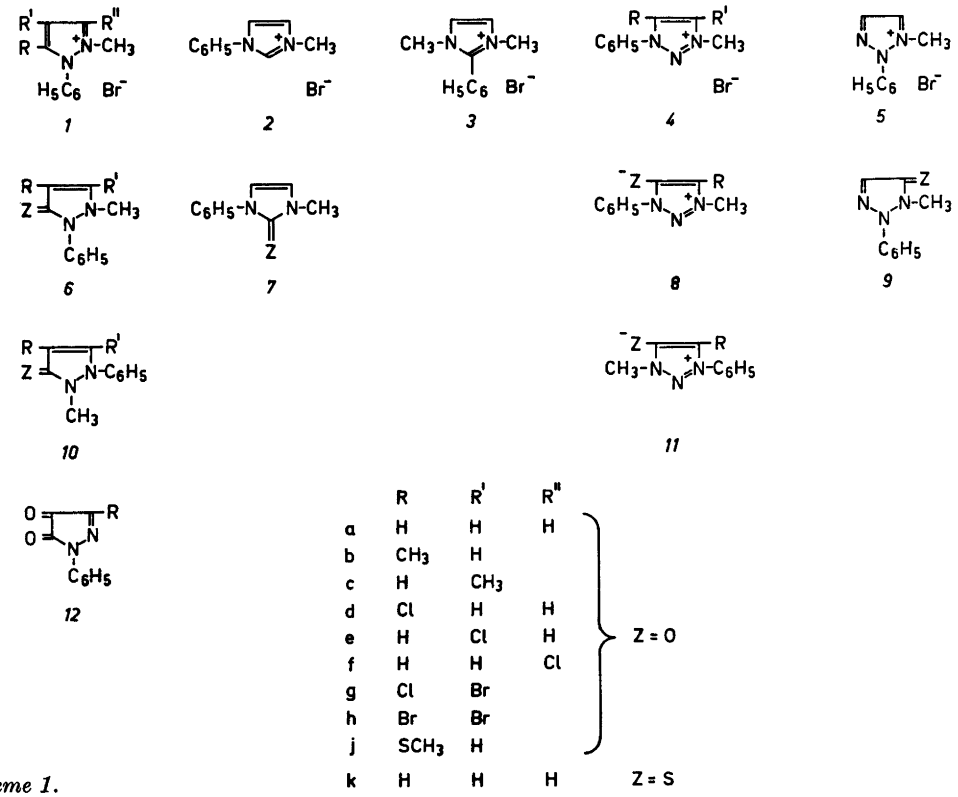
^{1}H- [1-6] and more recently ^{13}C-NMR-spectroscopy [7] has been employed for conformational studies of phenyl substituted heteroaromatic azoles. ^{1}H-NMR-spectra of unhindered compounds exhibit phenyl group multiplets, whereas phenyl substituted azoles with bulky substituents impeding interannular delocalization of the π-electrons give rise to phenyl group singlets or approximate singlets[1-3,5,6] When interannular conjugation in phenyl substituted azoles is extensive the electron density at C-2′ * increases leading to a high field shift of the C-2′ ^{13}C-NMR signal. Characteristic values for $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ were found when interannular conjugation was extensive or impeded, respectively.[7] The ^{13}C-NMR method was found to yield unambiguous results even in cases where the ^{1}H-NMR method fails.

So far, ^{1}H- or ^{13}C-NMR spectroscopy has not been applied systematically to conformational analysis of phenyl substituted azoles with charged, zwitterionic, or non-aromatic heterocyclic rings.

The marked deshielding of the *o*-phenyl protons in unhindered systems may be caused by several factors the relative significance of which is poorly understood.[1,2,4-6,9,10] Presumably, anisotropy effects induced by the heteroaromatic ring play a major role.[11,12] Since phenyl substituted azoles with non-aromatic heterocyclic rings are devoid of a heterocyclic ring current, ^{1}H-NMR would not be expected to be useful for conformational analysis in these systems. Even in conformational analysis of phenyl substituted azoles with charged or zwitterionic heterocyclic rings the values of ^{1}H-NMR-shifts should be interpreted with caution due to the uncertain origin of the shifts and to known exceptions from the rules.[7] In contrast, the ^{13}C-NMR method provides direct information about the extent of delocalization. Hence, reliable information about the conformation is expected in phenyl substituted azoles with charged, zwitterionic or non-aromatic rings.

Results of a ^{1}H- and ^{13}C-NMR investigation

* In the present paper the heterocyclic carbon atoms are numbered according to the IUPAC nomenclature.[8] The phenyl carbon atoms are denoted with a dash. Counting starts with the substituted atom (C-1′).



Scheme 1.

of such compounds are reported in the present paper. As representatives of charged azoles, phenyl substituted pyrazolium bromides *1*, imidazolium bromides *3*, and 1,2,3-triazolium bromides *4* and *5* have been studied. Zwitterionic azoles are represented by the phenyl substituted 4(1,2,3-triazolio)oxides and -sulfides *16*; Z = O and S. Finally, 1,2-disubstituted pyrazol-4-in-3-ones *13*; Z = O, -thiones *13*; Z = S, 1,3-disubstituted imidazol-4-in-2-ones *7*; Z = O, -thiones *7*; Z = S, 1,2-disubstituted 1,2,3-triazol-3-in-5-ones *9*; Z = O, and -thiones *9*; Z = S have been investigated as representatives of azole derivatives with reduced heteroaromaticity.*

* Undoubtedly, the azolinones and -thiones, like 2-pyridone and -thione,[13] are heteroaromatically stabilized to a certain extent through resonance structures such as *14* and *15*. Estimates of heteroaromatic stabilization are available only for pyrazolones *13*; Z = O, contradictory results being reported.[14–17]

RESULTS

Most of the pertinent ^{1}H-NMR-data have been published previously.[18–23] ^{13}C-Signals arising from the phenyl groups are summarized in the Tables 1, 2, and 3.

Proton noise-decoupled ^{13}C-NMR data for pyrazolium, imidazolium, and 1,2,3-triazolium bromides are presented in Tables 1 and 2.

In the non-decoupled spectrum of *1a* (Table 4), the two signals at lowest field and that at the next highest field exhibited the largest splittings. Hence these signals were assigned to the heterocyclic carbon atoms.[24a]

In the off-resonance proton-decoupled spectra of *1d*, *1e*, and *1f*, with irradiating upfield from the aromatic protons (see Experimental), the signals showing the largest residual coupling were ascribed to the proton-carrying C-3 and C-5 atoms. The (sharp) non-shifted singlets in *1d*, or *1e*, when compared to *1f*, were attributed

Table 1. ^{13}C-NMR chemical shifts of phenyl substituted azolium salts in aqueous solution.

Compound [a]		Position of carbon atoms in ppm C-2	C-3	C-4	C-5	C-1′	C-2′	C-3′	C-4′	$\frac{N\text{-}CH_3}{C\text{-}CH_3}$	[b]
1-Methyl-2-phenyl-pyrazolium bromide	*1a*		138.8	108.9	139.7	132.8	127.9	131.0	133.1	38.5	bs
1-Methyl-2-phenyl-3-chloro-pyrazolium bromide	*1d*		138.4	108.8	139.9	133.9	129.2	131.4	133.9	39.7	s
1-Methyl-2-phenyl-4-chloro-pyrazolium bromide	*1e*		136.9	113.6	138.0	132.3	127.9	131.0	133.5	39.1	s
1-Methyl-2-phenyl-5-chloro pyrazolium bromide	*1f*		139.0	109.2	139.4	133.1	128.0	131.1	133.5	36.1	nm
1-Methyl-3-phenyl-imidazolium bromide	*2a*	135.8		122.2	125.3	134.4	122.8	131.2	130.9	37.2	s
1,3-Dimethyl-2-phenyl-imidazolium bromide	*3*			123.6	123.6	121.7	130.2	130.8	133.1	36.5	bs
1-Methyl-3-phenyl-1,2,3-triazolium bromide	*4a*			129.5	132.5	135.3	122.0	130.8	132.5	41.2	m
1,4-Dimethyl-3-phenyl-1,2,3-triazolium bromide	*4b*			142.0	130.3	133.7	125.9	130.8	132.7	40.9 10.2	s
1,5-Dimethyl-3-phenyl-1,2,3-triazolium bromide	*4c*			127.0	142.2	135.2	121.8	130.9	132.3	38.4 9.4	m
1-Methyl-3-phenyl-4-chloro-1,2,3-triazolium bromide	*4d*			130.8	130.5	132.7	126.0	130.8	133.3	42.3	bs
1-Methyl-3-phenyl-5-chloro-1,2,3-triazolium bromide	*4e*			127.3	133.4	135.2	122.0	131.0	132.9	39.0	2+3
1-Methyl-2-phenyl-1,2,3-triazolium bromide	*5*			135.4	137.5		131.1	133.4	134.0	40.5	s

[a] The compounds were prepared as described in the experimental section. [b] Appearance of the phenyl group in the ^{1}H-NMR spectrum, bd (broad doublet), bs (broad singlet), m (multiplet), nm (narrow multiplet), s (singlet), 2+3 (a low field multiplet containing two protons plus a high field multiplet containing three protons).

to C-1′. In *1f*, the signal at lowest field, in *1d*, the signal at next lowest field, and in *1e*, the signal at next highest field appeared as (broad) singlets. Hence these signals were assigned to the chlorine-substituted carbon atoms C-5, C-3, and C-4, respectively. Consequently, the order $\delta_{C-5} > \delta_{C-3} > \delta_{C-4}$ was deduced for 1-methyl-2-phenyl-pyrazolium bromide *1a*.

Identification of the phenyl carbon atoms of *1a* through the fine structure of the undecoupled signals as described previously [7] proved unsuccessful since the spectra were indistinct and unresolvable and did no allow an unequivocal distinction between C-2′ and C-3′.* However, in all phenyl-substituted azole derivatives where a definite assignment, through undecoupled spectra, has been carried out (see Ref. 7 and below) the intensity of the signals decreases in the order C-3′ > C-2′ > C-4′. The same order of intensity was suggested by the identification of C-2′, C-3′, and C-4′ in *1a*, 1*d*, 1*e*, and

* The change in fine structure may be due to a change in the ratio between $^3J_{CCCH}$ and $^2J_{CCH}$ [7] or to the presence of a more strongly coupled spin system in the phenyl group of *1a* — and in other azolium salts as well — than in the azoles.

Table 2. ^{13}C-NMR chemical shifts of phenyl substituted azolium salts in deuteriochloroform solution.

Compound		Position of carbon atoms in ppm C-2	C-3	C-4	C-5	C-1′	C-2′	C-3′	C-4′	$\frac{N\text{-}CH_3}{C\text{-}CH_3}$	[b]
1-Methyl-2-phenyl-pyrazolium bromide	*1a* [a]		137.3	108.5	140.7	131.9	127.7	130.5	132.7	38.9	bd
1-Methyl-2-phenyl-3-chloro-pyrazolium bromide	*1b*		136.8	107.7	140.6	132.8	129.1	130.4	128.7	39.7	bs
1-Methyl-2-phenyl-5-chloro-pyrazolium bromide	*1f*		138.5	108.9	132.4		127.9	130.1	132.1	36.5	2+3
1-Methyl-3-phenyl-imidazolium bromide	*2a* [a]	135.8		120.7	124.5	134.2	121.7	130.3	130.0	37.2	m
1-Methyl-3-phenyl-1,2,3-triazolium bromide	*4a*			128.9	133.0	134.1	120.9	129.8	131.1	41.1	2+3
1,4-Dimethyl-3-phenyl-1,2,3-triazolium bromide	*4b*			140.4	131.2	132.7	125.7	129.9	131.9	40.3 10.0	s
1,5-Dimethyl-3-phenyl-1,2,3-triazolium bromide	*4c*			127.4	141.6	134.1	120.6	129.9	131.2	38.8	2+3

[a] Solution saturated at room temperature. [b] Appearance of the phenyl group in the ^{1}H-NMR spectrum.

1f, as well as in the other azolium bromides *2*, *3*, *4*, and *5*.

The ^{13}C-NMR-signals of 1-methyl-3-phenyl-1,2,3-triazolium bromide *4a*, its 4- and 5-methyl derivatives *4b* and *4c*, as well as its 4- and 5-chloro derivatives *4d* and *4e* (Table 1), were identified similarly. The off-resonance spectra indicated that δ_{C-5} in *4b* and *4d* was larger than δ_{C-4} in *4c* and *4e*, respectively. Conversely, δ_{C-5} in *4c* and *4e* was larger than δ_{C-4} in *4b* and *4d*, respectively. Hence, δ_{C-5} was assumed to be larger than δ_{C-4} in 1-methyl-3-phenyl-1,2,3-triazolium bromide *4a*, a supposition confirmed by its undecoupled spectrum (Table 4) in which C-4 appeared with sharp doublet fine structure due to coupling to H-5. The fine structure of C-5 was a doublet with unresolved hyperfine structure. The doublet part is explained by coupling to H-4, the hyperfine structure by coupling to the methyl protons.

In the undecoupled spectrum of 1-methyl-3-phenyl-imidazolium bromide *2a* (Table 4) the fine structure of the three signals with large splittings, from low to high field, was a narrow, quartet-like pattern, an extended double doublet with broad peaks, and an extended double doublet with narrow peaks. The two high field signals were attributed to C-4 and C-5, the extended fine structure was attributed to large $^2J_{CCH}$ couplings. The signal with the broad peaks was attributed to C-5, the broadening arising from long range coupling to the methyl protons. The narrow, fine splitting of C-2 is the result of small $^3J_{CNCH}$ couplings. The strong deshielding of C-2 is caused by the two adjacent nitrogen atoms.

C-4 and C-5 in 1-methyl-2-phenyl-1,2,3-triazolium bromide *5a* could be distinguished, since the latter signal in the undecoupled spectrum (Table 4) appeared with unresolved hyperfine structure due to weak coupling to the methyl protons.

The ^{13}C-NMR signals of [1-methyl-3-phenyl-4-(1,2,3-triazolio)]-oxide *8a* and of [1-phenyl-3-methyl-4-(1,2,3-triazolio)]oxide *11a* (Table 3) were identified through the proton undecoupled spectra (Table 5). The signal exhibiting solely doublet fine structure was attributed to C-4. The doublet arises from coupling to H-5. C-4 of *8a* and *11a*, like common carbonyl carbon atoms,[24b] absorb at very low field. The signal with the largest splitting was attributed to C-5. C-5 of *8a* and *11a* resonate at higher field than the other aromatic carbon atoms. The signals of the

Table 3. ^{13}C-NMR chemical shifts of phenyl substituted pyrazol-4-in-3-ones *13*; Z = O, pyrazol-4-in-3-thiones *13*; Z = S, imidazol-4-in-2-ones *7*; Z = O, imidazol-4-in-2-thiones *7*; Z = S, 4-(1,2,3-triazolio)oxides *16*; Z = O, 4-(1,2,3-triazolio)sulfides *16*; Z = S, 1,2,3-triazol-3-in-5-ones *9*; Z = O, and 1,2,3-triazol-3-in-5-thiones *9*; Z = S.

Compound [a]	Position of carbon atoms in ppm								$\frac{N\text{-}CH_3}{C\text{-}CH_3}$	$\frac{O\text{-}CH_3}{S\text{-}CH_3}$	[b]
	C-2	C-3	C-4	C-5	C-1′	C-2′	C-3′	C-4′			
2-Phenyl-5-methyl-pyrazol-5-in-3,4-dione *12b* [46]		183.9	148.7	144.0	136.5	117.4	128.7	125.8	11.0		2+3
2-Phenyl-4-methoxy-carbonyl-pyrazol-4-in-3-one *13l* [c,d]		166.3		138.2	137.2	121.1	128.8	126.7		51.5	m
1-Methyl-2-phenyl-pyrazol-4-in-3-one *6a* [13]		165.9	98.3	145.6	133.9	124.4	128.9	126.8	37.5		bs
1,5-Dimethyl-2-phenyl-pyrazol-4-in-3-one *6c* [48]		165.7	98.1	156.0	134.8	123.8	128.6	126.1	35.3 12.9		s
1-Methyl-2-phenyl-5-chloro-pyrazol-4-in-3-one *6e* [44,49]		164.1	99.6	147.5	134.3	123.9	128.9	126.9	36.7		bs
1-Methyl-2-phenyl-4-chloro-5-bromo-pyrazol-4-in-3-one *6g* [14]		159.7	108.1	134.0	133.8	123.8	129.1	127.3	38.7		s
1-Methyl-2-phenyl-4,5-dibromo-pyrazol-4-in-3-one *6h* [14]		156.1	95.7	136.5	134.1	123.8	129.0	127.3	38.7		s
1-Methyl-2-phenyl-pyrazol-4-in-3-thione *6k* [41]		170.1	111.1	136.2	133.2	128.4	129.2	129.9	37.2		s
1-Phenyl-2-methyl-pyrazol-4-in-3-one *10a* [13]		167.9	97.8	142.1	137.5	122.6	129.6	127.4	30.1		m
1-Phenyl-2,5-dimethyl-pyrazol-4-in-3-one *10c* [50]		167.1	97.8	152.5	136.9	126.3	129.4	128.6	30.0 13.2		m
1-Phenyl-2-methyl-5-chloro-pyrazol-4-in-3-one *10e* [44,49]		165.4	99.0	143.6	135.8	126.5	129.2	129.2	30.2		m
1-Phenyl-2-methyl-4-chloro-5-bromo-pyrazol-4-in-3-one *10g* [14]		161.0	107.7	130.0	136.6	126.9	129.4	129.7	31.1		m
1-Phenyl-2-methyl-4,5-dibromo-pyrazol-4-in-3-one *10h* [14]		162.0	95.2	132.6	136.7	126.8	129.3	129.7	31.3		m
1-Phenyl-2-methyl-pyrazol-4-in-3-thione *10k* [44]		170.0	112.2	134.8		125.6	129.7	129.9	33.3		m
1,2-Diphenyl-pyrazol-4-in-3-one *13m* [51]		166.6	98.9	145.3	138.9 135.1	120.8 123.2	129.3 128.4	126.2 125.9			nm nm
1,2-Diphenyl-4-methyl-pyrazol-4-in-3-one *13n* [e]		166.9	109.1	143.3	140.1 135.7	120.4 122.3	129.2 128.4	125.6 125.4	7.8		
1,2-Diphenyl-5-methyl-pyrazol-4-in-3-one *13o* [52]		166.0	98.9	155.9	138.6 135.4	125.1 123.2	128.9 128.2	127.6 125.4	 13.7		s m
1-Methyl-3-phenyl-imidazol-4-in-2-one *7a* [41]	152.1		108.8	112.4	137.0	121.1	128.7	125.3	30.4		m
1-Methyl-3-phenyl-imidazol-4-in-2-thione *7k* [41]	163.0		117.2	118.1	137.9	125.4	128.5	127.7	35.1		m

Table 3. Continued.

{1,3-Dimethyl-5-phenyl-4-(1,2,3-triazolio)}oxide	*16p*	155.6	126.7		127.8	128.6	127.8	38.8; 31.0		m
{1-Methyl-3-phenyl-4-(1,2,3-triazolio)}oxide	*8a* [16]	157.8	107.9	135.4	120.8	128.7	127.5	39.6		2+3
{1,5-Dimethyl-3-phenyl-4-(1,2,3-triazolio)}oxide	*8b* [16]	155.6	115.8	135.7	120.7	128.6	127.1	37.4 7.6		2+3
{1-methyl-3-phenyl-5-bromo-(1,2,3-triazolio)}oxide	*8h* [16]	154.1	92.8	135.7	120.3	128.8	128.6	38.9		2+3
{1-Methyl-3-phenyl-4-(1,2,3-triazolio)}sulfide	*8k* [43]	158.8	128.0	135.2	124.7	128.4	129.2	39.0		2+3
{1-Phenyl-3-methyl-4-(1,2,3-triazolio)}oxide	*11a* [16]	157.8	103.6	136.2	119.7	129.3	129.6	31.3		nm
{1-Phenyl-3,5-dimethyl-4-(1,2,3-triazolio)}oxide	*11b* [16]	156.3	115.0		124.1	129.3	130.0	31.0 8.6		bs
{1-Phenyl-3-methyl-5-bromo-4-(1,2,3-triazolio)}oxide	*11h* [16]	155.7	92.1	135.5	124.4	129.2	130.5	32.0		s
{1-Phenyl-3-methyl-5-methylthio-4-(1,2,3-triazolio)}oxide	*11j* [53]	157.6	111.5	135.6	124.5	128.8	130.1	31.7	17.9	s
{1-Phenyl-3-methyl-4-(1,2,3-triazolio)}sulfide	*11k* [53]	158.3	123.8	134.8	120.1	129.6	130.3	35.4		nm
1-Methyl-2-phenyl-triazol-3-in-5-one	*9a* [17]	130.1	160.0	137.6	123.4	129.5	128.7	30.4		m
1-Methyl-2-phenyl-triazol-3-in-5-thione	*9k* [41]	140.2	165.2	135.5	124.9	129.7	130.4	34.1		m

[a] The compounds were prepared as described in the references given. [b] Appearance of the phenyl group signal in the ^{1}H-NMR spectrum. See footnote *b*, Table 1. [c] The material was prepared analogous to the ethoxycarbonyl compound.[47] [d] The solution was saturated at room temperature. [e] The material was prepared analogous to *13m*.[51]

latter were identified through the undecoupled spectra as described previously.[7] The intensities of the proton noise decoupled signals of *8a* and *11a* decrease in the order C-3′ > C-2′ > C-4′ > C-5 > C-4 and C-1′. The order of intensities was used to identify the phenyl carbon signals of the 5-substituted 4(1,2,3-triazolio)oxides *8b*, *8h*, *11b*, *11h*, *11j*, and *16p* and those of the 4(1,2,3-triazolio)sulfides *8k* and *11k* (Table 3). The C-4 and C-5 signals of the 5-substituted 4(1,2,3-triazolio)oxides were assigned by their characteristic low field and high field positions, respectively. The 4(1,2,3-triazolio)sulfides *8k* and *11k* like the analogous oxygen compounds *8a* and *11a*, showed a low field and a high field ^{13}C-NMR signal (Table 3). Hence these signals were attributed to C-4 and C-5, respectively.

The ^{13}C-NMR signals (Table 3) of the pyrazol-4-in-3-ones *13*; Z = O, imidazol-4-in-2-ones *7*; Z = O, and 1,2,3-triazol-3-in-5-ones *9*; Z = O or S were identified analogously through undecoupled spectra (Table 5). Low field signals exhibiting only fine structure were ascribed to C = O carbon atoms and signals with large splittings to proton-carrying heterocyclic carbon atoms. Benzene carbon atoms were identified in the usual way.[7] C-5 of the pyrazol-4-in-3-one *6a* was identified as the heterocyclic carbon signal at the next lowest field since it appeared with quartet hyperfine structure due to a small long-range coupling to the methyl protons. In *10a* only broadening of the C-5 signal due to coupling to the methyl protons is observed.

C-3 and C-4 of the 4- and 5- substituted

Table 4. ^{13}C-^{1}H NMR coupling constants of phenyl substituted azolium bromides [a].

Compound		The carbon to which the coupling takes plane C-2	C-3	C-4	C-5	C-1′	C-2′	C-3′	C-4′	*N*-CH_3 / *C*-CH_3
				$^{1}J_{CH}$ / $^{2}J_{CCH}$ / $^{3}J_{CXCH}$ Hz						
				190			167	167	168	146
1-Methyl-2-phenyl-pyrazolium bromide	*1a*		7;7[b]	7;7					9	
1-Methyl-3-phenyl-imidazolium bromide	*2a*	220 5;10		12[b] 5	210[b] 6		166	165		145
1-Methyl-3-phenyl-1,2,3-triazolium-bromide	*4a*			211 13	212 13		165	166 8	165 8	146
1-Methyl-2-phenyl-1,2,3-triazolium-bromide	*5*			211 10	15			166		148

[a] All coupling constants have been obtained by first order analysis. [b] The $^{3}J_{CXCH}$ coupling constants were distinguished from the $^{2}J_{CCH}$ coupling constants since the latter are of the same order of magnitude as $^{2}J_{CCH}$ in the triazolium bromides *4a* and *5a* and from C-4 of the pyrazolium bromide *1a*.

Table 5. ^{13}C-^{1}H NMR coupling constants of phenyl substituted pyrazol-4-in-3-ones *13*; Z=O, imidazol-4-in-2-ones *7*; Z=O, 4-(1,2,3-triazolio)oxides *16*, Z=O, and 1,2,3-triazol-3-in-5-ones *9*; Z=O.[a]

Compound	The carbon to which the coupling takes place C-2	C-3	C-4	C-5	C-1′	C-2′	C-3′	C-4′
			$^{1}J_{CH}$ / $^{2}J_{CCH}$ / $^{3}J_{CXCH}$ Hz					
1-Methyl-2-phenyl-pyrazol-4-in-3-one *6a*		9[b] 7	183 6	186 8[b] 2	7	163 6	161 8	160 7
1-Phenyl-2-methyl-pyrazol-4-in-3-one *10a*			183 6	189 9		161 6	162 6	164 8
1-Methyl-3-phenyl-imidazol-4-in-2-one *7*			196 10	196 7[b] 2		163 6	161 7	161 7
{1-Methyl-3-phenyl-4-(1,2,3-triazolio)}oxide *8a*			11	200	9	165 6	162 9	161 9
{1-Phenyl-3-methyl-4-(1,2,3-triazolio)}oxide *11a*			11	201		165 5	163 4	162 5
1-Methyl-2-phenyl-1,2,3-triazol-3-in-5-one *9*			201		10	165	163	163

[a] All coupling constants have been obtained by first order analysis. [b] The $^{3}J_{CXCH}$ coupling constants were distinguished from the $^{2}J_{CCH}$ coupling constants since the latter are of the same order of magnitude as $^{2}J_{CCH}$ in the triazole-derivatives *8a* and *11a*.

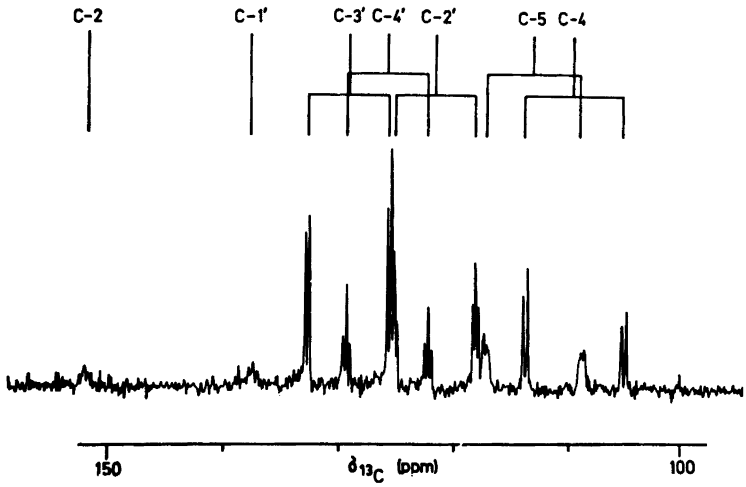


Fig. 1. Proton undecoupled ^{13}C-NMR spectrum of 1-methyl-3-phenyl-4-in-2-one *7a*.

pyrazol-4-in-3-ones *6c*, *6e*, *6g*, *6h*, *10c*, *10e*, *10g*, *10h*, *12b*, *13l*, *13m*, and *13o* were identified through their characteristic positions. C-4 in *12b* was identified as the second lowest field signal since its intensity, like that arising from C-3, was particularly low. Low intensity as the result of long relaxation time, T_1, is characteristic of carbonyl carbon atoms.[25a]

C-5 and the benzene ring carbon atoms of the substituted pyrazol-4-in-3-ones were identified, assuming the same order of intensities, C-3′ > C-2′ > C-4′ > C-4, C-5 > C-1′ > C-3, as observed in *6a* and *10a* and taking into account that substituent-carrying carbon atoms appear with strongly reduced intensity.[7]

The two sets of phenyl carbon signals of *13m* and *13n* were assigned by comparison with the phenyl carbon signals of *6a* and *10a*. For example, $\delta_{C-1'}$ of *10a* > $\delta_{C-1'}$ of *6a*. Hence, the low and high field C-1′ signals of *13m* were ascribed to the 1- and 2-phenyl groups, respectively. Similarly, the two sets of phenyl carbon signals of *13o* were identified by comparison with the phenyl carbon signals of *6c* and *10c*.

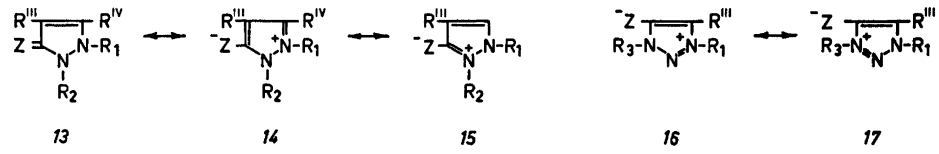
C-5 of 1-methyl-3-phenyl-imidazol-4-in-2-one *7a* was distinguished from C-4 as the signal which shows multiplet hyperfine structure due to coupling to the methyl protons (Fig. 1).

The ^{13}C-NMR signals of the thiones *6k* and *10k* were assigned comparing δ-values and relative intensity of signals with those of the corresponding oxygen analogues. The signals of the thiones *7k* and *9k* were identified through their undecoupled spectra as described for the corresponding oxygen analogues.

Superficially, δ^{13}_{C} of azolium ring carbon atoms is expected to increase when the number of ring nitrogen atoms, particularly in α-position or positively charged, increases. However, the observed ^{13}C chemical shifts are not in keeping with this assumption. (Thus δ_{C-5} of *1a* > δ_{C-5} of *5* > δ_{C-2} of *2a*). Nor are these shifts larger the faster the proton on the appropriate carbon atom is exchanged with deuterium in basic solution.[18,21,22]

According to calculations [26] and ^{13}C-NMR of the protonated azoles [27] or azines,[28] the electron density of α-carbon atoms increases when a heterocyclic ring nitrogen adopts a positive charge. This effect may account for the unexpected order in the present case.

In each of the methylphenylazolium bromides *1a*, *2a*, *4a*, and *5a* the chemical shift of a carbon atom adjacent to an *N*-methyl group is larger than that of a carbon atom adjacent to an *N*-phenyl group, indicating that the electron density is higher at the latter carbon atom. The protons at the appropriate carbon atoms absorb in the reverse order, which also corresponds to the relative acidity of the protons, as reflected by the rate of the base catalyzed deuterium exchange.[18,21] In *1a*, *2a*, *4a*, and *5a* $\delta^{13}_{CH_3N-1}$ is



13 14 15 16 17

	R_1	R_2	R^{III}	R^{IV}	
l	H	C_6H_5	$COOCH_3$	H	Z = O
m	C_6H_5	C_6H_5	H	H	
n	C_6H_5	C_6H_5	CH_3	H	
o	C_6H_5	C_6H_5	H	CH_3	
p	CH_3	CH_3	C_6H_5	H	

Scheme 2.

larger the more electron attracting the ring is. (Thus δ_{CH_3N-1} of *4a* and *5a* > δ_{CH_3N-1} of *1a* > δ_{CH_3N-1} of *2a*).

Methyl carbon atoms at N-1 in 1,3-disubstituted 4(1,2,3-triazolio)oxides *16*; Z = O, absorb at 6.8 – 8.7 ppm lower field than methyl carbon atoms at N-3. This indicates that N-1 is more electron deficient than N-3, suggesting that the resonance structure *16*; Z = O (Scheme 2) is the major contributor to the 4(1,2,3-triazolio)oxide hybrid. The chemical shifts of C-4, C-5, and the N-1 methyl carbon atoms in the 4(1,2,3-triazolio)sulfides *8k* and *11k* are similar to those in the corresponding 4(1,2,3-triazolio)-oxides *8a* and *11a*, respectively. In contrast, δ_{CH_3N-3} is 4.1 ppm larger in the 4(1,2,3-triazolio)sulfide *11k* than δ_{CH_3N-3} in the corresponding 4(1,2,3-triazolio)oxide *11a*. δ_{CH_3N-1} in *8k* is larger than δ_{CH_3N-3} in *11k*, again indicating that *16*; Z = S, is the major contributor to the hybrid. This has recently been confirmed in the solid by X-ray studies of [1,3-dimethyl-4-(1,2,3-triazolio)] sulfide *16*; $R_1 = R_3 = CH_3$, Z = S.[29]

Methyl carbon atoms at N-1 in 1,2-disubstituted pyrazol-4-in-3-ones *13*; Z = O, absorb at 7.4 – 8.2 ppm lower field than N-2 methyl carbon atoms. This indicates that N-1 is more electron deficient than N-2, suggesting that the dipolar structure *14*; Z = O, contributes appreciably to the pyrazol-4-in-3-one hybrid. Arguments for [14] and against [15–17] the importance of zwitterionic structures have been presented, and some of the arguments have been critically analyzed.[30,31] It should be emphasized that the present data are only suggestive.

The ^{13}C-NMR chemical shifts of the heterocyclic ring- and the *N*-methyl carbon atoms of the thiones *6k*, *10k*, *7k*, and *9k* are quite different from those of the corresponding oxygen analogues.

A methyl group deshields the methyl carrying carbon atom 10.4 – 11.5 ppm in 1,3-disubstituted 1,2,3-triazolium salts *4*, 1,3-disubstituted 4(1,2,3-triazolio)oxides *16*; Z = O, and 1,2-disubstituted pyrazol-4-in-3-ones *13*; Z = O. Carbon atoms adjacent to the methyl-substituted carbons are shielded 1.5 – 2.1 ppm in 1,2,3-triazolium salts *4* and 4(1,2,3-triazolio)-oxides *16*; Z = O.

The effect of chlorine on the ^{13}C chemical shifts of azolium salts depends on the type of ring and the position of the substituent. Thus chlorine deshields the substituted carbon atom 0.9 – 1.3 ppm in the 1,2,3-triazolium bromides *4d* and *4e*. The adjacent carbon atom is shielded 2.0 – 2.2 ppm. Chlorine in the 4-position of the pyrazolium salt *1e* has a similar effect but chlorine in the 3- or 5-position of the pyrazolium salts *1d* and *1f* affects the ^{13}C chemical shift of the substituted and the adjacent carbon atom less than 0.4 ppm (Table 1). Chlorine in the 5-position of the pyrazolones *13*; Z = O, deshields the substituted and the adjacent carbon atom 1.5 – 1.9 and 1.2 – 1.3 ppm, respectively.

Bromine shields the substituted carbon atom *ca* 14.6 ppm in the 4(1,2,3-triazolio)oxides *16*; Z = O. The adjacent carbon atom is deshielded *ca* 3.0 ppm.

The effects of the substituents are similar to

Table 6. The ^{13}C chemical shift of C-2′ and the ^{13}C chemical shift difference between C-3′ and C-2′ for *C*- and *N*-phenyl substituted azole derivatives.

Type of compound	Hindered/unhindered[a]		Representative compounds	$\delta_{C-2'}$[b] ppm	$\delta_{C-3'} - \delta_{C-2'}$[b] ppm
1-Phenyl-pyrazoles[c]	unhindered			118.5 – 118.8	10.5
	hindered			124.6 – 125.4	3.3 – 4.0
1-Methyl-2-phenyl-pyrazolium bromides *1*	hindered			127.7 – 129.1	1.3 – 2.6
1,2-Disubstituted pyrazol-4-in-3-ones *13*; Z = O	C_6H_5	*unhindered*	*13m*	120.8	8.5
	O		*13l*	121.1	7.7
	CH_3		*10a*	122.6	7.0
	$O + C_6H_5$		*13m*	123.2	5.2
	$O + CH_3$	↓	*6a*	123.8 – 124.4	4.5 – 5.3
	$CH_3 + CH_3$ or Br	*hindered*	*10c*	126.3 – 126.9	2.5 – 3.1
1,2-Disubstituted pyrazol-4-in-3-thiones *13*; Z = S	CH_3	*hindered*	*10k*	125.6	4.1
	$S + CH_3$	*hindered*	*6k*	128.4	0.8
1-Phenyl-imidazoles[c]	unhindered			121.0	9.4
1-Methyl-3-phenyl-imidazolium bromide *2*	unhindered			121.7	8.6
1-Methyl-3-phenyl-imidazol-4-in-2-one or -thione *7*; Z = O or S	O	*unhindered*	*7a*	121.1	7.6
	S	*hindered*	*7k*	125.4	3.1
2-Phenyl-imidazoles[c,e]	unhindered			125.2	3.2
	hindered			128.0	0
1,3-Dimethyl-2-phenyl-imidazolium bromide *3*[e]	hindered			130.2[d]	0.6[d]
1-Phenyl-1,2,3-triazoles[c]	unhindered			120	9.4
	hindered			124.5	4.6
1-Methyl-3-phenyl-1,2,3-triazolium bromides *4*	unhindered			120.6 – 120.9	8.9 – 9.3
	hindered			125.7	4.2
1,3-Disubstituted 4-(1,2,3-triazolio) oxides or sulfides *16*; Z = O or S	unhindered		*11a*	119.7 – 120.1	9.5 – 9.6
	O	*unhindered*	*8a*	120.3 – 120.8	7.9 – 8.5
	hindered		*11b*	124.1 – 124.5	4.3 – 5.2
	S	*hindered*	*8k*	124.7	3.7
{1,3-Dimethyl-5-phenyl-4-(1,2,3-triazolio)}oxide[e] *16p*	hindered			127.8	0.8
2-Phenyl-1,2,3 triazole[c]	unhindered			118.3	10.6
1-Methyl-2-phenyl-1,2,3-triazolium bromide *5*	hindered			131.1[d]	2.3[d]
1-Methyl-2-phenyl-1,2,3-triazol-3-in-5-one *9a*	CH_3	*hindered*		123.4	6.1
1-Methyl-2-phenyl-1,2,3-triazol-3-in-5-thione *9k*	CH_3	*hindered*		124.9	4.8

[a] See the footnote p. 71. The indications are typed in italics when the extent of hindrance has been estimated from the ^{13}C-NMR data and the substituents adjacent to the *N*-phenyl groups are stated in cases where a more exact specification is considered necessary. [b] When not otherwise stated the δ-values given are for deuteriochloroform solution with TMS as an internal standard. [c] The data have been published previously [7] but are shown for comparison. [d] The δ-values given are for deuterium oxide solution with *p*-dioxane as an internal standard. [e] Notice that the compound is *C*-phenyl substituted.

Table 7. $^1J^{13}_{CH_3N}$ coupling constants of *N*-methyl substituted azoles in deuteriochloroform.[a]

Compound	$^1J^{13}_{CH_3N}$ Hz
1-Methyl-pyrazole	140.0
1-Methyl-imidazole	140.9
1-Methyl-1,2,3-triazole	142.2
2-Methyl-1,2,3-triazole	142.0

[a] The values were determined by 60 MHz ^{1}H-NMR spectroscopy.

those observed in the phenyl substituted azoles.[7]

The $^1J^{13}_{CH}$ coupling constants of the heterocyclic carbon atoms in the azolium bromides *1*, *2*, *4*, and *5* are 12 – 20 Hz larger than the corresponding coupling constants of the parent 1-phenyl substituted azoles [7] (Table 4). The long range coupling constants are similar in *1*, *2*, *4*, and *5* and in the parent 1-phenyl-azoles. The $^1J^{13}_{CH_3N}$ coupling constants are 2 – 4 Hz larger in *1*, *2*, *4*, and *5* than in the parent 1-methyl substituted azoles (Table 7). The larger $^1J^{13}_{CH}$ coupling constants observed in the azolium salts, compared with the corresponding azoles, are explained by the increased electron deficiency of the azolium systems.

The $^1J^{13}_{CH}$ and $^2J^{13}_{CCH}$ coupling constants of the 1,2,3-triazol-3-in-5-one *9a*; the 4(1,2,3-triazolio)oxides *8a* and *11a*, imidazol-4-in-2-one *7a*, and the pyrazol-4-in-3-ones *6a* and *10a* (Table 5) are similar to the values found in the parent *N*-phenyl substituted compounds.[7] (Thus the $^1J^{13}_{CH}$ coupling constants decrease in the order $^1J_{CH-4}$ in *9a* and $^1J_{CH-5}$ in *8a* and *11a* > $^1J_{CH-4}$ in *7a* > $^1J_{CH-5}$ in *6a* and *10a* > $^1J_{CH-4}$ in *6a* and *10a*. Similarly, $^2J_{HCC-4}$ in *8a* and *11a* > $^2J_{HCC-4}$ in *7a* > $^2J_{HCC-3}$ and $^2J_{HCC-5}$ in *6a* and *10a* > $^2J_{HCC-4}$ in *6a* and *10a*).

DISCUSSION

The ^{13}C chemical shift of C-3′. In simple *N*-phenyl substituted azoles, $\delta_{C-3'} = 128.5 - 129.8$ ppm is the parameter least sensitive to ring type and substitution.[7] Similarly, C-3′ of the 4-(1,2,3-triazolio)oxides *16*; Z = O, 4-(1,2,3-triazolio)-sulfides *16*; Z = S, pyrazol-4-in-3-ones *13*; Z = O, pyrazol-4-in-3-thiones *13*; Z = S, 1,2,3-triazol-3-in-5-one *9a*, 1,2,3-triazol-3-in-5-thione *9k*, imidazol-4-in-2-one *7a*, and imidazol-4-in-2-thione *7k*, consistently resonate between 128.2 and 129.7 ppm (Table 3). The shifts of C-3′ of the azolium salts *1*, *2*, *4*, and *5* are slightly larger [129.8 – 130.5 ppm in deuteriochloroform solution (Table 2) and 130.8 – 133.4 ppm in deuterium oxide solution (Table 1)], presumably due to strong inductive electron attraction from the positively charged azolium ring.

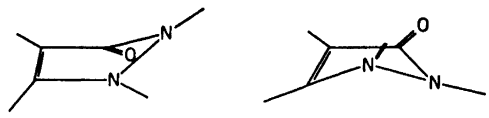
The ^{13}C chemical shift of C-2′. In simple *N*-phenyl substituted azoles, $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ are the parameters most susceptible to hindrance of interannular conjugation.[7] In Table 6, these parameters of simple phenyl substituted azoles are compared with those extracted from Tables 2 and 3 of the phenyl substituted derivatives with charged, zwitterionic, or partly aromatic heterocyclic rings. In the unhindered imidazolium salt *2* and triazolium salts *4a*, *4c*, and *4e*, $\delta_{C-2'}$ ($CDCl_3$) = 120.6 – 121.7 ppm and $\delta_{C-3'} - \delta_{C-2'}$ ($CDCl_3$) = 8.6 – 9.3 ppm. [In deuterium oxide solution $\delta_{C-2'} = 121.8 - 122.8$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 8.4 - 9.1$ ppm (Table 1)]. These values correspond to those of the unhindered azoles indicating that interannular conjugation is extensive in these salts. In the hindered * pyrazolium bromides *1a*, *1d*, *1e*, and *1f* and 1,2,3-triazolium bromides *4b*, *4d*, and *5a*, $\delta_{C-2'}$ ($CDCl_3$) = 125.7 – 129.1 ppm and $\delta_{C-3'} - \delta_{C-2'}$ ($CDCl_3$) = 1.3 – 4.2 ppm. [In deuterium oxide solution $\delta_{C-2'} = 125.9 - 131.1$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 2.1 - 4.9$ ppm (Table 1).] These values are similar to those of the hindered azoles. In the hindered *C*-phenyl substituted imidazolium salt *3*, $\delta_{C-2'} = 130.2$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 0.6$ ppm, *i. e.* values similar to those of the hindered *C*-phenyl substituted imidazoles.

In the unhindered [1-phenyl-3-methyl-4-(1,2,3-triazolio)]oxide *11a* and -sulfide *11k* the values of $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ correspond to those of the unhindered 1-phenyl substituted 1,2,3-triazoles indicating that interannular conjugation is extensive in *11a* and *11k*. Methyl, bromine, or methylthio in the 5-position of *11*; Z = O, impedes interannular conjugation as reflected by $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ which are

* In the following discussion it is assumed that the steric effects of substituents are similar to those in the azoles and that the steric effect of an *N*-methyl group and a *C*-methyl group are similar.

similar to those of the hindered 5-substituted 1,2,3-triazoles. In the [1-methyl-3-phenyl-4-(1,2,3-triazolio)]oxides *8*; Z=O, $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ deviate slightly from the values observed in the unhindered 1,2,3-triazoles indicating that the oxygen atom adjacent to the phenyl group impedes interannular conjugation insignificantly. In contrast, the sulfur atom of [1-methyl-3-phenyl-4-(1,2,3-triazolio)]sulfide *8k* impedes interannular conjugation as effectively as a methyl group, as evident from the similarity between $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ in *8k* (Table 3) and in 1-phenyl-5-methyl-1,2,3-triazole.[7] In the hindered *C*-phenyl substituted 4-(1,2,3-triazolio)-oxide *16p* $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values correspond to those of the hindered *C*-phenyl substituted azoles.[7]

In 2-phenyl-5-methyl-pyrazol-5-in-3,4-dione *12b* and 2-phenyl-4-carbomethoxy-pyrazol-4-in-3-one *13l*, $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values are similar to those of the unhindered 1-phenyl-pyrazoles indicating that the oxygen atom adjacent to the phenyl group does not impede interannular conjugation at all in *12b* and only slightly in *13l*. According to the ^{13}C-NMR data of 1-phenyl-5-methyl-pyrazole [7] and 1-methyl-2-phenyl-pyrazolium bromide *1a* (Table 2), the methyl group of 1-phenyl-2-methyl-pyrazol-4-in-3-one *10a* is expected to impede interannular conjugation. However, $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values are roughly between those of the unhindered and hindered heteroaromatic pyrazoles. This implies that interannular conjugation in *6a* is impeded to a minor extent, due to the fact that the extent of interannular conjugation is a cos^2-function of the angle between the 2*p*-orbitals of the *N*-atom and the π-orbitals of the attached phenyl group.[32-34] Inspection of Dreiding models reveals that minor impediment of interannular conjugation is possible only if the pyrazole ring of *6a* is not planar but in isotropic phase takes up a conformation like that shown in Fig. 2. By replacement of oxygen in *6a* with sulfur, $\delta_{C-2'}$ increases to values similar to those of the hindered heteroaromatic pyrazoles. The sulfur atom cannot influence the phenyl group through space. Hence, the hindrance must be due to the *N*-2 methyl group. This seems possible only if the pyrazole ring is planar or almost so in the pyrazolthione *10k*. By introduction of methyl or bromine in the 5-position of the pyrazolone *10a*, $\delta_{C-2'}$ increases, and $\delta_{C-3'} - \delta_{C-2'}$ decreases to values similar to those of hindered 1-phenyl-pyrazoles. A similar effect of a 5-methyl group is observed in *13o*. The effect of a 5-methyl group is much larger than that of the *N*-2 methyl group (compare $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values of *10a* and *10c*). This implies that the angle between the exocyclic bonds of *C*-5 and *N*-1 is smaller than that between the exocyclic bonds of *N*-1 and *N*-2. This in turn suggests that the 5-substituted 1-phenyl-2-methyl-pyrazol-4-in-3-ones *10b*, *10g*, and *10h* take up a skew conformation (Fig. 2). The strong impediment of 5-substituents indicates that the pyrazolones *6c*, *6e*, *6g*, *6h*, and *13o* in solution apparently do not adopt the conformation with a planar pyrazole ring and with the 5-substituent out of the plane, which has been observed by an X-ray study of 1,5-dimethyl-2-phenyl-4-bromo-pyrazol-4-in-3-one.[35]



18 *19*

Fig. 2. Possible nonplanear conformations of 1,2-disubstituted pyrazol-4-in-3-ones. *18* and *19* have been selected as the ideal envelope conformations with the least distorted conjugated carbonyl system. *18* seems more likely than *19* both for 1-methyl-2-phenyl-pyrazol-4-in-3-ones *6*; Z=O where the N-2 phenyl group is least hindered in the former, and for 1-phenyl-2-methyl-pyrazol-4-in-3-ones *10*; Z=O as discussed in the text.

In the 1-methyl-2-phenyl-pyrazol-4-in-3-ones *6a*, *6c*, *6g*, and *6h*, $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values are not far from those of the hindered 1-phenyl-pyrazoles, indicating that interannular conjugation is impeded in the pyrazol-4-in-3-ones *6a*, *6c*, *6g*, and *6h*. In these, however, the combined effects of the *N*-1 methyl group and the *C*-3 oxygen atom are smaller than that of the single methyl group in 1-phenyl-5-methyl-pyrazole.[7] This again may be explained by assuming that the heterocyclic ring in the pyrazol-4-in-3-ones *6a*, *6c*, *6g*, and *6h* is not planar (see Fig. 2). A conformation like that observed for 1,5-dimethyl-2-phenyl-4-bromo-pyrazol-4-in-3-one in the crystal phase [35] seems more improbable. $\delta_{C-2'}$ in the pyrazol-4-in-3-thione *6k* is larger and $\delta_{C-3'} - \delta_{C-2'}$ is smaller than those of the

oxygen analog *6a* (Table 3). This indicates that replacement of an oxygen atom adjacent to the phenyl group with a sulfur atom increases the impediment of interannular conjugation. In *6k* the combined effects of the *N*-1 methyl group and the *C*-3 sulfur atom are similar to the combined effects of a methyl group and a sulfur atom in heteroaromatic compounds. (Thus the difference between $\delta_{C-2'}$ of *6k* and 1-phenylpyrazole [7] is 9.7 ppm and the difference between $\delta_{C-2'}$ of 1-phenyl-5-methyl-pyrazole [7] and 1-phenyl-pyrazole [7] plus the difference between $\delta_{C-2'}$ of *8k* and 1-phenyl-1,2,3-triazole [7] is 10.0 ppm. The corresponding difference for $\delta_{C-3'} - \delta_{C-2'} = -9.5$ and -11.6 ppm, respectively). This suggests that the heterocyclic ring of the pyrazol-thione *6k* is probably planar, or nearly so.

According to $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values of *13m* and *13n*, an *N*-phenyl group impedes the interannular conjugation of an adjacent phenyl group to a smaller extent than an oxygen atom.

In 1-methyl-2-phenyl-1,2,3-triazol-3-in-5-one *9a*, $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values fall between those of the hindered and unhindered 1-phenyl-1,2,3-triazoles or 1,2,3-triazolium bromides *4*, implying that interannular conjugation is impeded to a smaller extent in *9a* than in the hindered aromatic triazoles. This may be explained assuming that the heterocyclic ring of *9a* is not planar but takes up a skew conformation like the pyrazol-4-in-3-ones *13*; Z = O (see Fig. 2). Like in *13*, interannular conjugation vanishes in *9* by replacement of oxygen with sulfur as indicated by $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ of *9k*.

$\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values of the imidazol-4-in-2-one *7a* correspond to those of 1-phenylimidazole indicating that interannular conjugation prevails in *7a*. When oxygen is replaced by the larger sulfur atom interannular conjugation vanishes as indicated by $\delta_{C-2'}$ and $\delta_{C-3'} - \delta_{C-2'}$ values of *7k*.

The ^{13}C chemical shift of C-4'. The chemical shift of C-4' varies much less than $\delta_{C-3'}$ with the extent of interannular conjugation. In the azolium bromides, the pyrazol-4-in-3-ones *13*; Z = O, and the pyrazol-4-in-3-thiones *13*; Z = S, C-4' is shifted to low field, and $\delta_{C-3'} - \delta_{C-4'}$ decreases when conjugation vanishes (Tables 1, 2, and 3). In the hindered and unhindered 4-(1,2,3-triazolio)oxides *16*; Z = O, only small variations in $\delta_{C-4'}$ and $\delta_{C-3'} - \delta_{C-4'}$ values are observed.

The ^{13}C chemical shift of C-1'. C-1' in *C*-phenyl substituted compounds is more shielded than C-1' of *N*-phenyl substituted compounds (compare $\delta_{C-1'}$ of *3* and *16p* with $\delta_{C-1'}$ of *2* and *11a*, respectively). $\delta_{C-1'}$ depends little on the extent of interannular conjugation. Generally, $\delta_{C-1'}$ and $\delta_{C-1'} - \delta_{C-3'}$ values decrease when conjugation vanishes. Conceivably, this high field shift of C-1' reflects increased electron density at the phenyl-substituted *N*-atom due to reduced delocalization of the *N*-lone pair in the hindered compounds. $\delta_{C-1'}$ and $\delta_{C-1'} - \delta_{C-3'}$ values of the unhindered 1,2,3-triazolium bromides *4a*, *4c*, and *4e* are slightly larger than those of the unhindered diazolium bromide *2*. Similarly, $\delta_{C-1'}$ and $\delta_{C-1'} - \delta_{C-3'}$ values of the hindered 1,2,3-triazolium bromides *4b*, *4d*, and *5* are slightly larger than those of the hindered diazolium bromides *1a*, *1d*, *1e*, and *1f* (Tables 1 and 2).

In the pyrazol-4-in-3-ones *13*; Z = O, $\delta_{C-1'}$, like $\delta^{15}_{CH_3N}$ (see above) depends primarily on the position of the phenyl group. Secondly, $\delta_{C-1'}$ depends on the extent of conjugation as described above.

Comparison between ^1H- and ^{13}C-NMR data. The phenyl groups of all of the hindered and unhindered *N*- and *C*-phenyl substituted azolium salts studied appear as singlets and multiplets, respectively, with two exceptions (Tables 1 and 2). The phenyl group of the hindered 1-methyl-2-phenyl-5-chloro-pyrazolium bromide *1d* appears as a multiplet both in deuteriochloroform and deuterium oxide solution. Secondly, the unhindered 1-methyl-3-phenyl-imidazolium bromide *2a* exhibits a phenyl group singlet in aqueous solution but a multiplet in deuteriochloroform solution. More inconsistencies between ^1H- and ^{13}C-NMR spectra are observed in the 4-(1,2,3-triazolio)oxides *16*; Z = O. Thus the unhindered [1-phenyl-3-methyl-4-(1,2,3-triazolio)]oxide *11a* and -sulfide *11k* exhibit phenyl group singlets or doublets, whereas the ^{13}C-NMR data indicate extensive interannular conjugation.

The hindered [1-methyl-3-phenyl-4-(1,2,3-triazolio)]sulfide *8k* exhibits a phenyl group multiplet, whereas the ^{13}C-NMR data imply that conjugation has vanished. In the 5-substituted [1-phenyl-3-methyl-4-(1,2,3-triazolio)]-oxides *11b*, *11h*, and *11i*, as well as in [1-methyl-3-phenyl-4-(1,2,3-triazolio)]oxide *8a*, the ^1H- and ^{13}C-NMR data are consistent. The observed

discrepancies between the ^{1}H- and ^{13}C-NMR data of 4-(1,2,3-triazolio)oxides and -sulfides may be explained by assuming that the major factor influencing the *o*-phenyl proton shifts is anisotropy by the *C*-oxygen or *C*-sulfur bonds. Hence, phenyl groups adjacent to these bonds appear as multiplets in the ^{1}H-NMR spectra, independent of the extent of conjugation. The appearance of the *C*-phenyl group of the hindered [1,3-dimethyl-5-phenyl-4-(1,2,3-triazolio)] oxide *16p* as a multiplet is in keeping with this assumption. In the [1-phenyl-3-methyl-4-(1,2,3-triazolio)]oxides (*11*; Z = O) or -sulfide *11k* the net anisotropy effect on the *o*-phenyl protons is zero, yielding phenyl group singlets in the ^{1}H-NMR spectra, independent of the extent of conjugation.

In compounds with reduced heteroaromaticity, ring current effects become of minor importance and other factors may determine the appearance of the phenyl group ^{1}H-NMR signal. The phenyl groups of the hindered 1-methyl-2-phenyl-pyrazol-4-in-3-ones *6c*, *6g*, and *6h* and -thione *6k* appear as singlets in the proton spectra. In contrast, the hindered 5-substituted 1-phenyl-2-methyl-pyrazol-4-in-3-ones *10c*, *10g*, and *10h* exhibit phenyl group multiplets in the ^{1}H-NMR spectra, while ^{13}C-NMR data reveal that conjugation has vanished. Like in the 4-(1,2,3-triazolio)oxide series, the position of the phenyl group and not the extent of interannular conjugation determines the appearance of the phenyl group in the ^{1}H-NMR spectra. A comparison of the ^{1}H-NMR spectra of *10a* and its 4,2′,4′,6′-tetradeuterio derivative (see Experimental) proved that it is the *o*-protons of *10a* which are deshielded relative to the *m*- and *p*-protons. Most likely, this is the case, too, in the 5-substituted derivatives *10*; Z = O, and the pyrazol-4-in-3-thione *10k*. The reason for the deshielding of the *o*-protons in *10*; Z = O, and *10k* is not clear.

The 1-methyl-2-phenyl-1,2,3-triazol-5-one *9a* and -thione *9k* behave like the analogous 1-phenyl-2-methyl-pyrazol-4-in-3-ones and -thione (*10*; Z = O or S), exhibiting a phenyl group multiplet in the ^{1}H-NMR spectra.

1-Methyl-3-phenyl-imidazol-4-in-2-one *7a* and -thione *7k* both exhibit a phenyl group multiplet in the ^{1}H-NMR spectra, whereas the ^{13}C-NMR data reveal that interannular conjugation is extensive in *7a* but vanishes in *7k*.

CONCLUSION

The results reveal that ^{13}C-NMR spectroscopy can be used for assessing the extent of interannular conjugation in *C*- and *N*-phenyl substituted azoles with charged, zwitterionic, or heterocyclic rings with reduced aromaticity. Comparison of the ^{1}H- and ^{13}C-NMR data demonstrates that ^{13}C-NMR spectroscopy apparently provides unambiguous information about the extent of interannular conjugation even in cases where ^{1}H-NMR spectroscopy leads to erroneous results or in azole derivatives with reduced heteroaromaticity where ^{1}H-NMR, as expected, provides no information about the extent of conjugation.

The ^{13}C-NMR data indicate that extensive interannular conjugation is present in unhindered *N*-phenyl substituted imidazolium salts *2*, 1,2,3-triazolium salts *4*, and 4-(1,2,3-triazolio)oxides *16*; Z = O, or sulfides *16*; Z = S. The data further imply that a given substituent in the partly heteroaromatic pyrazol-4-in-3-ones *13*; Z = O, or 1,2,3-triazol-5-ones *9*; Z = O, impedes interannular conjugation much less than in heteroaromatic systems. Therefore, the heterocyclic rings of *13*; Z = O and *9*; Z = O are believed to adopt a twisted conformation (Fig. 2).

An oxygen atom adjacent to a phenyl group impedes interannular conjugation only slightly, even in the heteroaromatic systems *8*; Z = O. In contrast, however, a sulfur atom adjacent to a phenyl group impedes interannular conjugation strongly. The ^{13}C-NMR data seem to indicate that the partly aromatic heterocyclic rings of the thiones *6k*, *10k*, *7k*, and *9k* are planar or nearly so, in contrast to the oxygen analogues.

The ^{13}C-NMR data for the unhindered and hindered charged or zwitterionic azoles, and species with reduced heteroaromaticity are summarized in Table 6. When these data are combined with those previously measured for phenyl-substituted azoles with aromatic uncharged heterocyclic rings, it may be concluded that in any *N*-phenyl substituted pyrazole derivatives of the types studied interannular conjugation is extensive if $\delta_{C-2'} = 118.5 - 118.8$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 10.5$ ppm but strongly impeded if $\delta_{C-2'} = 124.6 - 129.1$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 1.3 - 4.0$ ppm. Intermediate values signify a smaller hindrance to conjugation. Similarly, interannular conjugation is extensive in

imidazoles if $\delta_{C-2'} = 121.0 - 121.7$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 8.6 - 9.4$ ppm. In *N*-phenyl-1,2,3-triazole derivatives interannular conjugation is extensive if $\delta_{C-2'} = 118.3 - 120.9$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 8.9 - 10.6$ ppm but impeded if $\delta_{C-2'} = 124.1 - 125.7$ ppm* and $\delta_{C-2'} - \delta_{C-3'} = 3.7 - 5.2$ ppm. If these data are further combined it appears that in any *N*-phenyl substituted azole of the types studied, interannular conjugation is extensive if $\delta_{C-2'} = 118.3 - 121.7$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 8.6 - 10.6$ ppm but strongly impeded if $\delta_{C-2'} = 124.1 - 129.1$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 1.3 - 5.2$ ppm. In *C*-phenyl substituted azoles interannular conjugation is extensive if $\delta_{C-2'} = 124.7 - 126.4$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 2.1 - 3.8$ ppm but impeded if $\delta_{C-2'} = 127.8 - 128.1$ ppm ** and $\delta_{C-3'} - \delta_{C-2'} = 0 - 0.8$ ppm. Since the values in case of interannular conjugation do not overlap with values in case of strongly impeded conjugation the ^{13}C-NMR method is a powerful method for assessing the extent of interannular conjugation, and hence for conformational analysis, of phenyl substituted azole derivatives. In addition, the values presented in Table 6 may be useful for distinguishing between positional isomers. For example, 1-phenyl-3,4-disubstituted 1,2,3-triazolium salts *4c* may be distinguished from the 1-phenyl-3,5-disubstituted isomers *4b*. Furthermore, the size and possibly the orientation of a substituent adjacent to a phenyl group may be evaluated.

The compounds studied in the present and preceding papers may be taken as representative for a bulk of other compounds. Thus, the results become generally applicable to all *C*- and *N*-phenyl substituted azole derivatives. In the light of the results obtained, an investigation of the use of the ^{13}C-NMR method for conformational analysis of phenyl substituted azines with neutral, charged, zwitterionic, or with nonaromatic heterocyclic rings seems obvious.

* Extreme values ($\delta_{C-2'} = 131.1$ ppm and $\delta_{C-3'} - \delta_{C-2'} = 2.3$ ppm) have been observed for 1-methyl-2-phenyl-1,2,3-triazolium bromide *5* in aqueous solution.

** An extreme value ($\delta_{C-2'} = 130.2$ ppm) has been observed for 1,3-dimethyl-2-phenyl-imidazolium bromide *3* in aqueous solution.

EXPERIMENTAL

^{1}H-NMR spectra were obtained on a Varian A-60 instrument. Position of signals are given in ppm (δ-values) relative to tetramethylsilane (TMS) when deuteriochloroform was used as the solvent. When deuterium oxide was used as the solvent 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal standard.

^{13}C-NMR spectra were obtained using 0.695 mmole of compound dissolved in 1.20 ml of solvent (10 mm tube), when not otherwise stated. When deuteriochloroform was used as the solvent position of signals were measured relative to the center peak of the deuteriochloroform triplet (δ 76.9 ppm) [25b] and are given in ppm (δ-values) relative to TMS. When deuterium oxide was used as the solvent position of signals were measured relative to *p*-dioxane (δ 67.4 ppm) [25b] and are given in ppm (δ values) relative to TMS. The ^{13}C-NMR spectra were obtained on a Bruker WH-90 instrument using Fast Fourier Transform pulse technique. Unless otherwise stated, 1 000 scans were accumulated with 6000 Hz sweep using 8K computer memory. This corresponds to an accuracy of ± 0.07 ppm in the chemical shifts and of ± 3 Hz in the coupling contants. The repetition time was 3.0 sec. The decoupled spectra were obtained using proton-noise-decoupling. The undecoupled spectra were measured by the gated decoupling technique.[36] in order to maintain part of the Overhauser enhancement of the signals. Thus, the proton-noise decoupling was interrupted after 1.0 sec. After a delay of 0.4 sec, the pulse (4 μsec) was turned on again. This cycle was repeated every 3.0 sec, 6000 scans being accumulated. Off resonance decoupled spectra [24c,37-41] were measured irradiating with a low power (2 Watt) continuous wave radio frequency at 800 Hz to high field of the chloroform proton signal.

Preparation of azolium bromides

1-Methyl-2-phenyl-pyrazolium bromide 1a. 1-Methyl-2-phenyl-pyrazolium tosylate [42] (1.00 g) was dissolved in water and passed through Amberlite IRA 400 ion exchanger (65 ml) regenerated with aqueous hydrogen bromide. The eluate was filtered through activated carbon. The water was removed *in vacuo* and the residue was recrystallized from methanol-ether. This gave 0.70 g (98 %) of 1-methyl-2-phenyl-pyrazolium bromide *1a* as colourless crystals, m.p. 174°. The NMR-spectrum was identical with that of the corresponding pyrazolium tosylate, except that the tosylate ion signal had disappeared.

Similarly, the pyrazolium bromides *1d*, *1e*, and *1f* were prepared from the corresponding tosylates,[18] and the 1,2,3-triazolium bromides *4a*, *4b*, *4c*, *4d*, and *4e* from their tosylates.[21,43,44] The 1,2,3-triazolium bromide *5* was prepared in

an analogous manner from the corresponding fluorosulfonate.[22] Finally, the imidazolium bromides *2a* and *3* were prepared in the same way from the corresponding tosylates.[44] In all cases the yield was 95 – 100 %. The purity was controlled by ^{1}H-NMR spectroscopy. Further purification and combustion analysis of all azolium bromides was omitted since all of the corresponding tosylates (or fluorosulfonates) are well characterized compounds.

4,2′,4′,6′-Tetradeuterio-1-phenyl-2-methyl-pyrazol-4-in-3-one. 1-Phenyl-2-methyl-pyrazol-4-in-3-one *10a*[18] (142 mg) and conc. dideuterio sulfuric acid (99 % enriched) (0.44 ml) were heated with stirring to 140° for 3 h. Deuterium oxide (4.4 ml) was then added and the solution was neutralized with potassium carbonate, freshly dried at 140° for 24 h. The solvent was then removed *in vacuo* and the residue was extracted with boiling chloroform (5 × 10 ml). After removal of the chloroform the treatment with dideuterio sulfuric acid was repeated. After the chloroform extraction and removal of the chloroform the residue was extracted with boiling ethyl acetate (5 × 10 ml). The solution was filtered through activated carbon and the ethyl acetate was removed affording 69 mg (47 %) of crude 4,2′,4′,6′-tetradeuterio-1-phenyl-2-methyl-pyrazol-4-in-3-one, m.p. 93 – 96°. Recrystallizations from ethyl acetate-hexane raised the melting point to 109 – 112°. A comparison of the ^{13}C-NMR spectra of the starting material *10a* and the tetradeuterio derivative showed that the latter compound was devoid of absorptions due to C-4 and C-4′. The signal due to C-2′ was strongly reduced. In contrast, the signals due to C-3, C-5, and C-3′ showed no loss in intensity. Replacement of hydrogen with deuterium at a carbon atom gives rise to a prolongation of the relaxation time, T_1, and hence to loss in intensity of the ^{13}C-signal.[45] Consequently, the ^{13}C-NMR data indicate that H-4, H-2′, H-4′, and H-6′ have been replaced with deuterium. The ^{1}H-NMR spectrum of the starting material *10a* exhibits a phenyl group multiplet at 7.2 – 7.6 ppm. In contrast, the ^{1}H-NMR spectrum of the tetradeuterio derivative exhibits two broad singlets at 7.58 and 7.55 ppm corresponding to H-5 and the two *m*-protons, respectively.

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Correlations between Pantothenate Uptake, Phospholipid Synthesis and Pantothenate-binding Protein Formation in *Pseudomonas fluorescens* P-2

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Correlations were investigated between increased pantothenate uptake and the formation of pantothenate-binding protein and phospholipids in *Pseudomonas fluorescens* P-2. Ethanolamine, choline, sarcosine, glyoxylate, and serine increased pantothenate uptake in whole cells, even in the presence of chloramphenicol. On the other hand, pantothenate, glycerol, and glucose were the most effective of the compounds investigated in synthesizing phospholipids both in whole cells and in membrane vesicles prepared from *Pseudomonas fluorescens* P-2. Although growth on ethanolamine, choline, and sarcosine was negligible over several hours, incorporation of ^{32}P continued linearly from the very beginning. The concentration of total phospholipids and phosphatidyl ethanolamine followed cell growth quite closely.

Pantothenate-binding protein was formed only in the presence of pantothenate and pantoate.

Correlation was observed between pantothenate uptake and formation of pantothenate-binding protein when the cells were cultured on pantothenate and pantoate, whereas phospholipid synthesis was rapid on any of several good energy sources.

Active transport systems of several B-group vitamins and related compounds and their regulation in micro-organisms have been reported.[1–7] It is sometimes difficult to determine the type of regulation operating in the accumulation of compounds, because regulation of intracellular concentrations of the vitamins as well as other metabolites involves control not only of their biosynthesis but also of entry and exit of the compounds. It has long been recognized that this process is rarely one of passive diffusion: it involves specific carrier- or binding-proteins having affinities for their substrates.[8] Furthermore, suggestions have been made that a lactose transport system might require the synthesis of membrane lipids.[9,10]

In previous studies we have found pantothenate transport activities in *Pseudomonas fluorescens* P-2 to increase during growth on glyoxylate, glycolate, and glycine, even when growth was inhibited with chloramphenicol.[7] The present work investigates whether there are correlations between pantothenate transport activities in the whole cells, phospholipid synthesis and the formation of pantothenate-binding protein during the growth of *Pseudomonas fluorescens* P-2.

METHODS

Organism and growth conditions. Pseudomonas fluorescens P-2 was cultured aerobically at 30°C in media containing, per litre, 1.35 g of KH_2PO_4, 0.26 g of $MgSO_4.7H_2O$, 3 mg of $FeSO_4.7H_2O$, 2.1 mg Na_2MoO_4, 1.5 mg of $MnSO_4$ and 10 mmol of a carbon source.[11] 0.66 g of $(NH_4)_2SO_4$ was added as a source of nitrogen when carbon compounds containing no nitrogen groups were used as energy sources. The media were adjusted to pH 7.0 and applied immediately.

The strain was maintained on slopes of a pantothenate agar medium. Turbidity measurements were made with a Klett-Summerson colorimeter employing filter 62.

Preparation of protoplasts and membrane vesicles. Protoplasts and membrane vesicles were prepared by the lysozyme-EDTA method of Kaback.[12] Cells, taken from different growth

phases (20 mg of dry weight), were centrifuged at 10 000 *g* for 30 min and washed twice with 10 mM Tris-HCl (pH 8.0). The washed cells were suspended in 30 mM Tris-HCl buffer containing sucrose (20 %), dipotassium magnesium EDTA (15 mM), and lysozyme (0.5 mg/ml, Sigma Chemical Company, St. Louis, Mo., U.S.A.) and incubated for 20 min at 25°C. Protoplasts were collected by centrifugation for 15 min at 16 000 *g*. Lysis of protoplasts was produced by osmotic treatment in the presence of DNase II (Sigma Chemical Company, St. Louis, Mo., U.S.A.). Incubations were carried out at 30°C and centrifugations at 27 000 *g*. The purity of membrane vesicles was established by electron and phase contrast microscopy methods.

Measurement of pantothenate uptake into whole cells. An assay method for [1-^{14}C]-labelled pantothenate uptake into whole cells was described earlier by Mäntsälä.[13] The initial velocities of pantothenate uptake were determined by incubation of the cells (250 μg of dry weight), [1-^{14}C]-pantothenate (75 000 cpm, specific activity 4.75 mCi/mmol, The New England Nuclear Corporation, Boston, Mass., U.S.A.), potassium pantothenate (0.05 μmol) and $MgSO_4.7H_2O$ (0.5 μmol) in 0.2 ml of the reaction mixture at 30°C for various reaction times.

Measurement of pantothenate uptake into membrane vesicles. In total volume of 0.2 ml, the reaction mixtures contained 4 μmol of $MgSO_4.7H_2O$, 10 μmol of potassium phosphate buffer (pH 6.6), 75000 cpm of [1-^{14}C]-pantothenate, 0.01, μmol of potassium pantothenate, of 4 μmol ascorbate, 0.02 μmol of phenazine methosulfate, and 0.16 mg of membrane protein. Reactions were terminated by addition of 2 ml of 0.2 M NaCl, and membrane vesicles were washed immediately with 8 ml of 0.2 M NaCl on Millipore membrane filters (0.3 μm pore-size). The filters were dried and activities were measured in a scintillation spectrometer.

Phospholipid extraction and estimations. Samples (1 mg of dry weight) were taken at intervals during growth and centrifuged at 5000 *g*. The cell pellets were washed with phosphate-free medium and recentrifuged. The samples of whole cells and membrane vesicles (as prepared above) were inoculated into 20 ml of media containing about 2 μCi of [^{32}P]-labelled phosphate/ml, (The Radiochemical Centre, Amersham, U. K.), 10 μmol of potassium phosphate and other compounds, as described in the "Organism and growth conditions section". After incubation at 30°C, the samples were precipitated with 2 ml of cold 50 % TCA, filtered and washed twice with 3 ml of 5 % TCA and 3 ml of distilled water on a Millipore filter (0.3 μm pore-size). The filters were then dried and placed in extraction vessels from which phospholipids were eluted with 5 ml of a methanol-chloroform solution (1:2, by vol). The extraction was repeated twice at room temperature overnight. The solutions were poured into scintillation vials, the solvent removed *in vacuo* and activities counted in toluene-based scintillation fluid.

Chromatography was carried out on glass plates with a layer of silicic acid. Two-dimensional separations were made. The first solvent used was chloroform-methanol-water (70:30:5 by vol) and the second chloroform-methanol-7 M NH_4OH (60:35:5 by vol). After development the plates were sprayed with 0.2 % ninhydrin and then with molybdenum reagent.[14] The standard phospholipids were purchased from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, U.K. For quantitative determination of phosphatidyl ethanolamine and phosphatidyl glycerol, the spots were scraped from the plates and the phospholipids were eluted with 5 ml of scintillation fluid.

Protein was determined by the method of Lowry *et al.*[15]

Preparation of crude extract and shock fluid. Crude cell extracts were prepared by freezing and thawing as described by Mäntsälä and Nurmikko,[16] and shock fluid by the procedure of Neu and Heppel.[17] Samples (80 mg of dry weight) were taken from the cultures at different phases of growth. The shock fluid was concentrated by lyophilization.

Assay of pantothenate-binding activity. Binding activity towards crude extracts and shock fluids were assayed as described earlier[13] and by equilibrium dialysis. The dialysis sacs containing binding material (2 mg of protein) were shaken for 24 h at 4°C in tubes containing 600 000 cpm of [1-^{14}C]-pantothenate in 0.1 ml. After dialysis the samples were pipetted onto Millipore filters, dried and counted in a scintillation spectrometer.

RESULTS

Pantothenate transport into whole cells and membrane vesicles of Pseudomonas fluorescens *P-2.* In a previous study the uptake of pantothenate into whole cells of *Pseudomonas fluorescens* P-2 was found to increase in the presence of glyoxylate, glycolate, and glycine, even when growth was inhibited with chloramphenicol, but not in the absence of nitrogen.[7] Ethanolamine, choline, sarcosine, and serine without added pantothenate have now also been found to increase pantothenate transport activities (Fig. 1). As can be seen, while the most effective compounds in the early phases of growth are glyoxylate and ethanolamine, after several hours pantothenate and pantoate become the most effective. Activities begin to decrease on glyoxylate, ethanolamine, choline,

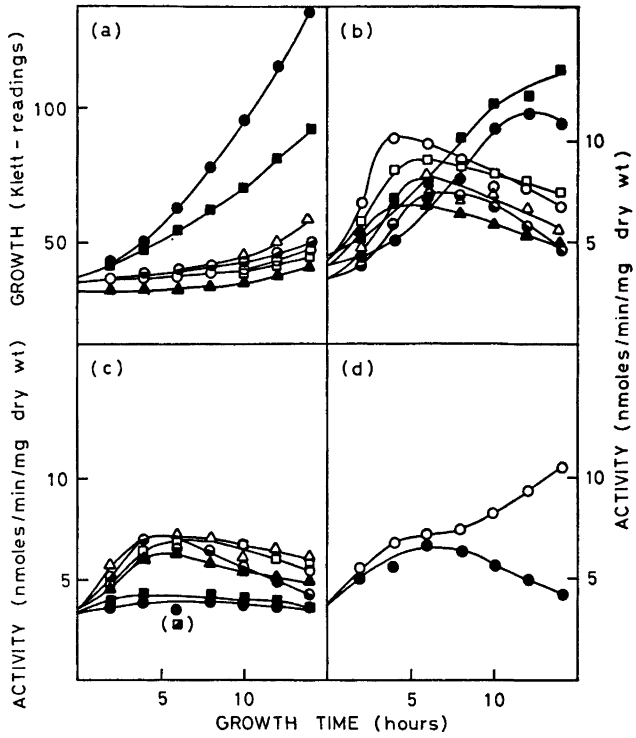

Fig. 1. Effect of pantothenate, pantoate, glyoxylate, ethanolamine, choline, serine, and sarcosine on pantothenate transport activity in *Pseudomonas fluorescens* P-2. Pre-cultivations were carried out on pantothenate. The reaction mixture contained [1-^{14}C]-pantothenate (75 000 cpm, specific activity 4.75 mCi/mmol), potassium pantothenate (0.05 μmol), $MgSO_4.7H_2O$ (0.5 μmol) and about 250 μg dry wt cell suspension in 0.2 ml. The reaction mixture was incubated at 30° and reactions were terminated at intervals of one minute by washing the cells on Millipore filters with 10 ml of 0.2 M NaCl solution.
(a) Turbidity of the culture (Klett-Summerson colorimeter, filter 62). The concentrations of the compounds were 10 mM. ●, pantothenate; ■, pantoate; ○, glyoxylate; □, ethanolamine; △, choline; ◒, serine; ▲, sarcosine.
(b) Specific activity of pantothenate transport. The symbols are the same as above.
(c) Specific activity of pantothenate transport in the presence of chloramphenicol (200 μg/ml of the culture). The symbols are the same as above.
(d) Specific activity of pantothenate transport. ○, ethanolamine + pantothenate; ●, ethanolamine + pantothenate + chloramphenicol.

sarcosine, and serine during the exponential phase of growth. In these cases moreover, the rise in transport activities was not prevented by added carbon compounds.

Membrane vesicles prepared from cells taken from the lag, acceleration, exponential, and stationary phases of growth on ethanolamine, choline, sarcosine, and serine accumulated [1-^{14}C]-pantothenate almost equally and without dependence on the growth phase (Fig. 2). Accumulation of pantothenate, negligible without an added electron donor system, is increased about 5 – 10 times when an artificial electron donor system, ascorbate-phenazine methosulfate, is used.

Incorporation of ^{32}P into phospholipid fractions of whole cells, protoplasts and membrane vesicles. Although ethanolamine, choline, sarcosine, and serine are the most effective compounds on pantothenate uptake into whole cells (Fig. 1), incorporation of ^{32}P is most rapid into cells grown on pantothenate, glycerol, and glucose and, in general, on compounds that allow rapid growth (Fig. 3). However, although growth

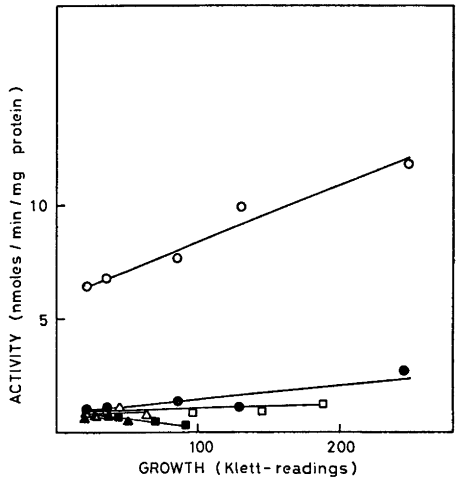

Fig. 2. Accumulation of [1-^{14}C]-pantothenate in membrane vesicles from *Pseudomonas fluorescens* P-2 grown on ethanolamine, choline, sarcosine, and serine. The reaction mixtures contained in a total volume of 0.2 ml, 4 μmol of $MgSO_4.7H_2O$, 10 μmol of potassium phosphate buffer (pH 6.6), 75 000 cpm of [1-^{14}C]-pantothenate, 0.01 μmol of potassium pantothenate and about 0.16 mg of membrane protein. Ascorbate-phenazine methosulfate was used as an artificial electron donor. Other experimental conditions were the same as in the legend to Fig. 1.
○, pantothenate (the reaction mixture contained ascorbate + phenazine methosulfate); ●, pantothenate; △, ethanolamine; □, choline; ▲, sarcosine; ■, serine.

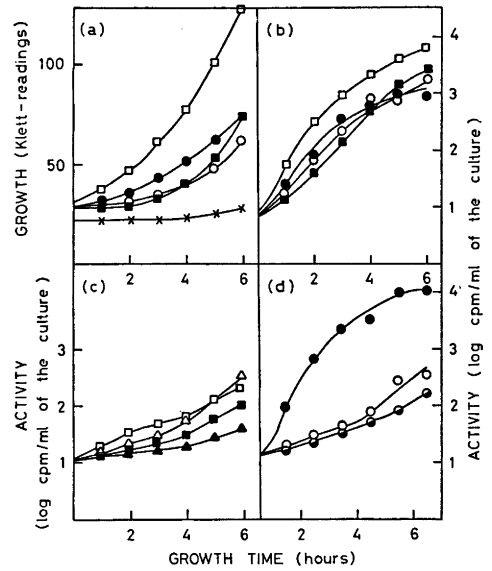

Fig. 3. Incorporation of ^{32}P into phospholipids of *Pseudomonas fluorescens* P-2. The reaction mixture contained 2 μCi [^{32}P]-labelled phosphate/ml, 10 μmol of potassium phosphate, 1 mg (dry weight) of the cell pellet and other compounds as described in the "Organism and growth conditions" section. The reaction mixtures were incubated at 30° and the samples were precipitated at intervals of 10 min with 2 ml of cold 20 % TCA, filtered and washed twice with 3 ml of 5 % TCA on Millipore filters (0.3 μm pore-size). The filters were then dried and phospholipids were extracted with methanol-chloroform solution (1:2, by vol).
(a) Turbidity of the culture. The concentrations of the compounds were 10 mM (Casamino acids 0.5 %). □, Casamino acids; ●, pantothenate; ○, glycerol; ■, glucose; ×, ethanolamine, choline, serine, sarcosine or glyoxylate.
(b) ^{32}P incorporation activity. The symbols are the same as above.
(c) ^{32}P incorporation activity. ■, ethanolamine; △, choline; □, serine; ▲, sarcosine.
(d) ^{32}P incorporation activity. ●, glyoxylate + pantothenate; ○, glyoxylate + 5 mM $(NH_4)_2SO_4$; ◒, glyoxylate without ammonium sulfate.

is almost negligible for several hours on ethanolamine, choline, sarcosine, and serine, incorporation of ^{32}P into phospholipids increases from the very beginning. When the growth reaches the acceleration phase, ^{32}P incorporation into phospholipid fractions also increases.

The results given in Table 1 are in good agreement with those presented in Fig. 3. Protoplasts and membrane vesicles prepared from the cells grown on pantothenate, glycerol, and glucose incorporate [^{32}P]-labelled phosphate more rapidly than the protoplasts and membrane vesicles prepared from the cells grown on ethanolamine, choline, sarcosine, and serine.

Because no correlation between total phospholipid synthesis and pantothenate uptake was observed when bacteria were cultured on glyoxylate, ethanolamine, choline, sarcosine, and serine, the synthesis of individual phospholipids during growth was investigated. It can be seen that concentrations of phosphatidyl ethanolamine are high throughout all growth phases in whole cells and parallel total incorporation of ^{32}P very closely (Fig. 4). In contrast, the synthesis of phosphatidyl glycerol, another common phospholipid in *Pseudomonas fluorescens* P-2, ceases very rapidly when the cells are transferred from pantothenate or

Table 1. Incorporation of [^{32}P]-labelled phosphate into phospholipids of protoplasts and membrane vesicles from *Pseudomonas fluorescens* P-2. Protoplasts and membrane vesicles were prepared by the method of Kaback (1971) [12] as described in the "Preparation of protoplasts and membrane vesicles" section. Activities were determined as described in the legend to Fig. 3.

Growth	[^{32}P] Incorporation (log cpm/mg protein)													
Klett$_{62}$	Pantothenate		Glucose		Glycerol		Ethanol-amine		Choline		Serine		Sarcosine	
	P[a]	M[a]	P	M	P	M	P	M	P	M	P	M	P	M
30	3.10	2.40	2.90	2.30	2.94	2.30	2.40	2.18	2.36	2.25	2.40	2.14	2.26	2.10
40	3.28	–	3.10	–	3.30	–	2.68	2.44	2.52	–	2.63	–	2.26	2.26
50	3.44	3.10	3.26	2.90	3.62	2.34	2.90	2.68	2.88	2.60	2.40	2.10	2.56	2.38
70	3.62	–	3.48	–	3.84	–	–	–	3.10	–	2.90	2.65	–	–
120	3.90	3.60	3.70	3.33	4.12	3.76	–	–	3.44	2.90	–	–	–	–
180	3.67	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Abbreviations: P = protoplasts; M = membrane vesicles.

Table 2. Incorporation of [^{32}P] labelled phosphate into phosphatidyl ethanolamine and phosphatidyl glycerol of protoplasts and membrane vesicles from *Pseudomonas fluorescens* P-2. The experimental conditions were the same as in the texts of Table 1 and Fig. 4.

Growth	[^{32}P] Incorporation (log cpm/mg protein)											
Klett$_{62}$	into phosphatidyl ethanolamine						into phosphatidyl glycerol					
	Pantothenate		Ethanolamine		Serine		Pantothenate		Ethanolamine		Serine	
	P[a]	M[a]	P	M	P	M	P	M	P	M	P	M
30	1.91	2.41	1.72	1.74	2.04	2.10	1.21	1.41	1.27	1.13	1.44	1.41
35	2.20	–	2.10	2.48	2.37	2.40	1.23	1.30	1.18	1.20	1.47	1.44
50	2.63	2.83	2.73	3.06	2.79	2.56	1.42	–	1.41	1.56	1.63	1.76
100	3.10	–	–	–	–	–	1.77	1.64	1.65	–	–	–
160	2.94	3.14	–	–	–	–	1.83	–	1.70	–	–	–

[a] Abbreviations: P = protoplasts; M = membrane vesicles.

glycerol media into ethanolamine, choline, sarcosine, or serine media. Phosphatidyl glycerol synthesis accelerates again when growth continues. At the end of the exponential phase of growth, phosphatidyl glycerol synthesis ceases and some degradation of the phospholipid is observed.

Table 2 shows the results of ^{32}P incorporation into phosphatidyl ethanolamine and phosphatidyl glycerol in protoplasts and membrane vesicles. The results resemble those obtained for whole cells (Fig. 4).

Formation of pantothenate-binding activity during growth on pantothenate, pantoate, ethanolamine and choline. Pantothenate-binding activity follows growth only in pantothenate and pantoate media (Fig. 5). In the course of two generations, the cells grown on pantothenate and pantoate lose their binding activity on ethanolamine and choline both in the shock fluids and the crude extracts. These results suggest the inducible nature of the pantothenate-binding protein and that there is a correlation between pantothenate transport activities in whole cells and pantothenate-binding activity on pantothenate and pantoate but not on ethanolamine and choline.

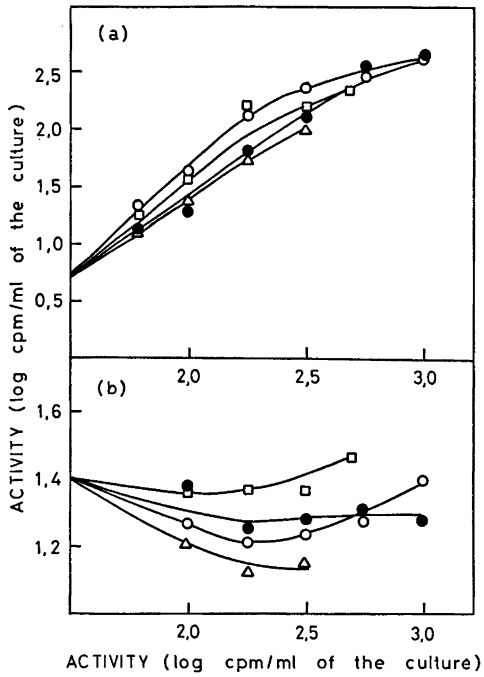

Fig. 4. Relative amounts of phosphatidyl ethanolamine and phosphatidyl glycerol in *Pseudomonas fluorescens* P-2 cells. Phosphatidyl ethanolamine and phosphatidyl glycerol were separated and quantitatively determined by thin-layer chromatography, radioactivity of the spots being measured in a scintillation spectrometer.
(a) ^{32}P incorporation in phosphatidyl ethanolamine *versus* ^{32}P incorporation in total phospholipids. ○, ethanolamine; □, choline; ●, serine; △, sarcosine. (b) ^{32}P incorporation in phosphatidyl glycerol *versus* ^{32}P incorporation in total phospholipids. The symbols are the same as above.

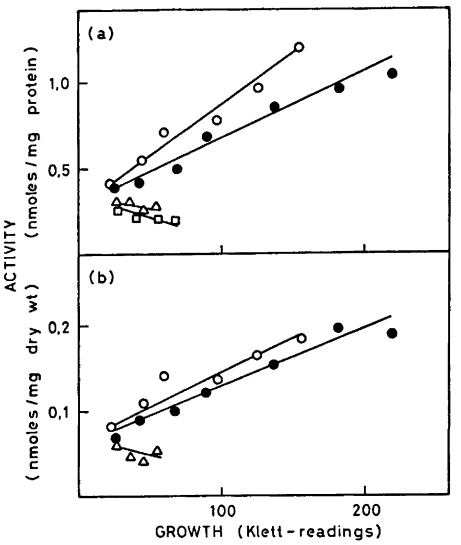

Fig. 5. Formation of pantothenate-binding protein during the growth of *Pseudomonas fluorescens* P-2. Pantothenate-binding activity was measured by gel filtration and equilibrium dialysis. The dialysis sacs contained 2 mg of protein and dialysis tubes 600 000 cpm of [1-^{14}C]-pantothenate (specific activity 4.75 mCi/mmol).
(a) Binding activity in shock fluid. ○, pantoate. ●, pantothenate; △, ethanolamine; □, choline (b) Binding activity in crude extracts. The symbols are the same as above.

DISCUSSION

Although the data presented in this paper demonstrate that there are no correlations between pantothenate transport activities in whole cells and phospholipid synthesis, they do reveal some general properties of the transport process and phospholipid synthesis in *Pseudomonas fluorescens* P-2. It has been found that pantothenate transport is an inducible process requiring cell multiplication.[7] The results concerning phospholipid synthesis in *Ps. fluorescens* P-2 are in good agreement with those of Ballesta and Schaechter[18] for *E. coli*. They found that the total phospholipid synthesis as well as the synthesis of phosphatidyl ethanolamine, the main phospholipid component, is related to growth and cell division. This may be due to the fact that phosphatidyl ethanolamine is normally rather stable compared, for instance, with phosphatidyl glycerol.[18–20] The linear incorporation of ^{32}P into phospholipids at the beginning of growth on the compounds mentioned in Fig. 3 probably reflects a turnover of phospholipids rather than a net synthesis because the transfer of the cells from a richer to a poorer medium causes strong inhibition of phospholipid metabolism. Concentrations of phosphatidyl glycerol decrease about 40 % during 4 – 6 h growth on ethanolamine, choline, sarcosine, and serine. These results and those of Ballesta and Schaechter[18] suggest that the metabolism of phosphatidyl glycerol is related not to growth and cell

division but to other cell processes. Cronan [21] reported that the phospholipid composition of the cells was altered when cultures of *E. coli* progressed from exponential growth to the stationary phase. Bertsch *et al.*[22] reported similar findings for *Bacillus magaterium* cells. When cells of *Pseudomonas fluorescens* are transferred from a rich medium to a poor one some phospholipids may be altered, thus preventing phospholipid degradation in the new growth conditions.

Several reports suggest a close relationship between the transport system and binding-protein.[8,23–26] Correlations were observed between pantothenate transport and formation of binding-protein in this work as well.

The finding that pantothenate uptake in *Pseudomonas fluorescens* P-2 is an inducible process [7] implies the possibility that growth on ethanolamine, choline, sarcosine, and serine stimulates the synthesis of pantothenate-binding protein. Nevertheless, binding activities remain low, at least on ethanolamine and choline. Unpublished results in our laboratory suggest that pantothenate uptake into membrane vesicles from *Pseudomonas fluorescens* P-2 is stimulated (except in the presence of the artificial electron donor system, ascorbate-phenazine methosulfate) in the presence of ethanolamine, choline, and sarcosine too. On the other hand, it is not clear whether the above-mentioned compounds can serve as direct electron donors in whole cells as well. Further results concerning pantothenate transport into membrane vesicles from *Pseudomonas fluorescens* P-2 will be published later.

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A Novel Iridoid Glucoside Isolated from *Lamium album* L.

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Whole plants of white dead-nettle (*Lamium album* L., Labiatae) contain, as the major glucoside, a novel iridoid. Its structure *1* is assigned mainly on the basis of PMR-data for the glucoside itself, its acetates, and bis-benzylidene derivatives. The aglucone, set free by hydrolysis with emulsin, is characterized as a triacetate. Proton coupling constants are utilized for estimating the most favoured conformations of the individual compounds. The results are in accord with those expected from considerations of steric interactions.

The genus *Lamium* is rich in iridoid glucosides.[1,2] In the white dead-nettle, *L. album* L. we have found the major glucoside to be a novel iridoid for which we propose the name lamiridoside** (for data, see Tables 1 and 3).

The PMR-spectrum of lamiridoside (*1*, see Table 1) exhibits absorptions typical for iridoid glucosides possessing a C-4 methoxycarbonyl grouping. Signals at 7.53, 5.68, and 3.82 ppm can be ascribed to H-3, H-1, and $COOCH_3$, respectively.[2] The doublet character ($J = 0.8$ Hz) of the signal at 7.53 ppm signifies the presence of a proton at C-5, a novelty in so far as all formerly known iridoids from *Lamium* species carry a hydroxy group at C-5.[1] Irradiation at 7.53 and 5.68 ppm ($J = 1.5$ Hz) revealed absorptions attributable to the H-5 and H-9 protons at 2.97 ppm and 2.89 ppm, respectively. A signal at 4.10 ppm was assigned to H-6 since double resonance experiments showed it to couple with both H-5 ($J = 3$ Hz) and another proton absorbing at 3.72 ppm ($J = 4.5$ Hz), *viz.* H-7. The last signal arising from the non-sugar moiety, a three-proton singlet at 1.27 ppm, could be assigned to a methyl group positioned at a tertiary carbon carrying an

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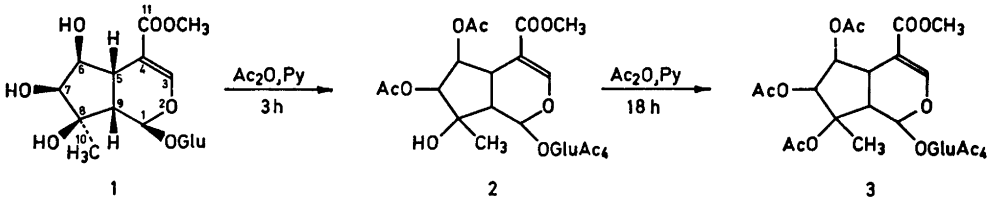
** See note, at the end of this paper.

Table 1. PMR-data[a].

Compound	δ-Values in ppm from internal TMS[b]								Coupling constants (Hz)[c]				
	H_1	H_3	H_5	H_6	H_7	H_9	CH_3-10	OCH_3	$J_{6,7}$	$J_{5,9}$	$J_{5,6}$	$J_{3,5}$	$J_{1,9}$
1	5.68	7.53	2.97	4.10	3.72	2.89	1.27	3.82	4.5	11	3	0.8	1.5
2	5.53	7.39	3.09	5.29	4.97	2.93	1.32	3.70	5	11	4	1.3	1.5
3	5.73	7.41	3.06	5.42	5.51	3.26	1.57	3.73	4.5	11	3.5	1.0	1.0
4	5.19	7.49	3.24	4.00	4.25	2.65	1.44	3.74	5	8	8	1	7.5
5	5.50	7.5	3.30	5.42	4.36	2.81	1.47	3.74	6	8.5	4	1.5	4
6	5.58	7.38	3.08	4.74	4.08	2.79	1.27	3.75	6	10	0.5	2	< 0.5
7	5.59	7.45	3.02	4.68	4.81	3.16	1.53	3.77	5.5	10	0.5	1.5	< 0.5
8	5.33	—	3.55	5.05	4.33	2.56	1.52	3.71	5.5	8	8	—	7.5
9	5.58	7.39	3.06	4.80	4.06	2.71	1.20	3.77	5	11	1	2	1
10	5.60	—	3.05	4.79	4.85	3.05	1.48	3.79	4.5	—	< 0.5	—	< 0.5
12	6.37	7.41	3.18	5.28	5.00	2.81	1.33	3.74	5	11	4	1.3	1.9

[a] The spectra were recorded on a Varian HA-100 instrument in $CDCl_3$, with TMS as an internal reference, except for *1*, recorded in D_2O, with DSS as a reference. [b] ± 0.02 ppm. [c] ± 0.5 Hz.



1 2 3

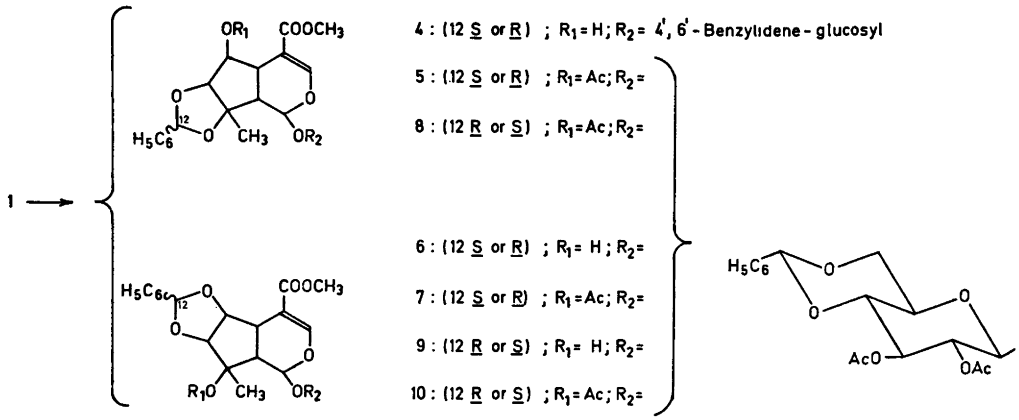
oxygen atom. Taken together, these data support the gross structure *1* (disregarding stereochemistry) for lamiridoside. In keeping herewith, acetylation afforded a hexaacetate *2* or a heptaacetate *3*, depending on conditions. The PMR-spectra of the acetates (Table 1) provided additional evidence for the proposed structure. Thus, acetylation of the tertiary hydroxyl group caused low field shifts of the absorptions of the neighbouring protons, the signals arising from H-7, H-9, and CH_3-10 being shifted 0.54, 0.33, and 0.25 ppm, respectively.

Assuming configurational similarity of lamiridoside with other iridoids,[2] H-1, H-5, and H-9 can be placed in α-, β-, and β-positions, respectively (*vide infra*), leaving the configurations at the three carbinol centers in the five-membered ring undecided. The coupling constants $J_{5,6}$ and $J_{6,7}$ of *1*, *2*, and *3* are of little help here, since they all fall within the range of 3 – 5 Hz, and hence are compatible with a *cis*- as well as a *trans*-junction.[4,5]

Other means were therefore sought to settle the stereochemistry. Treatment of *1* with benzaldehyde and zinc chloride yielded a mixture of bis-benzylidene derivatives. The PMR-spectrum of this mixture showed, within the region of the benzylic protons (5.5 – 6.5 ppm), a broad singlet attributable to the 4′,6′-benzylidene-glucosyl moiety and four different signals of varying intensity at lower field, signifying the presence of at least four isomers in the reaction mixture. From this mixture all four isomers were isolated either as such, or in acetylated forms.

Chromatography of the mixture afforded the amorphous compound *4*, converted upon acetylation under mild conditions into the crystalline triacetate *5*. A pronounced shift to low field (1.42 ppm) of the H-6 signal on passing from *4* to *5*, when compared to those of H-7 and CH_3-10 (0.11 and 0.03 ppm, respectively), securely positions the second benzylidene group at C-7 and C-8 in these compounds.

Acetylation under mild conditions of the remaining mixture afforded, after chromatography, a crystalline diacetate *6*. Acetylation of *6* under forcing conditions produced the triacetate *7*. On comparing the PMR-spectra of *6* and *7*, it appears that the low field shifts of H-7, H-9, and CH_3-10 (0.73, 0.37, and 0.26 ppm, respectively) are similar to those found by going from *2* to *3*. Consequently, *6* and *7* can be formulated as 6,7-benzylidene derivatives.



Additional to *6*, another crystalline compound could be isolated from the remaining mixture, namely the triacetate *8*, formulated as a 7,8-benzylidene compound mainly because of the low field position of H-6 (5.05 ppm), suggesting the presence of an acetoxy group at C-6.

The mother liquor from the crystallization of *8* was rich in the diacetate *9*, which was not obtained in the pure state (only a PMR-spectrum was recorded of this derivative). Acetylation of the latter, under forcing conditions, yielded the triacetate *10*. Comparison of the PMR-spectra of *9* and *10* revealed that H-7, H-9, and CH_3-10 were shifted 0.79, 0.34, and 0.28 ppm, respectively, towards lower field, shifts similar to those observed on going from *2* to *3*, and from *6* to *7*. Consequently, *9* and *10* must be 6,7-benzylidene compounds and hence epimers of *6* and *7*, respectively.

Since both 6,7- and 7,8-benzylidene compounds are formed in the reaction with benzaldehyde, it follows that all the hydroxy groups of the iridoid moiety in lamiridoside are positioned on the same face of the molecule.[3] The remaining problem, *viz.* on which side, is resolved by consideration of the coupling constants $J_{5,6}$ for the compounds *6*, *7*, *9* and *10*. The consistently low value of $J_{5,6}(\leq 1$ Hz) demands a dihedral angle close to 90° between H-5 and H-6, necessitating a *trans* relationship of these protons.[4,5] Consequently, the hydroxy groups at C-6, C-7, and C-8 in lamiridoside are all occupying β-positions.

Hydrolytic cleavage of *1* with emulsin afforded glucose, identified by paper chromatography with authentic glucose as a reference, and, in addition, the amorphous aglucone *11*, which was converted, under mild conditions, into a triacetate (*12*). By comparison of the PMR-spectra of *2* and *12*, the stereochemistry at C-1 in *12* appears to be identical with that of lamiridoside and its derivatives. The small value of $J_{1,9}$ (1.9 Hz) in *12* clearly places H-1 in an equatorial position, an arrangement prevailing also in the other derivatives, except in *4*, *5*, and *8*. In the latter, the overall conformation of the dihydropyrane ring is obviously altered, with H-1 assuming more or less an axial position, resulting in an increased value of the coupling constant $J_{1,9}$. This change in conformation is accompanied by low field shifts of H-5 and CH_3-10, and shifts to higher field of H-9. Assuming an α-situated acetoxy group at C-1 in *12*, an equatorial H-1 would require a conformation similar to that prevailing in *4*, *5*, and *8*; this is definitely not in accord with the observed PMR-data.

Turning to the glucose moiety of lamiridoside, the PMR-spectra of *1* and its derivatives clearly show the presence of a pyranose ring, attached to the aglucone through a β-glucosidic linkage, as evidenced by the large coupling constant $J_{1'2'}$ (7.5 Hz) observable in *1* and *4*.

In the above conclusions, the presence of a *cis*-fused ring system was assumed. Inspection of a Dreiding model shows the alternative *trans*-fused system to be far more strained, permitting only a single conformer for each of the two *trans*-fused forms. In both of these, the cyclopentane ring will adopt a twist form, namely $^5T_9$ for the 5β,9α- and $^9T_5$ for the 5α,9β-fusion.[6] These conformations demand dihedral angles between H-5 and H-6 of 170° or 20°, respectively, incompatible with the observed coupling constants ranging from 0.5 to 8 Hz. Moreover, the ring strain should be reflected in a shift of the UV-maximum when compared with that of the known *cis*-fused compounds, all absorbing within the 230 – 240 nm range.[2] Again, the compounds *1*, *2*, *3*, and *12* all exhibit UV-absorptions within the range 232 – 238 nm, in accord with the presence of *cis*-fused rings.

PMR has recently been shown to be of value in assessing the most favoured conformations of five-ring systems like furanosides.[6,7] Applying the treatment given by Hall *et al.*[6] for furanosides to the cyclopentane ring in the iridoid skeleton, we arrive at reasonable conclusions for lamiridoside and its derivatives. In the "cycle of pseudorotation" (*Cyclops*) of interchanging envelope (*V*) and twist (*T*) forms for this system,[6] we have substituted C-8, C-7, C-6, C-5, and C-9 with the corresponding atoms in the furanoses, *viz.* O, C-1, C-2, C-3, and C-4, respectively, and adopted the angle values measured by Hall *et al.* The error introduced by this approximation is considered to be negligible in the present approach.

The coupling constants listed in Table 1 can be used to divide the compounds into four groups as specified in Table 2 (*10* cannot be classified since $J_{5,9}$ is unknown because H-5 and H-9 have closely similar chemical shifts).

Table 2. Favoured conformers and data for estimation of these.

Group (compounds)	$J_{measured}$	Calculated dihedral angle ± 10°	Favoured conformers	Dihedral angle in favoured conformers
A (*1, 2, 3, 12*)	$J_{6,7}$ 4.5 – 5	30 – 55	^7V $^7T_8$	50
	$J_{5,6}$ 3 – 4	115 – 145		140 – 150
	$J_{5,9}$ 11	0 – 10		0 – 20
B (*4, 8*)	$J_{6,7}$ 5 – 5.5	25 – 50	V_6 $^7T_6$	50 – 60
	$J_{5,6}$ 8	150 – 170		170
	$J_{5,9}$ 8	10 – 30		20 – 30
C (*5*)	$J_{6,7}$ 6	20 – 45	V_8 $^7T_8$	30 – 50
	$J_{5,6}$ 4	120 – 145		120 – 140
	$J_{5,9}$ 8.5	10 – 30		20 – 30
D (*6, 7, 9*)	$J_{6,7}$ 5 – 6	25 – 50	V_7 $^6T_7$	50 – 60
	$J_{5,6}$ 0.5 – 1	65 – 120		70 – 90
	$J_{5,9}$ 10 – 11	0 – 10		0 – 20

Table 3. Optical rotations and elemental analyses of lamiridoside and derivatives.

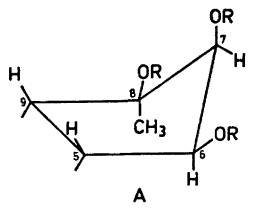
Compound	$[\alpha]_D^{21}$ (*c*, EtOH)	Formula	Analyses Calculated C	Calculated H	Found C	Found H
1	– 132(0.4)	$C_{17}H_{26}O_{12}$. $0.75H_2O$	46.85	6.36	46.83	6.33
					46.95	6.39[b]
2	– 107(0.6)	$C_{29}H_{38}O_{18}$	51.53	5.67	51.86	5.52
3	– 83(0.3)	$C_{31}H_{40}O_{19}$	51.96	5.63	51.82	5.72
4	– 104(0.3)	$C_{31}H_{34}O_{12}.H_2O$	60.39	5.89	60.67	5.81
					60.63	5.84[b]
5	– 142(0.1)	$C_{37}H_{40}O_{15}$	61.32	5.56	61.11	5.63
6	– 162(0.4)	$C_{35}H_{38}O_{14}$	61.57	5.61	61.40	5.68
7	– 122(0.9)	$C_{37}H_{40}O_{15}$	61.32	5.56	61.07	5.64
8	– 115(0.6)[a]	$C_{37}H_{40}O_{15}$	61.32	5.56	61.02	5.41
10	– 152(0.4)	$C_{37}H_{40}O_{15}$	61.32	5.56	61.51	5.71
11	– 108(0.2)	$C_{17}H_{22}O_{10}$	52.85	5.74	52.96	5.90

[a] In $CHCl_3$. [b] After additional drying.

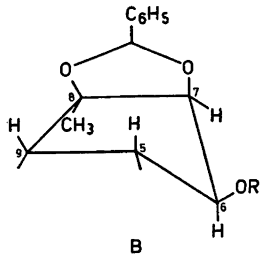
Using Karplus relationship * for calculating the dihedral angles and arbitrarily adding and subtracting 10° to the values found, a conservative estimate of the angles is obtained (se Table 2). Comparing the values with those derived from the *Cyclops* sequence and eliminating conformers which do not fit, first for $\angle H_6H_7$, then for $\angle H_5H_6$ and $\angle H_5H_9$, only a small number of conformers are left for each group of compounds (see Table 2).

* We have used the curve as given by Williams and Fleming.[8]

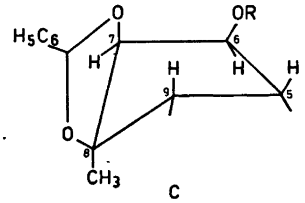
Thus, group A, comprising the compounds *1*, *2*, *3*, and *12*, is found preferentially to adopt the ^7V and $^7T_8$ conformations. Inspection of models of these conformers leaves only one possibility for the conformation of the dihydropyrane ring, namely a distorted half chair (HC_9^1) with an axial orientation of the glucose moiety and $\angle H_1H_9$ close to 70°. In the ^7V conformation (depicted as *A*) the interactions between the bulky groups are minimal, the oxygen substituents on C-6 and C-8 being pseudo-equatorial and that on C-7 together with the methyl group on C-8 pseudo-axial.



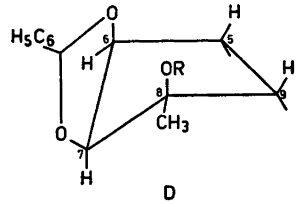
A



B



C



D

Group B, consisting of the 7,8-benzylidene compounds *4* and *8*, is found to favour the V_6 and ${}^7T_6$ conformers, of which V_6 is depicted as *B*. Again, this conformation introduces only minimal steric interactions of the bulky groups on the five-membered ring. The dihydropyrane ring in this case, however, is in an almost planar conformation, save for C-1 pointing downwards and giving rise to an equatorial position of the sugar moiety.

In group C, solely made up of *5* (the triacetate of *4*) the conformation of the molecule is less obvious. The above treatment leads to two sets of possible five-ring conformers, *viz.* ${}^8V$ and $V_8-{}^7T_8$. Models indicate, however, that the former requires an $\angle H_1H_9$ of either 180° (HC_9^1) or 90° ($Boat_{0,5}$), the latter agreeing with that calculated for *5*, namely 120 – 140°. Consequently, the ${}^8V$ conformation can be disregarded. The change observed in going from *4* to *5* is not easily explained, as a larger steric interaction prevails in $V_8-{}^7T_8$ (V_8 depicted as *C*). However, the effects of the benzylidene groups, with their unknown stereochemistry, are obscure.

Considering the last group of compounds, D, comprising the 6,7-benzylidene compounds *6*, *7*, and *9*, we find only one set of preferred conformers V_7 and ${}^6T_7$ for the cyclopentane ring (V_7 is depicted as *D*). The dihydropyrane ring in this case, as in group B, is almost planar except for C-1, here pointing upwards and giving rise to an axial position of the glucose moiety.

Obviously, the number of low-energy conformers of the five-ring is restricted through fusion with the dihydropyrane ring, which, on the other hand, is conformationally influenced by the presence of the five-membered ring. The present results indicate that in the mutual interactions between the two rings, the nature of the substituents in the five-ring play a dominant role.

However well the conformations arrived at by consideration of sterical interactions and analysis of coupling constants coincide, it is important to stress the element of speculation involved in the latter analyses, both in the present and previous work.[6,7] It is hoped, however, that considerations such as those described above may help in acquiring a more detailed picture of the actual state of iridoid molecules in solution.

EXPERIMENTAL

Melting points are uncorrected and were determined in capillary tubes in a heated bath. Preparative TLC was performed on 20 × 40 cm plates coated with 1 mm layers of silica gel PF_{254} (Merck); bands were detected by UV-light. Analyses were performed at Dr. A. Bernhardt, Mikroanalytisches Laboratorium, Elbach über Engelskirchen, Germany.

Isolation of lamiridoside (*1*). Fresh plant material * (850 g, collected close to the labora-

* A voucher has been deposited in the Botanical Museum of the University of Copenhagen under the file No. IOK 152/72.

tory) was homogenized in EtOH and worked up as previously described.[9] The resulting Me_2CO eluate (*ca.* 8 g) was chromatographed twice on SiO_2 (300 g), with $CHCl_3$-MeOH (3:1) as the eluent. On standing, the main fraction (3.2 g) deposited crystalline KNO_3 (400 mg, identified by the IR-spectrum).[10] Purification was achieved by adsorption on activated carbon (10 g), washing with H_2O, and elution of the glucoside fraction with MeOH. A colourless, amorphous product was obtained by evaporating the solvent (2.0 g; 0.24 %). λ_{max}(EtOH) 238 nm (ε 10 200). PMR: 4.83 ppm (*d*, $J_{1'2'} = 7.5$ Hz; H-1'), 3.35 – 3.95 ppm (absorptions corresponding to the sugar-protons in glucosides); further data in the tables.

Lamiridoside hexaacetate (2). Acetylation of *1* (490 mg), in pyridine (5 ml) and Ac_2O (3 ml) for 3 h at room temperature, gave, after chromatography on SiO_2 (Et_2O – EtOAc; 9:1), *2* (484 mg). Recrystallization from MeOH gave an analytical specimen, m.p. 126.5 – 128°. λ_{max}(EtOH) 234 nm (ε 10 650). Further data in the tables.

Lamiridoside heptaacetate (3) was obtained analogously, by prolonging the acetylation time to 18 h; m.p. 189 – 190°. λ_{max} (EtOH) 232 nm (ε 10 100). Further data in the tables.

Preparation of benzylidene derivatives. 1 (710 mg) was stirred with freshly distilled C_6H_5CHO (23 ml) and anhydrous $ZnCl_2$ (1.2 g) for 3 h at room temperature. The homogeneous, slightly yellow reaction mixture was poured into saturated $NaHCO_3$ solution (100 ml) and extracted with pentane (3 × 100 ml) which was then discarded. The aqueous solution was extracted with EtOAc (3 × 150 ml) which, after drying, was concentrated *in vacuo*. The residue (944 mg) was subjected to preparative TLC to give two bands by elution with $CHCl_3$ – EtOAc (1:1); a faster running band (*fract. A*, 490 mg) and a slower running band (*fract. B*, 165 mg). The latter was rechromatographed to give a homogeneous (PMR, TLC), amorphous product: *7,8 – 4',6'-dibenzylidene lamiridoside (4)*. (Elemental analyses showed this preparation to retain about 1 mol of H_2O, which could not be removed even by rechromatography followed by rigorous drying in high vacuum). PMR-spectrum: 7.30 – 7.60 ppm (*m*; 11 H, 10 arom. H and H-3); 6.20 and 5.44 ppm (*s*'s; 2 benzylidene H); 4.28 ppm (*d*; $J = 7.5$ Hz, H-1'). Further data in the tables.

Acetylation of *4* (Py/Ac_2O; 5 h, 25°) provided *6,2',3'-triacetyl 7,8 – 4',6'-dibenzylidene lamiridoside (5)*, which was crystallized from MeOH to give a colourless solid, m.p. 136 – 137°. PMR-spectrum: 7.30 – 7.60 ppm (*m*, 11 H, 10 arom. H and H-3); 6.03 and 5.52 ppm (*s*'s; 2 benzylidene H); *ca.* 5.4 ppm (*m*; H-3'); 5.02 ppm (*m*, H-1' and H-2'); 2.14, 2.05 and 1.95 ppm (3 × OAc). Further data in the tables.

Fraction A (see above) was acetylated (Py/Ac_2O; 5 h, 25°) and gave, after work up, a mixture (630 mg), which was separated into two bands (*fract. C* and *D*) by preparative TLC (C_6H_6 – Et_2O; 3:1). The slower running fraction *C* (126 mg) was recrystallized from MeOH to give *2',3'-diacetyl 6,7 – 4',6'-dibenzylidene lamiridoside (6)* as colourless crystals, m.p. 221 – 223° (softening and resolidifying at 141 – 145°). PMR-spectrum: 7.30 – 7.60 ppm (*m*; 10 arom. H and H-3); 5.79 and 5.49 ppm (*s*'s; 2 benzylidene H); 5.32 ppm (*m*; H-3'); 4.99 ppm (*m*; 2H, H-1' and H-2'); 4.37 ppm (*dd*; $J = 4$Hz and 10 Hz, H-4'); 2.02 and 1.90 ppm (2 × OAc). Further data in the tables.

Acetylation of *6* overnight at 80 – 90°, followed by preparative TLC (C_6H_6 – Et_2O; 1:1) gave the amorphous *8,2',3'-triacetyl 6,7 – 4',6'-dibenzylidene lamiridoside (7)*. PMR-spectrum: 7.30 – 7.60 ppm (*m*; 10 arom. H and H-3); 5.77 and 5.51 ppm (*s*'s; 2 benzylidene H); 5.33 ppm (*m*; H-3'); 5.01 ppm (*m*; H-1' and H-2'); 2.03, 2.01, and 1.91 ppm (3 × OAc). Further data in the tables.

From *fraction D* (465 mg) above a compound separated on crystallization from EtOH. Recrystallization gave *6,2',3'-triacetyl 12*-epi-*7,8 – 4',6'-dibenzylidene lamiridoside (8)* as colourless crystals (285 mg) m.p. 204°. PMR-spectrum: 7.30 – 7.60 ppm (*m*; 10 arom. H and H-3); 5.95 and 5.49 ppm (*s*'s; 2 benzylidene H); *ca.* 5.4 ppm (*m*; H-3'); 5.05 ppm (*m*; H-1' and H-2'); 4.40 ppm (*m*; H-4'); 2.03, 1.96 and 1.91 ppm (3 × OAc). Further data in the tables.

The mother liquors from the crystallization of *8* contained, according to PMR, *8* and another compound in the proportion 1:4. Repeated chromatography (Et_2O – EtOAc-pentane; 1:1:1) resulted in an improvement of the ratio to 1:10. This inhomogeneous preparation, mainly consisting of *2',3'-diacetyl 12*-epi-*6,7 – 4',6'-dibenzylidene lamiridoside (9)*, was not subjected to further purification; only a PMR-spectrum was recorded: 7.30 – 7.60 ppm (*m*; 10 arom. H and H-3); 6.25 and 5.51 ppm (*s*'s; 2 benzylidene H); 5.35 ppm (*m*; H-3'); 5.01 ppm (*m*; H-1' and H-2'); 4.42 ppm (*m*; H-4'); 2.04 and 1.92 ppm (2 × OAc). Further PMR-data in Table 1.

Acetylation of the preparation above (130 mg) for 3 days at 80° gave, after work-up and chromatographic separation (C_6H_6 – Et_2O; 1:1), *8,2',3'-triacetyl 12*-epi-*6,7-4',6'-dibenzylidene lamiridoside (10)* as a colourless foam. PMR-spectrum: 7.30 – 7.60 ppm (*m*; 10 arom. H and H-3); 6.14 and 5.56 ppm (*s*'s; 2 benzylidene H); 5.38 ppm; (*m*; H-3'); 5.04 ppm (*m*; H-1' and H-2'); 4.46 ppm (*dd*; $J = 4$ and 9.5 Hz, H-4'); 2.09, 2.07, and 1.95 Hz (3 × OAc). Further data in the tables.

Enzymic hydrolysis of lamiridoside. (1) (500 mg) was dissolved in H_2O (15 ml) and emulsin (200 mg) was added. After stirring for 3 h, *1* had disappeared (TLC), and the mixture was filtered through activated carbon (5 g) and celite (4 g). The filter cake was washed with H_2O (100 ml), and glucose was identified

in the filtrate by co-chromatography with authentic glucose on paper (solvent: BuOH-EtOH-H_2O; 4:1:4). Elution of the filter cake with MeOH (100 ml), and evaporation of the solvent gave an apparently stable, sirupy residue (196 mg), which could not be induced to crystallize. Its PMR-spectrum was in accordance with that expected for the aglucone. Without further purification, the aglucone was acetylated (Py/Ac_2O; 2 h, room temp.). Preparative TLC with $C_6H_6-Et_2O$ (2:1) as the eluent and extraction of the main band gave the *aglucone triacetate (11*, 70 mg). Crystallization from $EtOAc-Et_2O$ afforded the pure compound, m.p. 153–155°. λ_{max} (EtOH) 232 nm (ε 10 450). Further data in the tables.

Note added in proof. After the present paper was submitted, Brieskorn and Ahlborn[11] reported the isolation of the same glucoside, named 'lamalbid', from '*Flores lamii albi*'. We accept the latter name and hence consider the designation 'lamiridoside' as redundant.

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Synthesis and Properties of 3-(3-Carboxyphenyl)pyruvic Acid and 3-(3-Carboxy-4-hydroxyphenyl)pyruvic Acid

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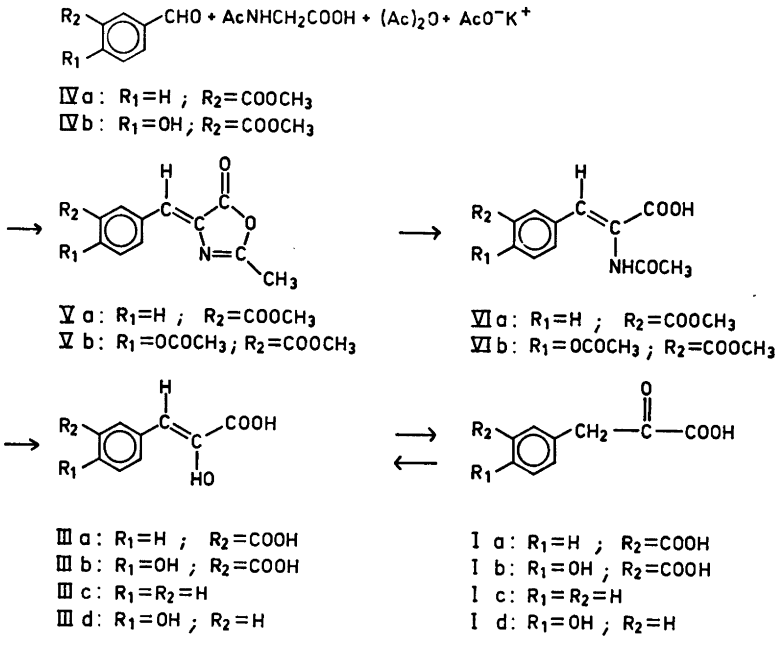
3-(3-Carboxyphenyl)pyruvic acid and 3-(3-carboxy-4-hydroxyphenyl)pyruvic acid have been synthesized from the corresponding benzaldehydes *via* azlactones and α-acetaminocinnamic acids. The equilibrium and equilibration rate between the two keto acids above, phenylpyruvic acid, and *p*-hydroxyphenylpyruvic acid and the corresponding enolic acids have been studied by PMR and UV spectroscopy. The configuration of the enolic acids and of the azlactones and α-acetamino acids have been determined.

3-(3-Carboxyphenyl)pyruvic acid (Ia) and 3-(3-carboxy-4-hydroxyphenyl)pyruvic acid (Ib) are the keto acids corresponding to 3-(3-carboxyphenyl)alanine (IIa) and 3-(3-carboxy-4-hydroxyphenyl)alanine (IIb). IIa and IIb occur free in various higher plants.[1–4] Biosynthesis of IIa and IIb has been proposed to proceed from chorismic acid *via* isochorismic acid [2-(3-carboxy-1,2-dihydro-2-hydroxyphenoxy)acrylic acid], isoprephenic



acid [3-(3-carboxy-4-hydroxycyclohexa-2,5-dienyl)pyruvic acid] and Ia and Ib on basis of incorporation studies with stereospecifically tritium-labeled shikimic acids.[5] Ia has been identified on spectroscopic evidence as a result of chemical rearrangement of isochorismic acid,[6] whereas Ib was unknown. For these reasons the synthesis and characterization of Ia and Ib were undertaken. The equilibrium of the keto forms Ia and Ib with the corresponding enol forms IIIa and IIIb was studied by PMR and UV spectroscopy and the results were compared with those obtained for the keto forms (Ic and Id) and the corresponding enol forms (IIIc and IIId) of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid.

The syntheses were accomplished by use of the classical Erlenmeyer method,[7] starting from isophthalaldehydic acid methyl ester (IVa)[8] or 5-formylsalicylic acid methyl ester (IVb)[9] and proceeding *via* 4-(3-carbomethoxybenzylidene)-2-methyl-2-oxazolin-5-one (the azlactone of α-acetamino-3-carbomethoxycinnamic acid) (Va) or 4-(3-carbomethoxy-4-acetyloxybenzylidene)-2-methyl-2-oxazolin-5-one (the azlactone of α-acetamino-3-carbomethoxy-4-acetyloxycinnamic acid) (Vb) and α-acetamino-3-carbomethoxycinnamic acid (VIa) or α-acetamino-3-carbomethoxy-4-acetoxycinnamic acid (VIb), respectively. Isolation of the intermediates VIa and VIb was not necessary. V and VI are tentatively assigned *Z*-configuration since the δ-values (see Experimental) for the olefinic protons in these compounds are similar to those reported (in $CDCl_3$) for *Z*-4-benzylidene-2-phenyl-2-oxazolin-5-one and *Z*-α-benzaminocinnamic acid methyl ester and differ from those of the corresponding *E*-isomers.[10–12] A reported δ-value (in D_2O) of 7.28 ppm for the vinylic proton in *Z*-α-(methylaminoacetyl)aminocinnamic acid[13] also supports this assignment. The syntheses were performed under acidic conditions to avoid base induced degradation. Phenylpyruvic acid and derivatives thereof substituted in the *o*- or *p*-position with substituents with negative mesomeric effects are decomposed by base to the corresponding substituted toluene and oxalate.[14] *p*-Hydroxyphenylpyruvic acid and other phenylpyruvic acids substituted in the *o*- or *p*-position with substituents with a positive mesomeric effect are decomposed by base to the corresponding substituted benzaldehydes.[14–16] Phenylpyruvic acids can also be synthesized in high yields from aromatic aldehydes and hydantoin.[14,16] The azlactone method was chosen in the present case, however, since a method was sought which subsequently could be adopted for synthesis of ^{14}C-labeled material, and since ^{14}C-glycine is easily available.

Phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid exist in the crystalline state solely as the enols IIIc and IIId, whereas the salts exist mainly as ketones, as established by chemical studies and ultraviolet spectroscopy.[17] As expected, therefore, the PMR spectra in DMSO of the freshly dissolved free acids only show the presence of singlets for the olefinic protons in IIIa (δ 6.50 ppm), IIIb (δ 6.41 ppm), IIIc (δ 6.41 ppm), and IIId (δ 6.31 ppm) besides the protons in the aromatic nuclei. The δ-values found are in close agreement with those previously reported for IIIa, IIIc, and IIId.[6] III is tentatively assigned the *Z*-configuration partly on the basis of the δ-values for the olefinic protons and partly on the basis of the UV-spectra (see below). The δ-values for the proton at C_3 with *E*- or *Z*-configuration can be calculated as 5.88 ppm and 6.40 ppm, respectively, assuming additivity of substituent effects on chemical shifts of olefinic protons[18] and using the same values for the effect of the hydroxy group as those reported[18] for alkoxy groups. The spectra of the lithium salts of the four acids in D_2O exhibited beside signals for the aromatic protons only benzylic protons as singlets, indicating that in the salts the anions corresponding to I are present. The signals for the benzylic protons slowly disappear as a result of exchange with D_2O.

In aqueous acid an equilibrium is established between I and III, as previously established for Ic – IIIc and Id – IIId.[17] The tautomerization of IIId follows reversible first-order kinetics, increasing in speed with increase in pH.[17] This was confirmed by UV measurements for all four acids as demonstrated in Table 1. Half times and rate constants for establishing the equilibrium at 25° both in 1 M HCl and in phosphate buffer at pH 6.35 are given. Determination of the amount of enol present at equilibrium in 1 M HCl permitted the calculation of the individual rate constants for the keto-enol tautomerization.

Table 1. Keto-enol tautomerization of phenylpyruvic acids at 25°. For designation of compounds see formula chart.

Compound	1 M HCl					Phosphate buffer pH 6.35	
	$t_{½}$[a] (min)	k[a] (min^{-1})	% enol at equilibrium	$k_{I→III}$ (min^{-1})	$k_{III→I}$ (min^{-1})	$t_{½}$[a] (min)	k[a] (min^{-1})
Ia – IIIa	10	6.9×10^{-2}	7	6.4×10^{-3}	6.3×10^{-2}	5	1.3×10^{-1}
Ib – IIIb	29	2.4×10^{-2}	12	2.9×10^{-3}	2.1×10^{-2}	16.5	4.2×10^{-2}
Ic – IIIc	16	4.4×10^{-2}	10	4.5×10^{-3}	4.0×10^{-2}	3.5	2.0×10^{-1}
Id – IIId	51	1.4×10^{-2}	17	2.4×10^{-3}	1.1×10^{-2}	11.5	6.0×10^{-2}

[a] For equilibration.

The values found in 1 M HCl for Id – IIId are in good agreement with those reported for this compound at pH 1.[17] For Ia – IIIa it has been stated that keto to enol conversion in strong acid does not occur readily,[6] but this is contradicted by the present results.

Presumably the conversion of keto to enol takes place without acid catalysis [19] and the rate is determined by the kinetic acidity of the protons on C_3. The order of the rate constants for I→III is that expected from the inductive and mesomeric effects of the substituents in the aromatic ring in I. The changed order of half-times and corresponding rate constants at pH 6.35 supposedly reflects the change of sign of the inductive effect of the aromatic carboxyl group by ionization.

In these considerations no regard is made to the hydration of I. It can, however, be expected that the degree of hydration is nearly the same for all four compounds and the effect on the order of the rate constants would be opposite to the effect described above.[20]

UV-data for the enols in 1 M HCl and for the anions of the enols at pH 6.35 are recorded in Table 2. The amount of enol at equilibrium at pH 6.35 supposedly is negligible. Therefore the UV-absorption recorded at equilibrium pre-

Table 2. UV-data for enol and keto forms of phenylpyruvic acids at 25°. For designation of compounds see formula chart.

Compound	1 M HCl		Phosphate buffer pH 6.35			
	Enol form		Anion of enol form		Anion of keto form	
	λ_{max} (nm)	ε_{max}	λ_{max} (nm)	ε_{max}	λ_{max} (nm)	ε_{max}
Ia – IIIa	286	23 000	284	23 000	275	1 600
	234	13 000	225	shoulder	225	shoulder
	204	6 000	199		199	29 000
Ib – IIIb	295	23 000	291	23 000	299	4 800
	233	15 000	227	shoulder	227	shoulder
	208	17 000	204	19 000	204	33 000
Ic – IIIc	289	25 000	285	25 000		
	208		197	14 000	197	13 000
Id – IIId	302	25 000	290	26 000	275	2 800
	224	7 400	220	shoulder	220	shoulder
			197	14 500	197	22 000

sumably represents the anions of the ketones as given in Table 2. The high ε-values for the high wavelength absorption band for the enols support the *Z*-configuration for these compounds since ε_{max} for the corresponding band for the similar configuration of cinnamic acid (*E*-cinnamic acid) is 20 000 whereas ε_{max} for the opposite configuration (*Z*-cinnamic acid) is only 9000.[21] The intensity and location of these bands are only changed very little in the corresponding anions of the cinnamic acids.[21] The spectral data for IIIa, IIIc, and IIId are in agreement with those reported in the literature.[6,16,22,23] In the spectrum of the anion of Ib the salicylic acid grouping is clearly visible.

EXPERIMENTAL

PMR spectra were measured on a JEOL-C-60 HL instrument. Chemical shifts are given in ppm downfield from TMS in deuterio-DMSO and from sodium 2,2,3,3,-tetradeuterio-3-(trimethylsilyl)propionate in D_2O. UV-spectra were measured on a Zeiss DMR-21 instrument at 25°. Transformations of enol to keto forms in 1 M HCl and in 0.06 M phosphate buffer pH 6.35 were observed at λ_{max} for the highest positioned absorption band of the enol form (see Table 1) in freshly prepared solutions and half-times for equilibration were determined as described in the literature.[17] Transformations of keto to enol forms in 1 M HCl were observed by acidification of neutral solutions. Extrapolations to $t=0$ permitted the determination of ε-values in acid for both keto and enol forms and the combined data permitted calculation of the equilibrium composition. Melting points are uncorrected. Microanalyses were performed by Mr. G. Cornali and his staff.

4-(3-Carbomethoxybenzylidene)-2-methyl-2-oxazolin-5-one (Va). A mixture of isophthalaldehydic acid methyl ester (IVa)[8] (5.01 g), acetylglycine (3.16 g), and potassium acetate (2.5 g) in acetic anhydride (5.5 ml) was kept with stirring at 100° for 3 h. After cooling overnight crystals of Va were collected by filtration and washed with methylene chloride. Yield 2.93 g (45 %), m.p. 185–187°. Recrystallization from benzene-benzine (80–110°) produced an analytical sample of yellow needles, m.p. 195–197°. (Found: C 59.64; H 4.33; N 5.33. Calc. for $C_{13}H_{11}O_5N$: C 59.77; H 4.24; N 5.36). PMR-spectrum in deuterio-DMSO: δ 2.41 (3 H) singlet, methyl; δ 3.95 (3 H) singlet, methoxyl; δ 7.36 (1 H) singlet, olefinic; δ 7.75–8.87 (4 H) multiplet, aromatic.

4-(3-Carbomethoxy-4-acetyloxybenzylidene)-2-methyl-2-oxazolin-5-one (Vb). A mixture of 5-formylsalicylic acid methyl ester sodium salt (IVb)[9] (406 mg), acetylglycine (234 mg), and potassium acetate (200 mg) in acetic anhydride (2 ml) was kept with stirring under nitrogen at 100–110° for 2 h. After cooling overnight crystals of Vb were collected by filtration and washed with ethanol. Yield 366 mg (60 %), m.p. 140–141°. Recrystallization from benzene produced an analytical sample of yellow needles, m.p. 140–141°. (Found: C 59.47; H 4.46; N 4.42. Calc. for $C_{15}H_{13}O_6N$: C 59.40; H 4.36; N 4.62.) PMR-spectrum in deuterio-DMSO: δ 2.34 (3 H) singlet, acetoxy; δ 2.42 (3 H) singlet, methyl; δ 3.87 (3 H) singlet, methoxyl; δ 7.33 (1 H) singlet, olefinic; δ 7.41–8.82 (3 H) multiplet, aromatic.

α-Acetamino-3-carbomethoxycinnamic acid (VIa). A suspension of Va (123 mg) in water (5 ml) and ethanol (5 ml) was refluxed for 2 h. After removal of part of the solvents by evaporation and cooling overnight crystals of VIa were collected by filtration. Yield 68 mg (52 %), m.p. 217–219° (decomp.). Recrystallization from methanol produced an analytical sample, m.p. 222° (decomp.). (Found: C 59.15; H 5.15; N 5.32. Calc. for $C_{13}H_{13}O_5N$: C 59.31; H 4.97; N 5.32.) PMR-spectrum in deuterio-DMSO: δ 2.05 (3 H) singlet, acetyl; δ 3.91 (3 H), singlet, methoxyl; δ 7.36 (1 H) singlet, olefinic; δ 7.42–8.3 (4 H) multiplet, aromatic.

α-Acetamino-3-carbomethoxy-4-acetyloxycinnamic acid (VIb). Vb (121 mg) in water (10 ml) and methanol (5 ml) was refluxed for 4 h. After removal of part of the solvent, filtration and cooling crystals of VIb were collected by filtration. Yield 94 mg (73 %). Recrystallization from ethyl acetate produced an analytical sample. m.p. 180°. (Found: C 55.69; H 4.83; N 4.28. Calc. for $C_{15}H_{15}NO_7$: C 56.08; H 4.70; N 4.35.) PMR-spectrum in deuterio-DMSO: δ 2.05 (3 H) singlet, acetyl; δ 2.34 (3 H) singlet, acetoxy; δ 3.87 (3 H) singlet, methoxyl; δ 7.34 (1 H) singlet, olefinic; δ 7.47–8.28 (3 H) multiplet, aromatic.

3-(3-Carboxyphenyl)pyruvic acid in enol form (IIIa). A solution of Va (980 mg) in ethanol (20 ml) and 2 M HCl (40 ml) was refluxed under nitrogen for 2.5 h. The solution was decolourised with charcoal and after cooling overnight colourless crystals of IIIa were collected, m.p. 222–224° (decomp.). Yield 670 mg (80 %). Two recrystallizations from 2 M HCl produced an analytical sample, m.p. 219–222° (decomp.). (Found: C 57.46; H 4.04. Calc. for $C_{10}H_8O_5$: C 57.69; H 3.87.) PMR-spectrum in deuterio-DMSO: δ 6.50 (1 H) singlet, olefinic; δ 7.34–8.48 (4 H) multiplet, aromatic. PMR-spectrum of lithium salt in D_2O: δ 4.18 (2 H) singlet, benzyl; δ 7.28–7.82 (4 H) multiplet, aromatic. For PMR-data, *cf.* Ref. 6.

3-(3-Carboxy-4-hydroxyphenyl)pyruvic acid in enol form (IIIb). A solution of Vb (366 mg) in ethanol (10 ml) and 4 M HCl (35 ml) was refluxed under nitrogen for 14 h. After cooling overnight and filtration the solution was left in the refrigerator for three days. Crystals of IIIb were collected by filtration. Yield 169

mg (68 %), m.p. 230 – 235° (decomp.). Recrystallization from 2 M HCl produced an analytical sample, m.p. 234 – 237° (decomp.). (Found: C 53.64; H 3.77. Calc. for $C_{10}H_8O_6$: C 53.58; H 3.59.) PMR-spectrum in deuterio-DMSO: δ 6.41 (1 H) singlet, olefinic; δ 6.9 – 8.45 (3 H) multiplet, aromatic. PMR-spectrum of lithium salt in D_2O: δ 4.08 (2 H), singlet, benzyl; δ 6.8 – 7.8 (3 H) multiplet, aromatic.

Ib – IIIb is rapidly decomposed at pH > 8 at room temperature. The corresponding aldehyde has been identified in the decomposition mixture by UV-spectroscopy.

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Mass Spectra of Substituted 1,2,3,4-Thiatriazoles

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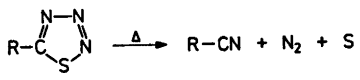
The mass spectra of 14 1,2,3,4-thiatriazoles have been recorded and interpreted with the aid of high resolution mass measurements and by the application of the metastable defocusing technique. The observed electron impact induced fragmentations are compared with the known thermal decompositions of these compounds.

The electron impact induced decompositions of heterocyclic compounds have received appreciable attention during the last decade, and mass spectrometry has as a result of these investigations become an important tool in the structure elucidation of this type of compounds.[1-3]

Although several classes of heterocyclic compounds thus have been investigated no detailed studies dealing with the mass spectra of thiatriazoles ** have been published. In connection with the study of the fragmentations of 1,2,3-thiadiazoles the mass spectrum of 5-phenylthiatriazole has been scrutinized.[4]

Thiatriazoles are known to be rather unstable compounds,[5] decomposing upon heating by the loss of N_2 and S according to the scheme:



The purpose of this investigation is twofold, partly to examine whether mass spectrometry can be used for the identification of this type of heterocyclic compounds, partly to compare the electron impact induced fragmentations with the known thermal decompositions of thiatriazoles.

Comparisons of thermally and electron impact induced processes are often complicated by the possibility of pyrolysis of the sample in the inlet system of the mass spectrometer. In order to prevent such decompositions prior to ionization the thiatriazoles were introduced through the direct inlet at a temperature below 100°C. Applying this technique it was possible to obtain a mass spectrum (Fig. 4) of 5-mercaptothiatriazole (XI) (known to explode at 50–60°C at atmospheric pressure) with a molecular ion peak of *ca.* 40 % relative abundance. The presence of an abundant molecular ion peak indicates that some of the sample has evaporized unchanged into the ion source, but it does not exclude that pyrolysis has also taken place. As the pyrolysis of the thiatriazoles leads to the formation of sulfur, the presence of peaks in the mass spectra at *m/e* n·32 corresponding to $S^{+\cdot}$ (n = 2, 3,...8) can be taken as evidence for a thermal destruction of the sample. Thus in the case of 5-mercaptothiatriazole (XI) the mass spectrum (Fig. 4) contains peaks at *m/e* 128 and 64, indicating that some of the sample decomposes during the evaporation.

This investigation deals with the mass spectra (Figs. 1–4) of 14 thiatriazoles (I–XIV). Depending on the substituent in the 5-position the compounds can be divided into three groups:

A: 5-Arylthiatriazoles (I–II)

B: 5-Aminothiatriazoles (III–X)

C: 5-Thiothiatriazoles (XI–XIV)

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** The positional numerals are for the sake of brevity omitted.

A:

I. R=-C_6H_5

II. R=-C_6H_4-p-CH_3

B:

III. R=-NH_2

IV. R=-$NHCH_3$

V. R=-$NHCH_2CH_3$

VI. R=-$NHCH_2CH_2CH_3$

VII. R=-$NHCH(CH_3)_2$

VIII. R=-NHC_6H_5

IX. R=-$N(CH_3)_2$

X. R=-$N(CH_2CH_2CH_3)_2$

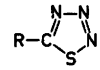
C:

XI. R=-SH

XII. R=-SCH_3

XIII. R=-SCH_2CH_3

XIV. R=-SC_6H_5



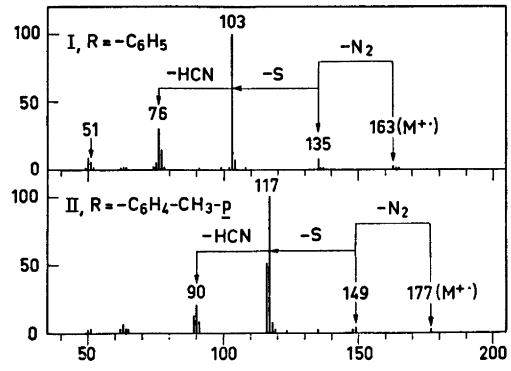


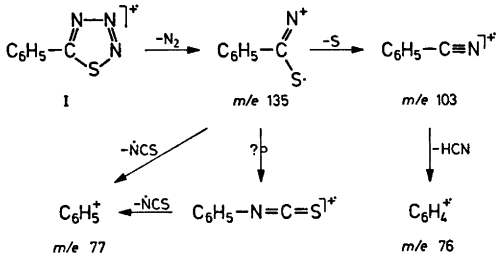
Fig. 1.

DISCUSSION

A. 5-Arylthiatriazoles

The base peak (*m/e* 103) in the mass spectrum (Fig. 1) of 5-phenylthiatriazole (I) is due to ionized benzonitrile and the peaks at *m/e* 76 and 75 correspond to the known decomposition products of $C_6H_5CN^{+\cdot}$ [6] (I) is known to decompose into benzonitrile upon heating,[5] and in order to ascertain whether 103^+ was the result of a pyrolysis prior to ionization or due to an electron impact induced process metastable defocusing technique (MDT) was applied. It was found that 103^+ is formed by the decomposition of 135^+ (by the loss of S) as suggested by Zeller *et al.*[4]

The peak at *m/e* 135 is due to ions formed by the loss of N_2 from the molecular ion. A similar thermal process is not known for this compound. The composition of the $M-N_2$ ion corresponds to that of phenylisothiocyanate and phenylthiocyanate. From the mass spectra of C_6H_5NCS [7] and C_6H_5SCN [8] we are most inclined to assign the $M-N_2$ ion to ionized phenylisothiocyanate. The formation of this ion requires a phenyl migration from C(5) to N(4).

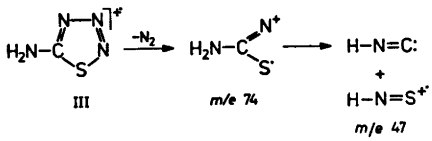


If the phenyl group is substituted by *p*-tolyl, the decomposition pattern observed for (I) is expanded by the loss of a hydrogen atom from $M-N_2$ as well as from $M-N_2S$, probably leading to the formation of isothiocyano- and cyanotropylium ions, respectively.[6]

B. 5-Aminothiatriazoles

The mass spectrum (Fig. 2) of 5-aminothiatriazole (III) displays rather abundant peaks at *m/e* 102, 74, 47, and 42, corresponding to $M^{+\cdot}$, $M-N_2$, $HNS^{+\cdot}$, and $H_2N-CN^{+\cdot}$, respectively. The molecular ion has an abundance of *ca.* 16 % Σ_{40}; in the mass spectrum of the methylamino analogue (IV) the abundance of $M^{+\cdot}$ is 18.5 % Σ_{40}, whereas the remaining compounds (V–X) from this group display molecular ion peaks with abundances less than 5 % Σ_{40}. The decrease of molecular ion intensity with increasing size of the alkyl substituent is a well known phenomenon, but the situation is normally reversed when the alkyl group is replaced by an aromatic moiety such as phenyl. In the mass spectrum (Fig. 3) of 5-phenylaminothiatriazole (VIII), however, the abundance of the molecular ion is only 0.8 % Σ_{40}.

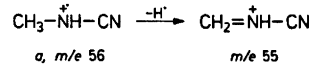
The decrease in molecular ion abundance with increasing size of the alkyl is normally due to the possibility for new reactions when the side chain is enlarged, and this is also the case here, as seen from below.



The $M-N_2$ ion from 5-aminothiatriazole (III) has the composition CH_2N_2S and this fragment decomposes into the ion $HNS^{+\cdot}$ and neutral HCN. In the mass spectra of the two 5-arylthiatriazoles (I–II) the $M-N_2$ ions corresponded to ionized arylisothiocyanates, but an analogous skeletal rearrangement (leading to $H_2N-N=C=S^{+\cdot}$) is neither reasonable from a mass spectrometric point of view, nor in agreement with the further loss of HCN and formation of $HNS^{+\cdot}$. The genesis of $HNS^{+\cdot}$ requires the formation of a S–N bond.

Although the mass spectrum of 5-methylaminothiatriazole (IV) displays an abundant peak at *m/e* 88, corresponding to $M-N_2$, the peaks due to the subsequent decomposition by loss of HCN (*m/e* 61) or formation of $HNS^{+\cdot}$ (*m/e* 47) are not as significant as in the case of III. Further, the $M-N_2$ ion from IV is found to decompose by the loss of ·H (*m/e* 87) and $\cdot CH_3$ (*m/e* 73 = CHN_2S, H. R.) as indicated by the presence of the appropriate metastable peaks. These differences between the decompositions of the $M-N_2$ ions from III and IV makes the assignment of a general structure to the $M-N_2$ ions impossible.

As in the mass spectrum of III that of IV displays an abundant peak corresponding to $M-N_2S$. This ion is assigned to the structure *a*. The peak at *m/e* 55 is due to $M-HN_2S$, formed by α-cleavage in *a*:



The dominating features in the mass spectra (Figs. 2–3) of the higher homologues are formation of *N*-alkyl cyanamides, which decompose further by α-cleavage, and formation of alkyl ions. Finally, these mass spectra display minor peaks corresponding to ionized alkylisothiocyanates ($M-HN_3$) and to the known fragments of these ions.[7]

The mass spectrum (Fig. 3) of 5-phenylamino-

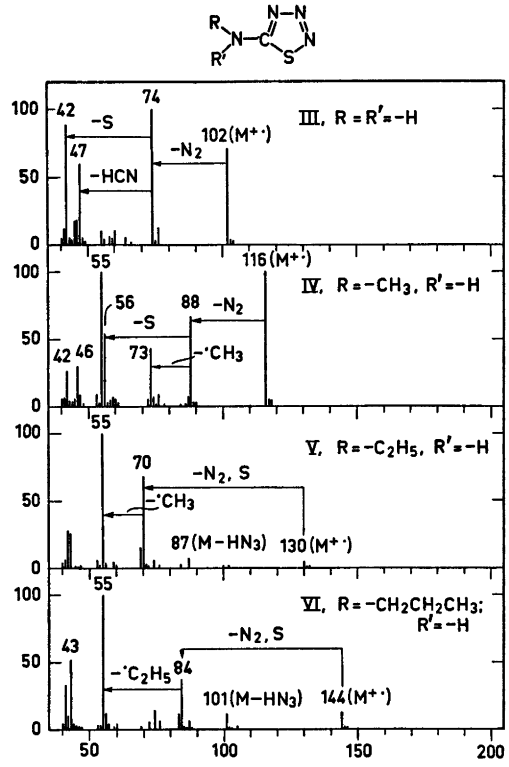
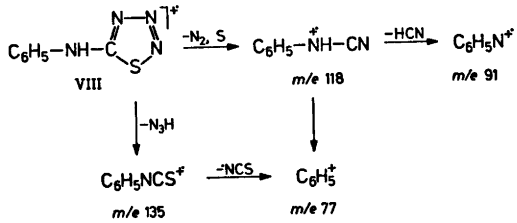


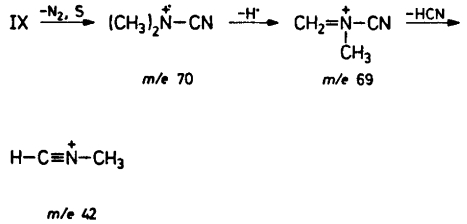
Fig. 2.

thiatriazole (VIII) reveals that the formation of phenylisothiocyanate (*m/e* 135) and phenylcyanamide (*m/e* 118) are important processes. Peaks corresponding to the decomposition products of the two compounds are found in agreement with the mass spectra of C_6H_5NCS [7] and C_6H_5NHCN. [9]

The mass spectra (Fig. 6) of dimethylamino (IX) and dipropylaminothiatriazole (X) display peaks corresponding to a fragmentation pattern very similar to that of the monoalkyl analogues (IV – VII), *i.e.* formation and decomposition of

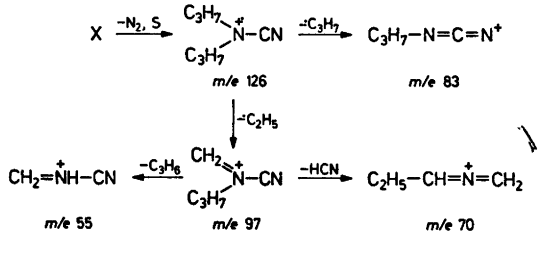


ionized cyanamides. In the case of IX the following sequence is observed:



The $M-N_2$ peak in the mass spectrum of IX is due to an ion undergoing subsequent decomposition through the expulsion of a methyl radical. A similar process was observed for the monomethyl analogue (IV).

The loss of N_2S from the molecular ion of dipropylaminothiatriazole (X) leads to the formation of ionized dipropylcyanamide (*m/e* 126), undergoing α-cleavage by loss of $\cdot C_2H_5$ (*m/e* 97) followed by the elimination of C_3H_6 (*m/e* 55). Minor peaks in the mass spectrum of X are due to loss of a propyl radical from the ionized cyanamide (*m/e* 83) and to the elimination of HCN from the α-cleavage product (*m/e* 70), as depicted in the Scheme:



C. 5-Thiothiatriazoles

The mass spectrum (Fig. 4) of 5-mercaptothiatriazole (XI) contains peaks corresponding to various $S_n^{+\cdot}$ ions. This finding is a strong indication of an initial pyrolysis of the sample in the ion source prior to ionization, whereas the

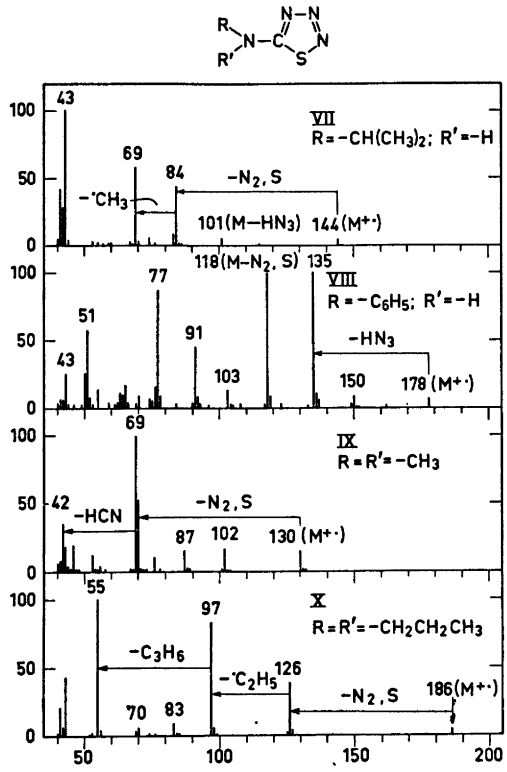
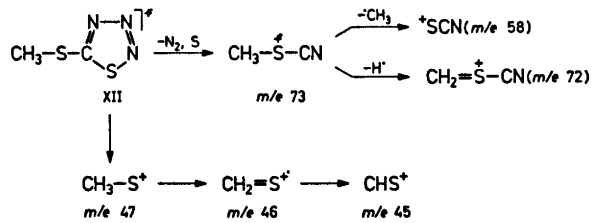


Fig. 3.

presence of a fairly abundant molecular ion peak clearly depicts that a large portion of the sample evaporizes unchanged from the probe tip. The peaks at *m/e* 76 and 59 are due to $CS_2^{+\cdot}$ and $HSCN^{+\cdot}$, respectively. These ions might be thermal in their origin.

The absence of any peaks, corresponding to $S_n^{+\cdot}$ in the mass spectra of the remaining compounds belonging to this class can be taken as evidence for the absence of thermal processes. The mass spectrum of methylthiothiatriazole (XII) displays peaks due to the processes listed below:



Processes of minor contribution to the total ion current include the loss of $\cdot CH_3$ (*m/e* 118), loss of N_2 (*m/e* 105), and a combined expulsion of N_2 and $\cdot CH_3$ leading to S_2CN^+ (*m/e* 90). As in the case of the mercaptothiatriazole (XI) $CS_2^{+\cdot}$ gives rise to a fairly abundant peak (*m/e* 76). The assignment of the $M-N_2S$ ion to the structure of ethylthiocyanate is supported by the decomposition pattern of this compound.[10]

The base peak (*m/e* 135) in the mass spectrum of phenylthiothiatriazole (XIV) is due to ionized phenylthiocyanate, the decomposition of which is known.[8] Minor peaks are due to $M-N_2$ and $CS_2^{+\cdot}$.

CONCLUSIONS

As it will be evident from the discussion above the electron impact induced decompositions of the thiatriazoles resemble the pyrolytic loss of N_2S. In all cases the $M-N_2S$ ion together with the fragmentation products from the $M-N_2S$ ion are responsible for the major part of the total ion current.

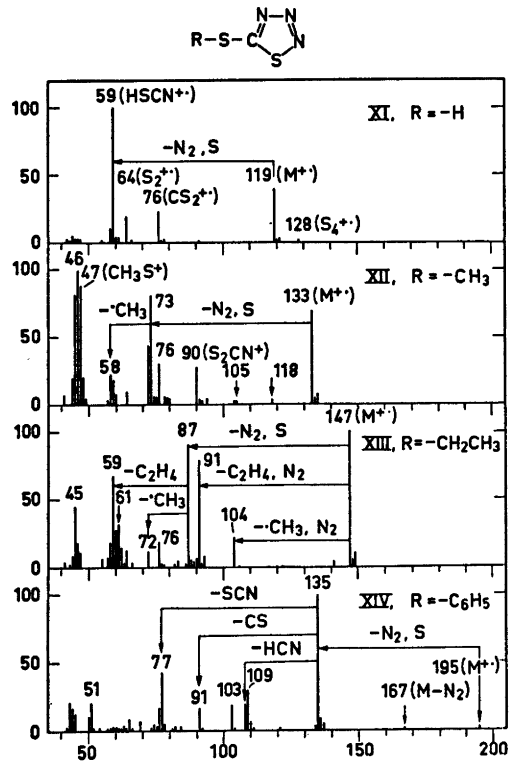
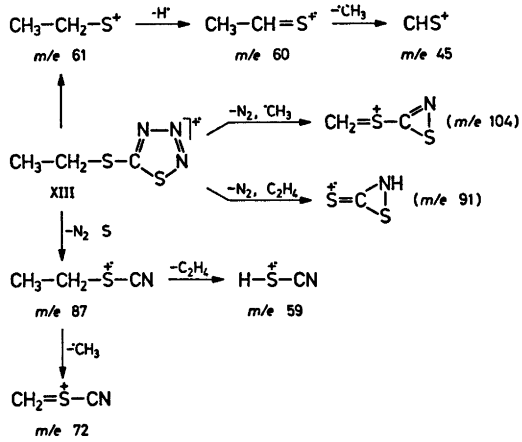


Fig. 4.



Rearrangement processes not observed upon pyrolysis include the formation of $M-N_2$ ions (observed in most spectra) and elimination of HN_3 (observed for some of the aminothiatriazoles).

EXPERIMENTAL

The mass spectra were recorded at an AEI MS902 mass spectrometer. The samples were introduced through the direct inlet at a temperature below 100°C. All thiatriazoles were prepared in accordance with previously published procedures.[5]

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Covalent Binding of Proteins to Polysaccharides by Cyanogen Bromide and Organic Cyanates. III. Structural Studies on the Linkage Region

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The reaction between cyanogen bromide activated methyl 4,6-*O*-benzylidene-α-D-glucopyranoside, Sephadex, or dextran and amino acids or peptides has been investigated. The coupling between the activated carbohydrate and the amino compound involves formation of isourea groups. The simultaneous release of ammonia is independent of the coupling reaction. Part of the ammonia is derived from hydrolysis of carbamate groups but most of it is derived from other groups in the activated carbohydrate.

Proteins can be covalently linked to polysaccharides if the latter are first treated with cyanogen bromide [1] or organic cyanates.[2] Previous proposals [3,4] concerning the nature of the linkage region in such complexes are not unambiguous. We have previously discussed the nature of the activated complex.[5–8] In the present communication, the bonds between the carbohydrates and the fixed amino compounds are re-examined.

EXPERIMENTAL

Apparatus and methods. IR-spectra in KBr were recorded with a Unicam 200 instrument. The amino acid analyses were made according to Spackman *et al.*[9] For the amino acid directly linked to the modified carbohydrate, this analysis gave low values. Corrections were therefore made assuming that the analysis of the second amino acid in a dipeptide linked to the carbohydrate was correct. The ammonia was determined by the Kjeldahl method. The molecular weights were determined by gel chromatography.[10] The melting points were determined in a pre-heated melting point apparatus. As the compounds investigated were amorphous, and further melted under decomposition, these values are not very accurate. Concentrations and dryings were performed under reduced pressure.

Materials. Sephadex® G-25 Superfine and Dextran T10 ($\overline{M}_w$9300, $\overline{M}_n$5700) from Pharmacia Fine Chemicals, Uppsala, were used. The cyanogen bromide (BrCN) was purchased from Fluka AG. Pre-coated TLC-plates Silicia Gel F_{254} and solvent system $CHCl_3/CH_3OH$ 1:1 were used. Cyanogen bromide activated Sephadex (SxOH*) was prepared as previously described.[2] Cyanogen bromide activated methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (MBGP*) was prepared as described previously except that $CHCl_3$ was exchanged for CH_2Cl_2 as extraction solvent.[6] In the experiments performed, the nitrogen content of MBGP* varied from 2.0 to 4.5 %. Sephadex-carbonate was prepared as described for cellulose-carbonate.[11] IR 1750 cm^{-1}(>C=O, acyclic), 1800 cm^{-1} (>C=O, cyclic).

1. *Coupling of glycine to MBGP** (*Ia*). To MBGP* (2.0 g, N 2,4 %) in acetone (40 ml) was added an aqueous solution (40 ml) of glycine (4.0 g) and triethylamine (2 ml). The mixture was stirred for 0.5 h at room temperature, evaporated to dryness and dried for 1 h at 80°. The residue was pulverized, dried again to ensure that all triethylamine had been removed, and thereafter stirred in water (50 ml) at room temperature for 20 min. The solid product was filtered off, washed with boiling acetone (10 ml), dried and precipitated from water (50 ml). After 20 h in a refrigerator (4°) the precipitate was filtered, dried at 110° for 0.5 h giving an amorphous product (0.2 g) that was hygroscopic. M.p. 176–177°d. (Found: C 52.2; H 6.0, N 6.9; glycine 2.25 mmol/g. Calc. for $C_{17}H_{22}O_8N_2$:

* To whom correspondence should be addressed.

C 53.5; H 5.7; N 7.25; glycine 2.40 mmol/g). The product was homogeneous on TLC. The glycine value is low, due to incomplete hydrolysis, and is not corrected. An IR spectrum showed a double peak at 1680, 1700 cm^{-1} (C=N, stretching).

2. *Coupling of glycylglycine to MBGP* (Ib).* To MBGP* (2.0 g, N 2.7 %) in acetone (30 ml) was added an aqueous solution (30 ml) of glycylglycine (2.0 g) and triethylamine (2.0 ml). The mixture was stirred for 20 min at 20° and evaporated at 50° for 3 h, whereupon the product was washed on a filter with acetone (3 × 20 ml) and finally dried for 1 h at 80°. Two precipitations from hot water (50 ml) and drying at 80° for 2 h afforded an amorphous product (0.4 g) which was homogeneous on TLC. M.p. 187°d. (Found: C 51.4; H 5.7; N 9.0; O 32.5; glycine 4.25 mmol/g. Calc. for $C_{19}H_{25}O_9N_3$: C 51.9; H 5.7; N 9.6; O 32.8; glycine 4.55 mmol/g). The glycine value is low for the same reason as above, and is not corrected. An IR spectrum showed a double peak at 1665, 1690 cm^{-1} (C=N, stretching).

3. *Sephadex-carbamate* was prepared by treating Sephadex-carbonate (0.5 g) with liq. NH_3 (2 ml) for 15 min. After evaporating excess NH_3, the product was washed with water (15 ml), dioxane, (20 ml), ether (20 ml) and finally dried at 50°. 0.53 g. IR 1715 cm^{-1} ($-CONH_2$). N 4.5 %.

4. *Hydrolysis of Sephadex-carbamate.* Sephadex-carbamate (0.250 g, from exp. 3) was stirred in 0.5 M aqueous $NaHCO_3$ (10 ml) at 23°. After various intervals, the reaction mixture was centrifuged, and the supernatant removed, the residue washed with water (10 ml), centrifuged, and the supernatant removed. The washing procedure was repeated twice. The NH_3 present in the combined supernatants was determined. After 1, 2, 4, 22, 28, and 51 h; 2.8, 5.6, 9.2, 26.8, 29.6, and 42.8 μmol NH_3 per g Sephadex-carbamate, respectively, were releazed.

5. *Hydrolysis of Sephadex-carbamate in the presence of different amino compounds.* The Sephadex-carbamate (3.0 g, from exp. 3) was reacted with methanol (5 ml) in 0.5 M aqueous $NaHCO_3$ (10 ml) for 18 h at room temperature to remove remaining carbonate groups. The reaction was followed by IR. The product was filtered and washed with water, water-methanol 1:1, methanol, ether and finally dried at 40° for 20 min. This product (0.250 g) in 0.5 M aqueous $NaHCO_3$ (10 ml) was stirred with glycine (0.075 g), glycylglycine (0.132 g) or glycyl-L-leucine (0.188 g), respectively, for 25 h at 20°. The pH of the solutions was adjusted to 8.4 by adding solid Na_2CO_3. The working up and the determination of NH_3 was carried out as in Exp. 4. After corrections for small amounts of impurities in the amino compounds used the NH_3 liberated was found to be 16 – 28 μmol per g Sephadex-carbamate.

6. *Hydrolysis of SxOH*.* SxOH* (N 1.9 %, 0.250 g) was treated as in Exp. 4. After 0.5, 1, 2, 6, and 21 h, 54, 72, 84, 120, and 132 μmol NH_3 per g SxOH*, respectively, were released.

7. *The reaction of amino compounds with SxOH*.* A. To glycine, glycine ethylester, and glycyl-L-leucine (1.00 g), respectively, in 0.5 M aqueous $NaHCO_3$ (2 ml) was added SxOH* (0.4 g) and the mixtures were stirred for 20 h at room temperature. After filtration, the products were washed with 0.5 M $NaHCO_3$, 0.01 M HCl, 1 M NaCl solution, water, water-acetone (3:1, 1:1, 1:3), acetone, and dried for 2 h at 40°. A blank, containing SxOH* but no amino compound was run parallel with the other samples. The results are given in Table 1.

B. To glycine, glycine ethylester and glycine-L-leucine (0.40 g), respectively, in 0.5 M aqueous $NaHCO_3$ (2.5 ml) was added SxOH* (0.40 g). The mixtures were stirred for 15 h at room temperature and worked up as above. A blank was run as above. The results are given in Table 2.

Table 1. Binding of amino compounds to SxOH*.

Amino compound	Nitrogen in the product %	Amino acid μmol/g	Calc. nitrogen in the product[a] % A	*B*	C
Glycine	7.3	1390(1348)[b]	7.3	5.4	5.4
Glycine ethyl ester.HCl	6.9	1276(1238)[b]	7.0	5.2	5.2
Glycyl-L-leucine	7.0	658, 680 gly leu	7.1	6.2	6.2
—	6.0				

[a] A, Calc. as isourea derivatives. B, Calc. as imidocarbonates. C, Calc. as *N*-substitued carbamates. [b] The values are corrected for incomplete hydrolysis. Non-corrected values in parentheses.

Table 2. Binding of amino compounds to SxOH*.

Amino compound	Nitrogen in the product %	Amino acid μmol/g	Calc. nitrogen in the product[a] % A	B	C
Glycine	7.6	1646(1463)[b]	7.6	5.3	5.3
Glycine ethyl ester.HCl	7.4	1575(1401)[b]	7.2	5.0	5.0
Glycyl-L-leucine	6.6	673, 768 gly leu	7.1	6.1	6.1
–	6.0				

[a, b] Compare Table 1.

Table 3. Binding of amino compounds to SxOH*.

Amino compound	Released ammonia μmol/g	Nitrogen in the product %	Amino acid μmol/g	Calc. nitrogen in the product, %[a] A	B	C
Glycine	252	6.5	680(632)[b]	6.6	5.7	5.7
Glycine ethyl ester.HCl	212	6.5	509(473)[b]	6.4	5.7	5.7
Glycyl-L-leucine	210	6.2	133, 144 gly leu	6.2	5.8	5.8
–		6.0				

[a, b] Compare Table 1.

C. Solutions of glycine, glycine ethylester and glycine-L-leucine (0.25 g), respectively, in 1 M aqueous $NaHCO_3$ (10 ml) were adjusted to pH 8.4 by addition of solid Na_2CO_3. SxOH* (0.250 g) was added and stirring was continued for 18 h at room temperature. The reaction mixtures were centrifuged, the supernatants removed, and the residues washed with water, centrifuged, and the supernatants removed. The washing procedure was repeated twice. The NH_3 content of the combined supernatants was determined. The products were washed and dried as above. The results are given in Table 3.

D. The reactions between SxOH* (N 5.65 %, 0.250 g) and glycine, glycine ethylester, alanine, glycylglycine, glycyl-L-leucine, glycyltyrosine (1 mmol) and aqueous ammonia (68 μmol), respectively, in 0.5 M aqueous $NaHCO_3$ (10 ml) at 25° were allowed to proceed for 24 h. Excees CO_2 was removed at 50° during 5 min. The pH of the buffer solutions was 8.2 – 8.3. The working up procedure was as described in Exp. C. The results are given in Table 4.

8. *Dextran cross-linked by BrCN (dextran*).* A. Dextran 10 (45 g) was dissolved in water (300 ml) in a 500 ml three-necked flask provided with a stirrer and a pH-electrode. BrCN (7.9 g) was added in portions over 90 min. The pH was maintained at 10.5 by adding 5 M NaOH. After neutralization with 5 M HCl, the solution was dialyzed against tap water for 3 days and thereafter in distilled water for 2 days. The solution was then concentrated to 200 ml at 65°, the cross-linked dextran precipitated, using 2.5 l ethanol, and dried for 2 h at 40°. $\overline{M}_w$ 229 000, $\overline{M}_n$ 17 000. N 1.40 %.

B. Conditions and working up procedure as above. BrCN (8.9 g) was added in portions over 90 min. $\overline{M}_w$ 1 080 000, $\overline{M}_n$ 23 000.

9. *Reaction of glycine and glycylglycine with dextran*.* A. Glycine (1 mmol) and dextran* (from exp. 8B, 0.5 g) were dissolved in 1 M aqueous $NaHCO_3$ (10 ml). After 15 h at 20°, the reaction mixture was dialysed for 5 days and freeze-dried. $\overline{M}_w$ 111 000, $\overline{M}_n$ 13 000, glycine 37.5 μmol/g. A parallel experiment using the same reaction conditions, was performed with dextran * (from exp. 8B, 0.5 g). $\overline{M}_w$ 148 000, $\overline{M}_n$ 17 000.

B. Glycylglycine (1 mmol) and dextran* (from exp. 8A, 0.5 g) were dissolved in 0.5 M aqueous $NaHCO_3$ (10 ml). After 23 h at 25°, the reaction mixture was dialysed for 5 days

Table 4. Binding of amino compounds to SxOH*.

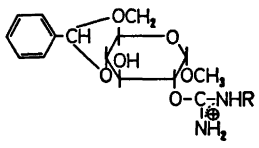
Amino compounds	Released ammonia μmol/g	Nitrogen in the product %	Amino acid μmol/g	Calc. nitrogen in the product, %[a] A	B	C
Glycine	304	5.9	701(637)[b]	5.8	4.8	4.8
Glycine ethyl ester.HCl	292	5.7	542(493)[b]	5.6	4.8	4.8
Alanine	288	5.4	387(352)[b]	5.5	4.9	4.9
Glycyl-L-leucine	280	5.6	275, 309 gly leu	5.7	5.2	5.2
Glycyl-L-tyrosine	320	5.5	399, 438 gly tyr	5.8	5.2	5.2
Glycylglycine	288	5.5	529(503)[b]	5.7	5.3	5.3
Ammonia[d]	484	5.2				
—	268	5.1				
—	—	5.65[c]				

[a,b] Compare Table 1. [c] Nitrogen content before treatment. [d] 272 μmol/g added to the system.

and freeze-dried. $\overline{M}_w$ 57 000, $\overline{M}_n$ 11 000, glycine 51 μmol/g. A parallel experiment, using the same reaction conditions, was performed with dextran* (from exp. 8A, 0.5 g). $\overline{M}_w$ 71 000, $\overline{M}_n$ 15 000.

RESULTS

1. *Studies with model compounds.* When MBGP* was reacted with glycine or glycylglycine in water-acetone, using triethylamine as catalyst, only one type of addition products could be isolated. These are isourea derivatives (I) according to evidence given below.



R: $-CH_2COO^{\ominus}$ $-CH_2CONHCH_2COO^{\ominus}$

Ia Ib

The products obtained contained impurities from the activation of MBGP and were purified by various washing procedures and repeated precipitations from hot aqueous solution. The purified products are amorphous and are only soluble in hot water and methanol, indicating a dipolar structure. The elemental analyses ruled out a carbamate or imidocarbonate structure but were in accordance with the proposed isourea formula (I). This was also confirmed by the characteristic double peak at 1660 – 1700 cm^{-1} in the IR-spectrum.[12] No attempts to separate the 2- and 3-isomers were performed.

II. *The reactions of amino compounds with SxOH* and dextran**. The products obtained by reacting SxOH* with amino compounds in dilute alkaline solution have been given structures represented schematically in II – IV.[3,13,14]

SxOCNHR (O) II SxOCNHR (NH) III $(SxO)_2C=NR$ IV

If products of type III or IV are formed at least one molecule of ammonia per molecule amino compound incorporated into the Sephadex matrix would be liberated. As shown in Table 4, the amount of ammonia liberated during the coupling procedure is 280 – 320 μmol/g SxOH* and the amount of amino compound incorporated 309 – 701 μmol/g SxOH*. Under the same conditions but with no amino compound, SxOH* releases 268 μmol/g SxOH* due to partial hydrolysis of carbamate and other groups in the SxOH* matrix. Thus, only a few percent of the released ammonia may come from reactions leading to *N*-substituted carbamates (III) and *N*-substituted imidocarbonates (IV), which shows that the main component is the isourea derivative (II).

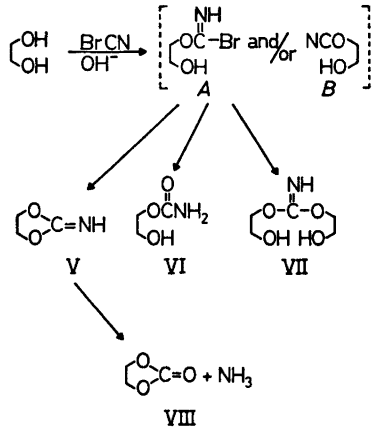
The carbamate groups in SxOH* are only slowly hydrolyzed under the weak alkaline conditions used, as was shown from studies with Sephadex-carbamate, prepared by reacting Sephadex-carbonate with liquid ammonia. The Sephadex-carbamate was hydrolyzed and the ammonia released determined. Only 15 – 30 μmol of ammonia per g Sephadex-carbamate was obtained (Exp. 4 – 5), thus demonstrating that only a small part (5 – 10 %) of the ammonia liberated could then arise from hydrolysis of carbamate groups in SxOH*. The main part of the ammonia liberated during the coupling procedure must originate from the hydrolysis of other groups in the SxOH* matrix. Thus, SxOH* was hydrolysed in 0.5 M aqueous $NaHCO_3$, and the ammonia determined as a function of time. Most of the ammonia was liberated within the first 6 h, giving after 21 h totally 132 μmol of ammonia per g SxOH* (Exp. 6). It may be noted that this SxOH* only contained 1.9 % N, while, in most experiments performed, the nitrogen content was 5.6 – 6.0 %.

Dextran * was obtained by reacting concentrated low molecular weight dextran solutions with BrCN in alkaline media. The product was kept in water for several days to make sure that the unstable five-membered *trans*-cyclic imidocarbonate groups,[5] which also are formed, were completely hydrolysed. The dextran* was then reacted with some amino compounds and the molecular weights and the amino acid contents of the products determined (Exp. 9A and B).

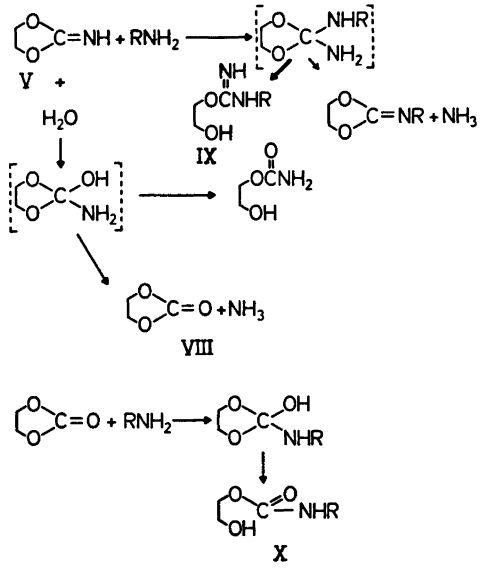
DISCUSSION

The reaction of BrCN in dilute alkaline solution with vicinal *trans*-hydroxyl groups as in Sephadex or dextran is represented schematically in Scheme 1. The structures A[15] and B are proposed as intermediated leading to end-products with the structures V – VIII.

Proof of the existence of the reactive groups V, the carbamate groups VI, and the carbonate groups VIII was presented in studies on a model compound, MBGP.[6] The acyclic imidocarbonate structures VII are formed mainly by cross-linking two glucose units in different dextran chains. This is indicated by the reduced swelling capacity of the SxOH* and by the increase in molecular weight when low molecular weight dextrans are treated with excess of BrCN [2] (Exp. 8A and B). The amount of VIII in SxOH* is low since its characteristic IR-absorption at 1800 – 1830 cm^{-1} is very weak. Under favourable activation conditions, it has been found that about 50 % of the nitrogen incorporated in the activated products is due



Scheme 1.



Scheme 2.

to imidocarbonate groups (V).[5] Thus, it can be concluded that the reactive groups V and the carbamate groups (VI) are the main structures present in BrCN activated carbohydrates, containing vicinal *trans*-hydroxyl groups and that V and VI both have IR-absorption at 1715 cm^{-1}.

The reaction of V with an amino compound in $NaHCO_3$ solution and the side reaction the alkaline hydrolysis of V, is shown in Scheme 2. The first step is presumably a nucleophilic addition of the amino compound to V, giving a five-membered unstable intermediate which subsequently rearranges to the stable isourea derivative IX. Investigations with a model compound (MBGP) have resulted in the isolation and characterization of only one type of addition product, the isourea derivative. Only a few percent of the total amount of ammonia anticipated, if *N*-substituted imidocarbonates are the only reaction products, has been found. It may also be pointed out that the amount of NH_3 released is rather constant and thus independent of the amount of amino compound incorporated in the Sephadex matrix (Tables 3 and 4). Furthermore, as shown in Tables 1 – 2, particularly where the amount of amino compound in the products is high, the calculated nitrogen content for the isourea structure agrees fairly well with the value found.

The hydrolysis of V under the coupling conditions used may compete with the addition of the amino compound to SxOH*. Thus, V may by addition of water be transformed into a reactive intermediate (Scheme 2), giving either the carbamate or the carbonate, the latter formed with concomitant release of ammonia.

Several products obtained by reacting SxOH* with amino compounds have been examined by IR-spectroscopy. No absorption at 1800 – 1830 cm^{-1}, characteristic of the carbonate group, has been found. If, however, some carbonate groups are formed, they may react with the amino compound, giving as end-products *N*-substituted carbamates (X), which as discussed above can only be present in a few percent.

When dextran*, in which the reactive *trans*-cyclic imidocarbonate groups have been removed, is treated with amino compounds under mild basic conditions, the molecular weight decreases more than when the dextran* is treated similarly but in the absence of the amino compounds. This indicates that the acyclic structures VII, which are likely to be the remaining reactive groups, react at least to some extent with the amino compounds giving structures IX and X. Stable, low molecular weight acyclic imidocarbonates, analogous to XI, are known,[16] and the formation of such structures cannot be excluded. The main part of the decrease in molecular weight arises from the hydrolytic cleavage of the acyclic imidocarbonates (VII) with the formation of carbamates (VI). Small amounts of the acyclic carbonates (XII) may also be formed. These may then react with amino compounds to give the *N*-substituted carbamates (X).

It has been shown that some of the ammonia released during the coupling procedure may arise from hydrolysis of carbamates and from the formation of imidocarbonates or carbonates. However, the main part was demonstrated to originate from hydrolysis of other groups in the SxOH*. At present we can only speculate as to which groups are responsible and suggest that during the activation procedure part of the imidocarbonate groups (V) may have reacted with BrCN to give cyanamide groups. During the mild alkaline conditions of the coupling reaction, nitrogen compounds may be released from these groups and give ammonia during the strong alkaline conditions of the Kjeldahl analysis.

The ammonia released from SxOH* may react with imidocarbonate groups in the SxOH*, yielding isourea groups. However, from the experiments with added ammonia (Table 4), it is evident that this reaction is of little importance.

XI

XII

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Two Dibenzofurans Obtained on Oxidative Degradation of the Moss *Polytrichum commune* Hedw.

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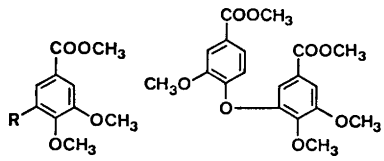
Stalks of the moss *Polytrichum commune* Hedw. were finely ground and the resulting meal was extracted and heated at 170° with aqueous NaOH-Na_2S for 3 h. The dissolved material was methylated with dimethyl sulfate, oxidized with $KMnO_4$-$NaIO_4$ in aqueous sodium hydroxide and with H_2O_2 in aqueous sodium carbonate. The crude mixture of products was treated with diazomethane. In addition to methyl veratrate and dimethyl isohemipate, two new esters were isolated and identified as methyl 4,7,9-trimethoxy-2-dibenzofurancarboxylate and methyl 3-(4,7,9-trimethoxy-2-dibenzofuranyl)-propanoate. Neither dimethyl 2′,5,6-trimethoxydiphenylether-3,4′-dicarboxylate nor dimethyl 5,5′-dehydrodiveratrate, two oxidation products characteristic of guaiacyl and guaiacyl-syringyl lignins, could be detected. This finding indicates that *Polytrichum commune* does not contain lignin.

A method for the structural characterization of lignins has recently been described.[1] This method (in this paper referred to as "oxidative degradation") comprises the solubilization of the lignin of the preextracted, finely ground plant material by heating with aqueous NaOH-Na_2S for 3 h at 170°, followed by methylation with dimethyl sulfate, and oxidation with permanganate-periodate in aqueous sodium hydroxide and with H_2O_2 in aqueous sodium carbonate. The resulting mixture of aryl carboxylic acids is treated with diazomethane in methanol-ether, and the major esters thus formed are measured by quantitative gas-liquid chromatography (GLC). Their relative amounts are indicative of the type of lignin originally present.

As the presence of lignin in non-vascular plants, notably the taxon Bryophyta, is still a matter of dispute (for a recent survey, see Ref. 2), we decided to investigate some mosses (Musci) and liverworts (Hepaticae) by this method. The present paper is restricted to the presentation of the results from the oxidative degradation of stalks of the moss *Polytrichum commune* Hedw.

Gas-liquid chromatography of the methyl esters obtained on oxidative degradation of preextracted *Polytrichum commune* meal (200 mg) indicated the presence of methyl veratrate (*1*) and dimethyl isohemipate (*2*), later confirmed by mass spectrometry (MS). Esters *1* and *2* have also been found to be the dominant members of the mononuclear ester fractions resulting from the oxidative degradation of a number of conifers [1,3] and ferns.[3]



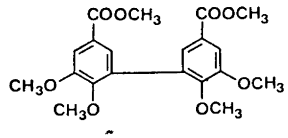
1 R = H
2 R = $COOCH_3$

3

The composition of the binuclear ester fraction, as roughly defined by the volatility properties of its members in GLC analysis, was completely different from the composition of corresponding fractions obtained from guaiacyl

* Part XIII of the series "Gas Chromatographic Analysis of Lignin Oxidation Products". Part XII, see Ref. 13.

lignins (the lignins of most gymnosperms and Pteridophyta). Dimethyl 2′,5,6-trimethoxydiphenylether-3,4′-dicarboxylate (*3*) and dimethyl 5,5′-dehydrodiveratrate (*4*), the two major binuclear esters obtained from guaiacyl lignins, could not be detected among the degradation products of *Polytrichum commune* Hedw.



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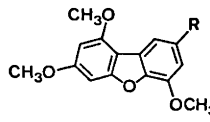
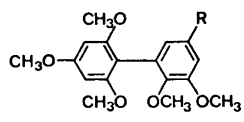
Instead, two unknown compounds, termed *A* and *B*, dominated the binuclear ester fraction from the moss. The mass spectra of *A* (molecular ion at $m/e = 316$, base peak) and of *B* (molecular ion at $m/e = 344$, base peak) were rather uninformative concerning the basic skeleton of these compounds. High resolution mass spectrometry indicated an elemental composition of $C_{17}H_{16}O_6$ for *A* and $C_{19}H_{20}O_6$ for *B*.

In order to obtain *A* and *B* in milligram quantities for NMR analysis, a total of 7 g of moss meal was oxidatively degraded. Separation of the products by preparative thin layer chromatography (TLC) yielded 15 mg of *A*, colourless crystals of m.p. 196 – 198°, and 19 mg of *B*, colourless crystals of m.p. 84.5 – 85.5°. The NMR spectrum of *A* revealed the presence of four methoxyl groups and four deshielded protons, coupled in pairs with coupling constants corresponding to *m*-positioned aromatic protons. The shift of the strongly deshielded proton at δ 8.27 ppm was indicative of in-plane deshielding by a neighbouring ring (*cf.* Ref. 4). On the basis of this spectroscopic evidence, structure *5*, methyl 4,7,9-trimethoxy-2-dibenzofurancarboxylate, was assigned to *A*. This assignment was confirmed by synthesis of *5 via* the biphenyls *8*, *9*, *10*, and *11*, and the dibenzofuran *7*.



5 R = $COOCH_3$
6 R = $CH_2CH_2COOCH_3$
7 R = CH_3



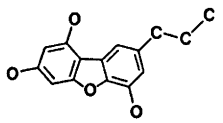
8 R = $COOH$
9 R = $COOCH_3$
10 R = CH_2OH
11 R = CH_3

The NMR spectrum of *B* indicated the same ring system as in *A*, but with the methoxycarbonyl substituent replaced by a 2-methoxycarbonylethyl side chain. This was supported by the prominent M – 73 fragment (CH_2-$COOCH_3$) in the mass spectrum of *B*. Furthermore, *B* was found to yield *A* on oxidative degradation. It thus could be concluded that *B* is represented by structure *6*, methyl 3-(4,7,9-trimethoxy-2-dibenzofuranyl)-propanoate. It seems very likely that, on oxidation of *Polytrichum commune* meal, *A* is formed *via B* or from a parent structure common to *A* and *B*.

It should be noted that this method for oxidative degradation originally had been optimized for the characterization of lignins in vascular plants. The reason why *B* is not completely oxidized to *A* in the experiments with moss meal must be due to an insufficiency of oxidant. This insufficiency may be caused by the comparatively large amounts of organic material dissolved by the NaOH-Na_2S pretreatment of the moss meal.

Since in the method used, methylation of phenolic hydroxyl groups precedes the oxidative degradation, nothing could be ascertained about the number of methylated phenolic hydroxyl groups originally present in the parent structures of type *12*. In a degradation experiment with *Polytrichum commune* meal, an answer to this question was found by using hexadeuterio-dimethyl sulfate in the methylation step. Mass spectra of deuterated components corresponding to *5* and *6* demonstrated the incorporation of three trideuterio-methyl groups (M + 9) per molecule. Thus all the methoxyl groups of *A* and *B* are derived from non-methylated phenolic hydroxyl groups originally present in parent structures of type *12*. By the same technique, it was furthermore demonstrated that the esters *1* and *2* are derived from non-methylated 4-alkyl and 3,5-dialkyl pyrocatechol units, respectively, rather than from the corresponding units of guaiacyl type as in guaiacyl and guaiacyl-syringyl lignins. The demethylation of methoxyl groups by the pretreatment with NaOH-Na_2S at 170° is comparatively slow and can be neglected.[5]

When *Polytrichum commune* meal was heated with NaOH-Na_2S, methylated with dimethyl



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sulfate, and treated with diazomethane, omitting the oxidation steps, compound *B* (*6*) could be detected while *A* (*5*) was absent. On the other hand, neither of them was found to be formed on mild alkaline hydrolysis of the plant material with aqueous KOH at 25°, or by boiling with 0.2 M HCl in dioxane-water (9:1, "acidolysis" [6]). To rule out the possibility of the precursor of *B* being an artefact formed from phloroglucinol and 4-alkylpyrocatechol structures eventually present, 4-methylcatechol and phloroglucinol were heated with aqueous NaOH-Na_2S at 170° and the reaction product methylated. No formation of *7* could be observed, however.

The biogenesis of the dibenzofuran structures yielding *A* and *B* remains to be properly explained. Obviously, one part of structure *6* is a phenylpropane, probably formed *via* a shikimic acid pathway. The substitution pattern of the other moiety is that of phloroglucinol and indicates formation by head–to-tail condensation of acetate units followed by cyclisation.

From the experiments described above it can thus be concluded that the moss *Polytrichum commune* Hedw. contains 3-(2-dibenzofuranyl)-propyl structures with oxygen functions in the 4-, 7-, and 9- positions (*12*). These entities are not connected with the bulk cell wall material by ester linkages. *Polytrichum commune* does not contain lignin, although structures partially derived from C_6C_3-units are present. This finding invalidates the postulation [7] that *Polytrichum commune* has a lignin of the *p*-hydroxyphenyl type.

EXPERIMENTAL

Plant material. Upper parts of stalks of *Polytrichum commune* Hedw., as long as covered with green leaves, were collected in Halland (southern Sweden). After removal of the leaves and fixation in ethanol, the stalks were ground and the resulting meal extracted and dried as described.[1]

Treatment of the moss meal with NaOH-Na_2S, methylation and oxidation. See Ref. 1.

GLC. See Ref. 1. *Retention times* (SE 30, relative to *4*, $t=240°$): *5*, 0.92; *6*, 1.43; *7*, 0.40; *9*, 0.60; *11*, 0.24.

MS. Identification: AEI MS 20. *High resolution:* AEI MS 902. *Electron energy:* 70 eV. *Source:* 200°. *Intensity relative to base peak (rel. int.):* ≥ 5 %. *Lower mass limit:* $m/e=100$.

NMR. 60 MHz, in $CDCl_3$, tetramethylsilane as internal standard, δ-values (ppm).

TLC. Silica gel HF 254, Merck. R_F*-values* (acetone-hexane 1:2): *1*, 0.29; *A*, 0.35; *B*, 0.25.

Isolation of compounds A *and* B. For this purpose, 7 g of preextracted moss meal was used. The permanganate oxidation was carried out in 7 batches. Appreciable amounts of MnO_2 precipitated during this reaction. The combined products were treated with H_2O_2 and then with diazomethane as described.[1] The resulting viscous oil (0.5 g) was chromatographed on preparative TLC plates (acetone-hexane 1:2). A major fraction contained compounds *1*, *A* and *B*. These components were separated by repeated TLC on analytical plates (toluene, acetone-hexane 1:2).

Compound *A*, *methyl 4,7,9-trimethoxy-2-dibenzofurancarboxylate* (5), colourless crystals (15 mg), m.p. 196–198° (ethyl acetate-hexane). (Found: C 64.67; H 5.16. Calc. for $C_{17}H_{16}O_6$ (316.32): C 64.55; H 5.10). A mixed m.p. with synthetic compound *5* showed no depression. *A* and synthetic *5* gave identical mass and NMR spectra. *NMR* (3 %): δ 3.86 (3) s, OCH_3; 3.96 (3) s, OCH_3; 4.00 (3) s, OCH_3; 4.07 (3) s, OCH_3; 6.42 (1) d, H_8; 6.71 (1) d, H_6; 7.56 (1) d, H_3; 8.27 (1) d, H_1. $J_{1,3}=1.5$ Hz. $J_{6,8}=1.9$ Hz. *MS. m/e, rel. int.*: 316, 100; 301, 11; 285, 22; 273, 19; 258, 11; 257, 9; 228, 5; 227, 8; 212, 10; 199, 5; 158, 11; 143, 10; 142.5, 24; 106, 11. *Exact mass of the molecular ion.* Found: 316.0936. Calc. for $C_{17}H_{16}O_6$: 316.0947.

Compound *B*, *methyl 3-(4,7,9-trimethoxy-2-dibenzofuranyl)-propanoate* (6), 19 mg, colourless needles, m.p. 84.5–85.5° (ethyl acetate). (Found: C 66.12; H 5.88. Calc. for $C_{19}H_{20}O_6$ (344.37): C 66.27; H 5.85). *NMR* (5 %): δ *ca.* 2.87 (4) m, CH_2CH_2; 3.68 (3) s, OCH_3; 3.85 (3) s, OCH_3; 3.98 (3) s, OCH_3; 4.01 (3) s, OCH_3; 6.35 (1) d, H_8; 6.69 (2) m, H_6 and H_3; 7.41 (1) broad d, H_1. $J_{6,8}=1.9$ Hz. *MS. m/e, rel. int.*: 344, 100; 316, 6; 313, 8; 301, 5; 285, 41; 284, 13; 271, 85; 270, 7; 256, 10; 241, 9; 227, 10; 226, 7; 213, 14; 167, 15; 149, 45; 142.5, 11. *Exact mass of the molecular ion.* Found: 344.1260. Calc. for $C_{19}H_{20}O_6$: 344.1260.

In addition to *A* and *B*, 37 mg of *methyl veratrate* (1), m.p. 56–57° (ether-hexane, Ref. 12, m.p. 58°) was isolated.

Oxidation of B. Dimethyl isohemipate (*2*, as internal standard) and *B* (4 mg) were oxidized with permanganate-periodate, the H_2O_2 step being omitted. After methylation of the products with diazomethane, analysis by GLC dem-

onstrated the formation of *A* in about 60 % yield and the presence of traces of *B* (identification by MS).

Acidolysis of moss meal. 200 mg of preextracted moss meal was acidolyzed.[6] The reaction product was methylated with diazomethane and analyzed by GLC. No *B* could be detected. Trimethylsilylation [Bis(trimethylsilyl)trifluoroacetamide, Pierce, in pyridine], followed by GLC, did not reveal the presence of binuclear degradation products.

Mild alkaline hydrolysis of moss meal. Moss meal (preextracted, 200 mg) was suspended in 25 ml of 2 M KOH under nitrogen for 26 h. After methylation with dimethyl sulfate, the solids were filtered off, the filtrate was neutralized, evaporated to dryness, and oxidized as usual. No *A* or *B* could be detected by GLC.

Treatment of moss meal with NaOH-Na_2S at 170°. The methylated (dimethyl sulfate) products were dissolved in methanol and methylated with ethereal diazomethane. GLC demonstrated the presence of minor amounts of *B*.

Treatment of phloroglucinol and 4-methylpyrocatechol with NaOH-Na_2S at 170°. A solution of 33 mg of 4-methylcatechol and 33 mg of phloroglucinol in 10 ml of aqueous NaOH-Na_2S was heated to 170° for 3 h. The solution was acidified, extracted with acetone-$CHCl_3$ (1:1), and the evaporation residue was methylated with dimethyl sulfate. *7* could not be detected by GLC.

Syntheses

2′,4′,5,6,6′-Pentamethoxybiphenyl-3-carboxylic acid (8). A mixture of bromophloroglucinol trimethyl ether[8] (3.8 g), methyl 5-bromoveratrate[9] (4.0 g), and copper bronze (10 g) was heated under nitrogen at 240° for 4 h. The ethyl acetate extract of the products was distilled *in vacuo* at 0.01 Torr. The fraction collected between 160 and 215° was saponified with KOH in MeOH-H_2O. From the alkaline solution, 1.1 g of *2,2′,4,4′,6,6′-hexamethoxybiphenyl*, m.p. 156° (Ref. 10, m.p. 156°), was isolated by extraction with ether. Neutralization afforded a mixture of 5,5′-dehydrodiveratric acid and *8*, from which *8* could be separated by soaking with acetone. Recrystallization from acetone yielded 480 mg of *8*, m.p. 238–239°. (Found: C 61.89; H 5.79. Calc. for $C_{18}H_{20}O_7$ (348.36): C 62.06; H 5.79).

Diazomethane and *8* (450 mg) gave *9* (440 mg), m.p. 145.5–146.5° (ethyl acetate-hexane). (Found: C 62.85; H 6.17. Calc. for $C_{19}H_{22}O_7$ (362.39): C 62.97; H 6.12). *NMR* (10 %): δ 3.65 (3) s, OCH_3; 3.68 (6) s, 2 OCH_3; 3.84 (6) s, 2 OCH_3; 3.92 (3) s, OCH_3; 6.19 (2) s, $H_{3'}$ and $H_{5'}$; 7.48 (1) d, H_4; 7.55 (1) broadened d, H_2. $J_{2,4}$ = 2.0 Hz.

2,2′,3,4′,6′-Pentamethoxy-5-hydroxymethylbiphenyl (10). Reduction of *9* (390 mg) with $LiAlH_4$ (180 mg) in tetrahydrofuran (40 ml) gave *10* as a colourless oil. *NMR* (10 %): δ *ca.* 2.3 (1) broad s, OH; 3.57 (3) s, OCH_3; 3.66 (6) s, 2 OCH_3; 3.83 (6) s, 2 OCH_3; 4.57 (2) s, $ArCH_2$; 6.18 (2) s, $H_{3'}$ and $H_{5'}$; 6.67 (1) d, H_6; 6.98 (1) d, H_4. $J_{4,6}$ = 2.0 Hz.

2,2′,3,4′,6′-Pentamethoxy-5-methylbiphenyl (11). Hydrogenolysis of *10* (Pd, 10 % on charcoal, in ethanol, 1 atm H_2) gave 243 mg of *11*, m.p. 122–123.5° (methanol). (Found: C 67.71; H 7.00. Calc. for $C_{18}H_{22}O_5$ (318.38): C 67.91; H 6.97). *NMR* (10 %): δ 2.30 (3) s, $ArCH_3$; 3.55 (3) s, OCH_3; 3.68 (6) s, 2 OCH_3; 3.82 (6) s, 2 OCH_3; 6.18 (2) s, $H_{3'}$ and $H_{5'}$; 6.52 (1) m, H_6; 6.67 (1) broad d, H_4. $J_{4,6}$ = 1.8 Hz.

1,3,6-Trimethoxy-8-methyl-dibenzofuran (7). For the method, see Ref. 11. The solution of 145 mg *11* in 10 ml HBr (48 %) and 10 ml glacial acetic acid in a thick walled, evacuated glass ampoule was heated to 110° for 24 h. The contents were neutralized with 40 % KOH and the solution extracted with ether (4 × 40 ml), then washed with dithionite and dried. The methylated (diazomethane) product was purified by TLC (acetone-hexane 1:2). Crystallization from methanol yielded 61 mg of *7*, m.p. 108.5–110.5. (Found: C 70.44; H 5.98. Calc. for $C_{16}H_{16}O_4$ (272.31): C 70.57; H 5.92). *NMR* (10 %): δ 2.46 (3) s, $ArCH_3$; 3.83 (3) s, OCH_3; 3.97 (3) s, OCH_3; 4.02 (3) s, OCH_3; 6.35 (1) d, H_2; 6.68 (1) m, H_7; 6.71 (1) d, H_4; 7.40 (1) m, H_9. $J_{2,4}$ = 1.9 Hz.

Methyl 4,7,9-trimethoxy-2-dibenzofurancarboxylate (5). 40 mg of *7* was oxidized, with omission of the H_2O_2 step. After methylation with CH_2N_2, 14 mg of *5*, m.p. 195–197°, were obtained (ethyl acetate). (Found: C 64.09; H 5.13. Calc. for $C_{17}H_{16}O_6$ (316.32): C 64.55; H 5.10).

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Thermodynamics of Vinyl Ethers. IV. Rotational Isomerism and the Relative Stabilities of the Geometric Isomers of Alkyl 1-Propenyl Ethers

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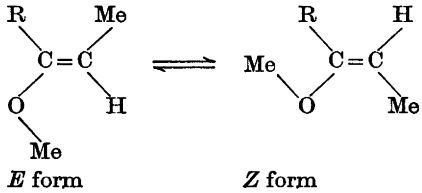
Equilibrium concentrations of the *E* and *Z* forms of some alkyl 1-propenyl ethers have been determined in the neat liquid at various temperatures. The values of $\Delta H°$ and $\Delta S°$ of these reactions suggest the presence of planar s-*cis* and s-*trans* (but not the nonplanar *gauche*) rotamers in the *E* forms of the compounds studied. An exception is *t*-butyl 1-propenyl ether which probably adopts only the s-*trans* configuration in each of the geometric isomers. The enthalpy of the s-*trans* rotamer of the *E* isomer of methyl 1-propenyl ether is about 4.0 kJ mol^{-1} higher than that of the s-*cis* rotamer. The relatively high enthalpy and entropy values of compounds of the type (*Z*)-MeOC(R_1)=CHR_2, where R_1 and R_2 are alkyl groups, are explained to be due to steric interactions between the methoxy group and R_1 in the planar s-*trans* rotamer.

In previous papers,[1-3] the relative stabilities of various α,β-unsaturated (vinyl) ethers differing in either the degree of double bond substitution by alkyl groups or geometric isomerism about the double bond have been studied. A considerable increase of enthalpy (as well as entropy) has been observed to result from the introduction of an alkyl group at the β carbon of the vinyl group in a *cis* position with respect to the methoxy group as in the reaction



E form ⇌ *Z* form (1)

for which, in case R=Me, $\Delta H°$ (g, 298.15 K)= 9.85±0.41 kJ mol^{-1} and $\Delta S°$ (g, 298.15 K)= 13.4±0.9 J K^{-1} mol^{-1}.[3] In the compounds studied previously in this series, the group R has always been larger than hydrogen and therefore it was felt necessary to obtain thermodynamic data for reaction (1) in the case R=H. Compounds of this kind have been the subject of several investigations[4-7] among which the most extensive work is that of Okuyama *et al.*[6] These authors reported the values of the thermodynamic functions of isomerization for reaction (1) (R=H) but, unfortunately, the values of $\Delta H°$, $\Delta S°$, T, and the equilibrium constant K given are in mutual disagreement. In order to check the results of the above authors, the relative stabilities of a number of alkyl 1-propenyl ethers were determined over a relatively wide range of temperature (more than 100 K). In the experimental performance of the measurements, the procedure proposed by Okuyama *et al.*[6] was used with slight modifications.

EXPERIMENTAL

Materials. Methyl 1-propenyl ether (I). 1-Chloropropyl methyl ether was prepared from methanol, propionaldehyde, and hydrogen chloride by the procedure described by Swallen and Boord.[8] The crude chloro ether was dropped in boiling pyridine after which I could be isolated from the reaction mixture by fractional distillation. After redistillation from metallic sodium, the yield of I boiling at 318.9–320.6 K/101.0 kPa was about 60 % (lit.,[5] I*Z* b.p. 316.7–317.2 K/101.9 kPa, I*E* 321.2–321.7

K/102.8 kPa). The product consisted of *ca.* equimolar amounts of the geometric isomers, and it was fractionated further to give the isomer mixtures used in the equilibrations.

Ethyl 1-propenyl ether (II). A 50 % yield of propionaldehyde diethyl acetal (b.p. 396.2–397.2 K/101.3 kPa) was obtained from propionaldehyde (0.5 mol), ethanol (1.75 mol) and concentrated hydrochloric acid (1 cm^3).[9] The acetal was heated with a small amount of sodium hydrogen sulfate in a distillation apparatus and the mixture of ethanol and II which was collected at 338–348 K was washed four times with water and the organic layer was dried over calcium chloride. The yield of II (b.p. 339–347 K/101.6 kPa, lit.,[10] II*Z* 342.2 K/100.6 kPa, II*E* 348.2 K/101.0 kPa) was about 23 %.

Isopropyl 1-propenyl ether (III). Propionaldehyde di-isopropyl acetal (b.p. 316.2 K/1.6 kPa) was prepared as described by Kulesza *et al.*[11] The yield was 34 %. The product was cleaved to a mixture of isopropyl alcohol and III by means of a catalytic amount of sulfanilic acid in the manner described above for II. The yield of III (b.p. 356.0–358.0 K/100.9 kPa, lit.,[10] III*Z* 356.2 K/100.0 kPa, III*E* 363.7 K/100.0 kPa) was 55 %.

s-*Butyl 1-propenyl ether* (IV). This product was prepared as described above for III. The di-*s*-butyl acetal of propionaldehyde (b.p. 340.2 K/1.3 kPa), which was obtained in 22 % yield, was cleaved to *s*-butyl alcohol and IV by distillation from sulfanilic acid. The yield of IV (b.p. 378.0–383.0 K/101.6 kPa) was about 20 %.

t-*Butyl 1-propenyl ether* (V). *t*-Butyl allyl ether was prepared from *t*-butyl alcohol, allyl alcohol and sulfuric acid.[12] The product (b.p. 373.2 K/101.3 kPa) was obtained in *ca.* 6 % yield. It was isomerized to *t*-butyl 1-propenyl ether by means of potassium *t*-butoxide in 1,2-dimethoxyethane.[4] After redistillation from sodium, the yield of V (pure *Z* isomer) was 25 %. The product boiled at 370.2–371.2 K/98.7 kPa.

Phenyl 1-propenyl ether (VI). Allyl phenyl ether was obtained from allyl bromide, phenol and potassium carbonate in acetone.[13] The product (b.p. 349.2–350.2 K/1.6 kPa) was isolated in 80 % yield. It was isomerized to VI (pure *Z* isomer) as described above for V. The yield of the product (b.p. 336.2 K/1.2 kPa, lit.,[14] 344 K/2.0 kPa) was 50 %.

Mercuric acetate (E. Merck AG, *pro analysi*) was used as received.

NMR spectra. The NMR spectra of the vinyl ethers studied were recorded on a 60 MHz Perkin Elmer Model R 10 spectrometer at 307 K. Carbon tetrachloride was used as solvent and tetramethylsilane as internal standard. The concentrations of the vinyl ethers were *ca.* 20 % (v/v). In general, mixtures of isomers were used for recording the spectra and because of the relatively low concentrations of the *E* isomers in the mixtures, accurate peak positions and coupling constants for the *E* forms could not be measured. An exception is I. The NMR spectra are shown in Table 1.

Procedure. The purities of the vinyl ethers used as substrates in the equilibrations were higher than 98 %. This was found by gas chromatography and NMR spectra. The impurity was mainly the corresponding acetal, the peak of which was easily separated from those of the vinyl ethers in the gas chromatographic analysis.

Table 1. NMR spectra of some alkyl 1-propenyl ethers in carbon tetrachloride at 307 K. Peak positions are given in τ values.

$$\underset{\text{d}}{\text{R}}-\text{O}-\underset{\text{a}}{\text{CH}}=\underset{\text{b}}{\text{CH}}-\underset{\text{c}}{\text{CH}_3}$$

R	Isomer	a	b	c	d	e	f	Coupling constants/Hz
CH_3	*E*	3.74	5.34	8.45	6.56	–	–	J_{ab} 12.9, J_{bc} 6.9
d	*Z*	4.20	5.71	8.45	6.46	–	–	J_{ab} 6.0, J_{bc} 6.9
CH_3CH_2	*Z*	4.14	5.74	8.47	8.78	6.27	–	J_{ab} 6.0, J_{bc} 6.9, J_{de} 7.1
d e								
$(CH_3)_2CH$	*Z*	4.11	5.71	8.48	8.80	6.14	–	J_{ab} 6.3, J_{bc} 6.9, J_{ac} 1.6,
d e								J_{de} 6.4
$CH_3CH_2(CH_3)CH$ [a]	*Z*	4.09	5.71	8.46	9.09	6.37	8.84	J_{ab} 6.0, J_{bc} 6.0, J_{de} 7.1
d e f g								J_{fg} 5.1
$(CH_3)_3C$	*Z*	3.93	5.71	8.49	8.75	–	–	J_{ab} 6.7, J_{bc} 6.0, J_{ac} 1.5
d								
C_6H_5	*Z*	3.75	5.29	8.31	2.7	–	–	J_{ab} 6.0, J_{bc} 6.9, J_{ac} 1.7
d					–3.3			

[a] The signal of the g proton was not detected.

During the equilibrations, the only detectable side reaction was a slight increase of the amount of the acetal. The isomerization experiments were conducted in the neat liquid with mercuric acetate as catalyst. The amount of the catalyst in the substrate varied from 5 to 10 mass %. The substrate-catalyst combinations were enclosed in small ampoules which were then kept at the appropriate temperatures. For the tracking of the progress of isomerization, ampoules were quickly transferred to an ice-water bath to "stop" the isomerization (this procedure was necessary only for equilibrations carried out considerably above room temperature; at lower temperatures, the slowness of the isomerization reaction made this step superfluous) after which the ampoules were broken and a small volume (*ca.* 20 % of the original sample size) of a suitable organic base (diethyl amine, triethyl amine) was added into the substrate-catalyst mixture to prevent isomerization during the analysis. Okuyama *et al.*[6] did not use any base in their experiments but it is our experience that the reproducibility of the subsequent gas chromatographic analysis was essentially better if the samples of the equilibrium mixture injected into the gas chromatograph contained sufficient amounts of some organic base. After the isomer ratios in successive samples were found to be unchanged, the state of equilibrium was considered having been attained and the following samples were analyzed over sufficiently extended periods of time (usually about three to five times the time necessary for the isomer ratio to become constant) to ascertain that the isomer ratio really remained unchanged. Moreover, in many cases the position of equilibrium was approached from both sides and the results of such experiments were in nice agreement. Peak areas, which were considered to be proportional to the molar concentrations of the isomers,[1] were determined by the cut-and-weigh method. The column used in the analyses contained 10 % Carbowax 1500 on Chromosorb G. The order of elution through this column was *Z*, *E* in each case.

Determination of normal boiling points. The normal boiling points of the compounds studied were determined by the gas chromatographic method described previously.[2] The following compounds were used for the preparation of the reference curve (compound, relative retention time, normal boiling point): ethyl vinyl ether, 0.166, 309.7 K; isobutyl vinyl ether, 0.299, 356.2 K; isobutylidene ethyl ether, 0.366, 366.7 K; 1-methoxycyclopentene, 0.622, 387.0 K; 1-ethoxycyclopentene, 1.000, 409.0 K. The following boiling points were obtained for the compounds I–V (compound, relative retention time, boiling point at 101.3 kPa): I*Z*, 0.181, 316.2 K; I*E*, 0.195, 321.4 K; II*Z*, 0.238, 337.2 K; II*E*, 0.263, 345.4 K; III*Z*, 0.299, 356.2 K; III*E*, 0.329, 363.0 K; IV*Z*, 0.487, 377.9 K; IV*E*, 0.564, 383.2 K; V*Z*, 0.409, 371.5 K; V*E*, 0.473, 376.8 K. For the determination of the boiling points of VI*Z* and VI*E*, the reference curve was made by means of the following data: 1-ethoxycyclopentene, 0.460, 409.0 K; 1-methoxycyclohexene, 0.540, 417.0 K; 1-ethoxycyclohexene, 0.697, 435.0 K; 1-propoxycyclohexene, 1.000, 455.9 K. The relative retention times of VI*Z* and VI*E* were 0.904 and 1.000 corresponding to normal boiling points of 450.9 and 455.9 K, respectively.

RESULTS

The primary results of the equilibration experiments are shown in Table 2. From the values of the equilibrium constant at various temperatures, the values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ at 298.15 K for each $E \rightleftharpoons Z$ isomerization were calculated as described earlier [1] and the results are collected in Table 3. In each case, a strictly linear relation between $\Delta G°$ and temperature was observed. By means of eqns (2) and (10) given in Ref. 2 the values of $\Delta H°$ and $\Delta S°$ in the liquid state were converted to those in the ideal gas state at 298.15 K (Table 4).

DISCUSSION

In general, our results (Table 3) are in qualitative agreement with those of Okuyama *et al.*[6] in the sense that the entropy change in each $E \rightleftharpoons Z$ isomerization is positive and that the enthalpy of isomerization decreases as the size of the alkyl group R increases. Quantitatively, the agreement between our results and those of Okuyama *et al.* is, however, poor. For example, in the reaction V*E*⇌V*Z* (R = *t*-Bu) the entropy change is about 0.4 J K^{-1} mol^{-1} according to Okuyama *et al.* but 6.3 J K^{-1} mol^{-1} according to our results. Similarly, in II*E*⇌II*Z* (R = Et), the values of $\Delta H°$ and $\Delta S°$ are *ca.* 1.6 kJ mol^{-1} and 7.9 J K^{-1} mol^{-1}, respectively, in the paper of Okuyama *et al.* but about −0.65 kJ mol^{-1} and 1.1 J K^{-1} mol^{-1} according to our results.

It seems that the simplest case in the $E \rightleftharpoons Z$ reactions studied is the reaction V*E*⇌V*Z* (R = *t*-Bu). This can be inferred from the bulky size of the *t*-Bu group, which makes the s-*cis* configuration (see below) highly unfavored even in the *E* isomer. In the *Z* isomer the s-*cis* configuration is completely out of question. Therefore, as the orientation of the *t*-BuO group apparently remains unchanged in the reaction, the values of $\Delta H°$ and $\Delta S°$ may be considered

Table 2. Values of the mean equilibrium constant K and its standard error for the $E=Z$ isomerization reactions of some alkyl 1-propenyl ethers in the neat liquid. Mercuric acetate was used as catalyst. c denotes catalyst concentration, t the time from the start of the equilibration when the first accepted sample was analyzed and n the number of independent determinations. Initial contents of the Z isomers in the equilibration mixtures (mol %): a 77.0; b 40.0; c 76.5; d 81.4; e 49.0; f 83.5; g 100.0.

R	T/K	c/mass %	Start from	t/d	n	$K(E \rightleftharpoons Z)$	R	T/K	c/mass %	Start from	t/d	n	$K(E \rightleftharpoons Z)$
Me	263.2	5–10	a	7	5	0.918±0.013	i-Pr	373.2	5	e	0.3	4	2.59±0.07
	283.2		a	4	4	0.964±0.012		373.2		d	0.3	4	2.61±0.05
	294.2		a	3	4	0.984±0.013		387.2		d	0.3	4	2.52±0.01
	313.2		a	1	2	0.989±0.001		399.2		e	0.3	4	2.51±0.03
	313.2		b	1	2	1.007±0.007		399.2		g	0.3	5	2.50±0.01
	328.2		a	0.7	4	1.023±0.006		423.2		d	0.2	4	2.42±0.03
	336.2		b	0.8	2	1.049±0.004							
	336.2		a	0.8	2	1.047±0.003	*s*-Bu	272.7	5	f	5	4	2.67±0.05
	343.2		b	0.3	5	1.042±0.017		284.2		f	3	5	2.65±0.07
	356.2		b	0.1	2	1.057±0.007		293.7		f	0.8	8	2.58±0.09
	357.2		a	0.2	2	1.066±0.003		303.7		f	0.8	4	2.53±0.07
	373.2		a	0.2	2	1.084±0.021		323.2		f	0.3	6	2.42±0.02
	373.2		b	0.2	3	1.085±0.010		347.2		f	0.8	6	2.37±0.02
	388.2		a	0.2	4	1.105±0.012		360.2		f	0.3	4	2.32±0.03
	403.2		a	0.1	3	1.138±0.005		370.2		f	0.8	4	2.27±0.03
								397.2		f	0.3	4	2.22±0.05
Et	283.2	5	c	7	6	1.495±0.036		413.2		f	0.7	3	2.17±0.02
	295.2		c	9	6	1.476±0.026							
	303.7		c	3	6	1.488±0.027	*t*-Bu	270.2	10	g	14	6	5.32±0.04
	333.2		c	0.3	6	1.438±0.020		293.2		g	6	6	5.00±0.17
	353.2		c	0.7	6	1.411±0.016		303.2		g	3	4	4.93±0.08
	373.2		c	0.3	5	1.403±0.016		323.2		g	1.9	6	4.65±0.08
	395.2		c	0.3	5	1.395±0.010		343.2		g	0.3	7	4.45±0.08
	410.2		c	0.2	9	1.371±0.031		355.2		g	1.7	7	4.29±0.06
								369.2		g	0.8	7	4.21±0.07
i-Pr	270.2	5	d	6	5	3.16 ±0.04		384.2		g	0.8	8	4.08±0.03
	283.2		d	5	8	3.10 ±0.09		406.2		g	0.8	8	3.95±0.09
	293.7		d	1.8	7	3.07 ±0.03							
	308.2		e	1.7	4	2.92 ±0.06	Ph	299.2	5	g	35	6	2.75±0.01
	308.2		g	1.6	6	2.93 ±0.07		308.2		g	22	5	2.74±0.01
	323.2		d	1.8	4	2.77 ±0.05		329.2		g	13	7	2.64±0.04
	323.2		e	1.8	3	2.80 ±0.01		347.2		g	27	5	2.58±0.02
	323.2		g	1.8	4	2.83 ±0.07		359.2		g	8	6	2.54±0.03
	345.2		e	0.9	4	2.71 ±0.01		373.2		g	8	6	2.49±0.01
	345.2		g	0.9	4	2.72 ±0.04		383.2		g	6	4	2.42±0.04
	360.2		e	0.8	4	2.63 ±0.02		397.2		g	2	5	2.39±0.02
	360.2		g	0.7	4	2.62 ±0.02		415.2		g	4	8	2.34±0.03

Table 3. Values of the thermodynamic functions $\Delta G°$, $\Delta H°$ and $\Delta S°$ for the $E \rightleftharpoons Z$ isomerization reactions of some alkyl 1-propenyl ethers in the neat liquid at 298.15 K. The errors are twice the standard errors.

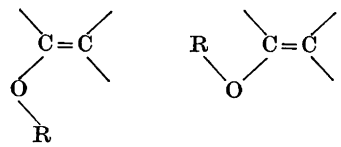
R	$\Delta G°$/kJ mol^{-1}	$\Delta H°$/kJ mol^{-1}	$\Delta S°$/J K^{-1} mol^{-1}
Me	0.044±0.019	1.26±0.12	4.09±0.35
Et	−0.969±0.018	−0.65±0.10	1.07±0.28
i-Pr	−2.702±0.023	−1.75±0.13	3.20±0.37
s-Bu	−2.324±0.013	−1.43±0.08	2.99±0.22
Ph	−2.541±0.026	−1.50±0.14	3.50±0.38
t-Bu	−3.959±0.018	−2.08±0.11	6.29±0.31

Table 4. Values of the thermodynamic functions $\Delta H°$ and $\Delta S°$ for the $E \rightleftharpoons Z$ isomerization reactions of some alkyl 1-propenyl ethers in the gas phase at 298.15 K.

R	$\Delta H°$/kJ mol^{-1}	$\Delta S°$/J K^{-1} mol^{-1}
Me	0.43 ± 0.32	2.85 ± 0.61
Et	-1.97 ± 0.31	-0.72 ± 0.57
i-Pr	-2.84 ± 0.33	1.81 ± 0.62
s-Bu	-2.28 ± 0.31	1.98 ± 0.55
Ph	-2.30 ± 0.33	2.73 ± 0.63
t-Bu	-2.94 ± 0.32	5.26 ± 0.59

reflecting only the enthalpy and entropy changes due to the altered position of the methyl group attached to the double bond. In the gas phase at 298.15 K, $\Delta H° = -2.94$ kJ mol^{-1}, which shows that there is a kind of attraction between the oxygen atom and the methyl group of the *Z* isomer. A similar situation prevails in 1-chloropropenes in which the enthalpy of the *Z* isomer is reported to be *ca.* 3.8 kJ mol^{-1} lower than that of the *E* isomer.[15] For a compilation of other similar examples, see Ref. 16.

Next let us consider the isomerization of I*E* to I*Z* (R=Me). It is generally accepted that methyl vinyl ether (MVE) exists as a mixture of at least two rotamers the most stable of which is the planar s-*cis* rotamer (see, for example, Ref. 17 and the references cited therein). Thus it seems justified to assume that I*E* also exists mainly in the s-*cis* configuration:



s-*cis* s-*trans*

On the other hand, this configuration is impossible for I*Z* because of steric hindrance. Hence a change in the spatial orientation of the methoxy group is a necessity in the reaction I*E*⇌I*Z*. Apparently, the orientation of the methoxy group in I*Z* is the same as in the less stable rotamer of MVE. Owen and Seip [18] and Aroney *et al.*[19] have suggested that the second rotamer of MVE has a nonplanar heavy atom skeleton which results from the rotation of the methoxy group about the O−C= bond. This view has been critisized by Katritzky *et al.*[20] and Cadioli and Pincelli.[17] The latter authors studied the electronic structure of MVE through SCF calculations. The second rotamer of MVE was predicted to be s-*trans* with an enthalpy about 8.4 kJ mol^{-1} higher than that of s-*cis*. The enthalpy of the nonplanar *gauche* rotamer (suggested by Owen and Seip [18] and Aroney *et al.*[19]) was calculated to be about 24 to 28 kJ mol^{-1} higher than that of the s-*cis* rotamer. The experimental difference in the enthalpies of the two most stable rotamers of MVE, 4.8 ± 1.0 kJ mol^{-1} in the gas phase at 298.15 K,[21] does not essentially differ from the value calculated by Cadioli and Pincelli.

The enthalpy change in I*E*⇌I*Z* is about 3.37 kJ mol^{-1} more positive than in V*E*⇌V*Z* (Table 4), which apparently is to be ascribed to the rotation of the methoxy group from the s-*cis* configuration (in I*E*) to the other configuration, *gauche* or s-*trans* (in I*Z*). It seems, however, that I*E* exists as a mixture of two rotamers (*cf.* MVE) and hence the enthalpy of the mixture of rotamers is higher than that of the pure s-*cis* rotamer, which has the lower enthalpy. The difference between the enthalpies of the rotamers may be estimated as follows. Assuming that the entropy difference between the rotamers is negligible, the mol fraction of the less stable rotamer may be estimated to be about 0.15 (at 298.15 K) if it is further assumed (as a first approximation) that the enthalpy difference does not essentially differ from that between the rotamers of MVE (4.8 kJ mol^{-1}). If the enthalpies of the rotamers of I*E* are denoted by H_t and H_c where H_t is the enthalpy of the less stable rotamer and equal to $(H_c + 4.8)$ kJ mol^{-1} (as a first approximation), the following equation can be written:

$$H_t - [0.85H_c + 0.15(H_c + 4.8)] = 3.37 \qquad (2)$$

Hence $H_t - H_c$ is *ca.* 4.1 kJ mol^{-1}. If this enthalpy difference is used to estimate the mol fractions of the rotamers of I*E* (assuming $\Delta S = 0$), the mol fractions are obtained as 0.84 (s-*cis*) and 0.16 (s-*trans* or *gauche*). Eqn. (2) may now be replaced by

$$H_t - [0.84H_c + 0.16(H_c + 4.1)] = 3.37 \qquad (3)$$

from which $H_t - H_c$ about 4.0 kJ mol^{-1}. The result achieved seems very reasonable.

The entropy change in the I*E*⇌I*Z* isomerization reaction, 2.85 J K^{-1} mol^{-1} in the gas phase at 298.15 K, is considerably less positive than

the entropy change in V*E*⇌V*Z*, 5.26 J K^{-1} mol^{-1}. This is probably due to the presence of two rotamers in I*E*, which, through the entropy of mixing, increases the entropy of I*E* by as much as 3.66 J K^{-1} mol^{-1}, if the mol fractions of the rotamers are taken as 0.84 and 0.16 (see above). Therefore, the entropy of isomerization of the pure s-*cis* rotamer of I*E* to I*Z* (which apparently exists as the pure s-*trans* rotamer, see later) should be about $3.66+2.85\cong6.5$ J K^{-1} mol^{-1}. The contribution of the entropy of mixing on the total entropy is, however, very sensitive to the values of the mol fractions of the rotamers and if the mol fractions are taken as 0.90 and 0.10, the "corrected" entropy change in I*E*⇌I*Z* is obtained as 5.5 J K^{-1} mol^{-1}. Hence the entropy change in this reaction is practically equal to that in V*E*⇌V*Z*, if the presence of rotational isomers in I*E* is taken into account.

It seems that the value of $\Delta S°$ in the reaction I*E*⇌I*Z* is reasonable only if it is accepted that the configuration of the methoxy group of I*Z* is s-*trans*, which may be deduced as follows. If I*Z* existed in the nonplanar *gauche* configuration, this would imply the presence of two enantiomeric isomers in which the C atom of the methoxy group lies either below or above the plane formed by the group of the other heavy atoms (O−C=C−C). In this case the value of the entropy change in the above reaction should be about 5.8 J K^{-1} mol^{-1} ($=R$ ln 2) more positive than can be explained by the reasoning given above. However, as shown above, the "corrected" entropy change is very close to the entropy change in V*E*⇌V*Z* in which no change in the configuration of the *t*-BuO group is expected. Hence only one rotamer is present in I*Z* and this rotamer must be s-*trans*. Thus it seems justified to assume that the configuration of the less stable rotamer of MVE is also s-*trans*, not *gauche*, in accordance with the predictions of Cadioli and Pincelli [17] and Katritzky *et al.*[20]

It is of interest to compare the values of $\Delta H°$ and $\Delta S°$ of the following reactions (gas phase, 298.15 K):

$$\underset{I\!E}{(E)\text{-MeOCH}=\text{CHMe}} \rightleftharpoons \underset{I\!Z}{(Z)\text{-MeOCH}=\text{CHMe}} \quad (4)$$

$\Delta H°=0.43$ kJ mol^{-1}, $\Delta S°=2.85$ J K^{-1} mol^{-1}

$$\underset{VII\!E}{(E)\text{-MeOC(Me)}=\text{CHMe}} \rightleftharpoons \underset{VII\!Z}{(Z)\text{-MeOC(Me)}=\text{CHMe}} \quad (5)$$

$\Delta H°=9.85$ kJ mol^{-1}, $\Delta S°=13.4$ J K^{-1} mol^{-1} (Ref. 3)

A similar change of structure is seen to lead to very different values of $\Delta H°$ and $\Delta S°$ and, curiously enough, the enthalpy change is more positive in the latter reaction despite the *cis* methyl-methyl interaction in VII*E*. As a possible explanation, it might be thought that the planar s-*trans* configuration is strongly unfavoured in VII*Z* because of the close approach of the hydrogen atoms of the methoxy group and those of the α methyl group in this rotamer. As also the planar s-*cis* configuration is impossible in VII*Z*, the methoxy group is forced to adopt a nonplanar configuration in which conjugation between the lone pair electrons of the oxygen atom and the π electrons of the double bond is essentially decreased, which leads to decreased stability. Thus it appears that VII*Z* might exist as a mixture of several rotamers. On the contrary, VII*E* probably exists only in the s-*cis* configuration because of the high enthalpy of the s-*trans* rotamer. The entropy of VII*Z* is increased by the presence of several rotamers and therefore the entropy change in VII*E*⇌VII*Z* is exceptionally positive.

As to the values of $\Delta H°$ in the other isomerization reactions studied, a continuous decrease in $\Delta H°$ is observed as the size of the alkyl group increases. This probably reflects an increase of s-*trans* rotamers in the *E* isomers, due to the increase of strain in the s-*cis* configuration as the alkyl group becomes bulkier. It seems, however, that also the entropy term might favor s-*trans* rotamers because of restricted rotation of the alkyl group in the s-*cis* configuration. This should be apparent from the high entropy change, 37.5 ± 8.3 J K^{-1} mol^{-1}, in the s-*cis* to s-*trans* reorientation of the *t*-BuO group in *t*-butyl formate.[22] The values of $\Delta S°$ in the above reactions are also qualitatively interpretable in terms of increased concentrations of the s-*trans* rotamers in the *E* isomers. A quantitative explanation of the enthalpy and entropy changes in these reactions is hardly possible.

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Thermodynamics of Vinyl Ethers. VI. The Stabilizing Effect of Methoxymethyl and (Methoxycarbonyl)methyl Groups on the Ethylenic Linkage of Vinyl Ethers

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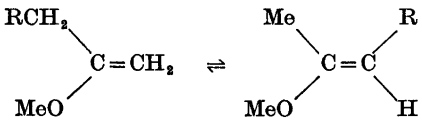
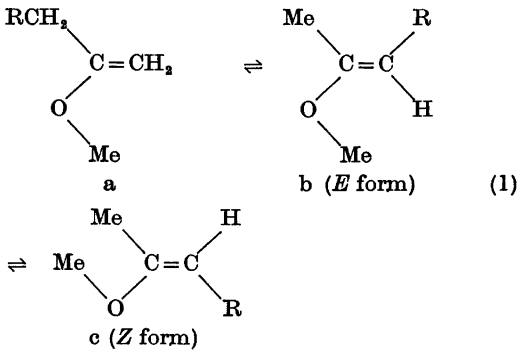
Thermodynamics of the following isomerization reaction have been studied by means of chemical equilibration:



The values of $\Delta H°$ (g, 298.15 K) (0.53 ± 0.47 kJ mol^{-1} for R = CH_3OCH_2 and 0.77 ± 0.39 kJ mol^{-1} for R = CH_3OCOCH_2) show that the stabilizing effect of a methoxymethyl or (methoxycarbonyl)methyl group on the double bond of a vinyl ether is equal within experimental error to that of an unsubstituted alkyl group, such as Me, Et, or i-Pr.

In part III of this series,[1] thermodynamics of the following isomerization reaction were studied:



(R = Me, Et, i-Pr)

The values of $\Delta H°$ for the a⇌b isomerization were found to be practically independent of the alkyl group R, $\Delta H°$ (g, 298.15 K) = 0.45 ± 0.38, 0.78 ± 0.39, and 0.60 ± 0.37 kJ mol^{-1} for the Me, Et and i-Pr derivatives, respectively, which shows that the stabilizing effect of the alkyl group on the double bond of the b isomer was practically independent of R. Excluding destabilizing steric interactions, the stabilizing energy of the alkyl group was evaluated as *ca.* 4.3 kJ mol^{-1}, which is considerably less than the stabilization, *ca.* 11 kJ mol^{-1}, brought about by R on the double bond of a corresponding olefinic hydrocarbon. This was assumed to be due to electron delocalization in vinyl ethers, which leads to a shift of negative charge to the β carbon of the double bond. Electropositive alkyl groups, when attached to the β carbon, were assumed to oppose this electron delocalization by their inductive effect, and thus their stabilizing effect on vinyl ethers is considerably weaker than on olefins.

However, it might be expected that if the alkyl group R were essentially less electropositive than Me, Et or i-Pr, electron delocalization in b would be restored and thus the group R should stabilize the double bond more effectively. To test the validity of this assumption, thermodynamics of reaction (1) were studied in cases R = CH_3OCH_2 and R = CH_3OCOCH_2. Both of these groups are noticeably more electronegative than Me, Et, or i-Pr.

EXPERIMENTAL

Materials. 2,4-Dimethoxy-1-butene (Ia) and isomers (Ib, Ic). 2-Methoxyethyl methyl ketone was prepared from methyl vinyl ketone and

methanol in the presence of mercuric oxide and boron trifluoride etherate.[2] The yield of the product boiling at 410 – 411 K/101.8 kPa was 60 %. It was converted to its dimethyl acetal by means of trimethyl orthoformate in methanol.[3] The yield of the acetal, b.p. 335 – 336 K/2.7 kPa, was 62 %. The acetal was distilled slowly from Al_2O_3 to give a mixture of methanol and the desired vinyl ethers.[4] The product was washed with water and dried over $CaCl_2$. After redistillation from metallic sodium the yield of the mixture of the isomeric vinyl ethers (b.p. *ca.* 401 K/101.6 kPa) was about 20 %.

Methyl 4-methoxy-4-pentenoate (IIa) and isomers (IIb, IIc). Levulinic acid (0.86 mol), methanol (2.7 mol), benzene (250 cm³), and concentrated sulfuric acid (24 cm³) were refluxed for 24 h, after which the mixture was poured into water and the organic layer was washed with sat. $NaHCO_3$ solution and water. After drying over $CaSO_4$, the product was distilled to give a 22 % yield of the methyl ester of levulinic acid, b.p. 362 – 364 K/2.1 kPa. The ester was converted to its dimethyl acetal, as described above for I. However, the acetal was not isolated but the crude acetal was distilled slowly from a small amount of *p*-toluenesulfonic acid. After a forerun of methanol, a 72 % yield of the isomeric vinyl ethers could be collected at 350 – 359 K/2.3 kPa.

NMR spectra. The NMR spectra were recorded in CCl_4 solutions (*ca.* 20 vol %) at 307 K with TMS as internal standard. NMR spectra could not be obtained for the *Z* isomers because of their low concentration in the synthesis products. For the spectra of Ia and Ib, the isomers were separated by preparative GLC; the spectra of IIa and IIb were recorded on a mixture containing equimolar amounts of IIa and IIb. Peak positions and some coupling constants:

Ia $CH_3OCH_2CH_2C(OCH_3)=CH_2$
a b c d ef
τ 6.72 (a), 6.56 (b), 7.71 (c), 6.48 (d), 6.14 (ef); $J_{bc}=6.9$ Hz.

Ib $(E)-CH_3-C(OCH_3)=CH-CH_2OCH_3$
a b c d e
τ 8.21 (a), 6.50 (b), 5.47 (c), 6.16 (d), 6.80 (e); $J_{bd}=7.5$ Hz.

IIa $CH_3OCOCH_2CH_2C(OCH_3)=CH_2$
a b c d ef
τ 6.39 (a), 7.62 (bc), 6.50 (d), 6.18 (ef); $J_{bc}=0.0$ Hz.

IIb $(E)-CH_3-C(OCH_3)=CH-CH_2COOCH_3$
a b c d e
τ 8.25 (a), 6.50 (b), 5.54 (c), 7.08 (d), 6.39 (e); $J_{cd}=7.4$ Hz.

Procedure. The details of the equilibration procedure have been described previously.[1,5,6] The equilibrations were carried out in cyclohexane solutions with iodine as catalyst. The column used in the gas chromatographic analyses was a 4 m column containing 10 % Carbowax 1500 on Chromosorb G. The isomers were eluted in alphabetical order. Of the geometric isomers b and c, the one with the higher thermodynamic stability (b) was taken as the *E* isomer.[1,6] Because of the relatively poor resolution of the peaks of IIb and IIc, the amount of the latter in the equilibrium mixtures could not be determined with sufficient accuracy. Hence isomerization data involving this compound have not been included in this work. However, the error in the area of the peak of IIb, caused by the poor resolution of the peaks of the geometric isomers, was negligible because of the low concentration of IIc. Peak areas, which were considered to be proportional to the molar concentrations of the isomers,[5] were determined by the cut-and-weigh method.

In the case $R=CH_3OCOCH_2$, the position of equilibrium could be approached from both sides. However, in the case $R=CH_3OCH_2$, only Ia was used for the equilibrations, for sufficient amounts of IIb could not be separated owing to the low yield of the synthesis. In the latter case, the progress of isomerization was followed by GLC and after the isomer composition ceased to change, the position of equilibrium was considered having been attained. To be sure that true equilibrium had been reached, the following samples of the equilibration mixture were analyzed over sufficiently extended periods of time to detect possible changes in the isomer ratios.

Determination of normal boiling points.[6] *Ia, Ib, and Ic:* Reference curve (compound, b.p. at 101.3 kPa, relative retention time): isobutyl vinyl ether, 356.2 K, 0.228; isobutylidene ethyl ether, 366.7 K, 0.279; 1-methoxycyclopentene, 387.0 K, 0.485; 1-ethoxycyclopentene, 409.0 K, 0.780; 1-methoxycyclohexene, 417.1 K, 1.000. The relative retention times of Ia, Ib, and Ic were 0.543, 0.745, and 0.745 leading to normal b.p.'s of 392.7 K, 406.2 K, and 406.2 K, respectively. *IIa, IIb, and IIc:* Reference curve: 1-methoxycyclopentene, 387.0 K, 0.294; 1-ethoxycyclopentene, 409.0 K, 0.391; 1-methoxycyclohexene, 417.1 K, 0.473; 1-ethoxycyclohexene, 435.0 K, 0.648; 1-propoxycyclohexene, 455.9 K, 1.000. The relative retention times of IIa, IIb, and IIc were 0.668, 0.793, and 0.812 leading to normal b.p.'s of 436.8 K, 444.9 K, and 446.1 K, respectively.

RESULTS

Table 1 shows the mean values of the equilibrium constant *K* and its standard error at different temperatures. Table 2 gives the values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ of isomerization at 298.15 K, which were calculated from the variation of the mean equilibrium constant with

Table 1. Values of the mean equilibrium constant K and its standard error for the following isomerization reaction in cyclohexane solution:
$R-CH_2C(OMe)=CH_2 \rightleftharpoons (E)-Me-C(OMe)=CHR \rightleftharpoons (Z)-Me-C(OMe)=CHR$
a b c

Catalyst (I_2) concentration/mol dm^{-3}: 0.004 (R = CH_3OCH_2), 0.008 (R = CH_3OCOCH_2); substrate concentration *ca.* 2.0 mol dm^{-3}; n denotes the number of independent determinations.

R	T/K	n	K_1(a⇌b)	$10K_2$(a⇌c)	$10K_3$(b⇌c)
CH_3OCH_2	303.2	3	1.162 ± 0.010	0.332 ± 0.008	0.285 ± 0.007
	323.2	5	1.128 ± 0.006	0.445 ± 0.009	0.394 ± 0.008
	340.2	5	1.061 ± 0.006	0.547 ± 0.011	0.514 ± 0.011
	361.2	3	1.032 ± 0.002	0.655 ± 0.008	0.634 ± 0.009
	373.2	4	1.035 ± 0.006	0.748 ± 0.013	0.723 ± 0.013
	397.2	4	1.014 ± 0.006	0.955 ± 0.017	0.943 ± 0.017
	415.2	4	0.961 ± 0.007	1.076 ± 0.013	1.121 ± 0.016
CH_3OCOCH_2	297.2	8	0.839 ± 0.004	...	...
	323.2	6	0.848 ± 0.006		
	348.2	4	0.834 ± 0.011		
	369.2	4	0.827 ± 0.012		
	398.2	6	0.802 ± 0.004		
	420.2	3	0.799 ± 0.006		

Table 2. Values of the standard free energy, enthalpy and entropy changes for the following isomerization reaction at 298.15 K:
$R-CH_2C(OMe)=CH_2 \rightleftharpoons (E)-Me-C(OMe)=CHR \rightleftharpoons (Z)-Me-C(OMe)=CHR$
a b c

The errors are twice the standard errors.

State	R	Reaction	$\Delta G°$/kJ mol^{-1}	$\Delta H°$/kJ mol^{-1}	$\Delta S°$/J K^{-1} mol^{-1}
Liquid	CH_3OCH_2	a⇌b	−0.395 ± 0.071	−1.65 ± 0.36	−4.2 ± 1.0
		a⇌c	8.599 ± 0.085	10.94 ± 0.43	7.8 ± 1.2
		b⇌c	9.002 ± 0.084	12.63 ± 0.42	12.1 ± 1.2
	CH_3OCOCH_2	a⇌b	0.393 ± 0.051	−0.53 ± 0.25	−3.1 ± 0.7
Gaseous	CH_3OCH_2	a⇌b	...	0.53 ± 0.47	−1.7 ± 1.1
		a⇌c		13.11 ± 0.52	10.3 ± 1.1
		b⇌c		12.63 ± 0.52	12.1 ± 1.3
	CH_3OCOCH_2	a⇌b		0.77 ± 0.39	−1.8 ± 0.8

temperature, as described previously.[5] The values of $\Delta H°$ and $\Delta S°$ in the ideal gas state at 298.15 K were evaluated as shown in part II of this series.[6]

DISCUSSION

According to Table 2, the values of $\Delta H°$ (g, 298.15 K) for the reaction a⇌b, in cases R = CH_3OCH_2 and R = CH_3OCOCH_2, are very similar and moreover, comparable to those for the same reaction in cases R = Me, Et, or i-Pr, as is seen from Table 3.

As it is apparent that steric repulsions between the two *cis* alkyl groups in the b isomer are very similar in each case, the results of this work reveal the rather unexpected fact that the stabilizing effect of a methoxymethyl or (methoxycarbonyl)methyl group on the double bond of a vinyl ether does not noticeably differ from that of an unsubstituted alkyl group. Thus one might be tempted to draw the conclusion

Table 3.

R	σ^* (Ref. 7)	$\Delta H°$(g, 298.15 K)/kJ mol^{-1}	Ref.
CH_3OCH_2	0.52	0.53 ± 0.47	This work
CH_3OCOCH_2	0.73 [a]	0.77 ± 0.39	This work
CH_3	0.00	0.45 ± 0.38	1
CH_3CH_2	−0.10	0.78 ± 0.39	1
$(CH_3)_2CH$	−0.19	0.60 ± 0.37	1

[a] Obtained by dividing the σ^*-value of the methoxycarbonyl group by 2.8, the factor that approximately corresponds to the attenuation of the inductive effect by one methylene group.

that the inductive effect of the group R has no effect on its stabilizing power. However, the similar stabilization energies may be due to a fortuitous compensation of two opposing effects. Damico [8] has found that in methyl octenoates, equilibrium distribution of the double bond disfavors the 3-octenoate with respect to the 4-, 5-, or 6-octenoates, the equilibrium ratio of the 3-isomer to any of the other isomers mentioned being about 0.3 (at 398 K). Similarly, in methyl pentenyl ethers, the ratio of the 2-pentenyl isomer to the 3-pentenyl isomer was about 0.6 (at 293 K). Thus methoxymethyl and (methoxycarbonyl)methyl groups do not stabilize the double bond of an ordinary olefin so effectively as unsubstituted alkyl groups. As the σ^*-values of the methoxymethyl and (methoxycarbonyl)methyl groups do not significantly differ from that of a hydrogen atom (0.49),[7] it might be thought that these groups restore the electron delocalization that is present in vinyl ethers with no substituents on the β carbon of the vinyl group, but because of their weaker "intrinsic" ability to stabilize olefinic double bonds, the net stabilization on vinyl ethers is comparable to that caused by an unsubstituted alkyl group. However, it seems amazing that the two opposite effects should completely compensate each other in both cases.

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The Conformations of Cyclotetradeca-1,8-diyne

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A conformational change takes place in the solid at 30° for cyclotetradeca-1,8-diyne. The high-temperature solid phase conformation is indistinguishable by IR- and Raman-spectroscopy from the solution conformation, which is identified by dynamic ^1H and ^{13}C NMR-spectroscopy. Conformational interconversion mechanisms are discussed.

When cyclotetradeca-1,8-diyne was first synthesized,[1] its high melting point and ease of formation were rationalized on the basis of a single strain-free chair-like conformation (A, Fig. 1) although the closely related boat-like conformation B could not be excluded. There are also other possible ways of constructing a ring conformation with staggered pentamethylene chains (C and D), but these contain a larger number of *gauche* bonds and were therefore disregarded. Nevertheless, the crystal conformation, as determined by King [2] using X-ray methods, turned out to be C and not A or B. This result remained unexplained, and conformation C had to be assumed also for the solution, since very similar IR-spectra were observed for the solid, the melt and the solutions (Fig. 2 c,b,a).

We have now found by calorimetry that there is a solid-solid transition point at 30° with relatively small enthalpy and entropy changes ($\Delta H_{tr} = 1.06$ kcal/mol; $\Delta S_{tr} = 3.5$ e.u.) compared with the changes at the melting point (98°; $\Delta H_m = 5.07$ kcal/mol; $\Delta S_m = 13.7$ e.u.). Since there is a considerable heating of the sample by the beam during the IR-measurement, it is clear that our earlier solid spectrum (Fig. 2c) referred to the high-temperature crystal phase, whereas the crystal structure was determined on the low-temperature phase. In fact, the IR-spectrum of a cooled sample (Fig. 2d) is markedly different, and it may be significant that the bands are more numerous and each individual band is no sharper than in the high-temperature solid. It therefore seemed possible that the upper crystal phase and the solution might after all contain the conformation A, and that by cooling the solid there is a change to the more compact conformation C (see Fig. 1). The latter has a lower symmetry in

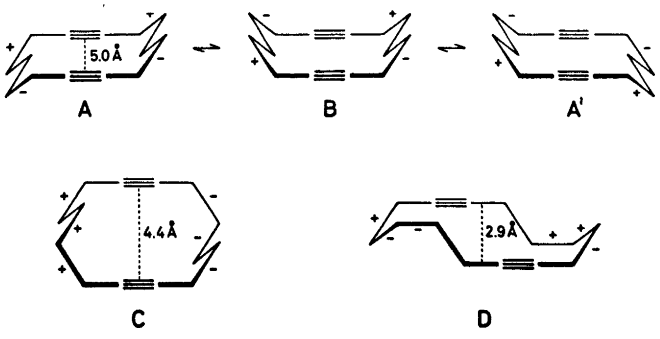

Fig. 1. Possible conformations of cyclotetradeca-1,8-diyne having staggered CH_2-CH_2 bonds. Right- and left-handed *gauche* bonds are indicated by + and − signs.

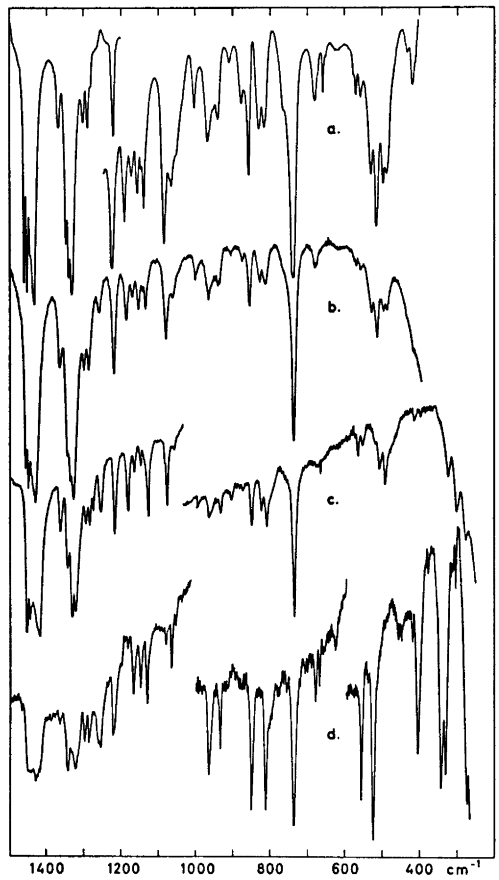

Fig. 2. Infrared spectra of cyclotetradeca-1,8-diyne (a) in solution in CCl_4 (left curve) and in CS_2 (right curve); (b) in liquid film at 110°; (c) in solid film at 40°; and (d) in solid film at −70°.

the pentamethylene chain and must give rise to a greater number of infrared active vibrations.

NMR-SPECTROSCOPY

The 251 MHz ^1H and 63.1 MHz ^{13}C NMR-spectra strongly suggest that A (or B) is in fact the solution conformation. In the compressed ^1H spectrum (Fig. 3) the three bands due to the α-, β-, and γ-protons on cooling broaden at −150° and each then splits up into two bands at −165° ($T_c \sim -160°$ (α-protons)); $\Delta G^{\ddagger} = 5.0 \pm 0.2$ kcal/mol. Only one process is observed. The ^{13}C spectrum on the other hand remains

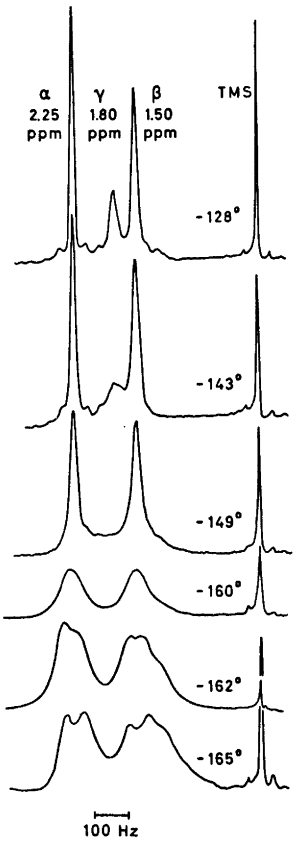

Fig. 3. 251 MHz ^1H spectra at various temperatures of a 1 % solution of cyclotetradeca-1,8-diyne in $CHCl_2F/CHClF_2$ (4:1).

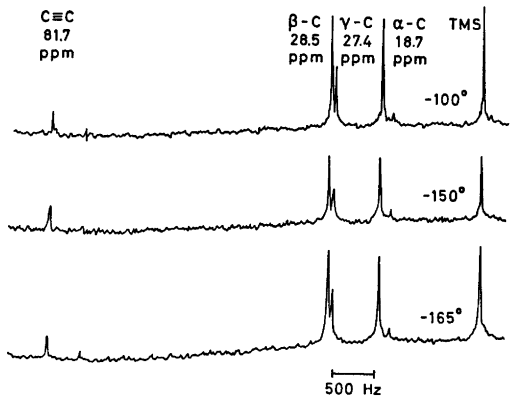

Fig. 4. Proton-decoupled 63.1 MHz ^{13}C Fourier transform spectra (500 transients) at various temperatures of a 4 % solution of cyclotetradeca-1,8-diyne in $CHCl_2F/CHClF_2$ (4:1).

unchanged on cooling through this temperature range (Fig. 4), so that there can be only one type each of all four constitutionally different carbon atoms. The most simple and immediate interpretation is of course that the solution contains the chair-like conformation, and that the geminally non-equivalent hydrogen atoms of each CH_2 become equivalent by direct flipping to the boat-like conformation B and further on to the inverted "chair" A′ (Fig. 1). However, this is an energetically expensive mechanism, since each step must involve a more or less concerted *syn*-eclipsing of two CC-bonds. Also, one cannot *a priori* exclude that the low-temperature spectrum only reflects the apparent symmetry of a rapidly exchanging species of still lower symmetry, such as C or D. We have therefore analysed the situation on the basis of a multistep conformational interconversion scheme constructed on the same principles as earlier [3] for cycloalkanes (Fig. 5).

CONFORMATIONAL INTERCONVERSION PATHS

Assuming that A is the lowest-energy conformation of cyclotetradeca-1,8-diyne, it can be converted over a series of barriers, each involving the full eclipsing of only one CC-bond, to the inverted conformation A′, and this can occur along two different paths (Fig. 5). One path goes through three intermediate conformational minima to B and then through three equivalent minima in the reversed order to A′. The other path goes through nine minima among which are both C and D and their equivalents D′ and C′. Each path, as well as a mixture of both paths in equal or unequal amounts, would satisfy the observed spectra revealing a single process, but it is difficult to estimate which of the several barriers on each is highest and therefore decisive kinetically, and it is equally difficult to guess which of the two paths is actually preferred.

An analogous scheme can be constructed on the assumption that B is the lowest-energy conformation; conformation A then plays the role of an intermediate on one path, and C and D are replaced by similar conformations on the other path. The NMR-spectra can make no distinction between A and B, and it is even possible that the chemical shifts are too close to

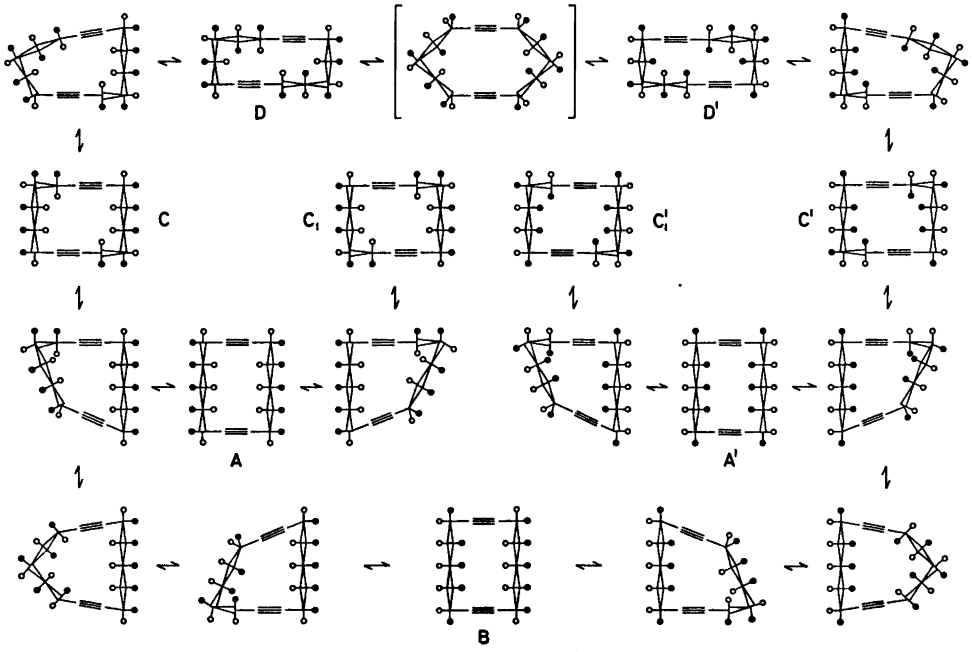

Fig. 5. Conformational interconversion paths for cyclotetradeca-1,8-diyne.

allow detection of a mixture of the two. Also the gas-phase electron-diffraction data, which have been discussed [4] on the basis of the chair-like conformation A of large vibrational amplitudes, would hardly allow a distinction to be made between A and B.

If one of the less symmetric conformations, C or D, had been of lowest energy, two separate processes should have been observed in dynamic NMR-spectroscopy. Thus, the interconversion scheme in Fig. 5 shows that C can either be converted to C′ along the path CDD′C′, whereby unequal α- and β-carbon atoms are fully exchanged, but not their geminally different hydrogen atoms, or it can be converted to C_1 along the path CAC_1, whereby again the carbon atoms but not the hydrogen atoms are exchanged. Only when both processes can occur, will complete exchange of all geminal hydrogen atoms take place, for example by going from C to C_1′ *via* DD′C′A′ or from C′ to C_1 *via* D′DCA. Since the decisive barrier on path CDD′C′ is different from that on path CAC_1, one should expect to observe the high-temperature process only by ^1H spectroscopy and the low-temperature process both by ^1H and ^{13}C spectroscopy. The possibility that the latter has such a low barrier that the coalescence temperature is below −165° seems unlikely. The observed value of 5 kcal/mol is already quite low, considering that full eclipsing of a CC-bond is involved in the barrier and that the conformational minima have completely staggered polymethylene chains and so must be very low in energy.

Thus, also the observation of one, and not two, dynamic processes in NMR-spectroscopy suggests that A (or B) is the solution conformer of cyclotetradeca-1,8-diyne.

CHAIR OR BOAT?

The chair conformation (A) and the boat conformation (B) are expected to have similar energies for the isolated molecule and in solution, since the two pentamethylene chains are too far apart to interact directly, and since there is hardly any rotational barrier for the methyl groups in dimethylacetylene and similar compounds.[5] On the other hand, the crystal lattice may well favour the centro-symmetric chair, as observed for the related molecules 1,6-dioxacyclodeca-3,8-diyne [6] and cyclotetradeca-1,3,8,10-tetryne.[7]

Not only the NMR-spectra are unsuited for settling this question. Also, the IR-spectra are expected to be very similar for the two conformations. The mechanical coupling between the pentamethylene chains being very weak, the hydrogen vibrations, which dominate the IR-spectrum, must obey the local symmetry, which is the same in both conformations. It is therefore dangerous to draw any conclusion from the observation (Fig. 2) that the IR-spectra of the upper solid phase, the melt and the solutions are very nearly identical. On the other hand, the Raman spectra should be dominated by the more strongly coupled skeletal vibrations, and by the C≡C stretching. Thus, if the upper solid contains conformation A, only the symmetric C≡C stretching mode should appear strongly in the Raman spectrum, while a mixture of A and B should show two additional bands due to both C≡C stretching modes of B.

The recorded Raman-spectra (Table 1), however, only confirmed what the IR-spectra had already shown, that the lower solid phase is different from the upper solid phase. Only very marginal differences exist between solution and upper solid phase spectra even at the lowest frequencies. The strongest band in all three phases is at 2230 cm^{-1} and there is no indication of its splitting in solution. The additional band at 2280 cm^{-1} is too far away compared with the very modest splitting expected for so weakly coupled triple bonds, and is probably due to an overtone in Fermi resonance, as observed for other acetylenes.[5] Thus, it can only be concluded that the solution and the upper solid phase contains either of the conformations A or B or an identical mixture of the two.

The possibility that the upper solid phase contains a mixture of A and B is rendered unlikely by the normal magnitude of the entropy of melting. Only expanded crystal lattices displaying a very low entropy of melting, are generally capable of accepting different molecules. Hence, also the solution contains only one conformation.

It remains to make a choice between the chair and the boat, of which only the former has a center of symmetry. This is of course in principle possible by a comparison of IR and Raman spectra, but in such large molecules numerous

Table 1. Raman spectral data (300 – 2400 cm^{-1}) for cyclotetradeca-1,8-diyne.

Solid Lower phase cm^{-1}	Upper phase cm^{-1}	Solution CCl_4 cm^{-1}
	327 vw	325 sh [b,c]
	353 vw	352 sh
367 s[a]		
	383 s	381 s
		415 w
433 m	432 w	428 w [b]
483 m	~500 w	~490 w [b]
	556 m	554 vw
583 m	576 w	574 w
~680 w	679 w	677 w
		737 vw [b]
764 m	~765 w	765 sh [b]
820 m	816 sh	818 sh
828 sh	830 m	830 sh
880 m	880 m	879 w
908 w	908 w	907 vw
950 m	~949 w	~951 w
1002 m	1001 m	1002 w
1049 m	1052 m	1051 w
1072 m	1068 m	1068 m
1080 sh	1082 m	1084 m
1102 w		
1136 m	1128 m	1129 w
~1160 w		1158 vw
		1190 vw
1224 m	1226 s	1224 m
1268 s	1267 s	1266 s
1301 sh	1300 s	1296 sh
1309 m		1304 m
1327 s	1327 vs	1330 vs
1337 sh		
1374 w	1370 w	1372 w
~1404 w		
1430 vs	1435 vs	1437 vs
1450 vs	1462 sh	1464 sh
2113 w		
2180 w		2178 sh
2232 vs	2226 vs	2232 vs
2269 sh	2259 w	2259 w
2281 vs	2281 vs	2286 vs
2331 m	2334 w	

[a] Abbreviations: s, m, w = strong, medium, weak; v = very; sh = shoulder. [b] Frequencies from CS_2 solution. [c] Additional low-frequency bands observed in solution: 311 w[b], 287 m[b] and ~180 w.

accidental coincidences must occur. It seems most likely, however, that the high-temperature solid contains the chair, and we have then to conclude that the chair is also alone in solution and in the melt. Such a conformational preference can only be attributed to a preference for staggering across the triple bond, as observed for simple acetylenes,[5] but the effect seems surprisingly strong in view of the very low barriers to methyl rotation in simple acetylenes.[5]

EXPERIMENTAL

Calorimetric data. A Perkin-Elmer Differential Scanning Calorimeter 1 B was used down to a temperature of −90°.

Infrared spectra. These were recorded with a Perkin-Elmer model 225 spectrometer. Saturated solutions in CS_2 and CCl_4 were measured using 0.5 mm KBr cells. The low temperature spectrum was obtained with a VLT-2 cell from RIIC, cooled with dry ice. The sample was contained between two pressed KBr discs. A heated cell from Perkin-Elmer equipped with KBr optics was used for studying the melt.

Raman spectra. These were measured with a Cary model 81 spectrometer equipped with a CRL 52G argon ion laser (5145 Å). Concentrated solutions in CS_2 and CCl_4 and the upper solid phase were studied using the 180° illumination technique and capillary cells. The low temperature spectrum was obtained under 90° illumination employing a cryostat with a copper tip cooled with liquid nitrogen.

NMR spectra. The spectra of $CHCl_2F/CHClF_2$ solutions were obtained on Prof. Anet's superconducting solenoid NMR spectrometer operating at 251 MHz for protons and at 63.1 MHz for ^{13}C. Acquisition and Fourier transform of free induction spectra were carried out with a Data General Nova computer.

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Short Communications

Naturally Occurring Lactones and Lactames. IV. Friedel-Crafts Acylation of the Tetronic Acid Nucleus and Synthesis of (*S*)-Carolic Acid

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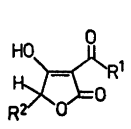
This note is concerned with the Friedel-Crafts acylation of the tetronic acid nucleus as part of our attempt to develop general methods for the synthesis of the more complex mold tetronic acids. The application of this reaction has previously been reported by Haynes and Jamieson,[1] who found that only 3,3-substituted tetronic acids and 3-phenyltetronic acid (in 20 % yield) could be acylated, whereas 5-methyltetronic acid, which is naturally occurring and a possible precursor to some mold tetronic acids, underwent no acylation. Recently Bloomer and Kappler [2] reported the acylation of 5-methyltetronic acid to 3-butyryl-5-methyltetronic acid and to (*RS*)-carolic acid, just as they have synthesized (*R*)-carolic acid by acylating (*R*)-5-methyltetronic acid obtained by degradation of naturally occurring (*R*)-carolic acid.[3]



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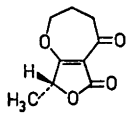

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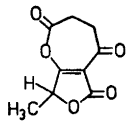

(3)

By modifying the acylation procedure of Haynes and Jamieson [1] we were able to acylate the tetronic acids (*1*) listed in Table 1. Furthermore, acylating (*S*)-5-methyltetronic acid, synthesized from ethyl (*S*)-lactate,[4] with 4-chlorobutyryl chloride, we obtained (*S*)-carolic acid identical in all respects with authentic carolic acid (*3*) isolated from *Penicillium charlesii* except for the specific rotation, which was of opposite sign.

It should be mentioned that in accordance with Haynes and Jamieson [1] cyclohexane-spiro-5-tetronic acid appeared not to undergo acylation.

Attempts to synthesize (*R,S*)-carolinic acid (*3*) from 5-methyltetronic acid by acylating with β-carbomethoxypropionyl chloride was not met with success. On working up the reaction mixture only succinic anhydride could be isolated.

Experimental. Standard acylation procedure. To 2.0 mmol tetronic acid and 2.2 mmol of the appropriate acid chloride was added 3 mmol of tin (IV) chloride. The solution was kept at room temperature for about 2 min to allow the

Table 1.

Compound	R^1	R^2	M.p.	M.p. lit. val.	Yield %
3-Acetyltetronic acid (*1*)	CH_3	H	78.0 – 80.2	79.5 – 80.5[7]	39
3-Propionyltetronic acid (*1*)	CH_3CH_2	H	94.0 – 96.0		64
3-Acetyl-5-methyltetronic acid (*1*)	CH_3	CH_3	56.0 – 57.5		44
3-Propionyl-5-methyltetronic acid (*1*)	CH_3CH_2	CH_3	64.5 – 65.5		75
3-Butyryl-5-methyltetronic acid (*1*)	$CH_3CH_2CH_2$	CH_3	40.0 – 42.0		93
3-Isobutyryl-5-methyltetronic acid (*1*)	$(CH_3)_2CH$	CH_3	50.0 – 51.5		70
3-Phenylacetyl-5-methyltetronic acid (*1*)	$C_6H_5CH_2$	CH_3	78.5 – 80.5		49
3-Propionyl-5-ethyltetronic acid (*1*)	CH_3CH_2	CH_3	55.0 – 57.5		68
3-Acetyl-5-phenyltetronic acid (*1*)	CH_3	C_6H_5	100 – 103	102 – 104[1]	86
(*RS*)-Carolic acid			116 – 118	117[5]	5
(*S*)-Carolic acid			130 – 132	132[6]	14

formation of an enol ester and was then heated to 110° and maintained at this temperature for 3 h. After cooling, the mixture was poured into ice-cold 4 N hydrochloric acid and extracted repeatedly with chloroform, and the extract was shaken with concentrated aqueous sodium hydrogen carbonate. The aqueous layer was acidified with conc. hydrochloric acid and extracted with chloroform. The dried chloroform extract gave on evaporation the pure 3-acylated tetronic acid. Only 3-acetyl- and 3-propionyl-tetronic acid had to be recrystallized from petrol ether.

(R,S)-*Carolic acid.* 5-Methyltetronic acid (4.38 mmol) was acetylated with 500 mmol of 4-chlorobutyryl chloride to give 0.47 g of dark red oil, which solidified when treated with ether. Four recrystallizations from ethyl acetate-light petrol gave 50 mg (5 %) of amber coloured crystals, m.p. 112.5–115.2°. Sublimation rised the m.p. to 116–118°. ^{1}H NMR in $CDCl_3$: 1.46 d (CH_3), 4.64 q (H), 3.45 t ($COCH_2$), 2.28 m ($CH_2CH_2CH_3$), 4.80 t (CH_2O). M_{MS} = 182.05843; M_{calc} = 182.0579 (Lit. [5] m.p. 117°).

(S)-*Carolic acid.* Acylation of 2 mmol of synthetic (*S*)-5-methyltetronic acid [4] resulted in the isolation of 70 mg (14 %) colourless crystals m.p. 130–132° (Lit.[6] 132°). $[\alpha]_{546}^{25}$ −70.8° (c = 0.49, H_2O) (Lit. val. for the enantiomeric form +84°) ^{1}H NMR as above.

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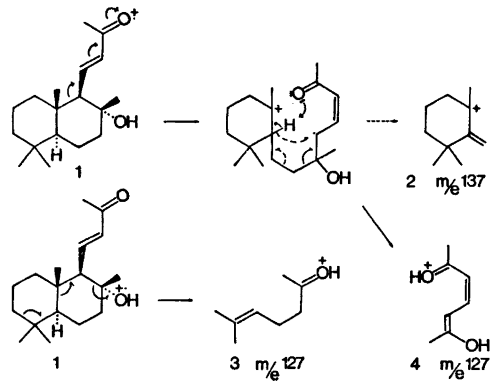
Tobacco Chemistry. 26. Synthesis of 14,15-Bisnor-8-hydroxylabd-11*E*-en-13-one, A New Tobacco Constituent

JOSEPH R. HLUBUCEK, ARNE J. AASEN, SVEN-OLOF ALMQVIST and CURT R. ENZELL*

Research Department, Swedish Tobacco Co., Box 17 007, S-104 62 Stockholm, Sweden

Current research in this laboratory has revealed that tobacco flavour comprises a large number of norisoprenoid constituents.[1,2] The present report deals with the identification and synthesis of a probable diterpenoid degradation product isolated from a medium-volatile, neutral fraction [3] of an extract [4] of sun-cured Greek *Nicotiana tabacum* L.

The elemental composition of this new tobacco compound, $C_{18}H_{30}O_2$, was established by high resolution mass spectrometry. The two oxygen atoms were accomodated by a *3E*-penten-2-oxo-1-ylidene moiety, >CH−CH=CH−CO−CH_3, [978 and 1661 cm^{-1}, 230 nm, δ 2.24 (3H, s), δ 6.16 (1 H, d, J 16 Hz), δ 6.82 (1 H, dd, J 10 and 16 Hz)], and a tertiary hydroxyl group (3450 cm^{-1}) attached to a methyl substituted [δ 1.26, (3 H, s)] carbon atom, >C(OH)−CH_3, respectively. Based on this evidence, the presence of three quaternary methyl groups (singlets at δ 0.83, 0.89, 0.99), its electron-impact induced fragmentation pattern being characteristic of labdane-type diterpenoids devoid of oxygen-substituents on ring A (*cf.* Scheme 1),[5] and no indication of further double bonds, 14,15-bisnor-8-hydroxy-labd-11*E*-en-13-one (*1*)** appeared likely as the structure for this new tobacco compound. This structure, including the stereochemistry inferred in *1*, was confirmed by total synthesis using drimenol [6] (*5*) as starting material.



Scheme 1.

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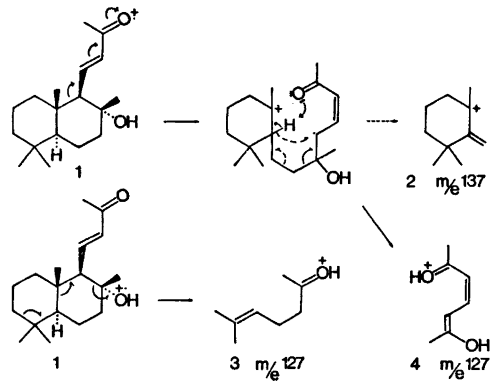

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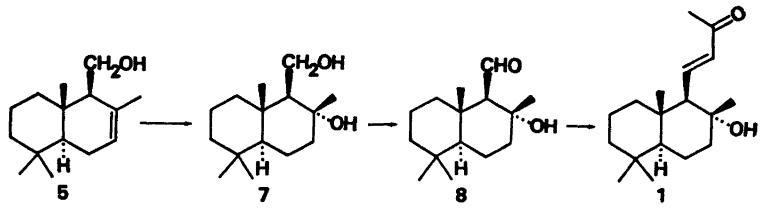

Scheme 2.

Drimenol (*5*) was converted to the diol *7* *via* the epoxide *6* as described elsewhere.[7] The diol *7* was oxidised employing Collins' reagent,[8] to the hydroxyaldehyde *8* which in turn was subjected to base-catalysed aldol condensation with acetone followed by *in situ* dehydration furnishing 14,15-bisnor-8-hydroxylabd-11*E*-en-13-one (*1*, Scheme 2), identical in all respects to the natural product. Very recently it has been reported that *1* could be obtained by oxidative degradation of the diterpenoid abienol.[9] 14,15-Bisnor-8-hydroxylabd-11*E*-13-one (*1*) has been isolated previously from the resin of *Abies sibirica* and the published [10] data are in agreement with ours except that *1* isolated by the Russian workers was reported to be optically inactive.

Experimental. NMR, IR, UV, and mass spectra were recorded on Varian XL-100, Digilab FTS-14, Beckmann DB-2A, and LKB 9000 (70 eV) instruments, respectively. Rotations were measured on a Perkin-Elmer 141 instrument, and accurate mass determinations were carried out at the Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm.

*14, 15-Bisnor-8-hydroxylabd-11*E*-en-13-one* (*1*, 40 mg) was isolated from a medium-volatile, neutral fraction [3] of an extract [4] of sun-cured Greek *Nicotiana tabacum*. L. leaves, employing liquid chromatography on silica gel. The fractionation of this medium-volatile material will be described elsewhere.[3] M.p. 126–127° (lit.[10] 126°); MS: *m/e* 278 (M^+, 7), 43 (100), 109 (56), 260 (33), 41 (32), 95 (27), 81 (26), 69 (26), 137 (25), 245 (25); accurate mass determination: $C_{18}H_{30}O_2$, found 278.2250, calc. 278.2246; $C_7H_{11}O_3$, found 127.0764, calc. 127.0759; $C_8H_{15}O$, found 127.1123, calc. 127.1123; δ ($CDCl_3$): 0.83 (3 H, s), 0.89 (3 H, s), 0.99 (3 H, s), 1.26 (3 H, s), 2.24 (3 H, s), 6.16 (1 H, d, *J* 16 Hz), 6.82 (1 H, dd, *J* 10 and 16 Hz); ν_{max}(KBr): 3450 (broad), 3026 (w), 1661 (s), 1633 (shoulder), 1268 (m), 1123 (m), 1084 (m), 986 (m), 978 (m), 970 (shoulder); λ_{max} (EtOH): 230 nm (ε 9300); $[\alpha]_D^{20}$ 15.8° (*c* 0.43, $CHCl_3$).

8-Hydroxydriman-11-al (*8*). 8,11-Dihydroxydrimane (*7*, 17 mg) [7] was added to a suspension of anhydrous CrO_3-pyridine complex (Collins' reagent,[8] 100 mg) in methylene chloride (2 ml). The dark mixture was stirred for 75 min at room temperature, diluted with ether and filtered through celite. The concentrated filtrate was applied in chloroform solution to a silica gel column (2 g) packed in 10 % ether/pentane. Elution with 50 % ether/pentane gave crystalline hydroxyaldehyde *8* (12 mg). δ ($CDCl_3$) 0.85 (3 H, s), 0.91 (3 H, s), 1.14 (3 H, s), 1.40 (3 H, s), 3.3 (1 H, broad s), 10.02 (1 H, d, *J* 2 Hz); ν_{max} (KBr): 3400 (broad), 2740 (w), 1710 (s); $[\alpha]_D^{20}$ 66.2° (*c* 0.58, $CHCl_3$).

*Synthetic 14,15-bisnor-8-hydroxylabd-11*E*-en-13-one* (*1*). 10 % aqueous KOH solution (0.5 ml) was added to a solution of the hydroxyaldehyde *8* (7 mg) in acetone (3 ml) and the mixture heated under reflux for 7 h. The cooled reaction mixture was concentrated *in vacuo*, diluted with ether, washed well with water and dried (Na_2SO_4). The residue obtained after evaporation of the solvent was chromatographed on silica gel (2 g) to yield crystalline *1* (4 mg), indistinguishable from *1* isolated from tobacco.

Acknowledgements. The authors are indebted to Miss Ann-Marie Eklund for skilful technical assistance, and to Prof. H. H. Appel and Prof. K. H. Overton for generous gifts of drimenol.

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The Structure of Methyl α-D-Galacturonic Acid Methyl Ester

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[a] Institutt for røntgenteknikk, Universitetet i Trondheim-NTH and [b] Institutt for marin biokjemi, Universitetet i Trondheim-NTH, N-7034 Trondheim-NTH, Norway

Uronic acids are constituents of a number of biologically important macromolecules. The physical and biological properties of these polymers are greatly influenced by the identity of the uronic acid, or acids, present and by conformational parameters for the uronic acid molecules. In contrast to other sugars such information is extremely scarce for the uronic acids. We have therefore initiated a program for the study of uronic acid conformation by X-ray crystallographic methods.

Methyl α-D-galacturonic acid methyl ester was synthesized according to the method of Jones and Stacey.[1] The yield was 20 % and the recrystallized product had a melting point of 148° C and $[\alpha]_D^{20} = 127°$ ($c = 1.0$, water.) Single crystals of suitable size were obtained by crystallization from a mixture of equal volumes of methanol and acetone at elevated temperatures.

Crystal data are: $C_6H_{14}O_7.H_2O$, orthorhombic space group $P2_12_12_1$, cell dimensions (with estimated standard deviations in parentheses) $a = 6.489(3)$ Å, $b = 8.004(3)$ Å, $c = 20.869(12)$ Å, calculated density 1.471 g cm^{-3} (measured 1.473 g cm^{-3}), number of molecules in the unit cell: $Z = 4$.

2386 observed reflexions were collected by an automatic Picker FACS-1 diffractometer using Mo$K\alpha$ radiation and the $\omega/2\theta$ scanning mode with max. $2\theta = 70°$. The structure was solved by a multisolution tangent refinement procedure using the program TANNY.[2] Refinement was performed by means of Fourier and least-squares techniques using programs from the XRAY system.[3] Refinement with isotropic temperature factors for all heavy atoms yielded the conventional R-factor of 0.060.

The pyranose ring has the expected $^4C_1$ chair conformation as shown in Fig. 1. The carbon atom C(8) of the glycosidic methyl group is *gauche* to the ring oxygen atom $O(5)$ and *trans* to C(2). The C(5) methyl ester group is nearly planar and equatorial to the ring, with $O(6)$ *cis* to O(5) and O(7) *trans* to O(5). The carboxyl C(6) – O(6) double bond is 1.190 Å, C(6) – O(7) is 1.326 Å and O(7) – C(7) is 1.443 Å. Other bond lengths and angles are similar to those of methyl α-D-galactopyranoside monohydrate[4] and other hexoses.[5]

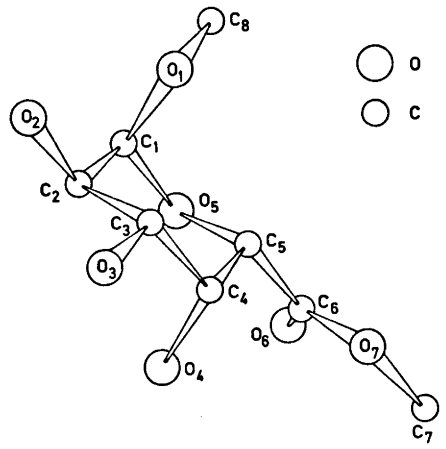

Fig. 1. Projection of the molecule, only with heavy atoms, on 100 plane.

Further refinement and a more detailed account of the structure will be published elsewhere. Additional work is in progress with the aim of obtaining information on the binding of cations to the uronic acids and their polymers. The results will also be valuable as basis for investigations of ionic bonds in solutions by other physical methods, *e.g.* NMR spectroscopy.

This work is part of S.T.'s thesis for a degree of Licentiatus Technicae (Lic. techn.) at The University in Trondheim-NTH sponsored by the Norwegian Agency for International Development (NORAD) which is gratefully acknowledged.

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New Syntheses of Substituted Indoles

JAN BAKKE

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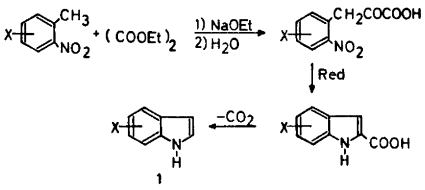
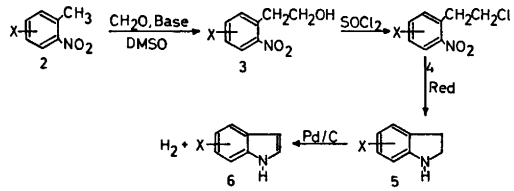
A number of syntheses of indoles are recorded.[1] Few of these, however, are suitable for the synthesis of indoles substituted in the benzene ring and unsubstituted in the pyrrole ring (*e.g. 1*). The Reissert synthesis [2] is one of the more used methods for the preparation of this type of indole, and has been applied in the case of number of substituted indoles:[3,4]



Although the yields are high for the first two steps, the decarboxylation in the last step gives highly varying yields.[3] A new synthesis for indoles of the type *1* could therefore be useful.

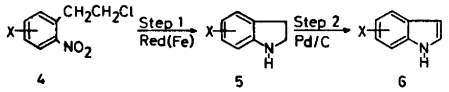
Some time ago, the addition of substituted and unsubstituted *o*- and *p*-nitrotoluenes to various aldehydes was reported.[5] The products (*3*) from the addition of *o*-nitrotoluenes (*2*) to formaldehyde were considered as potential starting materials for the synthesis of indoles as they contained a chain of two carbon atoms in the *ortho* position to a nitrogen atom. One possible way to the indoles would be *via* the 2,3-dihydroindoles (*5*). Ferber [6] obtained dihydroindole itself (*5*, X=H) by reduction and cyclization of 2-*o*-(nitrophenyl)ethyl chloride (*4*, X=H) prepared by nitration of 2-phenylethyl chloride.

Chlorination with thionyl chloride of 2-(*o*-nitrophenyl)ethanol obtained from *o*-nitrotoluene and formaldehyde gave 2-(*o*-nitrophenyl)ethyl chloride (*4*, X=H) in 80 % yield. Reduction of this compound by iron turnings activated by hydrochloric acid in aqueous suspension gave 2,3-dihydroindole (*5*, X=H) which was isolated by steam distillation (yield 85 %). The dehydrogenation of dihydroindole to indole was performed with palladium on carbon in refluxing toluene (yield 93 %). Volumetric experiments showed that 1 mol of hydrogen gas was evolved in the course of this reaction.



After success in the synthesis of indole itself, the reaction sequence was tried for some substituted indoles. The results are given in Table 1. The conditions are not optimalized and the results might be improved by using bromination instead of chlorination of the alcohol *3*. In some cases, catalytic hydrogenation may be of advantage instead of the reduction by iron turnings.

Table 1. Synthesis of substituted indoles from *o*-nitrotoluenes.



X (pos. in *4*)	Yield, Step 1, %	Yield, Step 2[a], %
H	85	93
4-Cl	55	52 [b]
6-Cl	71	73 (m.p. 84–86°)[c]
4-NO_2	59	64 (m.p. 105–107°)[d]

[a] Products were identified by comparison of IR, GLC, and m.p. (for crystalline compounds) with authentic samples. Products containing halogens gave satisfactory halogen analyses. [b] GLC showed the product to be 96 % pure and to contain 3 % indole. [c] M.p. lit.[3] 86–87°. [d] The products were 4-aminodihydroindole and 4-aminoindole, m.p. 4-aminoindole lit.[4] 108°.

This convenient preparation of dihydroindole itself also facilitates the synthesis of indoles substituted in the 5- or 7-positions. Terentév *et al.*[7] have synthesized such indoles by substitution of dihydroindole followed by dehydrogenation. The limitation of our procedure with respect to the preparation of 5- or 7-substituted indoles lies in the fact that the intermediate nitrotoluenes substituted at position 3 or 5 are not readily available.

When *o*-nitrotoluenes are added to arylaldehydes, 2-arylindoles (*9*) may be synthesized by oxidation of the alcohols *7* followed by reduction of the ketones *8*:

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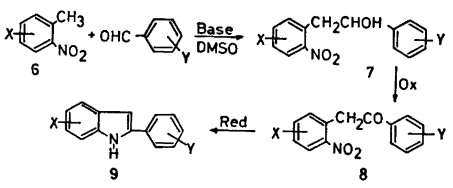

o-Nitrotoluene and benzaldehyde gave 1-phenyl-2-(*o*-nitrophenyl)ethanol (*7*, X = Y = H) which was oxidized by concentrated nitric acid in the presence of chloroform. *o*-Nitrobenzyl phenyl ketone (*8*, X = Y = H) was obtained in 98 % yield (m.p. 75 – 77°, IR (KBr): 3100, 2910, 1690, 1620, 1600, 1580, 1520, 1450, 1410, 1360, 1290, 1210, 1005, 995, 790, 730, 700, 690, 675 cm^{-1}).

Catalytic reduction of *8* gave 2-phenylindole in 85 % yield (m.p. 187 – 188°, lit.[8] 186°).

Our results thus show two useful routes to substituted indoles; one to indoles substituted in the benzene ring and unsubstituted in the pyrrol ring, and one to indoles with an aryl group in the 2-position.

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The Conformation of a *gem*-Dimethyl-substituted Cycloheptene

GERD BORGEN

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In a previous paper[1] the synthesis of a cycloheptene derivative, 2-carboxy-3,3,6,6-tetramethyl-cycloheptene is described. Variable-temperature NMR-spectra are now obtained of this compound, with a coalescence temperature as high as −40° in 100 MHz NMR.

In Fig. 1 the ^1H NMR-spectra of 2-carboxy-3,3,6,6-tetramethyl-cycloheptene in $CDCl_3$ at 35°, −41°, and −66° and in $CHFCl_2$ at −85° are shown.

The absorption for the *gem*-dimethyl protons at lowest field changes gradually into two sharp lines at low temperature, the other methyl signal is broad, but is not resolved at −85°C. The difference in chemical shift between the two methylene protons alpha to the double bond is *ca.* 50 Hz, their geminal coupling constant 14 Hz. The vicinal coupling, to the olefinic proton, of the methylene proton at lowest field is 7 Hz, the other is uncertain because of overlapping of lines from the other methylenes in the ring.

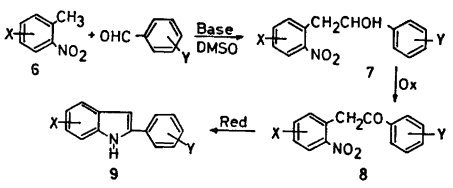
The triplet of the olefinic proton is not fully resolved at −66°, at −85° the signal is hidden by the signals of the proton in the solvent.

The free energy of activation for the exchange process was calculated[2] resulting in $\Delta G^{\ddagger} = 12.2$ kcal/mol.

The infrared spectra were taken of the crystalline solid in KBr and in CS_2 solution. IR-absorption bands at 1730 and 3510 cm^{-1} showed that the solution contained some monomeric acid together with the hydrogen-bonded dimer. Otherwise, the spectra of the two phases were alike which indicate that the cycloheptene skeleton takes the same conformation in solution as in the crystal, and that there is only one conformer in the solution.

The compound melted at 130° and no solid-solid transition was observed by differential calorimetry. The entropy and enthalpy of fusion were $\Delta S = 23.9$ cal/mol deg. and $\Delta H = 9.7$ kcal/mol.

As a result of independent calculations Favini *et al.*[3] found the chair and Allinger *et al.*[4] the boat to be the lowest energy form of cycloheptene. From NMR-data most investigators find the chair conformation in cycloheptene derivatives.[5,6] In 20 different benzocycloheptene derivatives, described by Kabuss *et al.*[7] including one with substituents in 3 and 6 positions analogous to our compound, only one conformer occurred in the low temperature NMR-spectra, presumably that of the chair form. Grunwald and Price[8] found two conformers by low temperature NMR of 3,3,6,6-



o-Nitrotoluene and benzaldehyde gave 1-phenyl-2-(*o*-nitrophenyl)ethanol (*7*, X = Y = H) which was oxidized by concentrated nitric acid in the presence of chloroform. *o*-Nitrobenzyl phenyl ketone (*8*, X = Y = H) was obtained in 98 % yield (m.p. 75 – 77°, IR (KBr): 3100, 2910, 1690, 1620, 1600, 1580, 1520, 1450, 1410, 1360, 1290, 1210, 1005, 995, 790, 730, 700, 690, 675 cm^{-1}).

Catalytic reduction of *8* gave 2-phenylindole in 85 % yield (m.p. 187 – 188°, lit.[8] 186°).

Our results thus show two useful routes to substituted indoles; one to indoles substituted in the benzene ring and unsubstituted in the pyrrol ring, and one to indoles with an aryl group in the 2-position.

Acknowledgements. Prof. M. Nilsson and my colleagues at AB Bofors, Nobelkrut, are all thanked for helpful discussions. Technical help from Miss G. Nyström and Miss B. Frisk is gratefully acknowledged. The work was supported by the Swedish Board for Technical Development. The English was checked by Dr. G. Francis.

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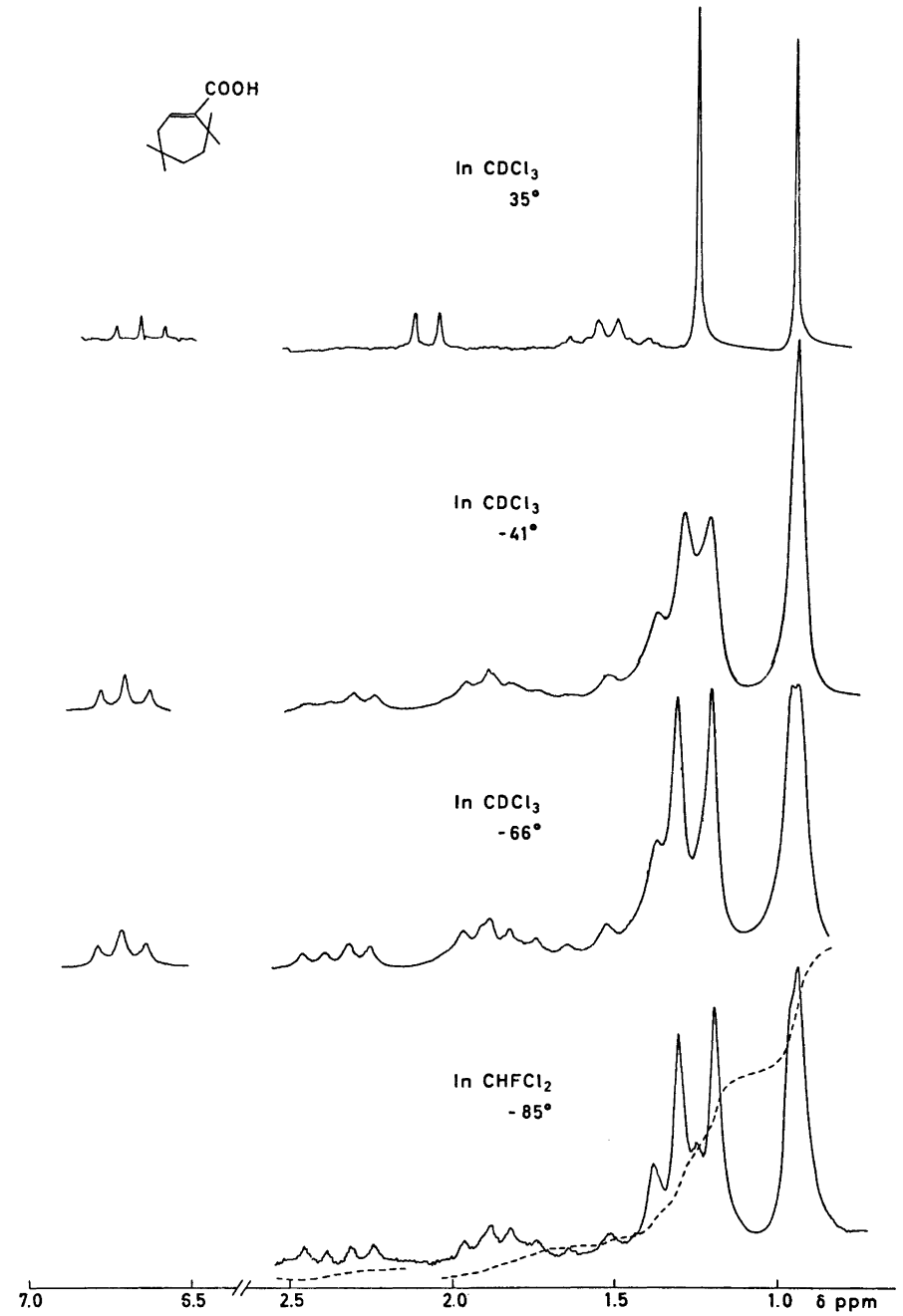

Fig. 1. Changes in the ^1H NMR-spectra of 2-carboxy-3,3,6,6-tetramethylcycloheptene at low temperatures.

tetramethylbenzocycloheptene, a conclusion based on new peaks arising in the methyl region by lowering the temperature. Our cycloheptene derivative has the *gem*-dimethyl groups in the same positions and new peaks arise in the low temperature spectra in lower part of the methyl region. This may be due to a second conformer but could also be explained by overlapping caused by the chemical shifts and the coupling of the methylenes in positions 4 and 5, which in the low temperature spectrum is extended over a region of at least 60 Hz. The fact that the infrared spectra show only one conformer in solution makes the last explanation the most probable and our assumption is that only one conformer is present.

From models it can be seen that the energetically possible forms of 2-carboxy-3,3,6,6-tetramethylcycloheptene are the two inverted chair forms and the two boat forms, however, only one of the two inverted twist boat forms. In the other the methyl-methyl interaction is too severe.

According to Favini *et al.*[3] the dihedral angle, ω_7, is the same in the chair and the boat forms of cycloheptene and = $-72.2°$. The π-contribution to the geminal coupling constant of the 7-protons in these two conformations should then be almost zero.[9] Our observed value $J_7^{gem} = 14$ Hz is as expected for this size ring, (cyclohexane = 13 Hz) and the same as the value found for the 7-protons in benzo-cycloheptene = 14.1 Hz,[6] which is found to take the chair conformation. The conclusion that may be drawn from the π-contribution to the coupling constant of the 7-protons is therefore that our cycloheptene derivative takes either the chair or the boat conformation.

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The result of the analysis of the low-temperature NMR-spectra of 2-carboxy-3,3,6,6-tetramethylcycloheptene is therefore that the conformation is either the chair or the boat.

The inversion barrier in cycloheptene itself is calculated from NMR-data[11] at $-160°$ to be 5.0 kcal/mol. The *gem*-dimethyl groups make it more difficult to find low-energy interconversion paths between the different forms, and this explains the considerably higher coalescence temperature, $-40°$C, and the corresponding higher barrier, 12.2 kcal/mol, in 2-carboxy-3,3,6,6-tetramethylcycloheptene.

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Isomerization of ε-Carotene to β-Carotene and of Lutein to Zeaxanthin

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More than 25 years ago Karrer and Jucker[1] reported the base catalyzed isomerization of α-carotene (*1*, β,ε-carotene by new nomenclature,[2] stereochemistry subsequently assigned[3]) by prolonged treatment with NaOEt/EtOH in benzene at elevated temperature. Extensive decolorization of the carotene occurred and from 30 mg of α-carotene (*1*) a small amount of β-carotene (*2*, β,β-carotene[2]) was isolated. The same authors reported the sodium ethoxide catalyzed isomerization of lutein (*3*, β,ε-carotene-3,3′-diol[2]) to zeaxanthin (*4*, β,β-carotene-3,3′-diol,[2] Scheme 1). More recently, Kargl and Quackenbush[4] reported the isomerization of δ-carotene (*5*, ε,ψ-carotene,[2] stereochemistry later assigned[5]) to γ-carotene (*6*, β,ψ-carotene[2]) by the procedure described by Karrer and Jucker.[1]

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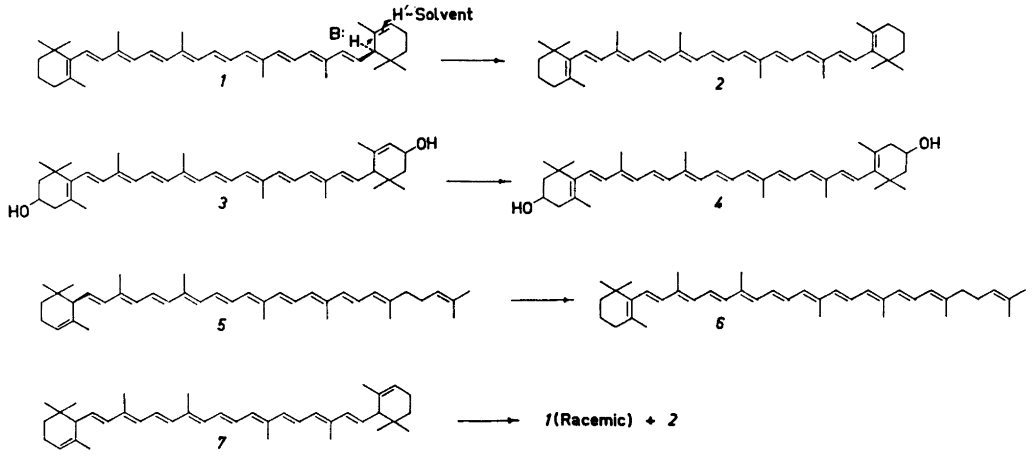

Scheme 1.

Attempts to utilize this procedure for the small scale (0.2 – 1.0 mg) isomerization of α-carotene (*1*) to β-carotene (*2*) or ε-carotene (*7*, ε,ε-carotene [2]) to α-carotene (*1*) and β-carotene (*2*) failed. Buchecker *et al.*[6] reported similar difficulties during attempts to isomerize lutein (*3*) to zeaxanthin (*4*). A modified base catalyzed isomerization was developed to allow small scale conversions of α-type (ε by new nomenclature [2]) end groups to β-type end groups.

Treatment of synthetic, racemic ε-carotene (*7*, 0.125 mg) with KOH/MeOH (20 %, 0.5 ml), benzene (0.5 ml) and anhydrous dimethyl sulfoxide (DMSO, 2 ml) in a sealed tube under an atmosphere of nitrogen at 118°C for 15 – 30 min gave, after the usual extractive isolation and chromatography, α-carotene (*1*, racemic, 16 – 28 %) and β-carotene (*2*, 16 – 21 %). Total pigment recovery including unreacted ε-carotene (*7*) was 51 – 68 %. The composition and total pigment recovery depended on reaction time. Shorter reaction times yielded higher pigment recovery, higher conversion to α-carotene (*1*) and lower conversion to β-carotene (*2*). Increased reaction times resulted in higher conversion to β-carotene but lower total pigment recovery. When NaOEt, KOEt, or *t*-BuOK were used as base the carotene was completely degraded in 10 min.

Attempted isomerization of lutein (*3*) to zeaxanthin (*4*) using the above optimum conditions failed. However, when a solution of lutein (*3*, 1.0 mg), KOMe/MeOH (5 %, 0.5 ml) and DMSO (2 ml) in a sealed tube under N_2 was heated at 118°C for 20 min, zeaxanthin was formed in 10 – 15 % yield. Stereochemical aspects of this reaction are treated separately.[7]

Instruments used were those commonly employed in this laboratory.[8] Identity of all products of the isomerization study was established by electronic absorption spectroscopy, mass spectrometry and chromatography including co-chromatography with authentic specimens. α-Carotene (*1*), β-carotene (*2*), and ε-carotene (*3*) were separated on Al_2O_3 plates developed with petroleum ether, ethyl ether (95 + 5). Lutein (*3*) and zeaxanthin (*4*) were separated on plates prepared from MgO, $Ca(OH)_2$, kieselgel G, $CaSO_4$ and H_2O (9 + 12 + 30 + 3 + 93) activated for 1 h at 100°C; developed with acetone-petroleum ether-isopropanol (20 + 77 + 3).

Acknowledgement. A.G.A. acknowledges the Royal Norwegian Council for Scientific and Industrial Research for a post-doctoral fellowship.

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Carotenoids of Higher Plants. 7.* On the Absolute Configuration of Lutein

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Lutein (*1*) possesses three chiral centers at C-3, C-3′, and C-6′. The absolute configurations at C-3 and C-6′ are generally agreed to be *R*.[1-3] However, the chirality at C-3′ of lutein has been assigned both the *R* and *S* configuration by different investigators. De Ville *et al.*[4] favoured the *S* configuration (*1a*) on the basis of biogenetic correlation of lutein (*1*) with (*R*)-β-cryptoxanthin. Later, Buchecker *et al.*[2,3] presented strong evidence for the *R* configuration at C-3′ of lutein (*1b*) on the basis of PMR analysis of (+)-3-methoxy-α-ionone, chemically derived from lutein (*1b*), and by chemical correlation with synthetic 3-methoxy-α-ionones of established stereochemistry.

Recently, Andrewes[5] has reported an improved isomerization of lutein (*1*) to zeaxanthin (*2*, Scheme *1*). Provided extensive racemization of the asymmetric centers at 3,3′ of *1* and *2* was avoided, the isomerization of lutein (*1*) to zeaxanthin (*2*) would represent an independent check of the chirality at C-3′ of lutein (*1*). This logical inference has already been pointed out by Buchecker *et al.*[3]

In the absence of racemization, isomerization of (3*R*,3′*S*,6′*R*)-lutein (*1a*) would provide (3*R*,3′*R*)-zeaxanthin (*2a*, identical to natural zeaxanthin, stereochemistry known[6]). On the other hand, isomerization of (3*R*,3′*R*,6′*R*)-lutein (*1b*) would give (3*R*,3′*S*)-zeaxanthin (*2b*, Scheme *1*). From the well tested additivity hypothesis,[6] the former should be optically active while the latter is an optically inactive *meso* compound. CD measurement of zeaxanthin obtained from lutein should then reveal the chirality of lutein at C-3′.

A plausible mechanism for the isomerization of lutein to zeaxanthin is given in Scheme 1. Various mechanisms may be considered to account for possible racemization at C-3,3′ of *1* and *2* via a ketone/enolate type intermediate in alkaline DMSO. To check the extent of racemization to be expected, natural zeaxanthin (*2*, 1.0 mg) was treated with KOMe/MeOH in DMSO as previously described for the isomerization of lutein[5] (no methyl ethers were formed).

The CD spectrum of recovered *trans* zeaxanthin showed the following $\Delta\varepsilon$-values in EPA solution at 285 nm in two separate experiments: $\Delta\varepsilon = -11.7 \pm 10\ \%$ (0.093 mg sample, spectrophotometrically determined using E (1 %, 1 cm) = 2280 at 452 nm in

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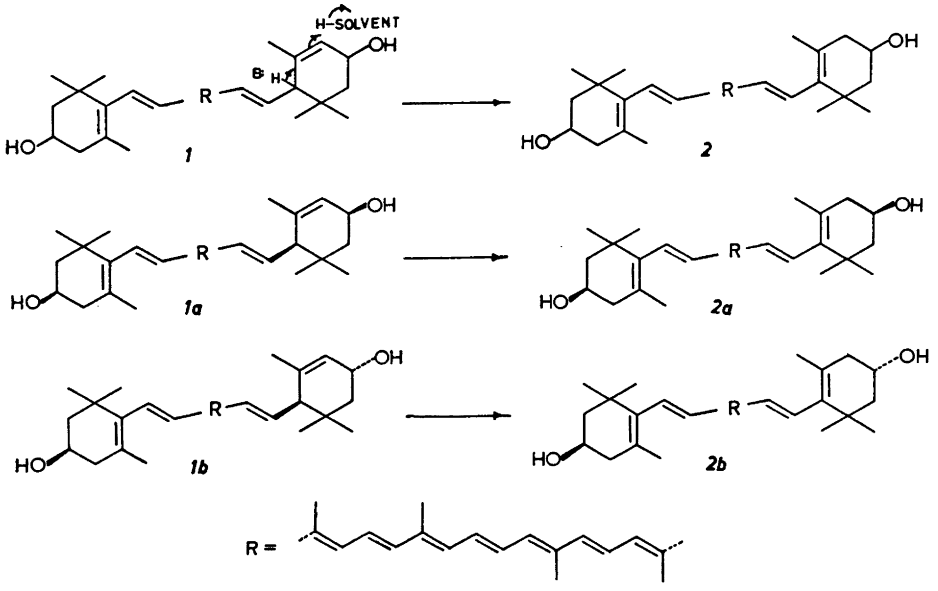

Scheme 1.

acetone) and $\Delta\varepsilon = -11.2 \pm 10$ % (0.158 mg sample), compared with $\Delta\varepsilon = -14.8 \pm 10$ % (0.360 mg sample) obtained in a parallel measurement for natural, untreated *trans* zeaxanthin *ex* alfalfa.

The latter value for natural zeaxanthin in EPA solution is in satisfactory agreement with values reported at 285 nm (dioxane) by Buchecker [7] ($\Delta\varepsilon = -16.2$) and by Bartlett *et al.*[6] ($\Delta\varepsilon = -11.8$). The value ($\Delta\varepsilon = -29$) in EPA solution reported by us [8] for zeaxanthin *ex* Flexithrix is now considered erroneously high by a factor of two.

The quantitative CD data for alkali-treated and untreated natural zeaxanthin thus show that 76 – 79 % (± 10 %) retention of optical activity (corresponding to *ca.* 11 % inversion or 22 % racemization) was obtained after alkali treatment of natural zeaxanthin.

Natural lutein (*1*, 5 mg) yielded after isomerization under identical conditions *trans*-zeaxanthin (0.521 mg). The CD-spectrum of zeaxanthin thus prepared showed no optical activity, which supports structure *1b* for lutein.

It might be argued that the allylic 3′-position in the ε-ring of lutein (*1*) may be more susceptible to racemization than the non-allylic 3-position in the β-ring. If this were the case, then zeaxanthin obtained from the isomerization of lutein should still show optical activity due to the residual asymmetry at C-3.

The *R*-chirality of lutein, previously assigned by Buchecker *et al.*,[2,3] is thus considered confirmed.

Lutein and zeaxanthin were separated and identified as described earlier.[5] Instrumentation was as commonly used in our laboratories.[9]

Acknowledgements. A.G.A. was the recipient of a post-doctoral fellowship provided by the Royal Norwegian Council for Scientific and Industrial Research.

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The Crystal Structure of Triose Reductone

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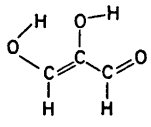
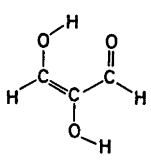
The crystal and molecular structure of triose reductone (2-propen-2,3-diol-1-one) has been determined from three-dimensional X-ray diffraction data measured by counter methods. The space group is $Pna2_1$; $a = 10.601(2)$ Å, $b = 3.647(1)$ Å, $c = 10.008(3)$ Å. The structure has been refined to $R_w = 0.04$ for 811 independent observed reflections. The estimated standard deviations in bond lengths involving non-hydrogen atoms are 0.002 Å. The compound crystallizes in a *trans*-enol form. Both hydrogen bonds are *inter*molecular and form a three-dimensional network.

Triose reductone ($C_3H_4O_3$) is a labile decomposition product which is formed when monosaccharides are heated with alkali in aqueous solutions at elevated temperatures.[1] It is known that the compound carries an aci-reductone group ($-CO-COH=COH-$); however, the molecule may exist in two enol forms:



I



II

In contrast to the acyclic β-diketones the *trans*-enol(I) may also be stabilized through *intra*-molecular hydrogen bonds. A previous X-ray investigation [2] of the rubidium salt of triose reductone has shown that the anion has a *trans* structure corresponding to I. Recent work based upon NMR studies has established the existence of the *cis*-isomer (II) in acetone solutions.[3] However, spectroscopic investigations (infrared, Raman) seem to indicate that in the crystalline state the molecule prefers the *trans*-enol form (I) with the two available hydrogen atoms engaged in *intra*-molecular hydrogen bonds forming two five-membered rings.[4]

The present X-ray diffraction study was undertaken in order to establish the structure in the crystalline state and to examine the hydrogen bonding properties of this compound. A comparative study of the cyclic homologue, reductic acid (2-cyclopentene-2,3-diol-1-one) is now being carried out in this laboratory.

EXPERIMENTAL

The compound was synthesized by the method described by von Euler and Eistert,[1] and purified by slow sublimation (55°C, 0.01 mmHg). Single crystals were obtained by recrystallization from a mixture of ether and hydrochloric acid. Preliminary photographic investigations implied the space group to be either $Pnma$ or $Pna2_1$, thus confirming the results of Aurivillius and Lundgren.[5] The space group $Pna2_1$ was tentatively assumed and proved correct by the successful outcome of the analysis. Unit cell dimensions were determined from measurements of 18 reflections using a manual Picker four-circle diffractometer (CuK radiation). For intensity measurements a crystal with approximate dimensions $0.39 \times 0.27 \times 0.43$ mm^3 was used. Due to instability in air and moisture, the crystal was mounted in a sealed glass capillary. Intensity measurements were made on a SYNTEX P$\bar{1}$ automatic four-circle diffractometer with graphite monochromated Mo$K\alpha$ radiation. The crystal was mounted with the b-axis near the goniometer head spindle axis. Intensities were measured within one octant for $2\theta < 75°$ with the $\omega - 2\theta$ scanning mode and a scan range in 2θ from 1.0° below 2θ (α_1) to 1.0° above 2θ (α_2). The scan speed for each reflection was calculated from a preliminary stationary-crystal stationary-counter measurement of the intensity and varied between 1.0 and 8.0°/min so as to main-

tain satisfactory counting statistics even for reflections of moderate intensity. Above $2\theta = 45°$ a rejection level was specified to avoid measurement of reflections with intensities less than the threshold value. A total of 957 independent intensities were recorded of which 811 had intensities greater than twice their standard deviations estimated from counting statistics. The intensities of three standard reflections measured for every 50 reflections showed no significant change in intensity. Lorentz and polarization corrections were applied. Atomic

Table 1. Observed and calculated structure factors. (The five columns list values of h, k, l, $10F_o$, and $10F_c$.)

Table 2. Fractional atomic coordinates and thermal parameters with estimated standard deviations ($\times 10^5$). The anisotropic temperature factor is given by $\exp -(B_{11}h^2+B_{22}k^2+B_{33}l^2+B_{12}hk+B_{13}hl+B_{23}kl)$.

Atom [a]	x	y	z	B_{11}(B)	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
O1	3467	28064	12802	516	10347	728	558	−97	−1065
	10	42	20	7	120	10	48	16	63
O2	28273	4680	12810	521	12893	469	202	−57	−279
	10	41	18	7	137	7	54	15	69
O3	37454	−13225	−12896	646	10352	580	209	66	−998
	12	41	20	8	116	10	52	15	56
C1	8747	17989	2363	603	7891	665	289	−222	−779
	13	46	22	10	119	13	60	20	79
C2	21456	6482	1426	563	6077	502	−169	−89	−245
	12	40	0	9	93	9	50	18	66
C3	25731	−2190	−10897	670	6614	536	195	−82	−413
	14	43	20	11	105	10	61	18	63
H1	4255	17975	−5913	2.93					
	205	546	226	.38					
H2	36679	7721	11513	5.88					
	288	780	369	.68					
H3	37930	−16225	19996	4.18					
	255	664	333	.55					
H4	20561	−257	−18368	2.90					
	188	504	220	.42					

[a] For numbering of atoms see Fig. 1.

Table 3. R.m.s. amplitudes $(\overline{u^2})^{\frac{1}{2}}$ and B-values along the principal axes of vibration given by the components of a unit vector **e** in fractional coordinates ($\times 10^3$).

Atom	$(\overline{u^2})^{\frac{1}{2}}$	B	e_x	e_y	e_z
	.270	5.76	13	262	−26
O1	.186	2.74	−18	78	94
	.169	2.26	92	−21	23
	.295	6.87	4	274	−4
O2	.174	2.38	−92	13	20
	.152	1.83	19	8	98
	.267	5.63	4	268	−21
O3	.191	2.89	92	1	22
	.166	2.18	−21	58	95
	.238	4.49	22	248	−36
C1	.191	2.89	−72	110	51
	.166	2.18	57	43	78
	.204	3.29	14	−270	11
C2	.180	2.56	88	28	−34
	.156	1.93	31	41	93
	215	3.65	27	257	−22
C3	195	3.00	−90	82	10
	161	2.05	15	52	97

Table 4. Bond distances, bond angles, and hydrogen bond lengths and angles (equivalent position numbers in parentheses as defined below). Estimated standard deviations in bond lengths between heavy atoms are 0.002 Å, in angles 0.1° (0.02 Å and 2° in bonds involving hydrogen bonds). Distances in parentheses are corrected for anisotropic thermal motion (see text).

Bond distances (Å)		Bond angles (°)	
C1 – C2	1.414 (1.423)	C1 – C2 – C3	116.7
C2 – C3	1.352 (1.356)	C1 – C2 – O2	117.9
C1 – O1	1.241 (1.244)	C2 – C3 – O3	121.7
C2 – O2	1.351 (1.360)	C2 – C1 – O1	125.0
C3 – O3	1.322 (1.327)	C3 – C2 – O2	125.4
C1 – H1	0.96	O1 – C1 – H1	120.3
C3 – H4	0.93	C2 – C1 – H1	114.7
O2 – H2	0.91	C2 – C3 – H4	121.2
O3 – H3	0.73	O3 – C3 – H4	117.1
		C2 – O2 – H2	113.5
		C3 – O3 – H3	105.2
Hydrogen bond distances (Å)			
O1···O2 (I)	2.744	Hydrogen bond angles (°)	
O1···O3 (II)	2.635	O1···H2 – O2 (I)	164.9
O1···H2 (I)	1.86	O1···H3 – O3 (II)	156.5
O1···H3 (II)	1.96	O1···O2 – H2 (I)	10.1
		O1···O3 – H3 (II)	17.2

I: $(x-\frac{1}{2},\frac{1}{2}-y,z)$
II: $(\frac{1}{2}-x,y-\frac{1}{2},z+\frac{1}{2})$

scattering factors were taken from Hanson *et al.*[6] except those for the hydrogen atoms.[7] All programs used are written or revised for CD-3300 by Dahl *et al.*[8]

CRYSTAL DATA

Triose reductone $C_3H_4O_3$, orthorhombic; $a=10.601(2)$ Å, $b=3.647(1)$ Å, $c=10.008(3)$ Å, $\varrho_{obs}=1.50$ g/cm³ (flotation). $\varrho_{calc}=1.49$ g/cm³, $Z=4$.

STRUCTURE DETERMINATION

The orientation of the molecule was found from a three-dimensional Patterson synthesis. The model was then translated stepwise through the unit cell and R over 50 low order reflections was determined for each step. The coordinates corresponding to the R-minimum were refined further by the "minimum residual method".[9] Least squares refinements assuming isotropic thermal parameters converged with $R_w=0.22$ and $R=0.17$. Introduction of anisotropic thermal parameters yielded $R_w=0.052$ and $R=0.047$. The hydrogen atoms were located from a difference Fourier map. Refinement of individual isotropic temperature factors for these atoms gave reasonable values, and refinements terminated with $R_w=0.043$ and $R=0.035$. The weight analysis based on the standard deviations from counting statistics showed only small intensity variations except for the smallest F-values. No secondary extinction effects were observed. A final difference Fourier map contained no larger density fluctuations than ± 0.35 e Å^{-3}. Observed and calculated structure factors are listed in Table 1 and atomic parameters in Table 2. Magnitudes and directions of the principal axes of the ellipsoids of vibration are given in Table 4. The r.m.s. discrepancy between the atomic vibration tensor components obtained in the structure analysis and those calculated from the rigid-body parameters found by analysis of the librational, translational, and screw motion of the molecule [10] is 0.0009, indicating that the molecule may be regarded as a rigid body. However, the ratio of "observed" to calculated parameters is small (1.8). The eigen values of **T** are 0.21, 0.17, and 0.16 Å while the r.m.s. libration amplitudes are 7.9, 4.1, and 2.1°. The two larger axes of libration lie approximately in the molecular plane (see below), the largest being nearly

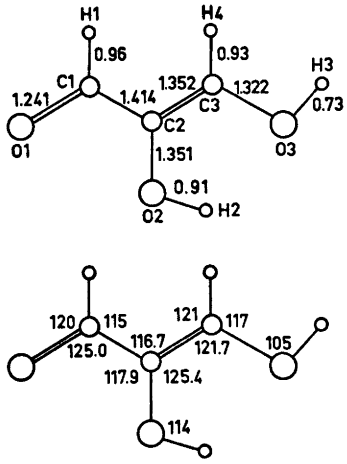


Fig. 1. Schematical drawing of the molecule showing bond distances and angles (uncorrected values).

parallel with the axis of minimum inertia. The translational vibration is largest in the direction perpendicular to the molecular plane. Corrections in bond lengths for librational motion range from 0.003 Å to 0.009 Å (Table 4).

DISCUSSION

Bond lengths and angles are listed in Table 4 and Fig. 1. The compound is found to have the *trans* structure (I). However, both hydrogen bonds are *inter*-molecular.

The molecular dimensions of triose reductone agree fairly well with those found in dialuric acid,[11,12] and thus there is also considerable conjugation in the alternating bond system of triose reductone. In both ascorbic [13] and dialuric acids [11,12] a large difference between the C–O bond lengths of the central and terminal hydroxyl groups is found. A similar difference is also observed in triose reductone (0.03 Å). It therefore appears likely that the terminal hydroxyl group carries the acidic proton, as it does in ascorbic acid.[14]

The molecule is nearly planar (Table 5). Deviations from planarity are mostly due to a slight rotation around the C1–C2 single bond, the dihedral angle O1–C1–C2–O2 being 3.3°. The enediol group (–COH=COH–), however, is exactly planar and the atoms H3 and H4 also lie in this plane, while H2 deviates significantly.

Table 5. Deviations from least squares planes (Å). Plane No. **1** is through all heavy atoms, plane no. **2** is through all heavy atoms except O1. Deviations of atoms not defining the planes in parentheses.

Atom	1	2
O1	0.019	(0.050)
O2	−0.006	−0.001
O3	0.011	−0.000
C1	−0.024	−0.001
C2	−0.006	0.003
C3	−0.002	−0.001
H1	(−0.050)	(−0.025)
H2	(0.380)	(0.377)
H3	(0.020)	(0.006)
H4	(0.003)	(0.007)

The two hydroxylic protons are both involved in *inter*-molecular hydrogen bonds to the carbonyl oxygen atom at distances 2.635(2) Å, and 2.744(2) Å. The shorter bond is that involving the terminal hydroxyl group. This bond lies approximately in the plane of the molecule, while the other bond is significantly out of this plane. A similar situation is also found in dialuric [11,12] and ascorbic acid.[13]

There are two short *intra*-molecular O···O contacts (O1···O2 2.76 Å, O2···O3 2.83 Å) which may be regarded as possible hydrogen bond contacts. There were, however, no maxima in the difference Fourier map that could be interpreted in terms of hydrogen bonding between these atoms. There is therefore no evidence of a disordered or bifurcated hydrogen bond system. The distance O3···H2 is 2.56 Å and must therefore be regarded as a normal van der Waals contact. The facile sublimation of triose reductone has been attributed to the existence of a double five-membered chelate ring structure.[15] Attempts to verify this kind of structure in the vapour phase by electron diffraction and microwave spectroscopy at this institute have so far been unsuccessful, due to the low stability and the low volatility of this compound.

As illustrated in Fig. 2 the molecules are hydrogen bonded to each other to form a three-dimensional network. The stronger hydrogen

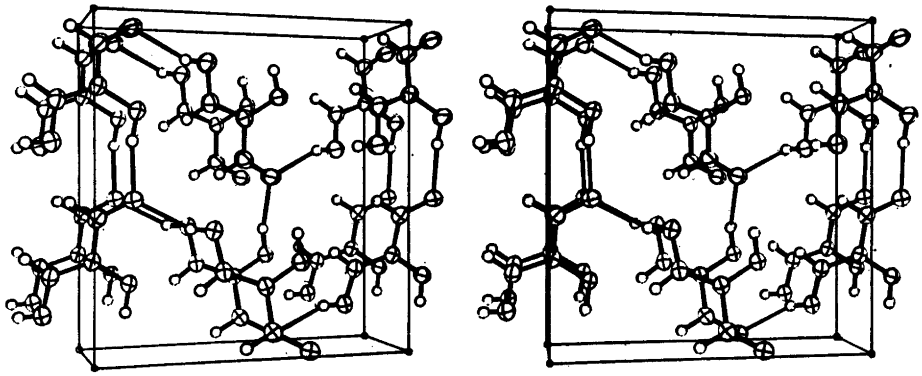


Fig. 2. Stereoscopic illustration of the structure of triose reductone. Thermal ellipsoids are scaled to include 50 % probability. Covalent bonds are filled, hydrogen bonds are open. (Johnson, C.K. (1965) *ORTEP*. ORNL-3794, Oak Ridge National Laboratory, Tennessee).

bonds between the terminal oxygen atoms form alternating zig-zag chains running parallel with the (011) and (0$\bar{1}$1) planes, respectively. These chains are cross-linked by the weaker hydrogen bonds along [100].

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Electrochemical Cleavage of Some Cyclic Sulphones

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A series of compounds having a sulphonyl group in a saturated ring annealed to a benzene ring has been synthesized and subjected to polarography, coulometry and preparative electrolysis at a mercury cathode. The compounds are found to be cleaved in a two-electron process to yield sulphinate ions, the structures of which indicate the cleavage mode. In the compounds having a four-, five-, six-, or seven-membered ring with the sulphonyl group bound to the aromatic ring, the cleavage takes place preferentially across this bond, in contrast to the behaviour of analogous open-chain alkyl aryl sulphones. The cleavage modes are discussed in terms of the different conformation and, in consequence, *d* orbital participation, of the sulphonyl group in the cyclic and open-chain sulphones. Also, the five-ring isomer in which the sulphonyl group is in a benzylic position, *o*-xylylene sulphone, has been found to undergo C—S cleavage.

In an earlier communication,[1] the cleavage mode of the three cyclic sulphones *2*—*4* (see Fig. 1) was described. The bond between the sulphonyl group and the aromatic ring was found to be broken, contrasting the behaviour of simple alkyl aryl sulphones. The latter type of compounds are cathodically cleaved between the sulphonyl group and the alkyl group.[2,3] Only in alkyl aryl sulphones carrying a strongly electron-attracting substituent, *e.g.*, *p*-cyano, is cleavage between the aromatic ring and the sulphonyl group observed.[4]

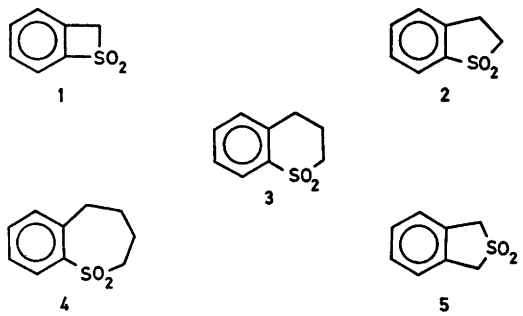

Fig. 1. Cyclic sulphones studied in the present work.

The present paper gives a full account of the electrochemical behaviour of the above-mentioned cyclic sulphones at a mercury cathode. In addition, the compound with a four-membered ring, *1*, has been included and also, the five-membered sulphone *5*. The four-membered ring compound was readily cleaved to give a mixture of the two possible isomeric sulphinate ions in the approximate ratio 4:1. The main product was α-toluenesulphinate ion, indicating that *1* follows the behaviour of *2*—*4*. The compound *5* was included since it is a cyclic sulphone in which the sulphur atom is not bound to an aromatic carbon atom. It was cleaved in wet *N*,*N*-dimethylformamide (DMF), with a tetraalkylammonium halide as the supporting electrolyte, at a potential close to the cathodic limit.

All of the compounds *1*—*5* are described in the literature, and the published syntheses were largely followed. Only with *1* were difficulties met with. The synthesis has been described by Dittmer and Davis [5] and involves several steps, the last of which gives only 3 % yield. In this work, the yield in the final step was increased to 15 %. An alternative route to *1*, reaction of *o*-chlorophenyl methyl sulphone with potassium amide in liquid ammonia, turned out to yield not *1*, but instead, *o*-aminophenyl methyl sulphone. The latter method, which involves a

benzyne intermediate, has been successfully employed for the synthesis of a benzocyclobutane derivative.[6]

The compounds *1*–*5* were polarographed in DMF with tetrabutylammonium iodide as the supporting salt. Coulometric experiments at a large-surface mercury cathode were also performed, and finally, preparative electrolyses on a gram scale. Identification of the sulphinate ions formed upon electrolysis was carried out by PMR after conversion to sulphones.

EXPERIMENTAL

General. Melting points have been determined on a Kofler Heizbank. Pressures are given in SI units. 1 Pa = 7.5×10^{-3} mmHg, 1 MPa = 145 psi.

Syntheses. 2H-*Benzo*[b]*thiete-1,1-dioxide* (*1*) was prepared essentially according to Dittmer and Davis.[5] However, silver oxide in boiling xylene was used in the last step instead of triethylamine in benzene to bring about a double dehydrobromination.[7] The yield in this step was 15 %. The compound had the same m.p. (126–128°) and PMR spectrum (in $CDCl_3$, 2 H singlet at δ 5.1 and 4 H singlet at δ 7.55) as previously found.[5]

Benzo[b]*thiophene-1,1-dioxide.* 1-Benzothiophene (Columbia Organic Chemicals Co.) was oxidized on a 0.2 mol scale to the corresponding sulphone with hydrogen peroxide in acetic acid solution.[8] After recrystallization from ethanol, a 69 % yield was secured, m.p. 142°, lit.[8] 142–143°.

2,3-Dihydrobenzo[b]*thiophene-1,1-dioxide* (*2*). The preceding compound was hydrogenated on a 50 mmol scale in tetrahydrofuran solution in a Parr apparatus,[9] using 10 % palladium on carbon as the catalyst. At an initial pressure of 0.31 MPa, the reaction required 15 min. After recrystallization from ethanol, the yield was 80 %, m.p. 92°, lit.[9] 91–92°.

3-Phenylmercaptopropionic acid. This compound was synthesized on a 0.5 mol scale from thiophenol and 3-bromopropionic acid.[10] The yield was 87 %, m.p. 54–56°, lit.[10] 59°.

Thiochroman-4-one-1,1-dioxide. Cyclization of the preceding compound was performed on a 0.1 mol scale in conc. sulphuric acid.[10] The crude thiochroman-4-one thus obtained was directly oxidized to the sulphone by a mixture of 20 ml 30 % hydrogen peroxide and 40 ml glacial acetic acid.[11] The crude product was recrystallized from ethanol. Needles were obtained, overall yield 49 %, m.p. 133°, lit.[11] 131–132°.

Thiochromane-1,1-dioxide (*3*). Of the preceding compound, 4.9 g (25 mmol) was dissolved in 100 ml glacial acetic acid. One drop of conc. hydrochloric acid and 0.1 g 10 % palladium on carbon were added, and the mixture was hydrogenated at room temperature in a Parr apparatus, using an initial pressure of 0.28 MPa. After 1 h, the expected amount of hydrogen (50 mmol) had been consumed, and the solution was filtered and vacuum evaporated to dryness. After recrystallization from ethanol, 3.45 g of product were obtained, yield 76 %, m.p. 88–89°, lit.[12] 87.5–88°.

5-Oxo-2,3,4,5-tetrahydrobenzo[b]*thiepin.* First, 4-phenylmercaptobutyric acid was prepared on a 1 mol scale from thiophenol and γ-butyrolactone according to Reppe.[13] The yield was almost quantitative. Of the crude product, 0.33 mol was cyclized in polyphosphoric anhydride according to Traynelis and Love.[14] Distillation yielded 39.7 g (67 %) of product, b.p. 98° at 53 Pa, lit.[14] 119–120° at 200 Pa.

5-Oxo-2,3,4,5-tetrahydrobenzo[b]*thiepin-1,1-dioxide.* Of the preceding compound, 24 g was oxidized with 30 % hydrogen peroxide and glacial acetic acid.[14] After recrystallization from ethanol, 20.2 g (71 %) of colourless plates were obtained, m.p. 153–155°, lit.[14] 155–156°.

2,3,4,5-Tetrahydrobenzo[b]*thiepin-1,1-dioxide* (*4*). Hydrogenation of the preceding compound was performed at about 40° starting with 10.5 g (0.05 mol) in 200 ml glacial acetic acid, 3 drops of conc. hydrochloric acid and 0.2 g 10 % palladium on carbon. The initial pressure was 0.33 MPa. After 14 h, 0.1 mol of hydrogen had been consumed. Filtration, vacuum evaporation and recrystallization from hexane yielded 8.7 g (89 %) of product, m.p. 78°, lit.[12,14] 77–78°.

1,3-Dihydrobenzo[c]*thiophene-2,2-dioxide* (*5*). α,α'-Dibromo-*o*-xylene, 100 g (0.38 mol), was allowed to react with sodium sulphide, following the directions of von Braun,[15] to yield 20.6 g (40 %) of the corresponding cyclic sulphide. Without undue delay, the sulphide was oxidized with peracetic acid according to Cava and Deana[16] to give, after recrystallization from ethanol containing 5 % tetrahydrofuran, 14.7 g (58 %) of the sulphone, m.p. 150°, lit.[16] 150–151°.

Benzyl o-tolyl sulphone, which was needed for identification, was synthesized from *o*-thiocresol and benzyl chloride in aqueous sodium hydroxide solution, the intermediate sulphide being oxidized to the sulphone by 10 % peracetic acid. In *Beilstein's Handbuch*, this compound is erroneously described as a yellow oil, but in fact forms colourless crystals. After recrystallization from ethanol, m.p. 94–94.5°, PMR $CDCl_3$: 3 H singlet at δ 2.53, 2 H singlet at δ 4.33, 9 H multiplet at δ 7.0–7.9.

Methyl phenyl sulphone and other sulphones needed for comparison purposes were either available from previous work or prepared by straightforward methods. Their melting points agree with literature values, and the structures were verified by PMR.

Polarography. The *N,N*-dimethylformamide (DMF) used for electroanalytical work was dried over anhydrous sodium sulphate and distilled through a 50 cm glass-helix column. After a 5–10 % forerun, a constant boiling

centre cut was taken at 94°/18.6. kPa. The solvent was stored in the dark and used within a week after distillation. Tetrabutylammonium iodide was from Fluka AG, polarography grade. A Tacussel Tipol 3-electrode polarograph was used. The sweep rate was 2.5 mV/s. The substrate concentration was 10^{-3} M, and that of supporting salt, 0.1 M.

Coulometry. A 0.25 M solution of tetrabutylammonium bromide in DMF was used as supporting electrolyte. The mercury cathode, about 10 cm^2, was agitated by a magnetic stirring bar, and the anode compartment was separated by a glass frit. A Tacussel type RM 04 vessel was used. The potentiostat and coulometer were Tacussel type ASA 4SHT and IG4 10A, respectively. The coulometric runs were performed on 20 – 30 mg amounts of substrate. In some runs, a tenfold excess of phenol was added after the initial current had decayed and the electrolysis resumed.

Macroelectrolyses. The procedure for compounds *2 – 4* has been published earlier.[1] For practical reasons (ease of workup, since extremely little material was available), compound *1* had to be electrolyzed in 1 M tetramethylammonium chloride in methanol. Compound *2* was electrolyzed in methanol as well, and gave the some product as previously obtained in DMF. Galvanostatic conditions were used for *1 – 4*. In contrast, *5* was electrolyzed at −2.2 V *vs.* Ag,AgI/0.25 M I^- using a Tage Juul type G-30 potentiostat. In this case, the supporting electrolyte was 0.25 M methyltributylammonium iodide in DMF containing 5 % water. Dry DMF was found to cause pumping of electrolyte into the cathode compartment, a greyish sludge (probably organomercury compounds) being also formed. This is thought to be caused by decomposition of the supporting electrolyte.

After the electrolyses, the catholyte was separated and allowed to react with benzyl chloride (*1 – 4*) or methyl iodide (*5*) by warming to 60 – 80° for 3 h and leaving overnight. The sulphinate ions were thus converted into sulphones, which were isolated by evaporation, dilution with water and extraction with methylene chloride or ether. For compounds *1* and *2*, the sulphones were compared with authentic material (m.p. and PMR spectra). For compound *5*, the reaction with methyl iodide yielded a sulphone which, after recrystallization twice from ether, had the m.p. 73 – 74° and PMR data in $CDCl_3$: 3 H singlet at δ 2.43, 3 H singlet at δ 2.80, 2 H singlet at δ 4.32, and 4 H singlet at δ 7.27. Elemental analysis (Microanalytical service of C.N.R.S., France): Found C 58.71; H 6.44; O 17.08; S 17.26. Calc. for $C_9H_{12}O_2S$: C 58.67; H 6.56; O 17.37; S 17.40. Based on these data, the compound was identified as methyl (*o*-methyl)benzyl sulphone. This compound has not been found in the literature. The PMR spectrum of the crude product revealed no other identifiable compounds, but only trace impurities.

RESULTS AND DISCUSSION

Electroanalytical work. The polarographic and coulometric results are given in Table 1. It has been shown earlier[3] that methyl phenyl sulphone gives rise to a two-electron, irreversible polarographic wave in DMF. Since the diffusion coefficients should be rather similar for the compounds studied in this work, it can be concluded from inspection of the $i/\sqrt{h}$-values in Table 1 that the cyclic sulphones give two-electron waves as well. As already observed for methyl phenyl sulphone, addition of a ten- to twentyfold excess of phenol as a proton donor increased the wave height by less than 15 % except for compound *2*, for which the height was increased by up to 40 %. The coulometric runs in absence of phenol give values indicating a two-electron process.

Addition of a ten- to twentyfold excess of phenol in the coulometric runs after the first

Table 1. Polarographic and coulometric data for sulphones studied in this work.

Compound	$E_{½}$ [a] V	$E_{½}$ [b] V	$i/\sqrt{h}$ μA $cm^{-½}$ [c]	Coulometry [c] F	Coulometry [d] F
1 (4-ring)	−1.62	−1.94	0.98	2.1	–
2 (5-ring)	−1.90	−2.22	1.40	2.0	3 – 4
3 (6-ring)	−1.90	−2.22	0.93	2.5	5.3
4 (7-ring)	−1.85	−2.17	0.74	2.2	2.6
5 (5-ring)	−2.18	−2.50	0.76	2.7	> 12
Methyl phenyl sulphone	−1.73	−2.05	1.00	2.04 [e]	–

[a] *Versus* Ag,AgI/0.1 M Bu_4NI, DMF. [b] *Versus* SCE in water, determined for methyl phenyl sulphone and calculated for the other sulphones. [c] No proton donor added. [d] Electrolysis continued after addition of excess phenol. [e] Taken from Ref. 3.

current had decayed to its background value caused the current to increase, and the experiments were continued until the current had again attained its background value. The last column in Table 1 thus represents the sum of the first and the second current-time integrals. For compound *1*, no phenol was added since the catholyte was saved for an attempted transformation to derivatives, and for compound *5*, which requires a much more negative potential than the others, phenol addition caused hydrogen ion discharge.

In an earlier study,[3] it was shown that no second wave is observed with methyl phenyl sulphone upon addition of phenol, the interpretation being that the benzenesulphinate ion initially formed is too weakly basic to be protonated by phenol. However, by titration with sulphuric acid, the free benzenesulphinic acid could be liberated, which then gave a polarographic wave.[18] With the cyclic sulphones in the present study, the preparative experiments show that araliphatic sulphinates are formed. A probable interpretation of our data is that the araliphatic sulphinic acids are weaker than benzenesulphinic acid, so that in the acid-base equilibrium

$$\text{Phenol} + \text{sulphinate} \rightleftharpoons \text{phenolate} + \text{sulphinic acid}$$

a sufficient sulphinic acid concentration prevails so as to permit a significant current.

Since the polarographic half-wave potentials for all compounds were found to be unaffected by phenol addition (*i.e.*, by the acidity of the medium), the potential-determining transition state in the irreversible reaction cannot contain a proton from the medium. The mechanism for cleavage of methyl phenyl sulphone has been already discussed,[3] and it appears probable that the same mechanism applies to the cyclic sulphones.

It is interesting to note the dependence of the half-wave potentials on the ring size. The four-membered ring is more easily cleaved than is methyl phenyl sulphone, whereas the five-, six-, and seven-membered rings are more difficult to cleave. Compound *5* is considerably more difficult to cleave than any of the others, since it is a benzylic sulphone. In fact, *5* does not give a polarographic wave in methanol, and an attempted preparative run in this medium, which has been so successfully used by Horner and Neumann [2] for other benzyl sulphones, failed completely, hydrogen evolution being the only reaction.

Preparative results. As is seen in Table 2, compounds *1*–*4* all give sulphinate ions resulting from cleavage between the sulphonyl group and the aromatic ring, though *1* is also cleaved to a minor extent between the sulphonyl group and the benzylic carbon atom. It was mentioned in Ref. 1 that impurity peaks in the PMR spectra of the products obtained from compounds *2*–*4* suggest 10–20 % cleavage to give *o*-alkylsulphinate ions. Reexamination by GLC indicates that none of this is formed for *2* and *3*, whereas for the seven-ring compound *4*, 15 % *S*-alkyl cleavage might have taken place. Comparison with authentic benzyl *o*-butylphenyl sulphone has not been carried out, though. The result for *5* is straightforward; carbon-sulphur rather than carbon-carbon cleavage takes place, the former alternative being *a priori* expected.

The main reason for including *1* in the present study was that Horner had suggested [2] that in

Table 2. Derivatives obtained upon alkylation of sulphinate ions in catholyte.

Compound	Solvent	Supporting salt	Alkylation agent	Product(s) formed,	isolated yield %
1	MeOH	Me_4NCl	$PhCH_2Cl$	$PhCH_2SO_2CH_2Ph$	35 [a]
				o-Tolyl-SO_2CH_2Ph	9 [a]
2	MeOH	Me_4NCl	$PhCH_2Cl$	$Ph(CH_2)_2SO_2CH_2Ph$	30
2	DMF	Et_4NClO_4	$PhCH_2Cl$	$Ph(CH_2)_2SO_2CM_2Ph$	80
3	DMF	Et_4NClO_4	$PhCH_2Cl$	$Ph(CH_2)_3SO_2CH_2Ph$	97
4	DMF	Et_4NClO_4	$PhCH_2Cl$	$Ph(CH_2)_4SO_2CH_2Ph$	68 [b]
5	DMF, 5 % H_2O	$MeBu_3NI$	MeI	*o*-$CH_3C_6H_4CH_2SO_2CH_3$	19

[a] As mixture. [b] Possibly also containing benzyl *o*-butyl sulphone; see the text.

Table 3. PMR data for derivatives obtained after alkylation of sulphinate ions in catholyte after electrolysis.

Compound No.	Alkylating agent	Product PMR data [a]	Product
1	Benzyl chloride	4.15 (4 H, s), 7.45 (10 H, s)	Dibenzyl sulphone [b]
		2.53 (3 H, s), 4.35 (2 H, s), 7.0 – 7.9 (9 H, m)	Benzyl *o*-tolyl sulphone [c]
2	Benzyl chloride	3.08 (4 H, s), 4.17 (2 H, s), 7.0 – 7.4 (10 H, m)	Benzyl 2-phenylethyl sulphone [d]
3	Benzyl chloride	1.8 – 3.3 (6 H, m), 4.17 (2 H, s), 7.1 – 7.5 (10 H, m)	Benzyl 3-phenylpropyl sulphone
4	Benzyl chloride	1.5 – 1.9 (4 H, m), 2.4 – 3.0 (4 H, m), 4.17 (2 H, s), 7.1 – 7.5 (10 H, m)	Benzyl 4-phenylbutyl sulphone
5	Methyl iodide	2.43 (3 H, s), 2.80 (3 H, s), 4.32 (2 H, s), 7.27 (4 H, s)	Methyl *o*-methylbenzyl sulphone

[a] Numbers in δ units rel. to TMS, solvent $CDCl_3$, s singlet, m multiplet. [b] Appr. 80 mol %. [c] Appr. 20 mol %. [d] M.p. after recryst. from ethanol 123°, lit.[17] 126°.

the cleavage of a mixed sulphone, the relative stability of the different possible radicals formed after transfer of one electron should determine the cleavage mode. At first sight, one might therefore expect *1* to give *o*-toluenesulphinate ion as the main product. However, our experimental results do not invalidate Horner's argument, because even if bond-breaking is significant in the critical transition state, the incipient benzyl radical is formed in the very conformation least suitable for mesomeric interaction with the benzene ring, the odd electron orbital being orthogonal to the aromatic π system.

Since the behaviour of *1* follows that of *2 – 4*, the arguments below apply to the whole series *1 – 4*. In a preliminary communication,[1] it was suggested that the vibrational pattern of the cyclic compounds might influence the cleavage mode, or that "the detailed mechanism of electron transfer" might be different. It was believed that tetramethylammonium amalgam is the reducing agent at the rather negative cleavage potentials for *2 – 4*. The results for *1* force us to dismiss the second possibility altogether, since the half-wave potential is *less* negative than that of methyl phenyl sulphone, which is directly reduced by the cathode.[3]

We feel that the most fruitful approach is to consider the resonance interaction between the sulphonyl group and the aromatic π system as a function of the orientation of the sulphonyl group. Gerdil and Lucken [19] have made an electron spin resonance and polarographic study of the sulphone group in diphenyl sulphone, dibenzothiophene-*S*,*S*-dioxide and thianthrene-*S*,*S*,*S'*,*S'*-tetroxide. The orientation of the sulphonyl group with respect to the aromatic rings is different in all these compounds. In the terminology originally introduced by Koch and Moffitt [20] the dibenzothiophene-*S*,*S*-dioxide is an example of Case I conjugation, whereas diphenyl sulphone represents Case II. The thianthrene tetroxide is intermediate between these two.

When one makes MO calculations of the interaction between the sulphur 3*d* orbitals and the aromatic π system, the symmetry properties of the *d* orbitals must be considered. Gerdil and Lucken [19] argued that since different *d* orbitals combine with the π system in Case I and II, respectively, the energy levels of the MO's are also different in the two cases, and in particular, the energy of the LVMO is lowered upon going from Case I to Case II conjugation.

The cyclic compounds in the present study all represent Case I conjugation, whereas methyl phenyl sulphone, the geometry of which is known *via* dipole moment measurements [21] and X-ray cristallography of a halogenated derivative,[22] is an example of Case II. The two cases are illustrated in Fig. 2.

If the energy of the LVMO (to which the initial electron transfer takes place in cathodic reactions) is higher in the Case I compounds *2 – 4* than in the Case II compound methyl

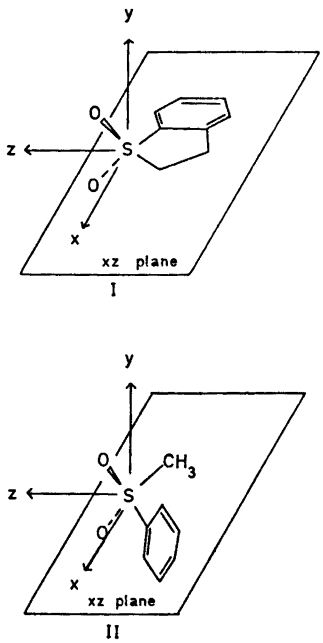

Fig. 2. Orientation of the sulphonyl group in 2,3-dihydrobenzo[*b*]thiophene-1,1-dioxide and methyl phenyl sulphone. The O–S–O bonds are in the *yz* plane. In the upper compound, all carbon atoms are in the *xz* plane. In the lower compound, the benzene ring is perpendicular to the *xz* plane.

phenyl sulphone, which is reasonable in view of Gerdil and Lucken's analysis,[10] we have a rationale for the observed bond-breaking as well as for the more negative half-wave potentials for the cyclic compounds. The four-membered compound *1* represents a special case, in which ring strain must be taken into account.

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Absolute Configurations of 2-Alkylmalic Acids

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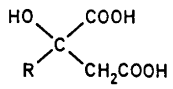
The absolute configurations of some naturally occurring 2-alkylmalic acids (V, VII–X) have all been shown to be *R* by asymmetric syntheses of (*S*)-III, -VI and -VIII (Fig. 1) and CD measurements on molybdate complexes of the synthetic and the natural acids. The key compound in the syntheses is the epoxide (*R*)-XII, obtained in 14 % enantiomeric excess by asymmetric synthesis.

Several 2-alkylmalic acids (I), as well as 2-benzylmalic acid (VIII), occur naturally. Thus, 2-methylmalic acid (citramalic acid, II) is produced by microorganisms and has been detected in or isolated from many fruits. Both (+)- and (−)-citramalic acid have been found. 2-Ethylmalic acid (III) and 2-propylmalic acid (IV) are also synthesised by microorganisms. 2-Isopropylmalic acid (V) is an intermediate in the leucine biosynthesis; its (−)-form has been isolated from different sources, *e.g.* a culture broth of a mutant of *Saccharomyces cerevisiae*.[1]



I:	R= alkyl	VI:	R= $CH_2CH_2CH_2CH_3$
II:	R= CH_3	VII:	R= $CH_2CH(CH_3)_2$
III:	R= CH_2CH_3	VIII:	R= $CH_2C_6H_5$
IV:	R= $CH_2CH_2CH_3$	IX:	R= $CH_2CH_2CH(CH_3)_2$
V:	R= $CH(CH_3)_2$	X:	R= $CH_2CH_2CH_2C(CH_3)_2OH$

2-Isobutylmalic acid [2] (VII) and 2-benzylmalic acid [3] (VIII) are components of two Orchidaceae alkaloids, and 2-(3-methylbutyl)malic acid [4] (IX) and 2-(4-hydroxy-4-methyl-pentyl)malic acid [4] (X) are components of alkaloids from *Cephalotaxus harringtonia*. The absolute configuration has been determined only for citramalic acid. By correlation with (*S*)-(+)-mevalolactone, (+)-citramalic acid has been shown to possess the *S*-configuration.[5,6] The fermentation product 2-hydroxymethylmalic acid (itatartaric acid) has been correlated with citramalic acid.[7] Strong evidence for the *R*-configuration of the natural forms of VII and VIII has recently been obtained from asymmetric synthesis coupled with CD studies.[8]

CD spectra of α-hydroxy acids as molybdate complexes give information on their absolute configurations at the α carbon atoms.[9] It was, however, uncertain whether the Cotton effects of (*S*)-2-methylmalic acid and the (*S*)-2-substituted malic acids with larger substituents would be strictly comparable. For that reason syntheses of the latter acids were performed.

Ethyl chloropyruvate was condensed with the lithium enolate [10] obtained from (−)-menthyl acetate and lithium diisopropylamide, and the resulting mixed ester was transformed into the partially racemic diethyl ester XI. Treatment of XI with lithium hydride in hexamethylphosphortriamide gave the epoxide XII. Opening of the epoxide ring by catalytic hydrogenation afforded, as predominant enantiomer, (*S*)-(+)-diethyl citramalate [10] the specific rotation of which showed that the epoxide (*R*)-XII was present in 14 % enantiomeric excess. The asymmetric syntheses using ethyl pyruvate [10] and ethyl chloropyruvate thus both yielded 2-substituted malic acids in which the *S*-forms predominated. The (*S*)-diethyl esters XIII, XIV, and XV (Fig. 1) were prepared by cleavage of the epoxide ring in (*R*)-XII with the ap-

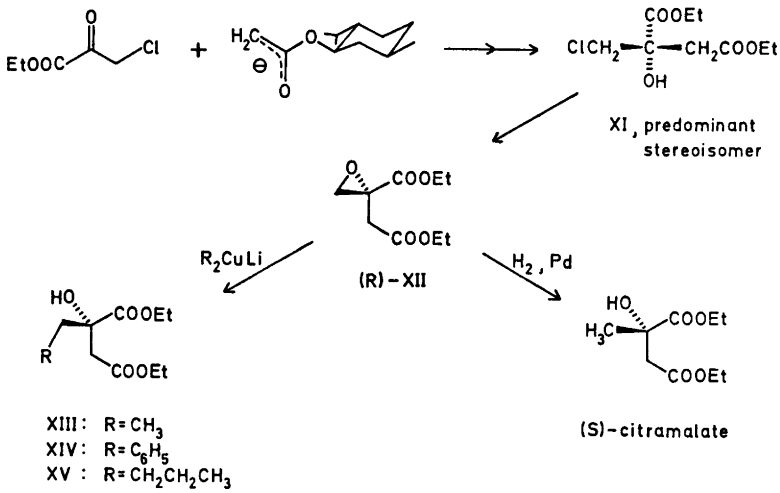


Fig. 1. Reaction scheme for the asymmetric syntheses of (*S*)-XIII, -XIV, and -XV (esters of (*S*)-III, -VIII, and -VI).

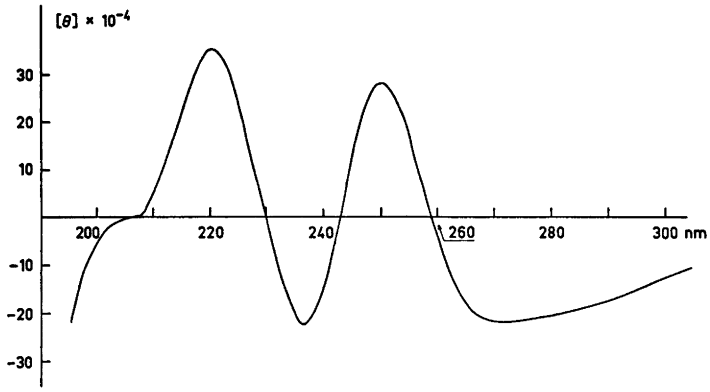


Fig. 2. CD spectrum of (*R*)-(−)-V molybdate complex, 2 mM solution in water, pH 3.3, cell 0.5 mm.

propriate cuprate reagents. Epoxide ring opening reactions with lithium dimethylcuprate and lithium diphenylcuprate have been described by Johnson *et al.*,[11] and the reactions between XII and these copper reagents gave the esters XIII and XIV (Fig. 1) in good yields. The diethyl ester of (*S*)-2-butylmalic acid (XV) was synthesised analogously. It seems reasonable to assume that the molybdate complexes of this acid and (*S*)-VII should give similar CD curves.

Molybdate complexes of the synthetic (*S*)-2-alkylmalic acids were prepared and investigated by CD. All these complexes and those of (*S*)-malic acid [9] and (*S*)-citramalic acid [8] show similar Cotton effects. A sample of (−)-2-isopropylmalic acid [(−)-V], isolated by Sai,[1] shows the opposite Cotton effects (Fig. 2) and consequently has the *R*-configuration. The natural forms of VII and VIII also show the opposite Cotton effects,[8] as do the natural forms of IX and X, and all these acids consequently also have the *R*-configuration.

EXPERIMENTAL

General methods were the same as previously described.[8] The diethyl 2-alkylmalates, purified by preparative GLC, were hydrolysed with 4 M hydrochloric acid (reflux 3 days). The solutions were concentrated (40°, 15 mm) and the acids were dried in a vacuum. Molybdate solu-

tions having pH values between 3.3 and 3.5 (2.0 mol of sodium molybdate per mol hydroxy acid) were prepared from these crude acids and CD spectra were recorded as previously described.[8]

Ethyl chloropyruvate was prepared according to Stekol,[13] starting with 144 g of ethyl pyruvate and 135 ml of sulphuryl chloride. In the distillation (55–58°, 7–8 mm) two fractions were taken. The first fraction (96 g) consisted of (NMR) 75 % ethyl chloropyruvate, 10 % ethyl pyruvate and 15 % ethyl dichloropyruvate. The second fraction (28 g) consisted of (NMR) 90 % ethyl chloropyruvate, 3 % ethyl pyruvate and 7 % ethyl dichloropyruvate.

Diethyl 2-(chloromethyl)malate (XI). A mixture of diisopropylamine (28.5 g, 0.28 mol) in tetrahydrofuran (150 ml) and butyllithium in ether (270 ml, 1.0 M) was stirred under nitrogen at −10° for 40 min. The resulting solution of lithium diisopropylamide was cooled to −78°, a solution of (−)-menthyl acetate (46.6 g, 0.25 mol) in tetrahydrofuran (250 ml) was added during 45 min, and the mixture stirred at the same temperature for 30 min. Ethyl chloropyruvate (42.8 g, 0.29 mol) was then added during 30 min, the temperature being kept at −78°, and the mixture was stirred for another 15 min before the temperature was allowed to rise to 0°. The mixture was poured into dilute hydrochloric acid-ice, the aqueous layer (pH 4–5) was extracted with chloroform, and the chloroform phase was dried (Na_2SO_4) and concentrated. NMR of the product showed comparable amounts of ethyl (−)-menthyl 2-(chloromethyl)malate and (−)-menthyl acetate. The crude product was treated with ethanol (1 l) and conc. sulphuric acid (30 ml) under reflux for 5 days. Part of the ethanol was evaporated. Partition between water and chloroform gave a product which on distillation (90–92°, 0.5 mm) yielded 16.1 g of an 80/20 mixture of XI and (−)-menthol. Redistillation yielded pure (GLC) XI, $[\alpha]_{578}^{24}$ −1.1° (*c* 6.6, chloroform). MS (*m/e*, relative intensity): M^+ = 238 (not observed), 189 (8), 167 (28), 165 (86), 121 (32), 119 (100), 79 (8), 77 (26), 29 (71). NMR (CCl_4): τ 5.70 (q, 2 H), τ 5.81 (q, 2 H), τ 6.00 (s, 1 H), τ 6.30 (s, 2 H), τ 7.12 and 7.28 (AB spectrum, 2 H, J = 16 Hz), τ 8.67 (t, 3 H), τ 8.73 (t, 3 H).

The *epoxide XII* was prepared by treatment of XI (7.2 g, 0.030 mol) with lithium hydride (0.48 g, 0.060 mol) in hexamethylphosphortriamide (HMPA, 15 ml, 65°, 1.5 h). The reaction mixture was poured into dilute hydrochloric acid-ice, extracted three times with ether, and the extract dried (Na_2SO_4) and concentrated. GLC indicated a 50 % conversion to XII (higher yields were obtained in some pilot experiments). As the distilled (60–62°, 2 mm) sample of XII contained approximately 10 % of XI, XII was purified by preparative GLC. The substance thus obtained was indistinguishable (GLC, MS) from a sample of XII obtained by epoxidation of diethyl itaconate.[7] MS: M^+ = 202 (not observed), 157 (27), 129 (40), 128 (29), 101 (95), 29 (100).

Hydrogenation of XII. The epoxide XII (100 mg) in ethanol (4 ml) was hydrogenated in the presence of palladium on charcoal (60 mg) and anhydrous potassium carbonate (50 mg). Diethyl citramalate, indistinguishable (GLC, MS) from an authentical sample was isolated by preparative GLC, $[\alpha]_{578}^{24}$ +2.9° (*c* 4.6, chloroform). MS: M^+ = 204 (not observed), 159 (3), 131 (87), 103 (17), 85 (73), 43 (100), 29 (35). Pure (+)-diethyl citramalate shows $[\alpha]_{578}^{24}$ +20.1°.[10]

2-Ethylmalic acid. A solution of lithium dimethylcuprate (2 mmol) in ether (10 ml) was prepared at 0°.[12] The epoxide XII (0.15 g, 0.75 mmol) in ether (2 ml) was added, and after 10 min at 0° the reaction mixture was poured into dilute hydrochloric acid. Separation of the ether layer and drying (Na_2SO_4) followed by preparative GLC (indicating 100 % ring opening) afforded diethyl 2-ethylmalate, $[\alpha]_{578}^{24}$ +1.3° (*c* 2.4, chloroform). MS: M^+ = 218 (not observed), 189 (2), 145 (63), 99 (50), 57 (100), 29 (50).

2-Butylmalic acid. Propyllithium in ether (2 ml, 0.75 M) was added to a stirred suspension of copper(I) iodide (150 mg, 0.80 mmol) in ether (10 ml), kept at −20° under nitrogen. After 1 min a solution of XII (72 mg, 0.36 mmol) in ether (5 ml) was added dropwise. The mixture was kept between −10° and −20° for 40 min and worked up as above. Preparative GLC, indicating 95 % purity of the product, gave diethyl 2-butylmalate, $[\alpha]_{578}^{24}$ +2.2° (*c* 0.7, chloroform). MS: M^+ = 246 (not observed), 189 (2), 173 (40), 127 (18), 85 (100), 57 (42), 29 (53).

2-Benzylmalic acid. Triethyl phosphite (830 mg, 5 mmol) in 5 ml of ether was added to a stirred suspension of copper(I) iodide (475 mg, 2.5 mmol) in 5 ml ether, and the mixture was stirred under nitrogen at room temperature for 15 min, a clear solution being produced.[14] The temperature was lowered to −60°, a solution of phenyllithium (6.7 ml, 0.75 M, 5.0 mmol) in ether was added, and after 10 min at −60°, a solution of XII (75 mg, 0.38 mmol) in ether was added. The temperature was then allowed to rise to −10°, and the reaction product isolated as above. The alkaloid phalaenopsine,[3] subjected to ethanolysis, afforded a sample of diethyl 2-benzylmalate which was indistinguishable (GLC, MS) from the sample obtained from XII. The latter showed $[\alpha]_{578}^{24}$ +1.8° (*c* 0.9, chloroform). MS: M^+ = 280 (not observed), 262 (13), 207 (25), 189 (20), 161 (16), 119 (27), 115 (44), 91 (100).

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and Dr. Rolf Håkansson, Kemicentrum, Lund, kindly measured the CD spectra and Dr. Lennart Kenne and Dr. Jörgen Lönngren the mass spectra. This work was supported by the *Swedish Natural Science Research Council.*

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Base- and Acid-catalyzed Prototropic Rearrangements of 1-Methyl-2-(*N*-piperidyl)indene

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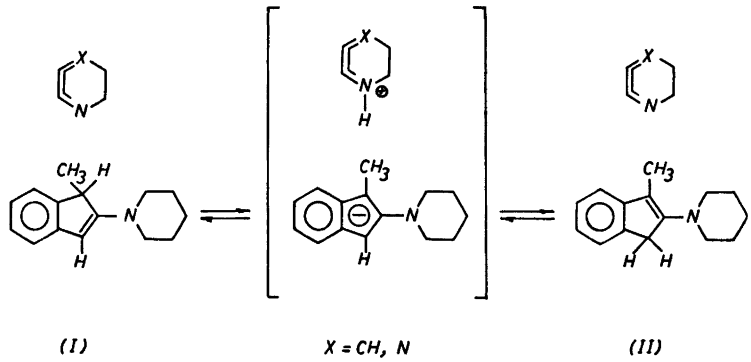
The 1,3-tautomerization of an amino-substituted indene I has been investigated by using basic or acidic catalysts in pyridine solution. Compared to 1,2-dialkylsubstituted indenes the isomerization rate found for I is considerably decreased when basic catalysts (DABCO or quinuclidine) are used. This retarding effect, caused by the 2-nitrogen, is in agreement with acidity differences obtained by simple Hückel calculation. Alkylsubstituted indenes do not undergo acid-catalyzed 1,3-isomerization. However, I rearranges rapidly in the presence of small amounts of strong acid due to the enamine character. Rate constants are given for the base- and acid-catalyzed isomerizations.

The alkylsubstituted indenes have, by kinetic and stereochemical studies, been found to undergo base-catalyzed rearrangements, which proceed in a suprafacial mode under certain conditions.[1,2] An alkyl substituent was found to decrease the rate constant for this reaction but the magnitude of this effect also depends on the nature of the catalyst.[1g] In connection with these studies of the 1,3-proton transfer reactions we wanted to investigate the influence on the rate of a nitrogen atom directly bonded to the carbon atom in the 2-position of the indene ring. Under similar conditions as those used in the indene studies, we have earlier reported that none or a very slow rearrangement could be observed using aliphatic amines as catalysts, like triethylamine.[1n] Base-catalyzed 1,3-proton shifts in enamines have not been studied earlier but a carbanion mechanism has been proposed to account for the *cis-trans* isomerizations of aliphatic enamines in basic media.[3] Furthermore, these compounds would be expected to undergo acid-catalyzed rearrangement owing to their enamine structure. This behaviour of enamines is a well-documented fact,[4] but no detailed investigation has yet been published. A few years ago Johnson mentioned that the presence of one thousandth percent of acid caused equilibration of substituted enamines in 5 min at room temperature.[5] Apparently, the observed thermodynamic equilibrium must be established through an immonium structure possibly preceded by an *N*-protonated salt.[4a]

RESULTS AND DISCUSSION

The present paper is limited to a study of the base-catalyzed and the acid-catalyzed tautomerizations of 1-methyl-2-(*N*-piperidyl)indene (I), (Scheme 1,3). Concerning the 1,2-dialkyl-substituted indenes the thermodynamic equilibrium favours almost completely the 2,3-substituted isomer since an alkyl substituent at the double bond afforded a more stable form.[1g] In the case of isomeric enamines the thermodynamic ratio is determined by a balance between steric and electronic effects which affect the overlap between the nitrogen lone pair and the double bond.[6] Thus we have found that the equilibrium constant between II and I (Scheme 1) is 0.41 ± 0.01 in pyridine solution.

We have studied the base-catalyzed proton transfer in pyridine, a protophilic solvent of moderate polarity, using effective catalysts like 1,4-diaza-bicyclo[2.2.2]octane (DABCO) and quinuclidine. These bases have been shown to be superior to aliphatic amines in prototropic

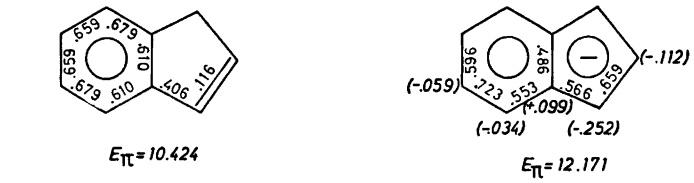


Scheme 1.

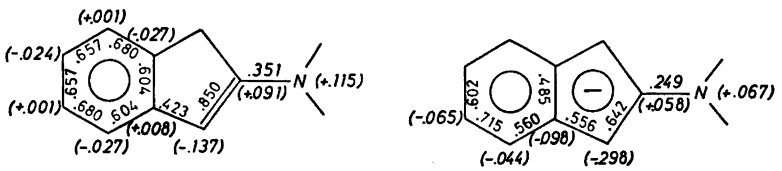
indene rearrangements owing to their rigidity and consequent lower activation entropy.[1j] The reaction was found to follow the kinetics of a pseudo-first order reversible reaction, the observed rate constant being proportional to the base concentration within the limits of experimental error. No significant isomerization could be noticed in the absence of base in a parallel experiment showing that pyridine is too weak a base to be responsible for any catalytic effect. The phenomenological rate constant k_1 for the forward reaction I $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ II was found at unit base concentration to be about 0.49×10^{-2} l mol^{-1} min^{-1} and 0.12×10^{-2} l mol^{-1} min^{-1} for quinuclidine and DABCO, respectively, at 35°C (*cf.* Table 1). Including the statistical factor this shows that quinuclidine is about eight times more effective than DABCO, a ratio close to that found for the rearrangement of 1-methylindene.[1j] Substitution of the hydrogen in the 2-position by the *N*-piperidyl moiety causes a very large decrease in the isomerization rate. A slower rate is also observed for 1,2-dimethylindene (Table 2) as well as for other 1,2-dialkylsubstituted indenes, but the difference between 1,2-dimethyl- and 1-methyl-2-ethylindene is small.[1g] Thus we conclude that in determining the exceptionally low rate for I the conjugation between the nitrogen lone pair and the indene π-system plays a major role. A Hückel π-electron energy

Table 1. Rate constants for the base- and acid-catalyzed isomerization of 1-methyl-2-(*N*-piperidyl)-indene (I) in pyridine. Concentration of substrate: 2 M.
I $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ II Equilibrium constant: 0.41 ± 0.01.

Temp. (°C)	Catalyst	Conc. (M)	$(k_1+k_{-1}) \times 10^2$ (min^{-1})	$(k_1+k_{-1}) \times 10^2$ / (catalyst) (l mol^{-1} min^{-1})	$k_1 \times 10^2$ / (catalyst) (l mol^{-1} min^{-1})
35.0	DABCO	0.500	0.212 ± 0.005	0.424 ± 0.010	0.12 ± 0.01
35.0	DABCO	0.750	0.315 ± 0.009	0.420 ± 0.012	0.12 ± 0.01
35.0	DABCO	1.00	0.398 ± 0.007	0.398 ± 0.007	0.12 ± 0.01
35.0	Quinuclidine	0.150	0.252 ± 0.005	1.68 ± 0.03	0.49 ± 0.02
35.0	Quinuclidine	0.200	0.335 ± 0.004	1.68 ± 0.02	0.49 ± 0.02
35.0	Quinuclidine	0.300	0.497 ± 0.010	1.66 ± 0.04	0.48 ± 0.02
27.0	Perchlorate	0.00040	0.290 ± 0.008	725 ± 20	211 ± 10
27.0	Perchlorate	0.00060	0.414 ± 0.007	690 ± 12	201 ± 7
27.0	Perchlorate	0.00100	0.703 ± 0.019	703 ± 19	204 ± 9



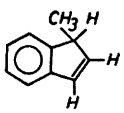
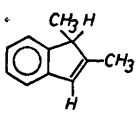
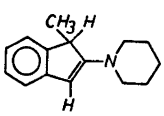
$\Delta E_\pi(\text{indene}) = 1.747 \Longrightarrow pK_A \approx 21$



$\Delta E_\pi(\text{aminoindene}) = 1.665 \Longrightarrow pK_A \approx 23$

Scheme 2.

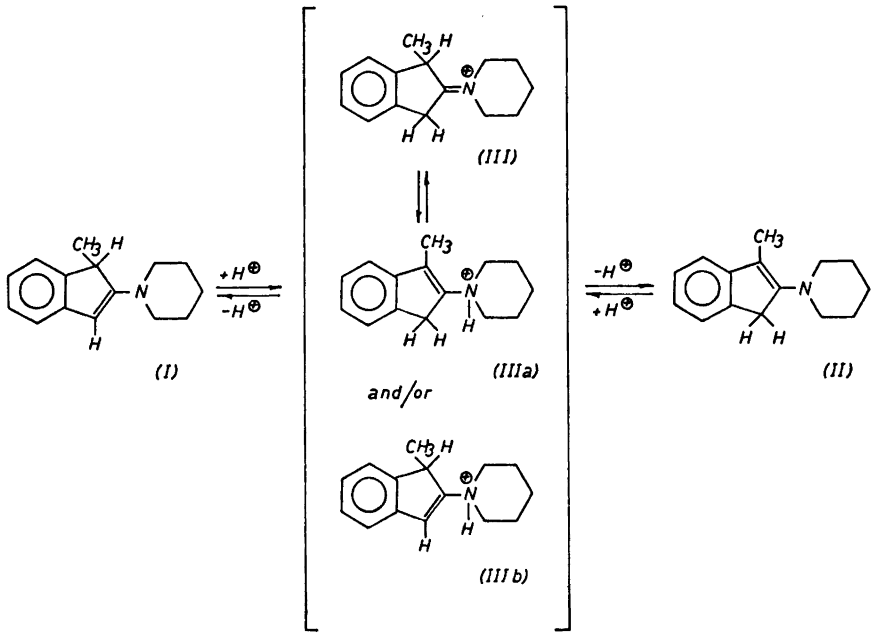
Table 2. Isomerization rate constants using DABCO as catalyst at 35°C in pyridine.

Compound	$k_1 \times 10^2$ l mole^{-1} min^{-1}
	216 [a]
	29 [b]
	0.12

[a] From Ref. 1j. [b] Estimated from rearrangement at 30°C. See Ref. 1g.

calculation (Scheme 2) predicts that 2-aminoindenes should be less acidic than indene by about 2 pK_A units. A lower acidity should, of course, result in a decreased isomerization rate due to correlation between the kinetic and thermodynamic acidity.[7a] The acidity of 2-aminoindene was estimated using the correlation given by Streitwieser [7b] between pK_A and the difference in Hückel total π-energy between the carbon acid and the corresponding anion. We used the same parameters in our calculation as recommended earlier [8] *i.e.* $h_N = 1.5$ and $k_{CN} = 0.8$. This implies, of course, that we have assumed maximum overlap of the nitrogen lone pair and the π-system in the indene ring. Thus our calculation gives an upper limit for the effect of the nitrogen atom in the π-electron system. Scheme 2 also shows the net charges and the bond orders in our systems. In Scheme 1 we have proposed that the rearrangement between I and II proceeds *via* an ion pair in analogy with the mechanisms put forward for the other 1,3-prototropic reactions.[1,2] An interesting question in this connection is whether there exists a discrete ion pair in which the substituted ammonium ion is situated above the nitrogen atom in the piperidine ring and whether an internal rotation within such an ion pair can be affected by rotation around the C_2-N bond. These questions cannot be answered since we have not as yet been able to prepare optically active substrates.

The acid-catalyzed isomerization between I and II is represented in Scheme 3. The reac-



Scheme 3.

tion was initiated by addition of a small amount (*ca.* 10^{-3} M) of the perchlorate salt III. Thus the intermediate III in the reaction also serves as the catalyst. As can be seen from Table 1, the reaction is strictly pseudo-first order within the limits of experimental error. The rate is remarkably rapid k_1 being about 200×10^{-2} l mol^{-1} min^{-1} at unit catalyst concentration. Thus very low catalyst concentrations must be used, but in pyridine the reaction is slow enough to permit a usual kinetic study probably due to the fact that pyridine interacts through hydrogen bonding to the catalyst. A preliminary experiment using acid-free chloroform as a solvent, where such bonding cannot occur, gives a rearrangement rate about fifty times as great as in pyridine solution. The *N*-protonated enamines IIIa and/or IIIb are possibly involved in the reaction since *N*-protonation is presumably favoured kinetically compared to *C*-protonation in our system.[11] However, the *C*-protonated form III is thermodynamically more stable than (IIIa)/(IIIb).

EXPERIMENTAL

All PMR-work was performed on a JEOL C-60 HL instrument. The mass spectrum was obtained with an LKB 9000 mass spectrometer. The GC-analyses were made on a PYE M64 with a flame ionization detector and nitrogen as the carrier gas.

1-Methyl-2-(N-piperidyl)indene (I). The contaminated 1-methyl-2-(*N*-piperidyl)indene (4 % of II) was prepared as earlier described.[11] To a solution of 3.99 g of this enamine (0.0187 mol) in 30 ml methanol a small amount of triethylamine was added to avoid fast isomerization. Crystallization at 0°C overnight yielded 1.22 g pure I (0.0057 mol, 19 % from 1-methylindan-2-one) as colourless large prisms, m.p. 33.0–33.5°C. PMR spectrum in tetrachloroethylene was free from any traces of isomeric impurities (Fig. 1).

The perchlorate salt (III). The perchlorate salt was synthesized in analogy with a method reported by Blomquist and Moriconi.[9] To an ethereal solution of 2.0 g of 1-methyl-2-(*N*-piperidyl)indene (0.0094 mol) a mixture of 25 ml 70 % perchloric acid and 25 ml absolute ethanol was added to the blue colour of Congo red paper (pH < 3). The immonium salt precipitates immediately. One recrystallization from acetone gave colourless needles of III. Yield: 1.8 g (61 % 0.0057 mol) m.p. 156–158°C.

Solvent, substrate and catalysts. Pyridine (Mallinckrodt analytical reagent) was kept over potassium hydroxide for two weeks before being fractionally distilled[10] and then, finally, stored over calcium hydride under nitrogen

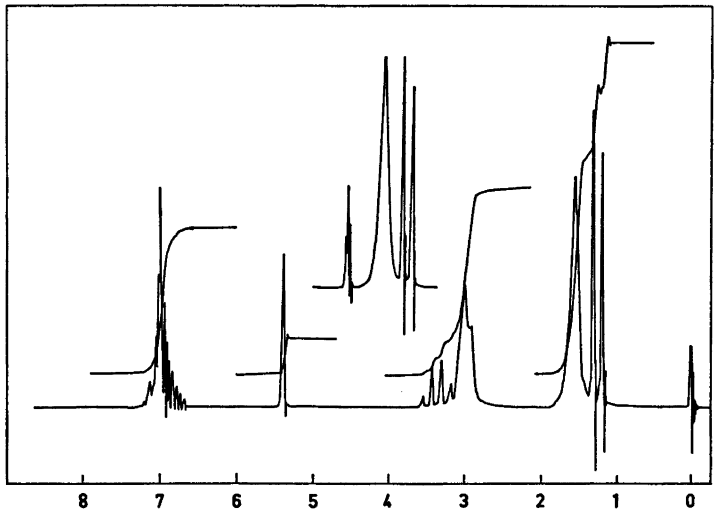


Fig. 1. PMR spectrum of pure 1-methyl-2-(*N*-piperidyl)-indene (I) in tetrachloroethylene with TMS as internal reference. Concentration: 2 M. Temperature: 27°C. (Compare the inset part of the spectrum from the thermodynamic mixture.)

atmosphere. GLC-analysis of the pure isomer I (1 % Apiezon L on Chromosorb W: 300 cm × 6 mm: Det. 180° C/col. 150° C/inj. 210°C. 1 % SE 30: 180 cm × 6 mm: Det. 180°C/col. 150°C/inj. 180°C. 1 % XE 60: 180 cm × 6 mm: Det. 140°C/col. 120°C/inj. 180°C) shows at least 99.5 % purity except for isomeric contamination, since attempts to resolve the two isomers have been unsuccessful. DABCO (Kebo *purum*) was recrystallized from hexane. Quinuclidine was generated from its hydrochloride (Fluka) and purified by sublimation.

Kinetics. All glassware including NMR-tubes, was dried at 150°C for at least 16 h. Pure I and the desired amount of the catalyst were weighed in a 5 ml volumetric flask and were then diluted with pyridine. Concerning the base-catalyzed runs, the rearrangement was performed at a constant temperature (35.0 ± 0.1°C) in a thermostat (Colora Ultra-Thermostat Type MB). From the reaction solution 0.5 ml aliquots were drawn and the appropriate PMR-signal measured.

In the acid-catalyzed runs a desired volume was drawn from a 100 ml 0.01 M solution of III in pyridine and injected to a given solution volume of I. From this mixture a 1 ml sample was withdrawn and filled into an NMR-tube under nitrogen. After careful sealing of the tube, the isomerization was performed at 27.0°C in the PMR-probe. The estimated errors in temperature measurements were ±0.3°C. The inaccuracies in weighing (±0.0001 g) and pipetting are considered to be small compared to those involved in the kinetic parameters and are therefore neglected in Table 1.

The rearrangement was followed by PMR-technique by measuring the increase in intensity of the 3-methyl proton peak at approximately 2 ppm. No significant rearrangement could be observed without any added catalyst. The equilibrium constant was determined by integration over the vinylic proton signal from the least substituted isomer and over the 3-methyl peak from the most substituted one. No change in the equilibrium proportions could be observed in the different runs. A least squares program PROGAEXP [11] has been used to evaluate the rate parameters. The residuals were randomly generated. The accuracy of the estimated values was set to two times the standard deviation ($\pm 2\sigma$).

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S. and Bergson, G. *Ark. Kemi 29* (1968) 351; k. Sörlin, G. and Bergson, G. *Ibid. 29* (1968) 593; l. Bergson, G. *Acta Chem. Scand. 22* (1968) 702; m. Bergson, G. and Ohlsson, L. *Ibid. 23* (1969) 2175; n. Edlund, U. and Bergson, G. *Ibid. 25* (1971) 3625; o. Ahlberg, P. *Chem. Scr. 4* (1973) 33; p. Ahlberg, P. *Ibid. 3* (1973) 183; q. Ahlberg, P. and Ladhar, F. *Ibid. 1* (1971) 31.

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Mass Spectra of α-Allenic Alcohols and Some Isomeric Acetylenic Analogues*

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The mass spectra of eleven α-allenic alcohols have been recorded. Deuterium labelling in some of the compounds studied made possible a structural interpretation of the principal fragments. The main fragmentation route consists of α-cleavage with loss of the allenyl group. A comparison has also been made between some isomeric tertiary allenic, α-acetylenic (propargylic), and β-acetylenic alcohols in order to investigate the possibility of using mass spectrometry to differentiate between isomeric allenes and acetylenes. In spite of the similarities of the spectra of the allenic and the β-acetylenic compounds it can be concluded that mass spectrometry is useful for such purposes.

Only scant attention has been paid to the mass spectra of allenes.[1-4] As part of an investigation currently in progress on the chemistry of allenes [5] it was necessary to use GLC and MS as major tools of identification. In the present paper we summarize our experience on the mass spectral behaviour of eleven α-allenic alcohols, I–XI.

Allene-acetylene isomerization is a well known problem encountered in most work with these types of compounds. Few attempts [6] have been made to use mass spectrometry to differentiate between isomeric allenes and acetylenes. Thus we found it of importance to include also this approach in the present investigation. We have compared two allenic tertiary alcohols (VII and XI) with their isomeric acetylenic compounds XII, XIII and XIV, XV respectively. Deuterium labelling in some of these compounds (*cf.* Table 1 and Fig. 1) made possible structural assignments to the principal fragments. The fragmentation discussed is supported by appropriate shift(s) in the deuteriated analogues. The mass spectra of compound I–XV are given in Table 2.

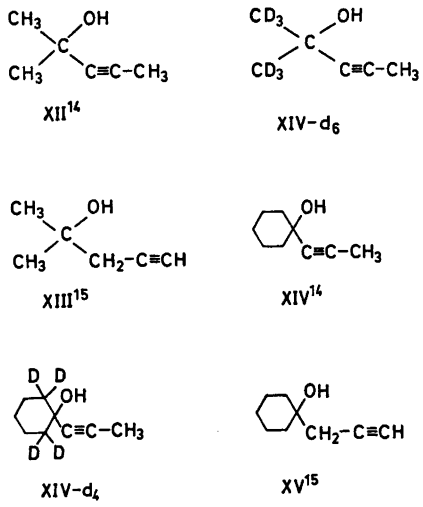
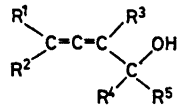


Fig. 1. Tertiary acetylenic alcohols and some deuteriated analogues.

α-Allenic alcohols. In general, the molecular peaks of the allenic alcohols studied are small (0.01 – 0.7 %, *cf.* Table 2). As expected, only the primary alcohols I and II exhibit a prominent $M^+ - H_2O$ peak while the secondary derivatives have smaller peaks of this kind (5 – 10 %). The molecular ions of the tertiary alcohols show no tendency to lose water on electron impact. However, this type of fission

* Allenes and Acetylenes V. Part IV, Ref. 5.

Table 1. α-Allenic alcohols and some deuteriated analogues.



Compound	R^1	R^2	R^3	R^4	R^5	B.p.°C/mmHg	Yield
I[9]	n-C_3H_7	H	H	H	H	85/20	73
II[10]	n-C_3H_7	CH_3	H	H	H	110/40	81
III[11]	H	H	H	n-C_4H_9	H	86/22	74
III-d_1	H	H	D	n-C_4H_9	H		
IV[y]	H	H	H	C_6H_5	H	127/13	55
V[z]	H	H	H	CH_3	$CH_2=CH$	54/22	61
V-d_1	H	H	D	CH_3	CH_3		
VI[11]	H	H	H	CH_3	n-C_3H_7	68/17	72
VII[12]	H	H	H	CH_3	CH_3	130/760	75
VII-d_6	H	H	H	CD_3	CD_3	130/760	71
VIII[14]	CH_3	CH_3	H	CH_3	$(CH_3)_2C=CH$	88/11	73
IX[13]	CH_3	CH_3	H	C_2H_5	C_2H_5	75/15	80
IX-d_1	CH_3	CH_3	D	C_2H_5	C_2H_5		
X[11]	H	H	H	$-(CH_2)_6-$		120/22	68
XI[13]	H	H	H	$-(CH_2)_5-$		98/22	75
XI-d_1	H	H	D	$-(CH_2)_5-$			
XI-d_4	H	H	H	$-CD_2(CH_2)_3CD_2-$		92/15	67

[y] Found: C 80.3; H 6.73. Calc. for $C_{10}H_{10}O$: C 82.2; H 6.90. [z] Found: C 75.6; H 8.98. Calc. for $C_7H_{10}O$: C 76.3; H 9.15.[14] Found: C 79.3; H 10.8. Calc. for $C_{11}H_{18}O$: C 79.4; H 10.9.

Table 2. Mass spectra of compounds I–XV and some deuteriated analogues.

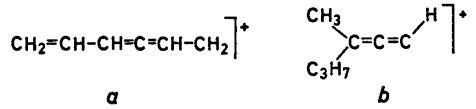
Compound	*m/e* (rel. int. % of the base peak)
I	112(0.1), 97(10), 95(7), 94(71), 93(10), 91(4), 85(4), 84(71), 83(58), 81(44), 80(7), 79(100), 78(4), 77(24), 73(5), 71(3), 70(29), 69(43), 67(47), 66(14), 65(20), 57(14), 56(14), 55(71), 54(19), 53(80), 52(16), 51(14), 50(3), 45(16), 44(9), 43(20), 42(17), 41(86), 40(14), 39(55).
II	126(0.01), 111(7), 108(17), 98(53), 97(43), 95(100), 93(40), 91(13), 83(33), 82(47), 81(27), 80(20), 79(33), 77(33), 73(13), 70(20), 69(47), 67(94), 66(13), 65(20), 56(20), 55(100), 53(53), 51(20), 45(40), 43(60), 41(80), 39(40).
III	126(0.2), 111(1), 107(6), 89(4), 87(75), 85(6), 84(7), 83(3), 79(4), 77(3), 73(5), 71(4), 70(32), 69(100), 68(5), 67(3), 59(4), 58(5), 57(11), 55(6), 53(4), 52(3), 51(4), 45(22), 44(8), 43(16), 42(24), 41(75), 40(7), 39(22).
III-d_1	127(0.03), 112(1), 87(18), 85(7), 79(5), 71(12), 70(100), 69(90), 68(3), 67(4), 59(3), 57(11), 56(5), 55(4), 54(3), 53(3), 45(11), 44(3), 43(13), 42(30), 41(60), 40(13), 39(10).
IV	146(2), 145(9), 131(4), 128(5), 127(3), 115(4), 108(8), 107(100), 105(8), 103(3), 91(3), 89(3), 80(4), 79(10), 78(8), 77(42), 75(3), 74(3), 73(3), 63(5), 53(3). 52(5), 51(25), 50(10), 45(9), 43(4),
V	110(0.6), 109(3), 95(26), 91(5), 77(5), 72(4), 71(100), 67(12), 65(5), 55(40), 53(9), 52(4), 51(7), 45(4), 44(3), 43(93), 41(21), 40(3), 39(23).
V-d_1	111(0.3), 110(4), 96(11), 92(5), 78(3), 72(4), 71(92), 68(19), 67(3), 66(4), 56(3), 55(15), 53(6), 52(3), 51(5), 45(4), 44(3), 43(100), 42(5), 41(15), 40(12), 39(13).
VI	126(0.1), 93(4), 87(46), 83(37), 79(4), 77(7), 71(7), 69(7), 67(3), 66(4), 65(4), 55(3), 53(4), 51(4), 45(55), 43(100), 41(24), 40(5), 39(28), 38(5).
VII	98(0.04), 83(4), 59(100), 53(3), 43(43), 41(9), 40(3), 39(18).

Table 2. Continued.

VII-d_6	104(0.2), 89(2), 88(3), 87(3), 86(3), 73(13), 66(3), 65(100), 64(4), 59(7), 58(4), 46(60), 45(65), 44(12), 43(16), 42(6), 41(8), 40(8), 39(11).
VIII	166(0.3), 151(2), 133(5), 105(3), 100(5), 99(70), 91(5), 83(4), 81(16), 79(7), 77(5), 73(4), 67(6), 65(5), 59(5), 55(11), 53(15), 51(7), 45(30), 44(5), 43(100), 42(3), 41(40), 40(5), 39(30).
IX	154(0.5), 87(100), 81(5), 69(11), 68(24), 67(11), 59(13), 57(24), 55(21), 53(10), 51(6), 45(40), 44(6), 43(33), 42(5), 41(45), 39(24).
IX-d_1	155(0.2), 88(5), 87(27), 73(3), 70(3), 69(33), 68(9), 67(3), 59(4), 58(3), 57(23), 56(12), 55(9), 54(5), 53(4), 52(3), 51(3), 46(3), 45(100), 44(8), 43(27), 42(15), 41(27), 40(9), 39(13).
X	152(1), 137(2), 124(3), 123(4), 114(7), 113(100), 109(13), 97(4), 95(62), 93(4), 91(7), 84(5), 83(6), 82(7), 81(5), 79(7), 77(9), 73(5), 69(19), 68(7), 67(32), 65(5), 64(4), 57(11), 56(4), 55(38), 54(9), 53(14), 51(7), 45(19), 43(24), 42(7), 41(57), 39(49).
XI	138(1), 123(3), 109(4), 100(6), 99(100), 96(8), 95(23), 91(4), 82(6), 81(63), 79(7), 77(7), 73(6), 69(4), 67(13), 65(5), 64(4), 63(3), 59(3), 57(14), 55(40), 54(10), 53(17), 51(8), 45(30), 43(37), 42(14), 41(53), 40(11), 39(73).
XI-d_1	139(1), 124(2), 110(4), 100(6), 99(100), 97(10), 96(31), 95(3), 92(4), 89(5), 87(5), 83(6), 82(7), 81(64), 80(6), 79(7), 78(6), 77(3), 73(17), 71(4), 70(4), 69(4), 68(15), 67(5), 66(4), 65(6), 59(8), 58(5), 57(17), 56(6), 55(43), 54(9), 53(13), 52(5), 51(6), 45(64), 44(11), 43(45), 42(21), 41(51), 40(38).
XI-d_4	142(1), 127(1), 104(5), 103(85), 97(8), 96(18), 95(3), 94(4), 93(3), 86(4), 85(60), 84(25), 83(9), 82(8), 81(7), 80(6), 79(5), 78(5), 71(5), 70(8), 69(10), 68(20), 67(8), 66(9), 65(6), 64(4), 60(5), 59(14), 58(16), 57(40), 56(60), 55(30), 54(20), 53(15), 52(15), 51(13), 50(4), 47(4), 46(19), 45(45), 44(60), 43(75), 42(60), 41(65), 40(75), 39(100).
XII	98(0.2), 84(3), 83(100), 82(4), 67(3), 53(5), 43(66), 41(5), 40(4), 39(9).
XII-d_6	104(0.1) 86(100), 64(9), 46(43), 45(3), 44(4), 42(6), 41(7), 40(33), 39(33).
XIII	98(0.03), 83(2), 60(3), 59(100), 55(4), 53(5), 51(4), 45(41), 44(43), 43(73), 42(27), 41(86), 40(19), 39(68).
XIV	138(3), 123(13), 110(8), 109(11), 105(3), 96(9), 95(100), 91(5), 83(5), 82(38), 81(16), 79(5), 77(5), 73(5), 69(5), 68(5), 67(22), 65(6), 63(4), 55(31), 54(8), 53(12), 52(4), 51(8), 50(3), 45(22), 44(3), 43(16), 42(8), 41(31), 40(9), 39(56).
XIV-d_4	142(2), 127(3), 124(3), 113(3), 112(3), 110(4), 102(3), 99(4), 97(8), 96(73), 95(5), 94(3), 85(17), 84(17), 83(20), 82(7), 81(4), 80(4), 79(3), 73(3), 72(7), 70(5), 69(12), 68(8), 67(33), 66(7), 65(4), 58(5), 57(13), 56(67), 55(15), 54(11), 53(8), 52(8), 51(5), 46(3), 45(13), 44(33), 43(40), 42(37), 41(50), 40(100), 39(90).
XV	138(0.1), 100(6), 99(100), 95(3), 89(3), 87(3), 82(4), 81(64), 79(6), 73(9), 69(4), 67(3), 59(4), 57(14), 55(43), 54(5), 53(10), 51(3), 45(31), 44(5), 43(28), 42(9), 41(36), 40(9), 39(39).

occurs as a secondary process in the fragmentation of compounds V, VI, and VIII.

Two primary alcohols (I and II) were examined, their mode of fragmentation being quite similar. Compound II is the methyl analogue of I which facilitates the interpretation of the spectra because of the possibility to exploit the shift technique. The spectra are complex and many peaks correspond to fragments formed after loss of alkyl moieties, *e.g.* *m/e* 97 ($M^+ - 15$), 84 ($M^+ - 28$), 83 ($M^+ - 29$), and 69 ($M^+ - 43$) in the spectra of I. This type of cleavage is sometimes combined with consecutive or preceding losses of water. Two common routes of importance are noticed: (*i*). The molecular ions lose H_2O and the species formed then expel a methyl radical as shown by a metastable peak. This decomposition pathway leads to the base peak at *m/e* 79 (*a*) in the spectrum of compound I while the analogous peak *m/e* 93 in the spectrum of II amounts to 40 %.



a *b*

(*ii*). A major mode of fission of I and II corresponds to $M^+ - CH_2OH$ and gives rise to the base peak *m/e* 95 (*b*) in the spectrum of II. This indicates that the positive charge of the

molecular ion can reside on the allenic function and trigger the ejection of the hydroxymethyl group. However, the secondary and tertiary alcohols exhibit this fragmentation to a less extent.

The decomposition of the secondary alcohols III and IV occurs mainly through cleavage α to the hydroxyl group. The base peak *m/e* 69 in the spectrum of III corresponds to loss of the butyl group, while the molecular ion splits off the allenyl group less easily. In the present investigation compound IV is the only alcohol containing an aromatic group. As expected, this group governs the decomposition, *e.g.* the α-fission occurs exclusively with loss of the allenyl group to form *m/e* 107 (base peak). The presence of the aromatic group is also reflected in the intensity of the molecular ion (9 %).

The tertiary alcohols V, VI, and VII all have a terminal allenyl group. In principle, three different types of groups, methyl, vinyl, and allenyl can be lost in an α-cleavage of compound V. However, the loss of the latter group dominates and the $M^+ - 39$ peak is the base peak in the spectrum. The α-cleavage of compound VI occurs to an equal extent with loss of the propyl (*m/e* 83) and the allenyl group (*m/e* 87).

A peak at *m/e* 67, which is shifted to *m/e* 68 in the deuteriated analogue V-d_1, is represented by species *c*. This fragment is also formed in the decomposition of compounds VI, X, and XI which likewise have a terminal allenyl group.

$CH_2=C=CH-C\equiv O^+$

c

The spectrum of compound V exhibits an abundant peak at *m/e* 43 (93 %) corresponding to an acetyl moiety. This species is associated with the methyl group located on the α-carbon to the hydroxyl group. A prominent acetyl fragment is also found in the breakdown pattern of compounds VI, VII, and VIII. The corresponding peak sometimes constitutes the base peak, *e.g.* in the spectrum of VI. A comparable ion having structure *d* is formed in the fragmentation of compound IX.

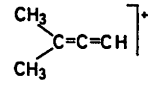
$CH_3-CH_2-C\equiv O^+$

d

The spectrum of compound VII shows a small peak at $M^+ - CH_3$ (*m/e* 83). In the spectrum of the hexadeuteriated analogue VII-d_6, which has an isotopic purity of at least 99 %, this peak is split into four peaks corresponding to $M^+ - CH_3$, $M^+ - CDH_2$, $M^+ - CD_2H$, $M^+ - CD_3$ of almost equal intensity. The reason for this behaviour may be an incomplete hydrogen scrambling, since the calculated values for a random loss of the above methyl groups from the molecular ion of VII-d_6 are 1:9:15:5, respectively.

Compounds VIII and IX do not have a terminal allenyl group. The main fragmentation pathway of these compounds is α-cleavage with loss of the allenic group. This behaviour is analogous to that of the other tertiary alcohols studied. Thus the $M^+ - 67$ peak corresponding to the loss of the allenic group is the base peak in the spectrum of compound IX. The formed moiety then expels H_2O, *e.g.* to rise to *m/e* 81 in the spectrum of VIII.

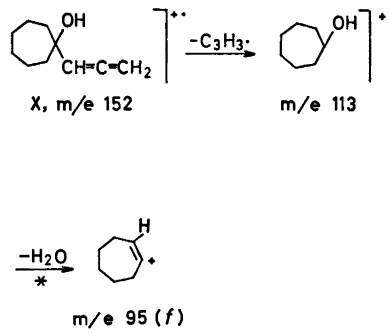
The allenic group is not a preferred site for the charge localization in the ionizing process of the secondary and tertiary alcohols studied. There is an abundant peak at *m/e* 39 in the spectra of the compounds having a terminal allenyl group (III, IV, V, VI, VII, X, and XI) but on the other hand, the analogous fragment *e* corresponding to *m/e* 67 in the spectra of VIII and IX is of minor importance.



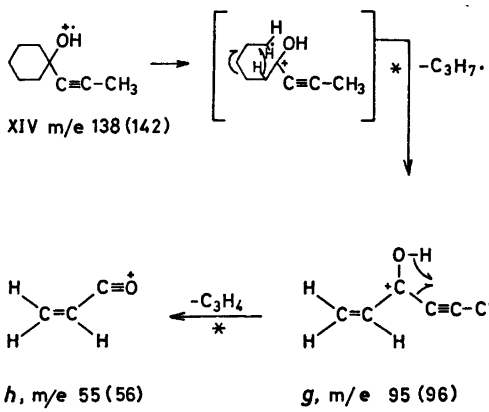
e

The breakdown pattern of compounds X and XI is dominated by peaks corresponding to ions derived from the cycloalkyl rings. The major mode of fragmentation is loss of the allenyl group and subsequent expulsion of H_2O from the formed ion to give a stable fragment, corresponding to *m/e* 95 (*f*) and *m/e* 81 in the fragmentation of X and XI, respectively (*cf.* Scheme 1).

Compounds XI and XIV are exposed to a dissociation route involving complex fissions of the ring as outlined in Scheme 2. It is substantiated by metastable ions, high resolution measurements, and by shifts in the spectra of the deuteriated analogues XI-d_1, XI-d_4, and



Scheme 1.



Scheme 2.

XIV-d_4, respectively, *e.g.* species *g* and *h* are shifted only one mass unit.

Acetylenic tertiary alcohols. Compounds XII and XIV exhibit modes of fragmentation which differ from those of their allenic counterparts VII and XI and their β-acetylenic isomers XIII and XV. The important difference is that no $M^+ - 39$ peak is present in the spectra, *i.e.* no fission occurs in the acetylenic chain. The acetylenic group behaves similarly to a vinyl group in this respect and analogous results have been noticed for acetylenic amines.[7,8] Instead, the major fragmentation route for XII implies loss of a methyl radical. The fragmentation of compound XIV occurs mainly in the cyclohexyl ring, *e.g.* the formation of $M^+ - 29$ and $M^+ - 43$. Deuterium labelling reveals that the $M^+ - 15$ ion is formed through loss of a methyl group from the acetylenic chain. The most important route of fragmentation is outlined in Scheme 2 and involves a site-specific hydrogen transfer to the expelled radical.

The β-acetylenic alcohols XIII and XV afford essentially the same breakdown pattern as their allenic isomers VII and XI (*cf.* Table 2). However, there are differences in the relative intensities of various peaks, which make it possible to differentiate between the isomers. Compared to compound XI, the fragmentation mode of XV seems to involve to a less extent the cyclohexyl ring, *m/e* 95 is only 3 %. To obtain an accurate identification of these two types of compounds only based on mass spectral data, a comparison with reference compounds is needed.

In conclusion, the allenic alcohols studied mainly fragment through α-cleavage. In this type of fission, the allenyl group can be anticipated to behave similarly to a vinyl or to a propargyl group. Judging from the similarity of the spectra of the β-acetylenic and allenic alcohols, the latter case seems to be relevant. α-Cleavage with loss of the allenyl group is the main fragmentation route of the tertiary alcohols. However, in some compounds, *e.g.* III, an alkyl group of similar size is lost to an equal extent.

The study of the isomeric allenic, α-acetylenic, and β-acetylenic tertiary alcohols shows that the α-acetylenic derivatives behave quite differently due to the fact that fission does not occur preferentially next to an acetylenic bond.

In spite of the similarities of the spectra of the allenic and β-acetylenic compounds it can be concluded that mass spectrometry is a useful method for distinguishing between isomeric allenic and acetylenic alcohols.

EXPERIMENTAL

Infrared and NMR spectra of all compounds are in full agreement with the proposed structures.

All reactions with $LiAlH_4$ or Grignard reagents were performed under a nitrogen atmosphere.

Elemental analyses were performed in the laboratories of Dr. A. Bernhardt, Mülheim, West Germany.

The mass spectra were recorded on an AEI MS-30 mass spectrometer, connected to a Pye 104 gas chromatograph. The ionizing energy was maintained at 70 eV, the accelerating voltage

at 4 kV and the temperature of the source at 200°. High resolution spectra were obtained on an Atlas MS 1 mass spectrometer in the laboratory of Dr. R. Ryhage.

High resolution measurements of 1-(1-propynyl)cyclohexanol (XIV).

Measured mass	Theoretical	Formula
55.0532	55.0547	C_4H_7
55.0170	55.0183	C_3H_3O (h)
67.0551	67.0547	C_5H_7
67.0184	67.0183	C_4H_3O
82.0418	82.0419	C_5H_6O
82.0775	82.0782	C_6H_{10}
95.0498	95.0450	C_6H_7O(g)
95.0858	95.0861	C_7H_{11}

3-Methyl-6-(tetrahydro-2-pyranyloxy)-1-hexen-4-yn-3-ol (XVI) was prepared as described for similar compounds[13] from 3-(tetrahydro-2-pyranyloxy)propyne (40.7 g; 0.29 mol) and methyl vinyl ketone (18.0 g; 0.26 mol). Yield 23 %. B.p. 108°/0.15 mmHg. (Found: C 68.3; H 8.83. Calc. for $C_{12}H_{18}O_3$: C 68.5; H 8.63).

1-Phenyl-4-(tetrahydro-2-pyranyloxy)-2-butyn-1-ol (XVII). Prepared as above from 3-(tetrahydro-2-pyranyloxy)propyne (77.1 g; 0.55 mol) and benzaldehyde (53.0 g; 0.50 mol). Yield 95 %. B.p. 130°/0.1 mmHg.

2,4,7-Trimethyl-7-(tetrahydro-2-pyranyloxy)-2-octen-5-yn-4-ol (XVIII). Prepared as above from 3-methyl-3-(tetrahydro-2-pyranyloxy)butyne[16] (67.0 g; 0.40 mol) and mesityl oxide (36.2 g; 0.37 mol). Yield 69 %. B.p. 100°/0.1 mmHg. (Found: C 72.4; H 9.61. Calc. for $C_{16}H_{26}O_3$: C 72.1; H 9.88).

2-(Methyl-d_3)-5-(tetrahydro-2-pyranyloxy)-3-pentyn-2-ol-1,1,1-d_3 (XIX) was prepared as above using acetone-d_6. B.p. 95°/0.15 mmHg. Yield 77 %.

1[3-(Tetrahedro-2-pyranyloxy)-1-propynyl]-cyclohexanol-2,2,6,6-d_4 (XX) was prepared as above using cyclohexanone-2,2,6,6-d_4. B.p. 128°/0.1 mmHg. Yield 40 %.

2-(Methyl-d_3)-3-pentyn-2-ol-1,1,1-d_3 (XII-d_6) was synthesized as described for the undeuteriated compound[14] using acetone-d_6. B.p. 140°/760 mmHg. Yield 69 %.

1-(1-Propynyl)cyclohexanol-2,2,6,6-d_4 (XIV-d_4) was prepared as described for the undeuteriated compound[14] using cyclohexanone-2,2,6,6-d_4. B.p. 115°/20 mmHg. Yield 86 %.

α-Allenic alcohols IV, V, VII-d_6, VIII and XI-d_4. Prepared according to Landor[9] from $LiAlH_4$ and the tetrahydropyranol derivatives XVI–XX.

Synthesis of the mono-deuteriated allenic alcohols III-d_1, V-d_1, IX-d_1 and XI-d_1. From $LiAlD_4$ (0.315 g; 7.5 mmol) and the appropriate tetrahydropyranyl derivative (6.0 mmol) (*cf.* the preparation of the undeuteriated alcohols). Micro distillation was applied for the isolation. The yields were between 30 and 50 %.

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The Crystal and Molecular Structure of Dimedone

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The crystal and molecular structure of dimedone, 5,5-dimethyl-1,3-cyclohexanedione, has been determined from three-dimensional single crystal X-ray diffraction data. The space group is $P2_1/c$, with cell dimensions $a = 10.040(1)$, $b = 6.823(1)$, $c = 12.984(2)$ Å, $\beta = 116.20(1)°$. There are four molecules in the unit cell. The structure has been refined by full matrix least-squares methods to a weighted R-factor of 0.046 for the 1428 independent reflections. The estimated standard deviations in bond lengths involving non-hydrogen atoms are 0.002 Å. The molecules crystallize in the enol form and are hydrogen bonded to each other forming infinite helices parallel to the b axis. The hydrogen bond length is 2.593 Å.

Dimedone (5,5-dimethyl-1,3-cyclohexanedione) is a white crystalline solid of melting point 148 – 149°C. It is commonly used as a reagent for separation and identification of aldehydes.[1] Unlike acyclic β-dicarbonyl compounds, enolization of dimedone gives a *trans*-enol where *intra*-molecular hydrogen bonding is impossible.[2,3] Nevertheless, dimedone is believed to exist largely in the enol form both in solution [5] and in the solid state.[4] In solvents incapable of forming hydrogen bonds the comparatively high enol content has been explained by assuming a hydrogen bonded dimeric enol form.[2,3] Several X-ray investigations of acyclic β-diketones have been carried out,[6–8] but there is a paucity of structural data for the cyclic analogs. An X-ray investigation of dimedone has therefore been undertaken.

EXPERIMENTAL

A commercial sample of dimedone was purified and crystallized from methanol. The observed extinctions, $h0l$ for $l = 2n + 1$ and $0k0$ for $k = 2n + 1$ uniquely determined the space group as $P2_1/c$. The cell dimensions were determined at room temperature on a manual four circle diffractometer using CuK radiation. The crystal used for the collection of intensity data was of nearly spherical form with diameter 0.35 mm. Data were collected on a SYNTEX P$\bar{1}$ diffractometer by the $\omega/2\theta$ scan technique using a graphite monochromator and Mo$K\alpha$ radiation ($= 0.71069$ Å) at 18°C. Each reflection was scanned from $2\theta_{\alpha 1} - 0.85°$ to $2\theta_{\alpha 2} + 0.85°$, at a variable scan rate ranging from 1°/min to 12°/min depending on intensity. Stationary-counter-stationary-crystal background counts were made at the upper and lower extremities of the 2θ scan range for each reflection. A rejection level was also specified to avoid measurement of reflections with intensities less than the threshold value. The intensities of three standard reflections, measured every 50′ reflections, showed no change during the data collection. A total of 1548 independent intensities were recorded, out to $2\theta < 70°$. Of these, 1428 reflections had intensities greater than twice their standard deviations. The intensities and their standard deviations were corrected for Lorentz and polarization effects and a 2 % uncertainty in diffractometer stability was included in the standard deviations. No corrections were made for absorption since intensity errors from this source are less than 1.5 % ($\mu = 0.09$ mm^{-1}). All programs applied are written or revised for CDC 3300 by Dahl *et al.*[11]

CRYSTAL DATA

Dimedone, $C_8H_{12}O_2$, F.W. 140.2, monoclinic; $a = 10.040$ (1), $b = 6.823$ (1), $c = 12.984$ (2) Å, $\beta = 116.20$ (1)°, $V = 798.3$ Å^3, F (000) = 444, $Z = 4$, $\varrho_{obs} = 1.15$ g cm^{-3}, $\varrho_{calc} = 1.15$ g cm^{-3}. Space group $P2_1/c$.

SOLUTION AND REFINEMENT

The phase problem was solved three-dimensionally by a computer program based on

symbolic addition methods. By permuting the signs of two structure invariant reflections with large unitary structure amplitudes, four sets of 350 signs were determined. From one of the resulting Fourier maps, peaks corresponding to all the heavy atoms were located. The trial structure was refined by full-matrix least-squares technique. A difference Fourier synthesis led to the location of the hydrogen atoms with the exception of those of the methyl groups. The coordinates for these atoms were calculated assuming staggered positions of the methyl groups relative to C5. Refinement of all positional and thermal parameters including a common isotropic thermal parameter for the methyl hydrogen atoms terminated at $R_w = 0.046$ and $R = 0.040$. Unobserved reflections were excluded throughout the refinement. Weight analysis showed that weighting based on standard deviations from counter statistics was satisfactory. No correction for secondary extinction was necessary. Atomic form factors were those of Hanson *et al.*[9] except for hydrogen.[10] A final difference Fourier map contained no larger density fluctuations than ± 0.3 e/Å^3. The parameters obtained in the last cycle of refinement are given in Table 1 together with their estimated standard deviations. Table 2 contains the observed and calculated structure factors.

Rigid-body analysis of translational, liberational, and screw motion [11] of the molecule gave a r.m.s. value of 0.0033 Å^2 when all the non-hydrogen atoms were included. When the two methyl carbon atoms were excluded, the r.m.s. value dropped to 0.0022 Å.^{2} The cyclohexene ring and the two oxygen atoms may therefore be regarded as a rigid body. Corrections in bond lengths generally exceed the standard devia-

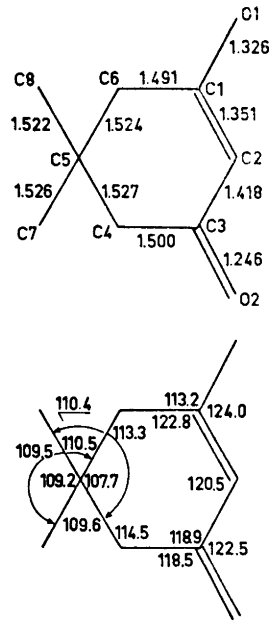

Fig. 1. Schematic drawing of the molecule showing bond distances and angles. (Bond distances uncorrected for librational motion).

Table 1. Fractional atomic coordinates and thermal parameters with estimated standard deviations ($\times 10^5$), isotropic temperature factor and positional parameters ($\times 10^4$) for the hydrogen atoms. The anisotropic temperature factor is given by $\exp -(B_{11}h^2 + B_{22}k^2 + B_{22}l^2 + B_{12}hk + B_{13}hl + B_{23}kl)$.

ATOM	X	Y	Z	B	β11	β22	β33	β12	β13	β23
O 1	26132(12)	65879(16)	35714(11)		1361(15)	2793(29)	833(10)	-818(33)	764(21)	549(26)
O 2	-8883(11)	19773(15)	35972(8)		1272(14)	2557(27)	831(9)	-971(31)	821(19)	-386(23)
C 1	21184(15)	51711(19)	48534(12)		1151(17)	2024(32)	732(12)	-11(39)	879(24)	-9(29)
C 2	8335(15)	41651(21)	34848(12)		1125(17)	2247(33)	628(11)	-77(40)	632(22)	-15(38)
C 3	3224(15)	28588(19)	48777(11)		1149(17)	1848(29)	734(11)	-45(40)	899(23)	-366(29)
C 4	12484(16)	25616(23)	63431(12)		1448(21)	2869(34)	699(11)	-337(45)	1081(25)	-107(38)
C 5	28991(15)	29271(21)	57624(12)		1294(19)	2246(32)	597(10)	-74(41)	584(22)	-68(28)
C 6	30788(17)	49165(23)	53854(12)		1268(28)	2492(39)	691(12)	-688(46)	651(25)	-232(33)
C 7	36671(24)	29562(34)	78718(14)		1971(38)	3517(53)	664(13)	-797(69)	505(32)	83(41)
C 8	35809(23)	13281(29)	53343(17)		1632(26)	3869(47)	1849(17)	1259(63)	944(35)	189(47)
H 1	229(15)	4352(28)	2677(12)	3,8(,3)						
H 2	835(17)	3444(22)	5751(13)	4,8(,3)						
H 3	1861(16)	1272(24)	5543(12)	4,4(,3)						
H 4	2833(16)	5968(22)	5733(12)	4,8(,3)						
H 5	4899(19)	5129(23)	5438(12)	5,1(,4)						
H 6	2818(21)	6664(25)	2865(16)	5,9(,4)						
H 7	4728(24)	3197(27)	7379(17)	7,8(,2)						
H 8	3484(21)	1789(29)	7382(15)	7,8(,8)						
H 9	3222(19)	4857(28)	7347(15)	7,8(,8)						
H10	4623(24)	1511(27)	5578(16)	7,8(,8)						
H11	3095(21)	1211(25)	4486(17)	7,8(,8)						
H12	3413(28)	27(28)	5684(15)	7,8(,8)						

Table 2. Observed and calculated structure factors. The columns are l, $10F_o$, $10F_c$ respectively.

Hk	0,Ke	0	6	101	97	- 6	289	281	- 1	32	33	2	58	51	Hk	2,Ke	8	- 8	9	10	9	31	32
2	486	489	7	37	33	- 5	202	198	0	82	85	3	182	174	- 9	23	21	- 4	15	15	11	28	28
4	304	293	8	41	38	- 4	251	250	1	49	52	4	102	103	0	30	30	- 3	83	80	12	20	16
6	16	16	9	52	54	- 3	242	241	2	19	19	5	37	38	Hk	2,Ke	10	- 2	33	31	Hk	4,Ke	2
8	20	14	10	56	54	- 2	798	822	3	67	64	7	125	126	- 3	23	20	- 1	14	15	-16	13	15
10	68	79	11	31	31	- 1	155	153	5	107	110	8	111	118	6	16	11	0	39	39	-15	37	35
12	30	23	13	47	46	0	353	351	6	61	61	9	35	34	Hk	3,Ke	0	1	55	56	-13	33	36
14	21	27	14	28	21	1	357	352	7	113	114	14	39	36	-18	26	22	3	78	79	-12	19	23
16	63	54	15	22	22	2	233	220	8	33	33	Hk	2,Ke	3	-12	23	19	4	27	27	-11	38	40
18	34	30	Hk	0,Ke	5	3	117	114	11	35	34	-15	56	51	-10	142	150	6	21	20	-10	20	18
Hk	0,Ke	1	-12	25	27	4	409	389	Hk	1,Ke	6	-14	55	54	- 8	60	61	7	30	32	- 9	87	89
-17	26	24	-11	42	42	5	246	232	- 9	51	54	-12	13	13	- 6	118	112	11	22	22	- 8	54	53
-14	18	15	- 9	40	39	6	49	46	- 8	28	27	-11	80	83	- 4	26	26	13	22	22	- 7	55	54
-10	37	36	- 8	23	24	7	67	65	- 5	59	60	-10	15	16	- 2	501	498	Hk	3,Ke	8	- 6	139	132
- 8	15	16	- 7	125	126	8	87	90	- 4	28	32	- 9	90	92	0	480	475	-17	22	19	- 5	18	19
- 7	9	5	- 6	48	49	10	30	41	- 2	57	56	- 8	92	97	2	205	197	-10	31	33	- 4	284	282
- 6	344	342	- 5	27	29	12	18	21	- 1	89	92	- 7	29	26	4	140	135	- 7	15	20	- 3	255	249
- 5	70	68	- 4	57	58	13	17	15	0	61	62	- 6	53	54	8	76	79	- 5	16	15	- 2	15	10
- 4	153	146	- 3	116	118	16	31	28	1	66	69	- 5	124	121	10	56	54	- 4	61	62	- 1	40	39
- 3	245	242	- 2	59	62	Hk	1,Ke	2	3	29	30	- 4	316	307	14	30	29	- 3	20	19	0	124	119
- 2	143	147	- 1	139	140	-15	22	22	5	34	33	- 3	259	259	Hk	3,Ke	1	- 2	62	61	1	172	164
- 1	475	484	1	139	140	-12	19	22	6	39	38	- 2	271	269	-16	58	53	- 1	78	78	2	18	15
1	473	484	2	62	62	-10	63	68	11	20	21	- 1	113	108	- 9	84	86	1	79	80	3	17	18
2	143	147	3	118	118	- 9	31	33	Hk	1,Ke	7	0	63	61	- 8	16	16	3	59	59	4	31	31
3	245	242	4	59	57	- 8	49	53	- 9	28	21	1	104	103	- 7	83	85	4	24	21	5	65	65
4	154	145	5	27	29	- 7	101	105	- 4	30	27	2	165	167	- 5	58	55	6	28	25	7	32	30
5	70	68	6	48	49	- 6	11	8	- 3	24	26	3	112	108	- 4	229	220	7	29	27	8	18	19
6	345	342	7	126	126	- 5	29	31	- 1	41	40	4	44	44	- 3	44	41	Hk	3,Ke	6	9	31	31
7	9	6	8	24	23	- 4	33	34	0	48	44	5	92	94	- 1	13	9	-12	51	50	10	16	14
8	14	16	9	41	39	- 3	116	114	2	26	27	6	53	50	0	310	314	-10	35	31	12	68	63
10	38	36	11	42	42	- 2	551	553	3	24	24	7	98	101	1	60	54	- 7	33	35	14	13	14
13	23	24	12	24	27	- 1	22	22	5	27	25	8	48	49	2	58	54	- 6	33	33	Hk	4,Ke	3
17	26	24	13	19	17	0	226	227	6	21	20	10	37	38	3	28	24	- 5	34	42	-18	28	22
Hk	0,Ke	2	15	15	11	1	215	217	Hk	1,Ke	8	13	50	47	4	26	26	- 4	10	9	-12	39	40
-14	38	34	Hk	0,Ke	6	2	17	13	-10	24	21	14	36	35	5	56	55	- 2	117	116	-11	82	83
-13	39	36	-10	13	15	3	24	24	- 9	21	19	Hk	2,Ke	4	6	67	67	- 1	47	44	-10	72	78
-12	19	18	- 9	21	21	4	94	93	- 4	29	29	- 9	34	37	7	55	59	0	30	30	- 9	111	114
- 8	22	22	- 7	27	27	5	24	25	- 1	55	50	- 8	118	121	9	33	35	1	23	25	- 8	36	35
- 7	53	57	- 5	33	34	6	11	9	0	12	14	- 7	28	29	11	27	26	2	44	46	- 7	124	126
- 6	31	31	- 4	23	26	7	44	44	2	15	17	- 6	67	69	12	37	35	3	30	31	- 6	79	73
- 5	149	149	- 3	39	38	8	96	101	3	45	42	- 5	27	29	Hk	3,Ke	2	4	55	53	- 4	10	4
- 4	114	110	- 1	47	47	9	42	42	5	20	19	- 4	64	60	-17	30	28	5	26	24	- 3	203	200
- 3	10	7	0	46	47	11	30	32	6	32	30	- 3	105	101	-13	67	66	6	75	74	- 2	100	106
- 2	81	78	1	46	47	14	31	26	8	37	33	- 2	80	78	-12	23	22	7	34	33	- 1	32	30
- 1	255	249	2	9	11	16	23	19	Hk	1,Ke	9	- 1	74	71	-11	39	40	Hk	3,Ke	7	0	56	61
0	436	428	3	37	38	Hk	1,Ke	3	- 6	16	17	1	32	32	-10	234	240	-11	54	49	1	202	202
1	256	249	4	24	26	- 9	160	167	2	35	34	2	56	56	- 9	13	20	- 8	15	15	2	30	28
2	82	78	5	30	34	- 8	169	174	4	14	17	3	44	46	- 8	183	187	- 6	41	45	3	46	43
3	11	7	6	13	16	- 7	125	132	Hk	2,Ke	0	4	24	25	- 7	189	186	- 5	14	13	4	21	20
4	113	110	7	27	27	- 6	138	142	-14	36	37	5	78	78	- 6	173	162	- 3	66	67	5	64	67
5	150	149	8	23	21	- 5	129	123	-12	16	16	7	61	61	- 4	435	421	- 1	22	22	6	75	79
6	32	31	Hk	0,Ke	7	- 4	12	12	-10	42	42	8	56	55	- 3	373	366	0	27	26	7	45	48
7	55	57	- 8	26	25	- 3	171	166	- 8	163	162	9	37	40	- 2	35	35	1	80	81	8	22	25
8	23	22	- 7	39	41	- 2	111	111	- 6	217	215	10	32	34	- 1	188	190	3	29	29	9	16	19
13	41	36	- 2	16	17	- 1	36	35	- 4	74	72	Hk	2,Ke	5	0	55	59	5	64	63	10	18	20
14	36	34	- 1	19	18	0	107	108	- 2	498	506	-10	52	52	1	203	195	7	44	43	13	21	20
Hk	0,Ke	3	1	19	18	1	111	114	0	400	403	- 9	21	23	2	193	197	Hk	3,Ke	8	Hk	4,Ke	4
-15	29	25	7	4,	41	3	204	203	4	54	54	- 8	43	44	3	313	310	- 6	23	24	-17	13	12
-13	30	31	8	26	25	5	92	89	6	161	170	- 6	57	55	5	64	64	- 4	26	25	-15	22	24
-12	22	18	Hk	0,Ke	8	6	32	31	8	51	54	- 4	104	103	6	117	123	- 2	16	16	-12	36	35
-11	20	19	- 5	17	18	7	96	96	12	22	19	- 3	58	59	7	106	105	Hk	3,Ke	9	-11	27	29
- 8	22	21	- 4	34	31	8	59	59	Hk	2,Ke	1	- 2	50	51	8	141	149	- 2	20	18	-10	55	57
- 7	66	69	- 3	28	27	9	133	137	-17	18	18	- 1	177	180	11	36	38	1	16	16	- 8	16	18
- 6	107	110	- 2	40	40	10	56	61	-15	17	17	0	71	70	12	47	45	Hk	4,Ke	0	- 7	34	39
- 5	25	23	- 1	20	21	11	24	27	-14	17	17	1	13	12	14	34	29	-16	28	26	- 6	103	101
- 4	31	32	1	21	21	13	20	22	-10	30	33	2	50	50	Hk	3,Ke	3	-12	130	133	- 5	79	84
- 3	186	186	2	40	40	15	22	21	- 9	36	34	3	66	66	-16	15	9	-10	111	111	- 4	23	19
- 2	25	25	3	27	27	Hk	1,Ke	4	- 7	11	9	4	19	21	-14	27	30	- 8	22	23	- 2	138	139
- 1	113	115	4	35	31	-13	23	25	- 6	164	157	5	32	31	-13	12	12	- 6	88	86	- 1	111	110
1	113	115	5	20	18	-12	25	23	- 5	163	163	8	33	33	-11	69	71	- 4	81	84	0	20	20
2	23	25	Hk	0,Ke	9	-11	54	58	- 4	52	46	11	31	28	-10	110	109	- 2	152	149	2	31	33
3	185	186	- 8	23	18	-10	136	137	- 3	85	85	Hk	2,Ke	6	- 9	91	92	0	92	93	3	14	11
4	30	32	- 2	37	37	- 9	73	77	- 2	814	837	-12	31	32	- 8	36	39	2	146	147	4	13	15
5	25	23	- 1	25	26	- 8	103	106	- 1	586	592	-10	15	16	- 7	13	13	4	264	275	6	42	42
6	107	110	1	28	26	- 7	130	134	0	284	278	- 9	52	51	- 6	34	37	6	150	159	7	13	18
7	67	69	2	38	37	- 5	55	55	1	20	22	- 8	67	67	- 5	202	197	8	18	17	8	31	29
8	24	21	Hk	1,Ke	0	- 4	45	44	2	312	308	- 6	33	38	- 4	276	279	12	34	28	9	15	13
12	19	16	-12	53	63	- 2	180	185	3	129	121	- 4	25	28	- 3	328	328	Hk	4,Ke	1	Hk	4,Ke	5
13	32	31	- 8	159	163	- 1	21	19	4	17	14	- 3	40	38	- 2	78	71	-18	25	21	-13	40	35
15	33	25	- 6	548	518	0	92	93	5	15	14	0	89	87	0	154	147	-16	37	38	-10	19	18
Hk	0,Ke	4	- 4	148	144	1	119	119	6	97	94	1	37	34	1	55	53	-15	15	13	- 8	34	36
-14	23	21	- 2	78	77	2	20	18	7	14	8	2	24	22	2	28	24	-13	37	43	- 7	60	63
-13	47	46	0	374	368	3	11	16	12	40	40	3	84	83	4	68	68	-12	143	147	- 6	12	12
-11	30	31	2	29	25	4	37	33	14	47	41	4	75	76	5	64	66	-11	51	52	- 5	47	44
-10	57	54	4	698	686	5	129	125	16	23	24	5	69	68	6	63	63	-10	99	102	- 4	59	60
- 9	50	54	6	343	335	6	51	52	Hk	2,Ke	2	6	49	49	7	95	98	- 9	65	66	- 3	10	7
- 8	39	38	8	29	27	7	37	39	-16	41	34	7	56	55	8	60	61	- 8	180	178	- 2	50	50
- 7	35	33	10	84	89	8	83	84	-14	28	23	9	36	36	11	18	15	- 6	160	162	- 1	87	86
- 6	99	97	12	17	9	9	98	96	-11	80	83	10	20	18	12	36	34	- 5	136	130	0	71	72
- 5	128	125	14	14	16	10	19	23	-10	128	131	Hk	2,Ke	7	13	30	26	- 4	254	247	1	37	36
- 4	36	38	16	30	25	12	19	20	- 9	36	40	-15	24	21	14	24	24	- 3	74	76	2	17	18
- 3	27	23	Hk	1,Ke	1	Hk	1,Ke	5	- 8	233	236	- 6	21	21	Hk	3,Ke	4	- 1	151	155	3	66	70
- 2	142	139	-15	23	23	-12	23	24	- 7	141	146	- 5	13	10	-14	15	13	0	147	146	4	33	35
- 1	227	225	-13	15	18	-11	43	42	- 6	101	104	- 4	22	22	-12	22	26	1	214	209	5	95	97
0	135	133	-12	30	30	-10	64	64	- 5	131	127	- 3	36	36	-11	32	30	2	173	161	Hk	4,Ke	6
1	225	225	-11	42	47	- 9	38	35	- 4	97	90	- 2	17	17	-10	31	31	3	56	54	-17	17	15
2	142	139	-10	47	48	- 8	16	16	- 3	98	102	0	63	62	- 9	68	70	4	53	54	-11	25	20
3	26	23	- 9	35	34	- 6	18	14	- 2	402	392	1	88	86	- 8	32	33	5	157	163	- 9	28	29
4	38	37	- 8	195	201	- 4	64	67	- 1	525	516	2	44	44	- 7	58	58	6	224	230	- 8	25	26
5	130	125	- 7	100	97	- 3	60	61	0	196	202	3	34	34	- 6	23	23	8	13	14	- 6	19	18

Table 2. Continued.

= 5	59	60	6	39	39	=11	11	5	= 9	10	0	= 4	13	15	Hx	8,Kx	6	3	19	20	= 6	35	35
= 4	12	11	10	25	25	=10	79	82	= 8	46	42	= 3	29	31	=14	27	25	Hx	9,Kx	8	= 4	29	32
= 2	76	81	Hx	5,Kx	4	= 9	69	71	= 6	56	59	= 1	32	31	=13	23	10	=12	28	25	= 3	30	31
= 1	134	138	=14	16	13	= 8	68	65	= 4	20	14	2	21	19	=12	36	30	= 8	51	51	= 2	17	18
0	62	61	=10	44	43	= 6	46	43	= 2	54	55	Hx	7,Kx	7	=11	22	22	= 2	32	29	0	37	35
1	39	39	= 9	25	26	= 5	60	60	0	73	71	= 7	31	28	= 8	21	19	Hx	9,Kx	7	1	21	16
2	43	42	= 8	60	66	= 4	43	46	2	69	73	= 5	56	50	= 7	44	39	= 9	35	33	Hx	11,Kx	2
3	23	24	= 7	64	63	= 2	59	59	4	60	59	= 3	15	14	= 6	21	20	= 8	13	14	= 7	14	14
4	23	25	= 6	81	78	= 1	33	35	6	16	18	= 2	12	12	= 5	45	47	= 7	43	41	= 6	12	12
5	22	19	= 5	114	116	0	57	63	8	46	46	3	13	11	= 2	25	27	1	18	15	= 2	17	16
7	23	21	= 4	155	157	1	62	62	Hx	7,Kx	1	Hx	7,Kx	8	2	13	12	Hx	10,Kx	0	0	19	18
9	16	16	= 3	183	186	2	9	4	=19	18	19	=11	31	27	4	31	28	=18	12	11	1	12	14
Hx	4,Kx	7	= 2	64	64	3	92	93	=16	16	18	= 9	18	17	5	23	18	=10	33	32	Hx	11,Kx	3
= 8	22	22	= 1	120	123	4	49	49	=15	25	24	= 5	22	18	Hx	8,Kx	7	= 8	105	103	=10	27	25
= 6	28	29	1	58	63	5	18	17	=14	20	21	3	18	14	=13	41	35	= 6	56	54	= 8	17	16
= 5	18	17	2	58	57	6	32	30	=13	32	34	Hx	8,Kx	0	=11	23	20	0	37	37	= 7	23	25
= 4	21	22	3	56	58	7	21	16	=12	83	84	=12	39	39	= 9	29	27	2	32	30	= 5	12	10
= 3	25	23	4	31	30	Hx	6,Kx	3	=11	57	60	=10	81	82	= 6	19	20	8	31	26	= 3	19	19
= 1	42	38	10	14	13	=14	28	25	= 9	57	56	= 8	35	34	0	23	21	Hx	10,Kx	1	0	37	33
2	29	28	11	28	27	=12	24	25	= 8	25	23	= 6	68	67	3	20	19	=18	18	16	2	19	18
3	21	21	12	23	20	=11	45	45	= 7	83	83	= 4	61	62	4	25	20	=14	27	28	Hx	11,Kx	4
4	27	28	Hx	5,Kx	5	=10	51	55	= 6	62	59	= 2	58	58	Hx	8,Kx	8	=13	27	25	=16	25	17
5	32	31	=14	18	19	= 9	46	46	= 4	165	164	Hx	8,Kx	1	= 7	23	20	=12	37	36	=13	20	17
Hx	4,Kx	8	=12	27	25	= 8	26	31	= 3	98	101	=15	42	41	Hx	9,Kx	0	=10	31	31	=11	15	18
=12	22	19	=11	36	35	= 7	16	18	= 2	114	116	=14	17	16	=16	39	38	= 9	61	62	=10	32	31
= 2	23	20	= 9	20	27	= 5	70	71	= 1	72	76	=13	29	26	=14	42	40	= 8	87	86	= 8	29	27
= 1	34	34	= 8	34	36	= 4	29	30	0	91	90	=12	40	41	=10	12	7	= 7	28	27	= 6	26	24
2	21	19	= 7	51	59	= 3	49	48	1	17	14	=10	27	28	= 8	24	20	= 6	26	22	= 3	33	32
3	29	27	= 6	72	72	= 2	42	42	2	61	60	= 9	14	16	= 6	68	71	= 5	37	36	= 2	26	28
4	21	22	= 5	61	63	0	9	13	3	23	23	= 8	82	83	= 2	23	27	= 4	68	68	0	39	31
Hx	4,Kx	10	= 3	159	161	1	47	50	4	56	57	= 7	21	24	2	49	47	= 3	27	27	1	44	40
= 2	20	19	= 2	34	37	2	87	84	5	33	36	= 6	95	93	4	29	29	= 2	39	41	Hx	11,Kx	5
Hx	5,Kx	0	0	55	53	4	43	43	6	41	42	= 4	60	62	6	15	13	= 1	54	53	=17	23	20
=20	21	19	1	49	51	6	21	19	8	38	35	= 3	21	23	Hx	9,Kx	1	0	27	25	=13	23	22
=18	62	55	3	61	62	10	21	18	9	33	30	= 2	63	51	=14	29	30	1	18	20	=10	25	21
=14	66	67	4	68	70	Hx	6,Kx	4	Hx	7,Kx	2	0	48	48	=13	13	16	3	12	14	= 8	17	13
=10	131	132	6	18	18	=16	16	14	=16	22	23	1	75	75	=12	64	60	Hx	10,Kx	2	= 5	18	18
= 8	111	110	Hx	5,Kx	6	=14	29	25	=15	16	17	2	66	67	= 7	62	61	=13	29	27	= 4	26	23
= 6	278	271	= 5	39	35	=13	26	30	=14	33	30	3	69	71	= 6	53	54	=12	16	16	= 2	20	21
= 4	63	59	= 1	24	24	=12	23	22	=12	24	22	4	49	48	= 5	83	83	= 9	21	21	Hx	11,Kx	6
= 2	427	423	0	23	24	=11	13	6	=11	35	36	6	24	23	= 4	10	10	= 6	19	20	= 5	20	14
0	310	302	3	53	53	=10	45	45	=10	17	17	8	13	15	= 2	44	46	= 5	36	36	Hx	12,Kx	0
2	125	127	5	33	33	= 9	102	109	= 9	36	36	10	24	20	= 1	17	18	= 4	43	45	=16	19	17
4	54	59	Hx	5,Kx	7	= 8	28	26	= 8	74	76	Hx	8,Kx	2	0	46	47	0	22	23	=14	25	22
6	51	51	=13	24	17	= 7	12	12	= 7	55	56	=16	38	36	3	14	12	3	30	26	=10	60	54
8	12	10	=11	25	22	= 5	108	110	= 6	126	125	=15	28	29	4	19	19	5	20	15	= 8	31	32
10	99	95	= 9	14	15	= 4	152	157	= 5	28	27	=14	55	55	8	20	23	Hx	10,Kx	3	= 6	65	65
Hx	5,Kx	1	1	19	12	= 3	42	43	= 4	104	107	=12	57	57	Hx	9,Kx	2	=16	25	25	= 4	28	28
=19	17	15	2	25	26	= 2	133	135	= 3	54	50	=11	67	66	=17	19	18	=14	27	24	= 2	47	42
=18	15	15	4	26	25	= 1	83	87	= 2	33	29	=10	44	37	=14	34	32	= 9	20	18	Hx	12,Kx	1
=15	18	21	5	24	20	0	38	40	0	37	44	= 8	14	13	=13	34	35	= 7	29	27	=11	28	27
=14	16	18	Hx	5,Kx	8	1	66	72	1	37	33	= 7	91	92	=11	35	38	= 6	34	34	=10	33	30
=12	122	125	= 9	24	19	2	87	93	2	104	106	= 6	102	101	=10	10	9	= 3	37	36	= 9	44	43
=11	71	68	= 7	20	16	3	69	72	3	25	28	= 5	48	49	= 9	52	53	= 1	13	18	= 8	16	14
=10	27	25	= 6	12	10	4	48	47	12	24	19	= 4	27	29	= 8	65	65	0	26	24	= 4	16	12
= 9	47	47	= 4	43	42	5	40	45	Hx	7,Kx	3	= 3	101	104	= 7	72	74	1	32	33	= 3	20	21
= 8	117	112	= 1	30	29	6	28	24	=14	22	22	= 2	63	61	= 6	164	166	Hx	10,Kx	4	= 2	28	26
= 7	52	52	1	15	14	8	21	19	=13	12	12	= 1	25	27	= 5	15	14	=15	23	23	Hx	12,Kx	2
= 6	66	62	3	26	26	Hx	6,Kx	5	=11	24	27	0	85	90	= 4	26	25	=14	19	19	=10	23	20
= 5	29	27	5	22	19	=15	23	20	= 9	39	43	1	22	20	= 3	48	47	=11	26	26	= 8	17	16
= 4	190	183	Hx	5,Kx	9	=14	18	14	= 8	20	22	2	99	98	= 2	80	85	= 8	43	40	= 7	17	18
= 3	83	80	=10	15	15	=13	26	25	= 7	71	72	3	41	42	= 1	42	38	= 6	43	43	= 4	20	16
= 1	182	180	= 8	28	24	=12	37	39	= 5	181	183	4	20	17	0	27	26	= 5	13	11	Hx	12,Kx	3
0	177	178	Hx	6,Kx	0	=10	16	14	= 4	189	195	5	41	37	1	53	53	= 4	16	10	=10	23	20
1	71	68	=18	18	17	= 9	14	14	= 3	35	37	7	31	30	2	43	44	= 3	17	18	Hx	12,Kx	4
2	23	25	=14	128	131	= 8	39	39	= 2	27	26	Hx	8,Kx	3	3	16	17	0	36	37	=16	25	21
4	145	150	=12	66	62	= 7	72	73	= 1	116	118	=16	14	10	4	13	12	2	36	34	=15	16	13
5	113	115	=10	162	162	= 6	13	14	0	18	23	=14	29	30	7	24	24	Hx	10,Kx	5	=12	23	21
6	103	105	= 8	23	25	= 5	32	35	1	41	41	=13	33	37	10	23	19	=15	21	21	= 9	20	17
7	45	47	= 6	179	180	= 3	135	138	3	25	27	=11	44	50	Hx	9,Kx	3	=14	26	23	0	30	27
8	15	14	= 4	31	35	= 2	83	80	Hx	7,Kx	4	=10	67	68	=13	36	35	=11	31	27	Hx	12,Kx	5
9	40	39	= 2	94	91	= 1	28	28	=16	16	15	= 9	40	44	=10	36	36	= 9	29	29	1	19	17
10	43	39	= 1	8	0	0	48	46	=14	26	26	= 7	73	76	= 9	28	31	= 1	12	14	Hx	13,Kx	1
Hx	5,Kx	2	0	146	140	1	76	76	=11	22	22	= 6	32	32	= 7	42	46	1	20	18	=14	16	14
=12	42	43	2	185	190	2	17	11	=10	37	37	= 4	90	85	= 6	29	27	2	33	25	=10	37	35
=10	13	16	4	280	290	3	42	41	= 9	42	46	= 3	58	57	= 5	33	33	3	29	26	= 8	36	32
= 9	18	21	6	54	53	4	24	23	= 8	24	26	= 2	25	28	= 4	31	30	4	23	21	= 5	21	23
= 7	46	43	8	47	49	5	29	29	= 7	17	17	= 1	27	32	= 3	21	20	Hx	10,Kx	6	Hx	13,Kx	2
= 6	27	25	10	22	21	6	13	13	= 6	60	60	0	93	97	= 1	48	45	=15	21	18	=16	18	15
= 4	127	123	Hx	6,Kx	1	Hx	6,Kx	6	= 5	106	109	1	44	47	0	52	51	=14	19	15	=12	19	16
= 3	180	174	=14	19	19	= 7	31	33	= 4	78	76	4	20	23	1	25	25	=11	21	18	=11	13	11
= 2	76	79	=13	85	85	= 6	23	24	= 3	45	45	6	27	25	3	37	35	= 9	17	12	= 8	24	22
= 1	100	99	=12	15	15	= 4	20	19	= 2	10	12	Hx	8,Kx	4	4	18	21	= 8	21	22	= 2	27	26
0	102	95	=11	20	18	= 3	20	19	= 1	56	57	=13	24	20	5	20	17	2	24	23	0	19	15
1	72	74	=10	12	10	1	21	23	0	31	32	= 9	17	15	Hx	9,Kx	4	3	23	20	Hx	13,Kx	3
3	24	23	= 9	65	64	2	26	29	2	89	90	= 7	31	29	=14	24	19	Hx	11,Kx	0	=17	15	9
5	62	64	= 8	13	9	Hx	6,Kx	7	4	22	22	= 6	23	23	= 9	15	12	=16	23	22	= 1	32	28
Hx	5,Kx	3	= 7	45	44	=11	20	17	Hx	7,Kx	5	= 5	19	22	= 7	32	32	=14	37	35	0	16	16
=14	30	29	= 6	26	26	= 9	30	24	= 8	45	46	= 4	40	41	= 5	20	24	=12	36	35	Hx	14,Kx	1
=12	47	47	= 5	111	110	= 7	23	21	= 7	48	53	= 3	25	24	= 4	43	44	=10	33	30	=10	20	16
=11	40	44	= 4	18	18	= 5	13	12	= 6	71	76	0	18	20	= 3	18	18	= 8	89	86	= 8	16	14
= 9	18	18	= 3	64	66	= 4	21	21	= 2	19	17	1	12	16	0	30	28	= 6	73	69	= 4	19	18
= 8	15	17	= 2	137	129	= 3	29	28	= 1	22	20	2	29	28	2	19	18	= 4	46	49	Hx	14,Kx	2
= 7	104	104	= 1	102	97	5	14	8	0	17	15	4	14	16	Hx	9,Kx	5	= 2	21	24	=16	16	17
= 6	36	40	0	42	45	Hx	6,Kx	8	1	27	26	Hx	8,Kx	5	=15	25	20	0	26	26	= 9	12	12
= 5	212	216	2	32	36	=12	19	14	4	18	15	=11	25	26	=13	24	22	2	32	28	= 7	27	24
= 4	77	79	3	123	128	=11	24	22	9	29	24	= 6	47	49	=11	12	8	4	14	15	Hx	14,Kx	3
= 3	38	34	4	55	55	Hx	6,Kx	9	Hx	7,Kx	6	= 4	22	20	= 8	13	12	Hx	11,Kx	1	=10	24	22
= 2	111	116	5	61	63	= 3	26	25	=11	41	37	= 3	13	12	= 6	21	18	=18	14	14	Hx	15,Kx	0
= 1	99	99	7	23	22	Hx	7,Kx	0	= 9	29	30	= 2	30	33	= 4	34	29	=10	20	19	=12	14	14
0	20	22	8	34	34	=16	25	22	= 7	28	27	0	29	29	= 3	19	16	= 9	22	28	Hx	15,Kx	2
1	83	79	10	25	22	=14	13	19	= 6	40	40	1	19	18	= 2	17	13	= 8	31	33	= 4	16	12
4	82	80	Hx	6,Kx	2	=12	31	34	= 5	35	37	3	35	34	2	14	11	= 7	54	53	= 3	16	13
5	23	20	=12	56	56																		

Table 3. Bond distances, bond angles and hydrogen bond lengths and angles. Estimated standard deviations in bond lengths between heavy atoms are 0.002 Å, in angles 0.1°, for bonds and angles involving hydrogen atoms 0.02 Å and 1.0°, respectively. Distances in parenthesis are corrected for librational motion.

Bond distances (Å)			Bond angles (°)	
C1 – C2	1.351	(1.357)	C6 – C1 – C2	122.8
C2 – C3	1.418	(1.425)	C1 – C2 – C3	120.5
C3 – C4	1.500	(1.510)	C2 – C3 – C4	118.9
C4 – C5	1.527	(1.534)	C3 – C4 – C5	114.5
C5 – C6	1.524	(1.530)	C4 – C5 – C6	107.7
C5 – C7	1.526	(1.534)	C4 – C5 – C7	109.5
C5 – C8	1.522	(1.531)	C4 – C5 – C8	110.3
C6 – C1	1.491	(1.495)	C5 – C6 – C1	113.3
C1 – O1	1.326	(1.329)	C6 – C5 – C7	109.5
C3 – O2	1.246	(1.249)	C6 – C5 – C8	110.5
C2 – H1	0.96		C7 – C5 – C8	109.2
C4 – H2	1.00		C2 – C1 – O1	123.9
C4 – H3	0.96		C6 – C1 – O1	113.2
C6 – H4	1.00		C2 – C3 – O2	122.5
C6 – H5	0.97		C4 – C3 – O2	118.5
C7 – H7	0.97			
C7 – H8	0.99			
C7 – H9	1.01			
C8 – H10	0.96			
C8 – H11	0.99			
C8 – H12	0.99			

Hydrogen bond lengths (Å)		Hydrogen bond angles (°)	
O1···O2	2.593	O1 – H6···O2	177
O1 – H6	0.87	C1 – O1···O2	112.7
O2···H6	1.74	C1 – O1 – H6	111
		C3 – O2···O1	129.7
		C3 – O2···H6	129

tions as shown in Table 3. The r.m.s. translational amplitudes corresponding to the discrepancy of 0.0022 Å are 0.22, 0.20, and 0.18 Å and the r.m.s. liberational amplitudes are 5.5, 3.9, and 2.9°.

DISCUSSION

Bond lengths and angles are given in Table 3 and Fig. 1. The molecular arrangement in the unit cell is shown in Fig. 2. The compound crystallizes in the *trans*-enol form as expected from spectroscopic investigations,[2,4] and there is an *inter*-molecular hydrogen bond (2.593 Å) between the hydrogen atom of the enol hydroxyl and the oxygen atom of the carbonyl group. These bonds tie the molecules in helices along the two-fold screw axes. The molecules thus form polymers rather than dimers as has been found in solution. However, formation of a dimer with two hydrogen bridges as suggested,[2,3] seems to be incompatible with normal hydrogen bonds if reasonable van der Waals distances are assumed. The O···O distance is significantly larger than corresponding distances found in analoguous *cis*-enolized β-diketones (2.43 – 2.50 Å).[6-8] In the open *trans* arrangement in dimedone, *inter*-molecular nonbonded repulsions may preferably give an expansion of the O···O distance, whereas in the *cis*-enolized β-diketones investigated,[6-8] there are varying amounts of *intra*-molecular nonbonded repulsions giving the opposite effect. The hydrogen atom (H6) is unambiguously located in an asymmetric position near O1 (O1 – H6 = 0.87 Å), and is situated very close to the least-squares plane through the conjugated enol system. The angle O1 – H6···O2 is 177°.

The distances in the enol system reveal a considerable degree of conjugation, although not as much as in the *cis*-analogues.[6-8] Similar conjugation effects have also been found in ascorbic,[13] dialuric,[14] and α-methyltetronic acids.[15] Changes in bond lengths due to hydrogen bond formation in the conjugated system in barbiturates have been discussed by Craven *et al.*[16] It is also reasonable to assume that formation of hydrogen bonds is partly responsible for the relatively large amount of conjugation in the above mentioned systems.

The conformation of the molecule might be described as an envelope. The carbon atom C5 is displaced by 0.61 Å from a least-squares plane through the other carbon atoms in the ring and the oxygen atoms (Table 4). The deviations from the plane are probably significant. The oxygen atoms and C4, and the other carbon atoms are displaced on opposite sides of the plane. The deviations thus give the cyclohexene ring a slightly curved boat-shaped form. The small deviation of O1 from a plane through C2, C1, and C6 is possibly associated with the formation of the hydrogen bond. The other deviations may be due to non-bonded interactions, expecially between O2 and C1′ across the two-fold screw axis in $x = 0$ and O2 and H1 across the centre of symmetry in $x = 0$. Very

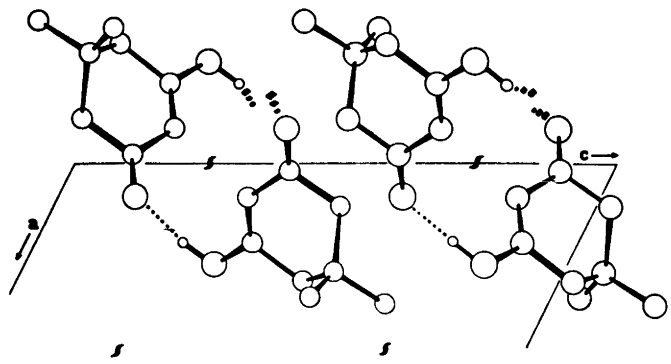

Fig. 2. The structure viewed along the *b* axis.

Table 4. Deviation (Å) of atoms from least-squares plane through parts of the ring system in dimedone. Distances to atoms not defining the plane in parenthesis.

O1	0.035
O2	0.025
C1	−0.024
C2	−0.049
C3	−0.017
C4	0.017
C6	−0.007
C5	(−0.61)
H1	(−0.063)
H6	(0.087)

similar deviations are found in the conformation of related six-membered rings.[17]

The sp^2-sp^3 and sp^3-sp^3 bonds have fairly normal values.[18] The angles inside the ring progressively decrease from values close to 120° near the double bond to a tetrahedral value at C5. The arrangements around C5, C7, and C8 are very regular, none of the angles departing much from tetrahedral values, and the hydrogen atoms at C4 and C6 are in staggered positions relative to the methyl groups at C7 and C8.

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Nuclear Magnetic Resonance of Aromatic Heterocyclics. VIII. A Comparative Study of ^{1}H and ^{13}C Spectra of Some 2-Substituted Furans, Thiophenes, Selenophenes and Tellurophenes

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A systematic investigation of the influence of the heteroatom on ^{1}H- and ^{13}C-NMR parameters in 2-substituted furans, thiophenes, selenophenes and tellurophenes has been carried out. In addition to the parent compounds, the formyl, acetyl, carboxyl, carbomethoxy, methylthio and hydroxymethyl derivatives were studied.

Linear correlations between the electronegativity of the heteroatoms and several of the NMR parameters were observed, as well as between the shifts of the corresponding protons and carbons in the four heterocyclic series. These can be used for predicting shifts of unknown selenophene and tellurophene derivatives, when those of thiophene are known.

$^{125}Te-H$ couplings were determined for the tellurophenes and compared with $^{77}Se-H$ couplings.

Extensive work on the effect of substituents on the proton NMR parameters of thiophenes,[1,2] furans,[3] and selenophenes [4] have previously been carried out by Gronowitz and coworkers. As early as 1962 a linear correlation between the 3-proton shifts in 2-substituted thiophenes and the corresponding shifts in 2-substituted furans was observed. The same was also found for the 5-proton shifts. We were therefore interested in extending this investigation to the selenophene and tellurophene series, as well as in extending our study to ^{13}C NMR. Only a few ^{13}C NMR investigations of these heterocyclic systems have been carried out,[5-9] from which basic information about chemical shift regions and most $^{13}C-H$ couplings may be obtained. Takahashi and coworkers,[9] who studied six 2- and six 3-substituted thiophenes, observed a linear correlation between the C_2 shift of 3-substituted derivatives and the C_3 shift in 2-substituted compounds, and also a linear correlation between C_5 shifts and H_5 shifts of 2-substituted thiophenes.

A systematic study of the influence of the heteroatom of these four related aromatic systems on the transmittance of substituent effects to different positions of the rings would perhaps provide some information related to the aromaticity of these rings and could contribute to an understanding of the chemical differences between these systems. Both experimental results and theoretical calculations have indicated that correlations exist between ^{13}C chemical shifts and charge densities.

In the present paper, due to synthetic difficulties in preparing 2-substituted tellurophenes, the series of derivatives studied had to be limited to the 2-formyl, 2-acetyl, 2-carboxyl, 2-carbomethoxy, 2-hydroxymethyl, and 2-methylthio derivatives of furan, thiophene, selenophene, and tellurophene, besides the parent compounds. This number and types of substituents are of course not representative enough for a test of the applicability of the Swain and Lupton two-parameter equation, which hitherto has been successfully applied by us to the chemical shifts of 5- and 4-substituted 2-fluorothiophenes and 5-substituted 3-fluorothiophenes,[10,11] as well as to proton chemical shifts of thiophenes [1,2] and selenophenes.[4]

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Table 1. ^{1}H NMR data for some furan, thiophene, selenophene, and tellurophene derivatives in deuterioacetone solution at 100 MHz using TMS as internal standard. X = heteroatom and r = correlation coefficient for correlation with electronegativity.

Substituent	X	δ_3	δ_4	δ_5	δ_{HX}	J_{34}	J_{35}	J_{45}
	O	7.45	6.74	7.94	7.72	3.45	0.83	1.73
	S	7.93	7.30	7.96	9.98	3.79	1.22	4.75
CHO	Se	8.17	7.54	8.68	9.86	3.90	1.22	5.44
	Te	8.62	8.05	9.56	9.58	4.10	1.32	6.77
	r					0.97	0.99	0.99
	O	7.32	6.65	7.81	2.41	3.58	0.76	1.74
	S	7.80	7.17	7.80	2.52	3.74	1.09	5.07
$COCH_3$	Se	8.02	7.43	8.52	2.51	3.96	1.14	5.54
	Te	8.44	8.00	9.41	2.52	4.22	1.16	6.78
	r					0.87	0.99	0.99
	O	7.24	6.59	7.76		3.53	0.88	1.72
	S	7.80	7.15	7.78	9.56	3.67	1.16	5.03
COOH	Se	8.03	7.37	8.42	10.53	3.90	1.26	5.50
	Te	8.53	7.93	9.40	10.81	4.20	1.34	6.76
	r					0.85	0.99	0.99
	O	7.23	6.61	7.79	3.86	3.50	0.83	1.74
	S	7.74	7.09	7.66	3.85	3.69	1.20	4.97
$COOCH_3$	Se	8.00	7.37	8.42	3.82	3.93	1.22	5.50
	Te	8.49	7.92	9.38	3.78	4.11	1.33	6.79
	r					0.90	0.99	0.99
	O	6.43	6.39	7.55	2.38	3.26	0.88	1.98
	S	7.07	6.96	7.40	2.45	3.60	1.26	5.36
SCH_3	Se	7.18	7.17	8.08	2.50	3.70	1.23	5.77
	Te	7.42	7.55	8.81	2.50	4.03	1.28	6.93
	r					0.94	0.97	0.99
	O	6.25	6.31	7.40	4.90	3.30	0.86	1.80
	S	6.91	6.88	7.21	4.67	3.40	1.11	5.14
CH_2OH	Se	7.05	7.11	7.90	4.82	3.50	1.18	5.59
	Te	7.41	7.64	8.77	4.74	3.88	1.25	6.83
	r					0.83	0.99	0.99

In the furan, thiophene, and selenophene series, we have recently finished an extensive study of representative 2- and 3-substituted derivatives, and have found that certain ^{13}C shifts can be excellently correlated with the reactivity constants $\mathscr{F}$ and $\mathscr{R}$ of Swain and Lupton.[12]

^{1}H NMR SPECTRA

The ^{1}H NMR spectra of tellurophene, 2-acetyltellurophene, and 2-tellurophene carboxylic acid in deuteriochloroform solution have been described earlier.[13] Assignments of chemical shifts in tellurophene were based on analogy with thiophene and selenophene and on shift effects caused by substituents. In order to obtain comparable ^{1}H NMR data for the six derivatives of the four heterocycles discussed in this paper, the spectra were recorded in deuterioacetone solutions.

In those cases when the substituent was electron-attracting the absorptions of the aromatic hydrogens were well separated, while the 2-hydroxymethyl and 2-methylthio compounds showed more strongly coupled spectra for the β-hydrogens. The refined δ and J values were obtained iteratively using an extended version of the QCPE program UEAITR (No. 188). The original version of UEAITR is given in Ref. 14. After a preliminary estimate of the parameters the calculations were performed until the best least squares fit was ob-

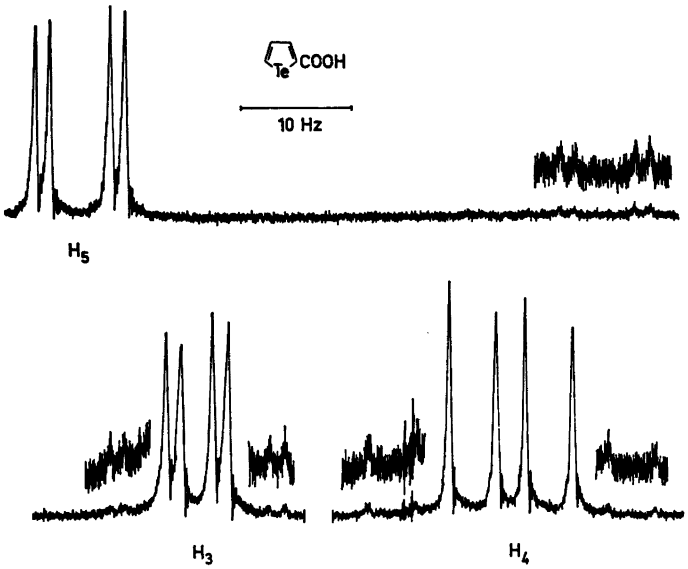

Fig. 1. ^{1}H NMR spectrum of 2-tellurophene carboxylic acid in deuterioacetone solution at 100 MHz showing tellurophene satellites.

Table 2. Heteroatom-hydrogen coupling constants for some 2-substituted tellurophenes and selenophenes.

Substituent	J_{Te-H_3}	J_{Te-H_4}	J_{Te-H_5}	J_{Se-H_3} [a]	J_{Se-H_4} [a]	J_{Se-H_5}
CHO	9.5	14.0	94.2	4.7	7.4	45.6
$COCH_3$	11.0	13.0	91.7	6.6	10.0	44.8
COOH	10.1	14.7	93.5	5.2	7.4	45.2
$COOCH_3$	14.1	16.7	97.6	–	–	44.8
SCH_3	9.7	14.9	94.7	–	–	46.1
CH_2OH	15.2	16.6	97.1	–	–	46.7

[a] Determined from ^{77}Se spectra.

tained (RMS 0.02) The procedure resulted in calculated proton spectra with average deviations from the observed frequencies of 0.01 Hz or less.

The shifts and coupling constants are given in Table 1.

The ^{1}H NMR tellurophene spectrum showed satellite spectra due to ^{13}C as well as ^{125}Te. A representative spectrum showing tellurophene satellites are given in Fig. 1. Thus, the magnitude of the coupling between the heteroatom and the three ring-hydrogens could be observed, as previously done for selenophene.[15] The measurement conditions for the two heteroatoms are comparable, they are of the same natural abundance and the sensitivity of selenium is about half that of tellurium. The absolute values of the tellurium-hydrogen couplings of the tellurophene derivatives are given in Table 2. The same relative order $|J_{X-H_5}| \gg |J_{X-H_4}| \sim |J_{X-H_3}|$ was found for some selenophene derivatives.[15] For each compound the magnitude of J_{X-H_4} is, however, larger than that of J_{X-H_3}.

The intervals for the largest heteroatom-hydrogen coupling, $|J_{X-H_5}|$, are 91.7 – 100.4 Hz and 44.8 – 48.5 Hz for the tellurophene and selenophene derivatives, respectively. There is a simple relation between coupling constants involving various isotopic nuclear species,

and the J value for a pair of nuclei.[16] The magnitudes of coupling constants between different nuclei are often compared by means of their reduced coupling constants, K,[17] defined as

$$K = (4\pi^2/h\gamma_A\gamma_B)J_{AB}$$

The products of the magnetogyric ratios $\gamma_{Se}\gamma_H$ and $\gamma_{Te}\gamma_H$ are of opposite signs. By introducing the visual α-coupling constants for unsubstituted tellurophene and selenophene, 100.4 Hz and 47.5 Hz, respectively, the reduced coupling constants were calculated to be $K_{Se-H} = |2.07|$ and $K_{Te-H} = |2.64|$. As the magnitudes are of the same order the same type of coupling mechanism seems to operate in both cases. The small difference might be due to the deviation in geometry for the two five-membered rings.

From Table 2 it is obvious that there is no systematic variation in the coupling constants due to the substituents.

From the ^{13}C satellite spectrum in the ^1H NMR spectrum of tellurophene, the $^{13}C-H_\alpha$ and $^{13}C-H_\beta$ cuplings were determined to be 183 Hz and 159 Hz, respectively. This is in agreement with the data for the other three heterocycles, where $^{13}C-H_\alpha$ is also found to be larger than $^{13}C-H_\beta$.[6]

^{13}C NMR SPECTRA

The ^{13}C NMR spectra were also recorded in deuterioacetone solution and the shifts were determined from the proton-decoupled spectra. The assignment of the α- and β-carbons in unsubstituted tellurophene was established from the undecoupled spectrum and based on the magnitude of the direct couplings. The smaller splitting was recognized in the low field ^{13}C absorption in the tellurophene spectrum. Thus the relative order of the carbon chemical shifts is opposite that of the hydrogens.

In the 2-substituted derivatives, the quaternary 2-carbon is directly identified by its lower intensity and the absence of the direct coupling, and the 5-carbon by the largest direct

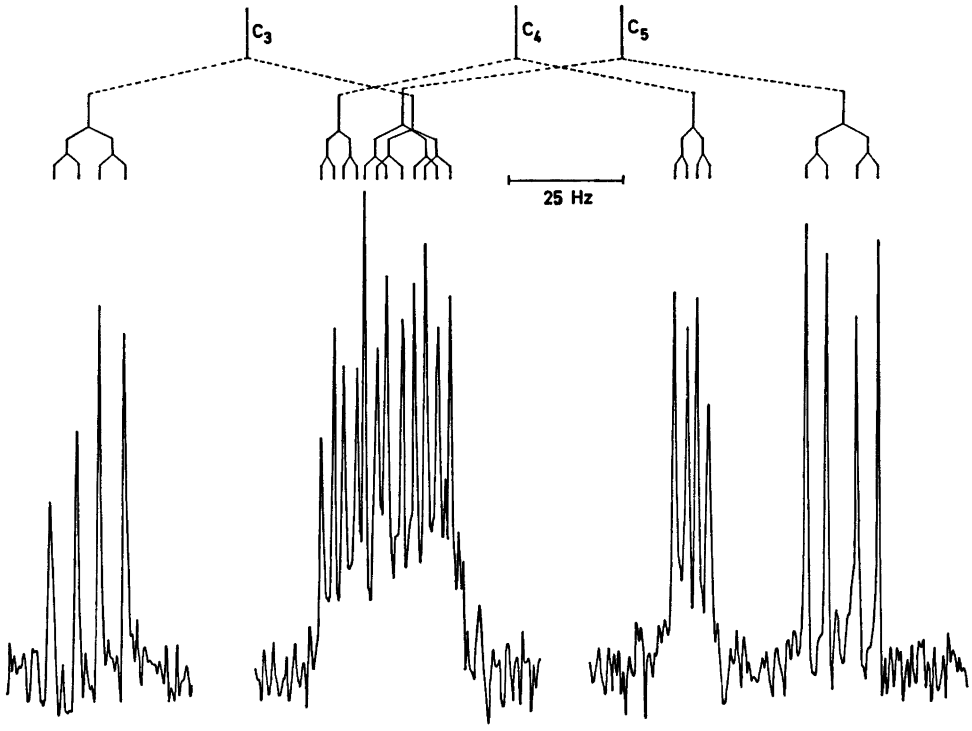

Fig. 2. ^{13}C spectrum of 2-methyl tellurophene carboxylic acid in deuterioacetone solution.

Table 3. ^{13}C-NMR data*for some 2-substituted tellurophenes in deuterioacetate solution at 25.14 MHz using tetramethylsilane as internal standard.

Substituent	C_2	C_3	C_4	C_5	$J_{C_2-H_3}$	$J_{C_4-H_4}$	$J_{C_5-H_5}$	$J_{C_3-H_4}$	$J_{C_3-H_5}$	$J_{C_4-H_5}$	$J_{C_4-H_3}$	$J_{C_5-H_4}$	$J_{C_5-H_x}$	C_x
CHO	151.5	148.1	139.4	138.7	161	163	184	5.6	11.0	2.5	4.5	4.9	11.2	C=O 188.3
$COCH_3$	153.5	143.4	139.8	137.8	161	163	182	5.3	10.7	2.5	4.7	4.6	10.8	C=O 194.7 CH_3 24.9
COOH	137.6	144.7	138.6	138.1	159	161	183	5.6	11.0	2.4	4.8	5.0	10.8	C=O 167.8
$COOCH_3$	139.0	144.5	138.6	137.4	164	163	184	5.6	10.7	3.0	5.0	4.8	11.2	C=O 167.1 CH_3 52.5
SCH_3	142.1	136.3	137.8	125.6	161	161	185	6.0	10.5	2.7	5.0	4.0	11.0	CH_3 23.5
CH_2OH	155.3	132.2	137.4	124.9	158	160	183	5.8	10.8	3.0	4.9	5.0	10.6	CH_2 65.2

coupling. The assignments of the 3- and 4-carbons are based on the expected substituent shift effects, as compared with those obtained for benzene,[18] thiophene,[12] and selenophene derivatives.[12] The assignments thus obtained for the 3- and 4-carbons are also supported by considerations of the long-range couplings. By other ^{13}C measurements it has namely been demonstrated that the long-range coupling over three bonds (^3J) is generally larger than ^2J.[8] This is also the case for 2-substituted thiophenes and selenophenes,[12] where it was proven by a study of deuterated derivatives. On the other hand, furans seem to be an exception to this rule.[12]

The undecoupled spectra of the tellurophene derivatives studied in this paper were of first order. In addition to the large splitting, J_{C-H}, each of the unsubstituted carbons appears as two quartets as a result of long-range couplings. These quartets overlap only in one case, namely that of methyl 2-tellurophenecarboxylate. Still all coupling could be obtained as shown in Fig. 2. The ^{13}C parameters are given in Table 3.

DISCUSSION

The difference in the proton chemical shifts of the α- and β-hydrogens in these five-membered heterocyclics has been suggested as a criterion of aromaticity. In the pure liquids the following differences were found: thiophene (0.132), selenophene (0.569), tellurophene (0.957), and furan (1.061).[19] This order is in agreement with that of decreasing aromaticity based on several criteria.[19]

The same general trend is observed in 20 % deuterioacetone solutions, except for an inversion between furan and tellurophene (*cf.* Table 4). However, a closer analysis of the shifts indicates that the β-hydrogen shifts vary systematically with the +M effect of the heteroatom O > S > Se > Te, and give a good correlation with the Pauling electronegativity data for the heteroatoms (O = 3.5, S = 2.5, Se = 2.4, Te = 2.1; $r = 0.99$). The α-hydrogen shifts, on the other hand, vary irregularly, and this is probably due to the fact that in addition to electronic effects, the anisotropy of the heteroatom also plays an important role in determining the α-shifts. In all four systems, however, the α-hydrogen resonances occur at lower field than the corresponding β-hydrogen resonances.

Table 4. α- and β-Hydrogen shifts (ppm) for furan, thiophene, selenophene, and tellurophene in deuterioacetone solution using TMS as internal standard.

Heteroatom	δ_α	δ_β
O ($E = 3.5$)	7.46	6.36
S ($E = 2.5$)	7.40	7.10
Se ($E = 2.4$)	8.10	7.33
Te ($E = 2.1$)	8.97	7.79
Correlation coefficient	0.81	0.999

The shift differences are even more pronounced in the ^{13}C spectra, but it is only the magnitudes of the differences between the α- and β-carbon shifts that follow the expected aromaticity order: thiophene |1.7|, selenophene |1.7| tellurophene |11.0|, and furan |33.2|. (*Cf.* Table 5, where the ^{13}C parameters for benzene also are given.) The real differences of the ^{13}C shifts, however, show no relation to aromaticity. The resonances of the α-carbons of furan and selenophene occur at lower field than those of the β-hydrogens, while the opposite is true for thiophene and tellurophene.

Table 5. α- and β-^{13}C NMR parameters for furan, thiophene, selenophene, and tellurophene in deuterioacetone solution using TMS as internal standard.

Heteroatom	C_α	C_β	$^1J_{C-H\alpha}$	$^1J_{C-H\beta}$
O	143.6	110.4	201	175
S	125.6	127.3	185	168
Se	131.0	128.8	189	166
Te	127.3	138.0	183	159
Benzene	128.7		159	

Table 6. Chemical shifts (ppm) of the ring protons of some furans, thiophenes, selenophenes, and tellurophenes relative to the α- and β-hydrogens in the parent compounds in deuterioacetone solution.

Substituent	CHO			$COCH_3$			COOH			$COOCH_3$			SCH_3			CH_2OH		
Heteroatom	$\Delta\delta_3$	$\Delta\delta_4$	$\Delta\delta_5$	$\Delta\delta_3$	$\Delta\delta_4$	$\Delta\delta_5$	$\Delta\delta_3$	$\Delta\delta_4$	$\Delta\delta_5$	$\Delta\delta_3$	$\Delta\delta_4$	$\Delta\delta_5$	$\Delta\delta_3$	$\Delta\delta_4$	$\Delta\delta_5$	$\Delta\delta_3$	$\Delta\delta_4$	$\Delta\delta_5$
O	1.09	0.37	0.48	0.96	0.29	0.36	0.87	0.23	0.30	0.86	0.25	0.33	0.07	0.03	0.09	−0.12	−0.06	−0.05
S	0.84	0.20	0.56	0.71	0.07	0.40	0.70	0.05	0.38	0.65	0.00	0.25	−0.03	−0.13	−0.01	−0.19	−0.22	−0.19
Se	0.85	0.21	0.58	0.70	0.11	0.41	0.71	0.05	0.32	0.67	0.04	0.32	−0.14	−0.16	−0.02	−0.28	−0.21	−0.20
Te	0.83	0.26	0.59	0.65	0.21	0.44	0.74	0.14	0.43	0.70	0.13	0.41	−0.37	−0.24	−0.16	−0.38	−0.15	−0.20
Correlation Coefficient	0.97	—	0.99	0.99	—	0.97	—	—	—	—	—	—	0.84	0.99	0.88	—	—	0.97

Furthermore, the α-carbon shifts vary irregularly and are quite similar for thiophene, selenophene, and tellurophene. The β-carbon shifts, on the other hand, are systematically shifted upfield with increasing +M effect of the heteroatom, and are again linearly correlated with the electronegativity of the heteroatoms ($r = 0.998$: $C_\beta = -18.9\ E_x$).

Good correlations were also obtained between the electronegativity of the heteroatoms and the magnitude of the couplings $^1J_{C-H\alpha}$ ($r = 0.96$) and $^1J_{C-H\beta}$($r = 0.94$). It is generally accepted that the direct $^{13}C-H$ couplings are related to the amount of *s*-character on the carbon nucleus and to the electronegativity of the substituents, the coupling constant increasing with electronegativity. This is for instance evident for the *trans* J_{CH} coupling in vinyl derivatives,[20] and has also been observed in acetylenic systems.[21]

Good linear correlations are also observed between shifts and coupling constants. Both C_α *versus* $^1J_{C-H\alpha}$ and C_β *versus* $^1J_{C-H\beta}$ give $r = 0.98$. For the proton shifts, an acceptable correlation was only obtained for C_β *versus* $^1J_{C-H\beta}$.

The absence of a linear correlation between the α-proton shift ($\delta_{H\alpha}$) and $^1J_{C-H\alpha}$ could be expected. Goldstein and Reddy [22] have used deviations from linear correlations between proton chemical shifts and $^{13}C-H$ coupling constants to estimate diamagnetic anisotropy effects. They assumed that in the absence of diamagnetic anisotropy effects and medium effects a linear correlation would be obtained. This was also the case for vinyl derivatives, while benzene and thiophene showed deviations of 78 Hz and 40 Hz, respectively (at 40 MHz). Goldstein and Reddy ascribed these deviations to anisotropy effects of the ring-current type. A preliminary study of the other five-membered heterocycles showed that the deviation form the "vinyl" line was even greater for selenophene, and for tellurophene especially great. This fact and the different behaviour of α- and β-hydrogens indicate strongly that other contributions to anisotropy than ring-current effects are responsible for the deviations. The good linear correlation between ^{13}C shifts and direct $C-H$ couplings again illustrates the fact [23-25] that carbon shifts are much less influenced by diamagnetic anisotropy effects

than the proton shifts.

Galasso [26] has recently carried out CNDO/2 calculations on the four chalcogen heterocyclics. The total charge densities at the α- and β-carbons obtained by him gave the following good linear correlations against the ^{13}C shifts: $C_\alpha = 74.3q + 129.3$ ($r = 0.974$); $C_\beta = 532.8q + 161.7$ ($r = 0.998$).

The proton-proton coupling constants J_{45} and J_{35} are very sensitive to a change of the heteroatom, and excellent linear correlations are obtained for all six derivatives (*cf.* Table 1). Similar observations have previously been made in vinylic systems [27] and 2-substituted thiophenes.[28] Also the coupling J_{34} increases with decreasing electronegativity. However, the correlations are not as good as for J_{45} and J_{35} and the sensitivity to changes in electronegativity is not as great.

The proton shifts relative to those of the α- and β-hydrogens of the parent compounds are given in Table 6, and the corresponding ^{13}C shifts in Table 7. The ^{13}C values for the furans, thiophenes, and selenophenes are taken from Ref. 12.

Even if the data unfortunately are limited, interesting differences between the four ring systems can be observed. The ease of transmission of the substitution resonance effects to the 5-hydrogen and 5-carbon increased in the series furan < thiophene < selenophene < tellurophene. As good correlations were obtained for the 5-proton shifts against the 5-carbon shifts in all four systems ($r > 0.97$), the same transmission mechanism seems to be operating both for carbon and proton. This sensitivity of the 5-position to substituent effects from the 2-position indicates that the heavier chalcogens transmit the substituent effects more efficiently, which may be due to the fact that the *d*-orbitals are easily polarisable. Obviously more material is needed in order to place this hypothesis on a firmer basis. However, the efficient transmission of the heavier heteroatoms to the 5-position can hardly be related to the aromaticity in these systems (*cf.* Ref. 3).

The resonance of the 2-carbon was shifted as a function of both the substituent inductive effect and the +M effect of the heteroatom, giving good correlations (*cf.* Table 7). The shifts of the 3- and 4-carbons caused by the substituents decreased steadily on going from furan to tellurophene. For each type of substituent we carried out linear correlations between the ^{13}C shifts and the electronegativity of the heteroatom. Very good correlations were observed for all ring carbons in compounds containing −I−M substituents, except for C_4 in the 2-acetyl series. For electron-donating substituents, correlations with electronegativity were only observed for the C_2 and C_4 resonances (*cf.* Table 7). Our data are of course too limited to decide if these deviations are due to different transmittance mechanisms for electron-attracting and electron-donating groups. It has been suggested that such differences exist.[2]

A comparison of Tables 6 and 7 shows that the 3- and 4-proton did not vary in the same systematic way as the corresponding carbons with the electronegativity of the heteroatom. This observation reflects the difference between ^1H and ^{13}C with regard to the ring current effect, anisotropy contribution and shift range.

Good correlations between the carbonyl ^{13}C shifts with electronegativity of the heteroatom have been obtained. This was also the case when the methyl of the methylthio and carbomethoxy group and the hydroxymethyl group were correlated with electronegativity. Only the methyl of the acetyl group fails to give such correlation (*cf.* Table 7).

Attempts were made to correlate the various ^1H and ^{13}C shifts with each other. The linear correlations obtained when the relative shifts of the furans, selenophenes, and tellurophenes reported in Tables 6 and 7 were plotted *versus* the relative shifts of the thiophenes are given in eqns. 1−9 for protons and 10−21 for carbons.

^1H

$\Delta H_O^3 = 1.16\Delta H_S^3 + 0.10$; $r = 0.99$ (eqn. 1)

$\Delta H_{Se}^3 = 1.11\Delta H_S^3 - 0.08$; $r = 0.99$ (eqn. 2)

$\Delta H_{Te}^3 = 1.29\Delta H_S^3 - 0.22$; $r = 0.99$ (eqn. 3)

$\Delta H_O^4 = 1.07\Delta H_S^4 + 0.19$; $r = 0.98$ (eqn. 4)

$\Delta H_{Se}^4 = 1.06\Delta H_S^4 + 0.01$; $r = 0.99$ (eqn. 5)

$\Delta H_{Se}^4 = 1.24\Delta H_S^4 + 0.06$; $r = 0.91$ (eqn. 6)

$\Delta H_O^5 = 0.68\Delta H_S^5 + 0.10$; $r = 0.98$ (eqn. 7)

$\Delta H_{Se}^5 = 1.02\Delta H_S^5 + 0.00$; $r = 0.99$ (eqn. 8)

$\Delta H_{Te}^5 = 1.17\Delta H_S^5 - 0.02$; $r = 0.97$ (eqn. 9)

Table 7. Chemical shifts (ppm) of the carbons of some furans, thiophenes, selenophenes, and tellurophenes relative to the α- and β-carbons in the parent compounds in deuterioacetone solution, and ^{13}C-shifts of side-chain carbons. (X = heteroatom and r = correlation coefficient for correlation with electronegativity.)

Substituent	X	ΔC_2	ΔC_3	ΔC_4	ΔC_5	$C_{C=O}$[a]	C_{CH_3}[a]
	O	10.7	12.1	3.2	5.9	178.6	
	S	19.3	10.1	1.9	10.0	183.4	
CHO	Se	20.1	10.5	1.8	10.6	184.8	
	Te	24.2	10.1	1.4	11.5	188.3	
	r	0.99	0.96	0.99	0.99	0.97	
	O	10.3	7.9	2.8	4.3	186.7	26.2
	S	19.8	6.2	1.6	8.8	190.4	26.7
$COCH_3$	Se	21.0	5.9	1.6	9.4	191.4	26.0
	Te	26.3	5.4	1.8	10.5	194.6	24.9
	r	0.99	0.99	0.90	0.99	0.99	–
	O	2.3	8.8	2.5	4.3	160.2	
	S	9.1	7.0	1.3	7.9	163.4	
COOH	Se	9.1	6.9	1.2	8.8	164.8	
	Te	10.3	6.7	0.6	10.9	167.8	
	r	0.99	0.99	0.99	0.98	0.94	
	O	2.1	8.4	2.5	4.2	159.5	52.1
	S	8.8	6.9	1.5	8.0	162.9	52.4
$COOCH_3$	Se	9.3	6.6	1.1	8.6	163.9	52.3
	Te	11.7	6.4	0.6	10.1	167.1	52.5
	r	0.99	0.99	0.99	0.99	0.95	0.94
	O	4.7	4.1	1.8	2.1		18.7
	S	12.0	4.2	0.8	3.0		22.0
SCH_3	Se	13.2	2.1	0.4	2.3		22.5
	Te	14.8	1.7	−0.2	−0.7		23.5
	r	0.99	–	0.97	–		0.99
	O	12.1	−2.6	0.6	−0.8		57.0
	S	20.4	−2.2	−0.1	−0.3		59.5
CH_2OH	Se	23.0	−3.7	−0.5	−0.7		51.9
	Te	28.0	−5.8	−0.6	−2.4		65.2
	r	0.98	–	0.97	–		0.90

[a] Relative to TMS.

^{13}C

$\Delta C_O^2 = 0.81\Delta C_S^2 - 5.05$; $r = 0.99$ (eqn. 10)
$\Delta C_{Se}^2 = 1.11\Delta C_S^2 - 0.62$; $r = 0.99$ (eqn. 11)
$\Delta C_{Te}^2 = 1.40\Delta C_S^2 - 1.76$; $r = 0.99$ (eqn. 12)
$\Delta C_O^3 = 1.22\Delta C_S^3 - 0.11$; $r = 0.99$ (eqn. 13)
$\Delta C_{Se}^3 = 1.17\Delta C_S^3 - 1.57$; $r = 0.99$ (eqn. 14)
$\Delta C_{Te}^3 = 1.37\Delta C_S^3 - 3.84$; $r = 0.95$ (eqn. 15)
$\Delta C_O^4 = 1.28\Delta C_S^4 - 0.75$; $r = 0.99$ (eqn. 16)
$\Delta C_{Se}^4 = 1.18\Delta C_S^4 - 0.43$; $r = 0.99$ (eqn. 17)
$\Delta C_{Te}^4 = 1.13\Delta C_S^4 - 0.71$; $r = 0.89$ (eqn. 18)
$\Delta C_O^5 = 0.58\Delta C_S^5 + 0.28$; $r = 0.98$ (eqn. 19)
$\Delta C_{Se}^5 = 1.14\Delta C_S^5 - 0.50$; $r = 0.99$ (eqn. 20)
$\Delta C_{Te}^5 = 1.56\Delta C_S^5 - 3.05$; $r = 0.97$ (eqn. 21)

In most cases both the proton and ^{13}C shifts give good correlations ($r > 0.97$), the exceptions were observed for the tellurophenes. It is, however, difficult to understand why some of these lines do not pass through the origin. These relations can anyhow be used for predicting shifts of unknown selenophene and tellurophene derivatives, when those of thiophene are known. It is thus obvious that the NMR parameters of the four heterocycles show certain systematic regularities, which should be verified by an investigation of a larger and more representative number of substituted derivatives.

EXPERIMENTAL

All NMR spectra were recorded with a Varian XL-100-15 NMR spectrometer, equipped with frequency sweep, proton wide band decoupler, Fourier transform operation, and frequency counter. The transmitter frequency for ^{13}C was 25.142 MHz and for ^{1}H 100.01 MHz. The shifts were determined with an accuracy of ± 0.1 ppm and ± 0.005 ppm relative to TMS. The ^{13}C shifts were measured on proton decoupled spectra on 20 % solutions in CD_3COCD_3. The coupling constants were obtained with an accuracy of ± 0.02 Hz from proton spectra and ± 0.25 Hz from the ^{13}C spectra.

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Studies on the Bacteriolytic Activity of *Streptomyces albus* Culture Filtrates. 1. The Effect of Variations in Cultivation Conditions and the Screening of Various Enzyme Specificities

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Optimal conditions for production of bacteriolytic enzymes of *Streptomyces albus* G have been studied, and found to depend on stimulation and selection of strain, composition of medium, inoculum, and incubation. The culture filtrates lysed whole cells and mucopeptide/cell wall of *Staphylococcus aureus*, *Planococcus*, *Micrococcus luteus*, *M. conglomeratus*, *M. lysodeikticus*, *Sarcina lutea*, and *Bacillus megaterium*. Results are presented to show that the lytic activity of the cultures is composed of an endo-*N*-acetylmuramidase, an *N*-acetylmuramyl-L-alanine amidase, several endopeptidases with specific activity on mucopeptide linkages, and caseinolytic activity. The specificities likely to be present are discussed in relation to apparently broken mucopeptide bonds.

Bacteriolytic enzymes have been isolated from a variety of sources including plants, animal tissues, and various microorganisms and, specially in the latter source, a great spectrum of specific lytic enzymes has been demonstrated.[1] Such enzymes, which are capable of solubilizing mucopeptide networks, the basic structure of all bacterial cell walls, belong to three classes: (1) Glycosidases or hexosaminidases which hydrolyse the polysaccharide (glycan) chains, (2) endo-peptidases, splitting the bonds within the peptides and their cross-links, and (3) acetylmuramyl-L-alanine amidases which cleave the junction between polysaccharides and peptides.[1-3] Proteolytic activity of soil microorganisms was discovered a long time ago,[4,5] but the first systematic study was carried out by Welsch [6] working with *Streptomyces albus* strain G. Subsequent works,[7-9] using fractional precipitation, ion exchange resin, and various test bacteria revealed that *Streptomyces* strains produce a complex system of lytic enzymes. This was confirmed by Ghuysen and collaborators (*cf.* Ref. 1) in their extensive work on the enzymes from *Streptomyces albus* G.

Bacteriolytic enzymes are of considerable interest in various fields; as potentially useful chemotherapeutics, in biochemical and genetical studies, and in the study of mucopeptide chemistry.[1]

Streptomyces strains are reported to vary on cultivation in both growth and pigmentation [10] as well as in the ability to produce lytic enzymes.[7,11] Therefore, the aim of the present study was to examine the growth conditions and to select strains with high capability for synthesis of lytic enzymes.

MATERIALS AND METHODS

Chemicals. Bacto beef extract (B 126), bactopeptone (B 118), special agar Noble, and trypsin were purchased from Difco Laboratories, Detroit, Mich., U.S.A.; hen egg-white lysozyme (3× crystalline) from Calbiochem. Inc., Los Angeles, Calif., U.S.A.; proteose peptone (code L46) and peptone bacteriological (L37) from Oxoid Ltd., London, England, and trypticase-soy from Baltimore Biological Laboratories, Baltimore, Md., U.S.A. Standard amino acids were obtained from Eastman Organic Chemicals Department, Rochester, N.Y., U.S.A.; dinitrophenyl(DNP)-amino acids from Nutritional Biochemicals Co., Cleveland,

Ohio, U.S.A.; casein, muramic acid, and glucosamine from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England; penta-glycine and ethylene-oxide from Fluka, CH-9470 Buchs, Switzerland, and Amberlite IRC-50 ion-exchange resin from the British Drug Houses Ltd., Poole, Dorset, England. Dialyzer tubing was purchased from A. H. Thomas Co., Philadelphia, Pa., U.S.A. Thin-layer plates (polygram Cel 300 and polygram sil N-HR) were from Machery, Nagel & Co., 516 Düren, BRD; Tabsorb (ethylene glycol adipate coated on chromosorb W), used as column material on gas chromatography, from Regis Chemical Co., Chicago, Ill, U.S.A., and trifluoroacetic acid anhydride from Pierce Chemical Co., Morton Grove, Ill, U.S.A. Trichloroacetic acid, 1-fluoro-2,4-dinitrobenzene (FDNB), diethylether, *p*-dimethylaminobenzaldehyde, acetic acid, chloroform, benzylalcohol, butanol, pyridine, ninhydrin, and all inorganic chemicals (reagent grade) were obtained from E. Merck, Darmstadt, BRD.

Organisms. *Streptomyces albus* G, *Staphylococcus aureus* Copenhagen (Sa), *Streptococcus pyogenes* A (Sp), *Planococcus* 2389 (Pc), *Micrococcus luteus* 144 (M-144), *Micrococcus conglomeratus* 84 (M-84), *Micrococcus lysodeikticus* NCTC 2665 (Ml), and *Sarcina lutea* (Sl) were from the laboratory stock of organisms at the Institute. *Bacillus megaterium* KM (Bm) was kindly provided by Dr. J.-M. Ghuysen, Service de Bactériologie, Université de Liège, Belgium.

Media. The following media were made up to 1 l in deionized water: nutrient broth, pH 7.2, containing 3 g bacto-beef extract, 10 g bacto-peptone and 5 g NaCl. For cultivation of the halofilic *Planococcus* 60 g NaCl were added. Mucopeptide medium contained 2.5 g mucopeptide from Sa, 1 g K_2HPO_4 and 1 g $MgSO_4.7H_2O$. Agar-mucopeptide medium contained in addition 10 g special agar Noble. The peptone media contained 10 g peptone, 1 g K_2HPO_4, 1 g $MgSO_4.7H_2O$, 0.5 g KCl, and 2 g $NaNO_3$.

Growth and harvesting of the test-bacteria. The test-bacteria were grown aerobically on nutrient broth; 4 h inoculum (100 ml) was transferred to 900 ml fresh medium and incubated on a shaking machine at 37°C. The growth was followed spectrophotometrically at 600 nm on a Unicam SP 800 and the bacteria were harvested at the end of the exponential growth-phase, using a Sorvall RC-2 centrifuge (10 000 *g*). After washing in buffer of 0.15 M NaCl, 0.01 M phosphate, pH 7.2, the bacteria were stored at −25°C.

Heat-killed bacteria. Portions of test-bacteria were suspended in distilled water and placed in a boiling water bath for 15 min to denature autolytic enzymes,[12] then washed three times in water and freeze-dried.

Preparation of mucopeptide. Mucopeptides were prepared and analyzed as described earlier.[13] To remove possible *O*-acetyl groups at the 6-position of muramic acid, the mucopeptide preparations were further extracted for 20 min with 9 N NH_4OH at 20°C.[14] After thorough washing with distilled water the mucopeptides were freeze-dried and examined by thin-layer and gas chromatography.

Preparation of cell wall. Isolation of mucopeptide from Bm was difficult since the bacteria clotted on suspension in trichloroacetic acid. Therefore, cell wall material was prepared from Bm according to the method used previously.[15] As for mucopeptides, the cell wall was further trypsinated and extracted with 9 N NH_4OH, to remove *O*-acetyl groups [14] and ester-linked D-alanine from teichoic acid,[16] then washed thoroughly, freeze-dried and examined chromatographically.[15]

Ethylene-oxide treatment of mucopeptides/cell wall. To mask free amino groups of mucopeptide/cell wall preparations, a hydroxyethylation procedure described by Ghuysen *et al.*[2] was employed.

Analytical procedures

Thin-layer chromatography in one and two dimensions was performed on cellulose and silica plates in the following solvent systems: (A) Chloroform:benzyl alcohol:HAc (70:30:3, by vol.),[17] (B) benzyl alcohol:chloroform:methanol:H_2O:NH_3 (25 %) (30:30:30:6:2, by vol.),[2] (C) butanol:HAc:H_2O (4:1:1, by vol.),[2] and (D) pyridine:H_2O (4:1, v/v).[2] Ninhydrin [18] and silver nitrate [18] were used as colour reagents.

Gas chromatography of trifluoroacetylated amino acid butyl esters [19] was carried out on a Perkin-Elmer 900 chromatograph fitted with flame ionization detector and a disc integrator. Tabsorb (80 − 100 mesh), in columns of 200 cm length and 2 mm inner diameter, was used as supporting material. The temperature was increased linearly (4°C/min) from 100°C to 200°C and was then held for 10 min at 210°C, the carrier gas (N_2) having a flow rate of 33 ml/min.

Tests for free groups. Estimation of the increase in free amino groups of enzymatically degraded mucopeptides/cell wall and the identification of *N*-terminal amino acids were carried out by the use of 1-fluoro-2,4-dinitrobenzene (FDNB) according to the description of Ghuysen *et al.*[2] Soluble *N*-acetyl amino sugars were detected by the Morgan − Elson reaction [2] and the increase in reducing groups was measured by the method of Park and Johnson with modifications.[2,20]

Reducing end-group determination. Samples of digested mucopeptide/cell wall, isolated oligosaccharides from preparative thin-layer chromatography and standard amino sugars (glucosamine and muramic acid) were reduced with $NaBH_4$.[21] Reduced and hydrolyzed (3 N HCl for 4 h at 95°C in sealed tubes) preparations were desalted by extraction with pyridine and analyzed by thin-layer chromatography.

Selection of lytic strain and its cultivation. *Streptomyces albus* G was plated on agar-

mucopeptide medium and incubated at 28°C, separate colonies being then inspected. The diameter of the clearing zone around the colony, read after 4 days, was taken as a measure of the lytic activity. Colonies showing high lytic activity were replated on agar-mucopeptide medium every fourth day and incubated at 28°C. For production of enzymes the selected strain (a colony with high lytic activity) was replated once more on agar-mucopeptide medium, grown for 2 days and then transferred to 20 ml of the mucopeptide medium. After 30 h at 28°C on a shaker (Microid Flask Shaker, Griffin & Tatlock Ltd., London, England) operating at a rate of 50 – 60 rotations/min the inoculum was transferred to 1 l of proteose peptone medium and further incubated with shaking for 50 – 60 h.

Concentration and purification of enzymes by the use of Amberlite.[14,22] After removal of mycelia by filtering through glass wool, the culture filtrate was mixed with one half its volume of distilled water and then 0.5 % (by weight) of Amberlite IRC-50 (H^+) was added. After 1 h with stirring at 4°C, the pH of the suspension was adjusted to 5.0 with concentrated acetic acid and the stirring continued for 10 h. The ion-exchange resin was then isolated by centrifugation, washed in distilled water, resuspended in 10 % K_2HPO_4 (approx. 10 ml/g of dry resin) and, while stirring, 25 % NH_4OH was added until the pH was stable at 8.0. The suspension was further stirred for 5 h, centrifuged to remove the resin, and finally dialyzed against 0.01 M Tris-HCl pH 8.0. The dialyzed supernatant is further referred to as crude enzyme preparation (CEP).

Quantitative determination of mycelia. Duplicates of culture (20 ml each) were centrifuged at 3000 *g*. The mycelia were thoroughly washed in distilled water, dried at 90°C and weighed.

Test for bacteriolytic activity. Bacteriolytic activity was expressed as reduction in turbidity of suspensions of bacteria or mucopeptide/cell wall compared to equal suspensions without enzyme. Heat-killed bacteria were washed twice in 0.01 M Tris-HCl buffer pH 8.0, and suspensions (0.5 ml) of bacteria and mucopeptide/cell wall in this buffer were adjusted to give a turbidity of 1.0 at 600 nm with 1.0 cm cell thickness after the addition of enzyme (0.25 ml). This corresponds to a cell concentration of about 5×10^8 cells/ml or a concentration of mucopeptides/cell wall of approximately 1 mg/ml. The suspensions were incubated at 37°C, the lytic activity, followed on a Unicam SP 800, being expressed as reduction in turbidity after 60 min (ΔT_{60}) when $\Delta T_{60} = (\Delta T_t/t) \times 60$. All measurements of activity were carried out before 50 % reduction in turbidity was reached.

Definition of bacteriolytic units. One lytic unit is defined as the activity, at the conditions described above, that gives a $\Delta T_{60} = 1.0$ in a suspension of Sa. Lytic units/ml is then referred to as $\Delta T_{60} \times 4$.

Test for caseinolytic activity. This was performed according to the method described by Petit *et al.*[14] and expressed as reduction in turbidity after 60 min (ΔT_{60}) as for bacteriolytic activity.

EXPERIMENTS AND RESULTS

Substrates for lytic enzymes. The results of gas chromatographic examination of mucopeptides/cell wall with respect to amino acids are given in Table 1, showing a dominance of the amino acids characteristic of mucopeptides. The molar ratio of these amino acids (Table 2) and the absence of teichoic acids in the mucopeptide preparations, as found serologically by double diffusion in agar and chromatographically by

Table 1. Amino acid composition (mμmol/mg) of the mucopeptides/cell wall.

Amino acids	Mucopeptide of M-84	M-144	Ml	Sa	Pc	Cell wall of Bm
Ala	1810	1070	1860	1080	720	1000
Gly	180	640	160	2430	210	60
Glu A	450	590	500	520	740	480
Lys	400	700	520	520	470	0
DAP	0	0	0	0	0	480
Val	20	40	60	60	240	0
Ile	70	30	50	50	210	0
Leu	50	100	120	80	370	30
Pro	100	20	30	30	160	0
Thr	60	40	40	50	160	0
Ser	90	20	20	100	100	0
Phe	0	40	50	80	150	0
Asp A	50	90	0	0	150	0
Tyr	0	0	0	30	0	0

Table 2. Molar ratios between the main amino acids of the mucopeptides/cell wall (Glu A = 1.0).

Amino acids	Mucopeptide of M-84	M-144	Ml	Sa	Pc	Cell wall of Bm
Glu A	1.0	1.0	1.0	1.0	1.0	1.0
Ala	4.0	1.8	3.7	2.1	1.0	2.1
Gly	0.4	1.1	0.3	4.7	0.3	0.0
Lys	0.9	1.2	1.0	1.0	0.6	0.0
DAP	0.0	0.0	0.0	0.0	0.0	1.0

search for glycerol and ribitol,[18,15] indicate that the preparations are satisfactorily pure.

Selection of lytic populations of Streptomyces albus *G.* Colonies with various lytic zones (0–0.5 cm diameter) appeared on agar-mucopeptide medium, each colony giving rise to new ones with variable lytic ability. However, upon further selection the extension of the highest lytic zones reached after 4 months a diameter of approximately 2.5 cm. There was no observable change in the maximal lytic activity of these colonies after 4 months, when the necessary replating and selections had been performed.

Influence of type of peptone on growth and enzyme production. Two litre flasks containing 0.5 ml of peptone medium made from various types of peptone were inoculated with 20 ml of *Streptomyces albus* G cultures and incubated with shaking at 28°C for 60 h. Five cultivations, a total of 5 × 4 flasks, were prepared for each peptone type, and the amount of mycelia and maximum lytic activity, measured on heat-killed cells of Sa and Bm, was determined. Considerable variations in lytic activity were observed within each peptone type and even in the flasks of the same cultivation. The variation in the amount of mycelia was small. Culture filtrates with low lytic activity appeared yellow-brownish in colour, whereas those with high activity were dark brown. The mean values of mycelia and lytic activity are listed in Table 3, indicating that the type of peptone apparently is of importance as far as lytic activity is concerned, proteose peptone being superior in this respect.

Effect of variation in the volume of inoculum. Three series of proteose peptone medium, each consisting of 0.25, 0.50, 0.75 and 1.0 l, were prepared in 2 l flasks, then inoculated with 20, 40 and 60 ml of culture and cultivated at 28°C with shaking for 60 h. The lytic activity in the different series, measured on heat-killed cells of Sa and Bm, showed rather small variations.

Table 3. The amount of mycelia and bacteriolytic activity in cultures of *Streptomyces albus* G using various types of peptone.

Type of peptone	Mycelia g/l	ΔT_{60} on Sa	ΔT_{60} on Bm
Bacto peptone	3.0	0.2	0.3
Proteose peptone	2.9	1.2	1.6
Peptone bacteriological	2.7	0.4	0.5
Trypticase Soy	2.6	0.2	0.3

Table 4. Bacteriolytic activity in cultures of *Streptomyces albus* G after direct inoculation and after preculture.

Inoculum	ΔT_{60} on Sa	ΔT_{60} on Bm
150 ml preculture	0.2	0.3
250 ml preculture	0.2	0.3
20 ml direct inoculum	1.2	1.6

Effect of precultures on peptone medium. Precultures were prepared by transferring 20 ml inoculum to 150 and 250 ml of proteose peptone medium. After 15 h of incubation the precultures were used to inoculate 1 l portions (2 l flasks) of proteose peptone medium. The resultant lytic activities, taken as the mean values of two cultivations each of 4 flasks, are listed in Table 4, showing a remarkably lower activity after use of precultures than after a direct inoculum.

Growth and lytic spectrum. In accordance with the results obtained in the varied culture conditions, all further cultures were prepared using

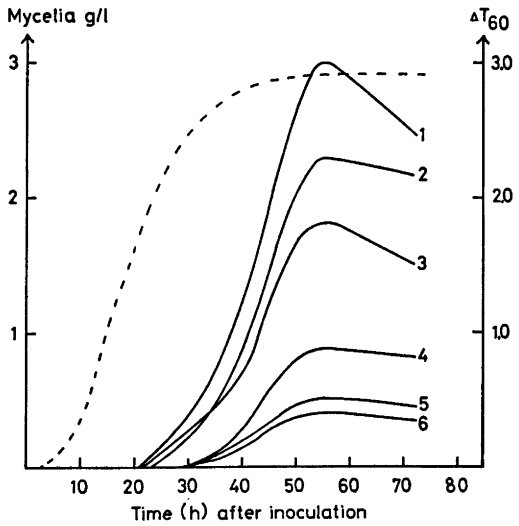

Fig. 1. The amount of mycelia (- - -), bacteriolytic activity tested on Bm(1), Sa (3), Ml and Sl (4), M-144 and M-84 (5) and Sp (6), and caseinolytic activity (2) of a culture of *Streptomyces albus* G at various time intervals. Samples for testing were taken every fifth hour.

20 ml 30 h inoculum to 1 l proteose peptone medium in 2 l flasks, which were then incubated with shaking (50 – 60 rotations/min) at 28°C for 55 h.

Fig. 1 illustrates the growth (expressed as the amount of mycelia) and lytic activity (tested on all test-bacteria as well as casein) of a representative culture. Samples for testing were taken every fifth hour. Extracellular lytic activity was detected in the culture at the end of the exponential growth-phase and reached a maximum in the stationary phase of growth. Lytic activity was demonstrated by all test-bacteria, and the bacteriolytic as well as caseinolytic activities reached maximum at the same time interval. Although the total lytic activity varied from one culture to another, the ratios between the degree of lysis of different test-bacteria were found to be nearly constant. Solubilization of mucopeptides/cell wall with culture filtrates (taken at the time of maximum lytic activity) revealed smaller differences in the lytic spectrum compared to that tested on whole cells (Table 5). Mucopeptides were, however, rather variable as substrate for testing of lytic activities, probably due to heterogeneity with regard to molecular size. Incubation of

Table 5. Bacteriolytic activity of culture filtrate (at maximum) as measured on heat-killed bacteria and on mucopeptides/cell wall.

Bacteria	ΔT_{60} on heat-killed cells	ΔT_{60} on mucopeptide	ΔT_{60} on cell wall
Sa	1.8	1.2	–
M-144	0.5	0.7	–
M-84	0.5	0.9	–
Ml	0.9	0.6	–
Bm	3.0	–	1.7

ethylene-oxide-treated mucopeptides of Sa, M-144, M-84, Ml, and Pc and cell wall of Bm with the culture filtrate increased the amount of free amino groups and reducing groups in all samples, indicating the presence of both peptidases and hexosaminidases.

Lytic activities adsorbed to and eluted from Amberlite. Dialyzed eluate from the resin (CEP) lysed heat-killed bacteria and casein similar to the culture filtrate, indicating that all activities observed were adsorbed to and eluted from the resin. However, only about 50 % of the total bacteriolytic activity and 25 % of the caseinolytic activity were recovered. The remaining activity of the culture filtrate was not adsorbed by the addition of new resin. In spite of low recovery, a rapid concentration and marked purification of the enzymes were obtained. Thus, the absorbance at 280 nm of the culture filtrate (10 × diluted) and CEP was 0.5 and 0.31, respectively. The ratios between degree of lysis on the various test-bacteria seemed similar for culture filtrate, for culture filtrate absorbed with Amberlite, and for CEP.

Samples of ethylene-oxide treated mucopeptides/cell wall (2 parts of 2 mg/ml) were digested with CEP (1 part) and egg-white lysozyme (1 part of 50 μg/ml), the free amino groups were traced with DNP and the mixtures then hydrolyzed. Chromatography of ether extracts in solvent system A and of water-saturated butanol extracts in system B revealed the amino acid derivatives listed in Table 6, which indicates that several types of linkages have been split on digestion with CEP.

Determination of hexosaminidase specificity. Bm cell wall (2 mg/ml in 0.01 M Tris-HCl, pH 8.0) was incubated with CEP at 37°C for 12 h.

Table 6. Dinitrophenyl-amino acids detected chromatographically after treatment of mucopeptides/cell wall with crude enzyme preparation (CEP) and egg-white lysozyme (E.L.).

	Mucopeptide [a]										Cell wall [a]	
	M-84		M-144		Ml		Sa		Pc		Bm	
	CEP	E.L.	CEP	E.L.	CEP	E.L.	CEP	E.L.	CEP	E.L.	CEP	E.L.
DNP-Ala	+++	+	+++	−	+++	−	+++	+	++	+	+++	−
DNP-Gly	−	−	−	−	−	−	+++	−	−	−	−	−
DNP-Glu A	−	−	−	−	−	−	−	−	+++	−	−	−
DNP-ε-Lys	−	−	+++	+	+++	+	−	−	−	−	−	−
DNP-DAP	−	−	−	−	−	−	−	−	−	−	++	++

[a] + to +++: weak to strong colour reaction.

Samples of digested cell wall were reduced with $NaBH_4$, and both reduced and non-reduced samples were hydrolyzed and chromatographed in two dimensions using solvent systems C and D. A spot in the chromatogram of non-reduced samples corresponding to muramic acid was strongly reduced after treatment with $NaBH_4$, while a spot corresponding to muraminitol appeared on the chromatograms. The intensity of the spot corresponding to glucosamine remained unchanged and glucosaminitol was not detected in the reduced samples. This indicates that the only hexosaminidase present is an *N*-acetyl-muramidase.

Test on amidase activity. Mucopeptide of Sa (4 mg) was suspended in and digested with 2 ml (2 lytic units) of *N*-acetylglucosaminidase isolated from lysostaphin,[23] for 12 h at 37°C, pH 8.0. Undissolved material was removed by centrifugation and the supernatant further digested with CEP. Thin-layer chromatography in system C gave a ninhydrin and silver nitrate positive spot with an R_F of 0.25. Preparative chromatography in system C, elution of the material at $R_F = 0.25$, and chromatography of a 3 N HCl hydrolysate (4 h at 95°C) showed two spots corresponding to standard glucosamine and muramic acid. Neither in thin-layer nor gas chromatography could any amino acid be observed in 6 N HCl hydrolysate (18 h at 105°C) of a corresponding eluate. The presence of an oligosaccharide, free of amino acids, thus suggests that the CEP contains amidase activity in accordance with the observed free *N*-terminal alanine in digests of all mucopeptides/cell wall (Table 6).

Test on glycyl-glycine splitting. Treatment of penta-glycine (0.15 mg/ml) with CEP (enzyme/substrate = 1/1 by vol., pH 8.0, 37°C for 12 h) was without effect as judged by free amino group determination and chromatography in system C.[14]

DISCUSSION

The selection of *Streptomyces albus* G gave colonies with increasing ability to produce bacteriolytic enzymes. However, the lytic activity of separate colonies varied after a number of successive selections. Without a continuous selection, the lytic activity of organisms from a colony with high activity diminished with time. This is in accordance with earlier observations on *Streptomyces albus* strains grown on nutrient broth.[7,11] Consequently, continuous selection was found necessary to keep a stable lytic population, although this includes a risk of variation in the strain during the study. The experiment with precultures on peptone medium showed that a selected population rapidly loses its bacteriolytic activity on peptone medium. A direct inoculation from mucopeptide medium therefore seems necessary to obtain maximal lytic activity of the culture. The production of lytic enzymes apparently varied with type of peptone, this phenomenon not being explicable on the basis of the peptone composition.

The strain selections were performed on medium with mucopeptide from *S. aureus* Copenhagen, but the resultant CEP (Crude enzyme preparation) also lysed all the other test-bacteria. The lytic activity, however, varied considerably from one type of bacteria to another. This may be due to differences in mucopeptide structures, chemistry and/or thickness, and secondary effects of other com-

ponents present.[2,8,24] Variations in the lytic spectrum of various cultures were not observed. Although the total activity varied, the ratios between activities on the different bacteria seemed nearly constant. This indicates that the production of lytic enzymes directed against different bacteria is intimately correlated. Of the characterized Streptomyces strains, only *Streptomyces albus* G [6,11] is found to lyse all the test bacteria used in this study. In contrast to earlier observations on *Streptomyces albus* G [25] and *Streptomyces* S-35,[26] that lytic activity of the culture supernatant parallelled the amount of mycelia, the lytic activity in the present experiments appeared at the end of the exponential growth-phase. This may indicate that the lytic activity is due to endoenzymes released by autolysis.

The mucopeptide exhibits the mechanical strength of cell wall structures, and enzymatic hydrolysis of either the glycan chain or the peptide subunits (*cf.* Ref. 1) will usually cause lysis of the cell. The prepared mucopeptides/cell wall seemed pure and apparently suitable as substrates for enzymes as far as component composition and molar proportions are concerned (Tables 1 and 2). Except for a high content of alanine and small amounts of glycine in the *M. lysodeikticus* mucopeptide, the results of analyses of the other preparations agree fairly well with earlier reports. However, insoluble materials like these must be heterogeneous with respect to aggregation, content of natural free groups and groups set free during preparation and treatment.[2] Solubilization by enzymes [12] may reveal free groups not susceptible for reaction with reagents before digestion. The complex and macromolecular structure makes a complete masking of groups impossible.[14] In spite of existent uncertainty, however, some suggestions as to the enzyme specificities of *Streptomyces albus* G culture may be made.

N-Acetylhexosaminidase, amidase, and endopeptidase activities have clearly been demonstrated, the latter activity most probably being composed of several enzymes. Since only muramic acid was found to be reduced by $NaBH_4$, the presence of only one hexosaminidase is likely, *i.e.* *N*-acetyl-muramidase. *M. conglomeratus* 84 has a high content of alanine [13] and is comparable with *M. roseus* Thr− [27,14] having an alanine tripeptide bridge between the terminal D-alanine of one tetrapeptide chain and ε-lysine of an adjacent tetrapeptide. According to the results of digestion with CEP, followed by DNP-tracing, two linkages, D-alanyl-L-alanine and L-alanyl-L-alanine, may have been hydrolyzed. This corresponds to the activities of the earlier observed SA and MR endopeptidases.[14,22] Also *Streptococcus pyogenes*, found to have an alanine dipeptide bridge,[28] is lysed by the same endo-peptidases as *M. roseus*.[1]

The lack of activity of CEP on a pentaglycine preparation and the absence of detectable ε-DNP-lysine on examination of *S. aureus* mucopeptide digest indicate that, in the lysis of *S. aureus*, peptidase activity is mostly located to D-alanyl-glycine linkages.[14,24] The tetrapeptides of *B. megaterium* KM mucopeptide contain diaminopimelic acid (DAP) instead of lysine and a direct linkage between D-alanine in one peptide to DAP in another is proposed.[29,30] This linkage is apparently broken on digestion with CEP (Table 6), indicating the presence of the KM-endopeptidase activity,[22,31] which has also been demonstrated in the culture filtrate of *Streptomyces* L_3.[32]

In *Planococcus* 2389 mucopeptide the linkage between tetrapeptides is shown to be effected through D-glutamic acid (D-alanyl-γ-D-glutamyl-ε-lysine).[33] Results of DNP-tracing indicate that the D-alanyl-γ-D-glutamyl linkage is broken by digestion with CEP. This linkage has been found to be split by the KM-endopeptidase of *Streptomyces albus* G.[31]

Mucopeptides of both *M. lysodeikticus* NCTC 2665 [14,34] and *Sarcina lutea* [35] are found to contain D-alanyl-ε-lysine linkages. Increase of DNP-ε-lysine after treatment with CEP therefore, indicates splitting of the D-alanyl-ε-lysine bridge and an activity corresponding to the ML endopeptidase.[14,22] The structure of mucopeptide of *M. luteus* 144 has not been revealed, but analyses indicate a structure similar to that of *M. lysodeikticus*. However, mucopeptide from *M. luteus* ATCC 398 has been found to contain the amino acids alanine, glutamic acid, glycine, and lysine at a ratio of 2:2:1:1 and a cross-bridge of γ-L-glutamyl-glycine [36] is proposed. Accordingly the result of DNP-tracing may indicate that the CEP splits the glycyl-ε-lysine bond of this

bridge, an activity which to our knowledge has not previously been observed in *Streptomyces* cultures.

An attempt to separate the observed endopeptidase, *N*-acetylmuramidase, *N*-acetylmuramyl-L-alanine amidase, and caseinolytic activities will be reported in a subsequent paper.

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Studies on the Bacteriolytic Activity of *Streptomyces albus* Culture Filtrates. 2. Fractionation on Ion-exchange Chromatography and Gel Filtration

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Experiments in fractionation of the various lytic activities of *Streptomyces albus* cultures have been carried out using Amberlite IRC-50 and Carboxy methyl cellulose cation-exchangers, electrophoresis in agarose and polyacrylamide gel, and gel filtration on Sephadex. Apparently pure *N*-acetylmuramyl-L-alanine amidase and endo-*N*-acetylmuramidase were isolated on carboxy methyl cellulose columns, whereas all endo-peptidase activities, and one caseinolytic activity were eluted together. Neither on electrophoresis nor gel filtration were the latter activities separated. These results, in addition to optimal pH and salt concentration, indicate that apparently different enzyme specificities had very similar molecular properties.

In a preceding paper [1] the study of strain selection, optimal conditions for enzyme production, and a screening of the enzyme specificities in the culture filtrates of *Streptomyces albus* G were described. Degradation of mucopeptide/cell wall releasing oligosaccharides with the C-1 position of muramic acid free, without detectable free reducing endgroup of glucosamine, showed the presence in the culture filtrates of an endo-*N*-acetylmuramidase. Amidase activity, *i.e.* *N*-acetylmuramyl-L-alanine amidase, was demonstrated by the release of oligosaccharides free of amino acids, and dinitrophenyl tracing of *N*-terminal amino acids of digests of mucopeptide/cell wall of various bacteria indicated the presence of several endopeptidases. In addition caseinolytic activity was demonstrated.

The present paper comprises the results of the attempts to separate highly purified, specific enzymes from the culture filtrate.

MATERIALS AND METHODS

Chemicals. Amberlite IRC-50 (H^+), analytical grade, and *N,N'*-methylene-bisacrylamide were obtained from British Drug Houses Ltd., Poole, Dorset, England; Carboxy methyl cellulose (CMC) cation exchanger (lot no. 39B-04701, medium mesh, 0.66 mequiv./g), cytochrome C and β-alanine from Sigma Chemical Co., Mo., U.S.A., and acrylamide from Eastman Organic Chemicals Department, Rochester, N.Y., U.S.A. Acrylic acid, polyethylene glycol (20 000) and 2,4-lutidine were purchased from Fluka AG, CH-9470 Buchs, Switzerland; agarose from Behringwerke AG, Marburg a.d. Lahn, BRD, Sephadex G-75 and blue dextran 2000 from Pharmacia, Uppsala, Sweden, and dialyzer tubing from A. H. Thomas Co., Philadelphia, Pa., U.S.A. Coomassie brilliant blue was obtained from Serva, D-6900 Heidelberg, BRD; *N,N,N',N'*-tetramethylethylene diamine, riboflavin, and ammonium persulphate for disc electrophoresis from Canalco, Rockville, Md., U.S.A., and Freund's complete adjuvant from Difco Laboratories, Detroit, Mich., U.S.A. Hen egg-white lysozyme (3× crystalline) was from Calbiochem. Inc., Los Angeles, Calif., U.S.A.; myoglobin from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England, and chymotrypsinogen from Boehringer, Mannheim, BRD. The other chemicals used were as described previously.[1]

The *organisms*, *Staphylococcus aureus* Copenhagen (Sa), *Streptococcus pyogenes* A (Sp), *Planococcus* 2389 (Pc), *Micrococcus luteus* 144 (M-144), *M. conglomeratus* 84 (M-84), *M. lysodeikticus* NCTC 2665 (Ml), *Sarcina lutea* (Sl) and

Bacillus megaterium KM (Bm), *media, growth* and *harvesting, mucopeptides/cell wall, thin-layer chromatography, tests for free groups, tests for bacteriolytic and caseinolytic activities* were as described or referred to in Ref. 1.

Absorption to and elution from Amberlite IRC-50.[1-3] A detailed description of the procedure has been given earlier,[1] and the dialyzed eluate (0.01 M Tris-HCl pH 8.0) is referred to as crude enzyme preparation (CEP). Experiments were also carried out in which washed, enzyme-loaded resin was transferred to columns and eluted with a salt-gradient (0 – 1 M NaCl) or stepwise with buffers of increasing pH (4.5 – 9.0). Concentration of enzyme solutions was obtained using polyethylene glycol in dialyzer tubing.

Fractionation on carboxymethyl cellulose.[2-5] CMC was pretreated with 0.2 M NaOH and 0.2 M HCl, washed in 0.05 M Tris-HCl, pH 8.0 to constant pH, equilibrated against 0.01 M Tris-HCl, pH 8.0, evacuated to remove air-bubbles, and then applied to a column (3.6 cm diam. × 3.0 cm) which was given a hydrostatic pressure of 20 cm. Enzyme solutions were applied to the column at a rate of 10 ml/h, then washed with 500 ml of 0.01 M Tris-HCl pH 8.0, the same buffer being used for elution arranged to give an NaCl-gradient from 0 to 0.2 M in 1 l elution buffer. The elution rate was 20 ml/h and 10 ml fractions were collected.

Gel filtration on Sephadex G-75. Sephadex G-75 in 0.01 M Tris-HCl, pH 8.0, containing 0.02 % Na-azide, was applied to a column (1.8 × 95 cm) and the void volume determined using blue dextran. After application of 2 ml of material, elution was performed with 0.01 M Tris-HCl pH 8.0 at a rate of 20 ml/h using a peristaltic pump (LKB Produkter, Stockholm, Sweden). Fractions of 5 ml were collected.

Apparent molecular weight was determined by the method of Andrews [6] using the Sephadex G-75 column described above and cytochrome C, egg-white lysozyme, myoglobin, and chymotrypsinogen as references.

Electrophoresis in agarose was performed according to the method of Scheidegger [7] using an LKB 6800 A immunoelectrophoretic apparatus (LKB Produkter).

Detection of bacteriolytic activity was attained by sandwiching approx. 0.5 mm of 1 % special agar Noble in 0.01 M Tris-HCl pH 8.0 containing 5 mg/ml of heat-killed bacteria, over the electrophoretic slides followed by incubation at 37°C.

Caseinolytic activity was similarly detected using 0.3 % casein in 1 % agar. After incubation the slides were placed in 5 % trichloroacetic acid to visualize undigested casein.

Polyacrylamide gel (disc) electrophoresis was carried out essentially according to the method of Ornstein [8] using two running systems: I as described by Broom [9] and II as described by Joice and Klemperer,[10] both previously used for separation of basic proteins. Ten µl of enzyme solutions dialyzed against the electrodic buffer (diluted 1/4) [11] and made up to 50 % in glycerol, were applied to the top of the gels. The equipment used was a Shandon disc electrophoresis apparatus (Shandon Scientific Company Ltd., London, England). The staining procedure was as described by Maurer.[12] Detection of bacteriolytic and caseinolytic activities in the gels were principally carried out as described above. The buffer used for gels run in system II was 0.05 M Tris-HCl pH 8.5. The gels were divided along the long axis and placed on agar containing heat-killd bacteria/casein.

Antisera. Rabbits were injected intramuscularly with enzyme solutions, 80 units/ml [1] dialyzed against 0.07 M sodium phosphate, pH 7.2, containing 0.15 M NaCl, mixed with an equal volume of Freund's complete adjuvant. Two injections (1 ml of enzyme solution/dose) were given 3 weeks apart and the animals were bled 2 weeks after the last injection. Sera were heated at 56°C for 30 min to inactivate complement, 1 % merthiolate was added as preservative (one drop/10 ml serum) and the sera were then stored at −25°C.

Serological techniques. Examinations for precipitating antibodies were performed by ring test [13] and double diffusion in agar.[14] Immunoelectrophoresis was carried out with an LKB apparatus as described by the manufacturer, and inhibition of bacteriolytic activity by serum was tested after incubation at 37°C for 2 h of 1/1 mixtures of serum, diluted in the phosphate-buffered saline, and enzyme in 0.01 M Tris-HCl pH 8.0.

RESULTS

Amberlite. The CEP (dialyzed eluate from the resin) was as previously [1] found to contain the same lytic spectrum as that of the culture filtrates. In spite of a low yield (approximately 50 % of the total bacteriolytic activity of the culture filtrate) the enzymes of large volumes were rapidly isolated in relatively small volumes. At the same time a 100 fold purification was obtained [1] in relation to the absorbance at 280 nm. The fractionation experiments using salt-gradient or stepwise elution with increasing pH were both unsuccessful. The lytic material was in both cases released in a large elution volume without detectable separation of different activities. All further fractionation experiments were carried out on CEP.

CM-cellulose. Some caseinolytic but no bacteriolytic activity was observed in the washings. Every second fraction of the eluate

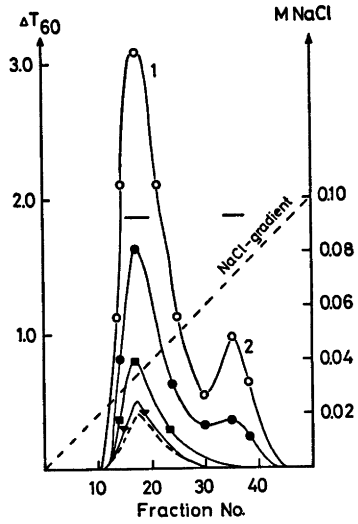

Fig. 1. Lytic activity in fractions from the CMC-column, eluted with NaCl-gradient, as tested on heat-killed cells of Bm (○), Sa (●), Ml and Sl (■), M-84 and M-144 (▼), and on Sp (- - -). ΔT_{60} (reduction in turbidity after 60 min) = $(\Delta T_t/t) \times 60$.

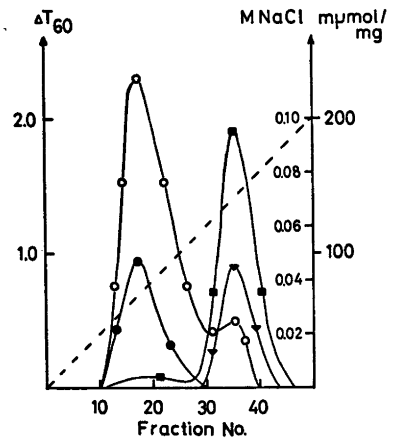

Fig. 2. Fractions from the CMC-column, eluted with NaCl-gradient (- - -), showing caseinolytic activity (●), increase in free amino groups (○) tested on Sa mucopeptide, and increase in reducing groups (■) and *N*-acetylamino sugars (▼) tested on Bm cell wall. ΔT_{60}: See legend Fig. 1.

was tested for bacteriolytic activity on heat-killed bacteria, caseinolytic activity and increase of free groups after digestion of Sa-mucopeptide and Bm-cell wall (Figs. 1 and 2). Two peaks with lytic activity were revealed; one (1) with lytic activity on all the test bacteria, the other (2) only for Sa and Bm when incubation time was 90 min. Longer incubation time showed trace of lysis on the other bacteria as well. Fractions were bulked as indicated by the horizontal lines in Fig. 1. The caseinolytic activity was eluted in one peak (Fig. 2) which overlapped peak 1. Tests on ethylene-oxide treated Sa-mucopeptide and Bm-cell wall showed the same elution profile. Fractions increasing the amount of free amino groups corresponded to peak 1, whereas fractions increasing the amount of reducing groups and of free *N*-acetylamino sugars corresponded to peak 2. The fractions showed no absorbance at 280 nm.

The occurrence and degree of activity of peak 2 varied considerably from one culture to another. When peak 2 did not appear, digestion of mucopeptide/cell wall with culture filtrate showed no increase in reducing groups or *N*-acetylamino sugars. The yield of bacteriolytic and caseinolytic activities was 75–

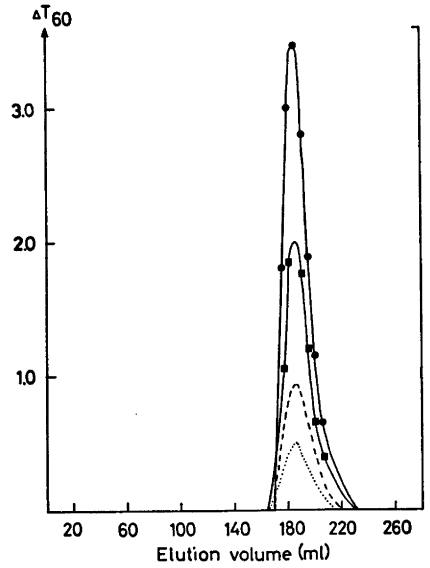

Fig. 3. Lytic activity in eluate from Sephadex G-75 on heat-killed cells of Bm (●), Sa (■), Ml and Sl (- - -), M-84, M-144 and Sp (···). Each fraction was 5 ml. ΔT_{60}: See legend Fig. 1.

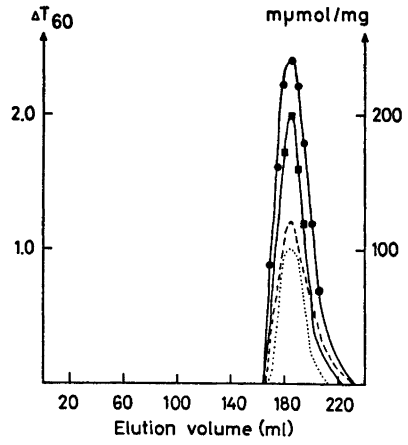

Fig. 4. Fractions (5 ml) from Sephadex G-75 column showing caseinolytic activity (- - -), increase in free amino groups (●) tested on Sa mucopeptide, and increase in reducing groups (■) and *N*-acetylamino sugars (···) as tested on Bm cell wall. ΔT_{60}: See legend Fig. 1.

80 % and about 30 %, respectively. Other dimensions of column or changes in the buffer system and salt gradient did not give better separation of different activities or the two peaks.

Sephadex G-75. Figs. 3 and 4 show the result of filtration on Sephadex G-75 of 10 ml CEP which had been concentrated to 2 ml following dialysis against 0.01 M Tris-HCl pH 8.0. All bacteriolytic and caseinolytic activity was eluted in one peak, the fractions of which also increased the amount of free amino groups, reducing groups and *N*-acetylamino sugars upon digestion of ethylene-oxide treated Sa-mucopeptide and Bm-cell wall. No absorbance was detected at 280 nm. Apparent molecular weight of the material in the peak was calculated to be 16 000.

Maximal release of free groups by various enzyme fractions. The increase in the amount of free amino groups, reducing groups, and *N*-acetylamino sugars of ethylene-oxide treated mucopeptide/cell wall after digestion with CEP, CMC-peak 1 and CMC-peak 2, is given in Table 1. All enzyme solutions were concentrated to about 30 units/ml and dialyzed before incubation with substrate until constant values were observed. All the three enzyme solutions gave a 100 % reduction in turbidity of all mucopeptide/cell wall preparations. In comparison egg-white lysozyme gave about 80 % reduction in turbidity under the conditions employed. It appears from Table 1 that there is a general increase in free amino groups in all digests, but that the effect of the CEP is markedly higher than that of the two other enzyme solutions. The CEP and CMC-peak 2 showed a significant increase in the amount of reducing groups and *N*-acetylamino sugars, whereas only a very small increase was observed in these after treatment with CMC-peak 1.

Specific activities. Dinitrophenyl-tracing of ethylene-oxide treated mucopeptide/cell wall digested with CMC-peak 1 revealed a significant increase of DNP-alanine only in M-84 (Table 2). The digests of M-144 and Ml mucopeptides contained DNP-ε-lysine, Sa mucopeptide DNP-glycine, Pc mucopeptide DNP-glutamic acid and Bm cell wall contained DNP-diaminopimelic acid. This indicates the presence of the same peptidase activities as demonstrated in culture filtrates,[1] whereas the amidase seems to be absent. Reduction with $NaBH_4$, hydrolysis

Table 1. Maximal increase (mμmol/mg) of free amino groups (DNP), reducing groups (P-J) and *N*-acetylamino sugars (M-E) after digestion of mucopeptide/cell wall with crude enzyme preparation (CEP), CMC-peak 1 and CMC-peak 2.

Substrate	CEP			CMC-peak 1			CMC-peak 2		
	DNP	P-J	M-E	DNP	P-J	M-E	DNP	P-J	M-E
M-84 mucopeptide	400	200	85	240	20	0	150	200	80
M-144 »	120	90	40	80	0	0	70	80	30
Ml »	110	80	20	75	0	0	70	80	25
Sa »	450	220	90	260	30	0	200	210	85
Pc »	310	150	70	210	35	0	150	145	75
Bm cell wall	200	230	100	50	25	0	35	210	100

Table 2. Dinitrophenyl(DNP)-amino acids detected chromatographically after treatment of mucopeptides/cell wall with CMC-peak 1.

	Mucopeptide [a]					Cell wall [a]
	M-84	M-144	Ml	Sa	Pc	Bm
DNP-Ala	+++	−	−	(+)	+	−
DNP-Gly	−	−	−	+++	−	−
DNP-Glu A	−	−	−	−	+++	−
DNP-ε-Lys	−	+++	+++	−	−	−
DNP-DAP	−	−	−	−	−	++

[a] (+) to +++: weak to strong colour reaction.

and chromatography of mucopeptide digested with CMC-peak 2 revealed appearance of muraminitol and disappearance of muramic acid, contrary to the findings with non-reduced digest. This shows that the muramidase, previously demonstrated in the culture filtrate,[1] is separated from the major portion of peptidases on CMC and located to peak 2. Search for the amidase activity observed in the culture filtrate [1] showed that, under the conditions described, the amidase was not bound to the CMC-column and could be obtained from the effluent.

Electrophoresis in agarose. Samples of culture filtrate, CEP, and CMC-peak 1, all concentrated to approximately 40 units/ml, were subjected to electrophoresis in agarose at pH 8.6 and 250 V for 120 min. Lytic activity was tested on all test bacteria and casein, a representative picture being shown in Fig. 5. The bacteriolytic activity seemed electrophoretically homogeneous, showing activity on all test bacteria in the same spot, and identical mobility of this activity of both culture filtrate, CEP, and CMC-peak 1. Several spots with caseinolytic activity were revealed in CEP (Fig. 5A) and culture filtrate, whereas only one caseinolytic spot, having identical mobility with that of bacteriolytic activity, was demonstrated with CMC-peak 1.

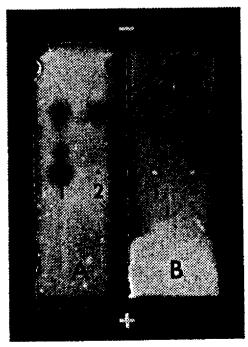

Fig. 5. Electrophoresis in agarose, pH 8.6, 250 V, 120 min, of CEP (1) and CMC-peak 1 (2) tested for caseinolytic (A) and bacteriolytic activity (B).

Disc electrophoresis. Samples of CEP, CMC-peak 1, and a 1/1 mixture of CMC-peaks 1 and 2, all concentrated to approximately 40 units/ml and dialyzed, were run at pH 8.3 and pH 4.3. For all enzyme solutions only one active area, having lytic activity on all test bacteria and casein, was observed. Fig. 6 shows the position of caseinolytic activity (A), bacteriolytic activity (B) and stained lysozyme (C) after electrophoresis in systems I and II.

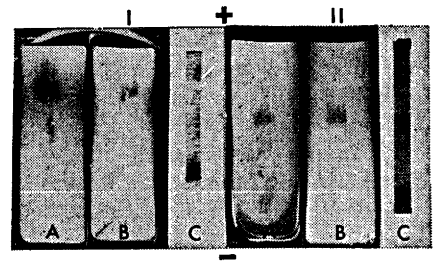

Fig. 6. Mobility of caseinolytic (A) and bacteriolytic (B) activity on (I) disc electrophoresis in system I (1 ma/tube for 10 min + 3 ma/tube for 50 min) and (II) system II (50 V for 10 min + 150 V for 50 min). Egg-white lysozyme (C) was included for comparison and visualized by staining. In system I, lysozyme was first run for 10 min with 1 ma/tube then for 25 min with 3 ma/tube.

Immunology. In contrast to the preimmune sera, rabbit antisera prepared against CEP and

CMC-peak 1 contained precipitating antibodies against the enzyme solutions. Double diffusion in agar using undiluted sera and enzyme solutions concentrated to about 40 units/ml, showed reaction of identity between CEP and CMC-peak 1 with only one detectable precipitin line. A comparison between bacteriolytic activity (Fig. 7A) and serological activity (Fig. 7B) after immunoelectrophoresis (250 V, 120 min) of CEP and CMC-peak 1 (40 units/ml), showed that the electrophoretic movement of the antigen responsible for the precipitin line was less than that of lytic activity. Experiments in inhibition of lytic activity by sera showed that both immune sera and preimmune sera gave complete inhibition, the immune sera being most effective, *i.e.* inhibiting at the highest dilution.

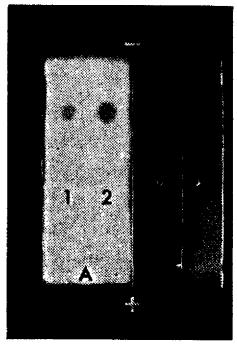

Fig. 7. Immunoelectrophoresis (250 V for 120 min) of CEP (1) and CMC-peak 1 (2) following tests for bacteriolytic (A) and serological (B) activities.

Kinetics. Measurements of the decrease in turbidity at 600 nm of suspensions of heat-killed bacteria and enzyme solutions as a function of time showed a linearity for all enzyme preparations and all test bacteria until approximately 65 % reduction. Thereafter the curves became flatter.

Serial dilutions (in 0.01 M Tris-HCl pH 8.0) of the enzyme preparations all showed the same relative reduction in activity on all test bacteria. Fig. 8 illustrates the results of CEP tested on heat-killed Sa and Bm cells.

The influence of variation in pH on the lytic activity of CEP and CMC-peak 1 was tested between pH 3.5 and 9.5 using Na-acetate (pH 3.5 – 5.5), Na-phosphate (pH 5.5 – 7.5) and Tris-HCl (pH 7.5 – 9.5) buffers. Heat-killed bacteria (1 mg/ml of water), buffer (0.04 M) and enzyme solution (dialyzed against 5 mM Tris-HCl pH 7.5) were mixed at a ratio of 2:3:1. All enzyme solutions showed maximal activity on all test bacteria at a pH of approximately 8.5.

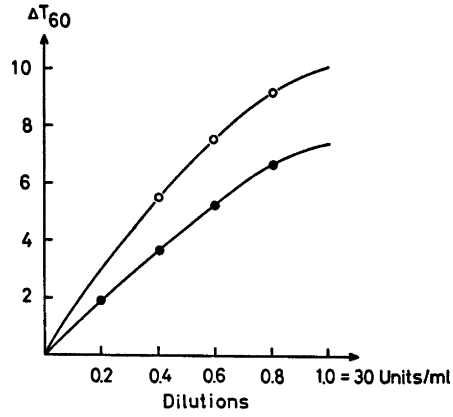

Fig. 8. Lytic activity of dilutions of CEP on heat-killed Bm (O) and Sa (●) bacteria. ΔT_{60}: See legend Fig. 1.

Lytic activity on heat-killed bacteria of both CEP and CMC-peak 1 in 0.01 M Tris-HCl pH 8.0, showed maximum at a NaCl concentration of approximately 0.01 M. Higher concentrations of NaCl inhibited the lytic activity, which was nearly zero at 0.16 M NaCl. The same conditions, pH 8.5 and 0.01 M NaCl, were also found to be optimal for digestion of mucopeptides.

The bacteriolytic activity of CEP or CMC-peak 1, tested on all bacteria in 0.01 M Tris-HCl pH 8.0, was not influenced by the presence of casein (0.5 mg/ml).

Stability. Enzyme solutions stored at 4°C in 0.01 M Tris-HCl pH 8.0, 0.02 % Na-azide, showed a high degree of stability. After one month about 5 % loss in lytic activity was observed. The enzymatic activities were also stable at −25°C.

DISCUSSION

The crude enzyme preparation (CEP) used in the present fractionation experiments corre-

sponds to the materials in other studies of *Streptomyces albus* G.[2-5] The bacteriolytic activity observed was completely bound to CM-cellulose, and fractionation on columns usually gave two peaks with lytic activity (Fig. 1). The material of peak 1, starting elution at 0.02 M NaCl and thus being weakly bound, contained practically all the endopeptidase activities. Digestion of mucopeptides/cell wall increased the amount of free amino groups, and the ratio between the degree of activity on the different test bacteria was approximately the same as that of CEP. Removal of the activity in CMC-peak 2 thus seemed to have little influence on the total lytic activity. Under the conditions imposed, peak 2 lysed mainly *B. megaterium* and *S. aureus*, and varied quantitatively from one culture to another. The material (peak 2) lysed mucopeptides/cell wall and increased the amount of free reducing groups and *N*-acetyl-amino sugars, whereas the increase of free amino groups was very small, the accessibility of these groups for reaction with FDNB probably being due to solubilization. This indicates that the activity of peak 2 is mostly dominated by an endo-*N*-acetylhexosaminidase which is shown to be an endo-*N*-acetylmuramidase. The properties of this agree well with those of the F_1-endo-*N*-acetylmuramidase [4] which also showed a somewhat stronger adsorption to CM-cellulose than the endopeptidases.

Optimal pH of the F_1-enzyme for lysis of different bacteria has been found to vary considerably (pH 3.5 – 8.0).[4] This may explain the difference in lytic activity and in the release of reducing groups on testing different bacteria with fractions of CMC-peak 2 and other solutions supposed to contain muramidase activity.

Mixtures of certain lytic activities from *Streptomyces albus* G have been found to act synergistically,[15,16] and the activity peaks of eluates from CMC-columns may therefore be due to favourable mixtures of enzymes and not to real quantities. Due to the low protein content indicated spectrophotometrically (280 nm), this was difficult to determine.

The *N*-acetylmuramyl-L-alanine amidase demonstrated in the CEP [1] was not adsorbed to CM-cellulose under the conditions imposed. This was also found in earlier studies.[2,3] The non-adsorbed material, containing the amidase, showed no lytic activity, thus suggesting, in agreement with an earlier supposition,[17] that the amidase is non-lytic on whole bacteria.

Electrophoresis in agarose and polyacrylamide and also gel filtration on Sephadex showed only one zone of bacteriolytic activity, indicating that the molecular properties of the apparent different enzyme specificities are very similar. Separation of several activities of *Streptomyces* culture filtrates on CMC-columns has been reported.[2-5] However, although all endo-peptidases reported, including MR, SA, ML, and KM, also seemed to be present in the CEP,[1] no separation was obtained.

Several caseinolytic enzymes were present in the CEP and could be separated on electrophoresis in agarose and by adsorption to CMC. The caseinolytic activity bound to CMC was eluted together with bacteriolytic activity in peak 1, these activities being also associated after electrophoresis and gel filtration. This suggests the possibility that the same enzyme might have bacteriolytic and caseinolytic activity, but casein in the test-solution did not seem to influence the degree of bacteriolysis. Caseinolytic activity has previously been found in association with the MR endo-peptidase,[2,3]

Most bacteriolytic enzymes isolated have been found to be basic proteins with a high isoelectric point.[2,3,18] The results of electrophoresis in gel in the present experiments also indicated basic proteins with pI-values above 8.6. According to the apparent molecular weight (16 000) it is reasonable to expect that the observed difference between the lytic enzymes and egg-white lysozyme (pI 11.0, Mw 14 000) [19] in electrophoretic mobility on polyacrylamide gel (Fig. 6) is due to difference in charge. On the other hand, due to the basic character of the enzymes, a possible retardation on Sephadex which influences the molecular weight determination,[20] cannot be excluded.

With a few exceptions,[4,21] all lytic enzymes previously isolated from *Streptomyces albus* G have been shown to have pH optima in the same range as found in this study,[15,16,22] and the low optimal salt-concentration is also in accordance with earlier findings.

The immunoelectrophoretic experiment showed that the material responsible for precipitation with antiserum in agar was different from that with lytic activities. However, this antigenic material does not need to be present

in high amounts since the ability of proteins to evoke immunological response may vary considerably.[23] Although the sera showed high activity of non-specific inhibitors,[24,25] the inhibition studies may suggest that the antisera contained specific inhibiting antibodies.

The reported results of fractionation studies on the lytic activities of *Streptomyces* cultures show great diversity, to some degree probably due to treatment and selection of the strain for enzyme production. In the present experiments fractionation on CMC-columns separated the CEP into three portions: (1) the material not adsorbed, containing non-lytic *N*-acetylmuramyl-L-alanine amidase, (2) CMC-peak 1 containing endo-peptidases, free of detectable amidase and hexosaminidase activities, and (3) CMC-peak 2, consisting mainly of endo-*N*-acetylmuramidase.

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Thermodynamics of Vinyl Ethers. VII.* The Relative Stabilities of 1,4-Dimethoxy-1,4-cyclohexadiene and 1,4-Dimethoxy-1,3-cyclohexadiene

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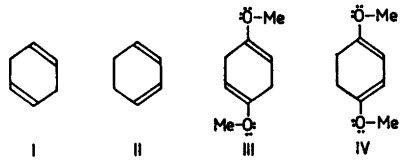
The relative stabilities of 1,4-dimethoxy-1,4-cyclohexadiene and 1,4-dimethoxy-1,3-cyclohexadiene have been determined at various temperatures between 273.2 and 422.2 K by means of chemical equilibration. In cyclohexane solution at 298.15 K, the following values were obtained for the changes of the thermodynamic functions on going from the former to the latter: $\Delta G^\circ = -2.061 \pm 0.014$ kJ mol^{-1}, $\Delta H^\circ = -0.17 \pm 0.18$ kJ mol^{-1}, $\Delta S^\circ = 6.4 \pm 0.6$ J K^{-1} mol^{-1} and $\Delta C_p^\circ = -9.2 \pm 4.2$ J K^{-1} mol^{-1}. In the ideal gas state at 298.15 K, the values of ΔH° and ΔS° were calculated to be -1.14 ± 0.35 kJ mol^{-1} and 5.5 ± 0.8 J K^{-1} mol^{-1}, respectively.

From the thermodynamic point of view, the relative stabilities of the isomeric carbocyclic dienes, 1,4-cyclohexadiene (I) and 1,3-cyclohexadiene (II), form an interesting subject of



I II III IV

investigation. The latter is a conjugated diene and thus it might be expected *a priori* that it should exhibit at least some of the enhanced thermodynamic stability characteristic of open-chain conjugated dienes, such as 1,3-butadiene. Against this background it seems surprising that in the liquid phase at 368 K, the equilibrium mixture of I and II contains only 68.9 % of the conjugated diene (II).[1] Thus, at equilibrium, II is favored over I by a factor of only 2.22, and moreover, if the effect of statistical factors on the equilibrium in question is considered, it is observed that the preponderance of II is mainly due to the fact that II is statistically favored over I by a factor of 2. Hence the equilibrium constant for the reaction I⇌II should be written as 1.11 if the effect of statistical factors on the position of equilibrium is eliminated. Accordingly, the Gibbs free energy difference between the isomers I and II is practically zero. As to the enthalpy difference, the enthalpy of hydrogenation (acetic acid, 298.15 K) of I to cyclohexane is reported [2,3] to be *ca.* 1.3 kJ mol^{-1} more negative than that of II, which shows that the enthalpy of the conjugated diene is only 1.3 kJ mol^{-1} lower than that of the unconjugated diene (assuming, of course, that I and II are solvated to the same extent in acetic acid). Thus it appears that either conjugation is weak in 1,3-cyclohexadiene or it is much more strained than I. As a continuation of this author's investigations on the effect of structural factors on the stability of vinyl ethers it was of interest to find out the effect of substitution of methoxy groups for the 1,4-hydrogen atoms of I and II on the relative stabilities of the conjugated and nonconjugated diene. Hence the isomeric vinyl ethers 1,4-dimethoxy-1,4-cyclohexadiene (III) and 1,4-dimethoxy-1,3-cyclohexadiene (IV) were prepared and thermodynamics of their interconversion were studied.

* Part VI: Taskinen, E. and Mäkinen, A. *Acta Chem. Scand. B 28* (1974) 121.

EXPERIMENTAL

Materials. A 76 % yield of a mixture of III and IV was obtained when 0.089 mol of 1,4-cyclohexanedione was treated with 0.196 mol of trimethyl orthoformate in methanol.[4] The cleavage of the tetramethyl acetal of 1,4-cyclohexanedione to methanol and the isomeric vinyl ethers was effected by fractionation from a small amount of *p*-toluenesulfonic acid. The boiling temperature of the product containing 79 mol % of IV was 474–475 K/101.8 kPa.

NMR spectra (*ca.* 20 % solutions in CCl_4 at 306 K). III: τ 5.49 (C=C–H), 6.50 (O–CH_3), 7.25 (–CH_2–); IV: τ 5.26 (C=C–H), 6.50 (O–CH_3), 7.70 (–CH_2–).

Determination of normal boiling points.[5] Reference curve (compound, normal boiling point, relative retention time): 1-methoxycyclopentene, 387.0 K, 0.245; 1-ethoxycyclopentene, 409.0 K, 0.298; 1-methoxycyclohexene, 417.1 K, 0.347; 1-ethoxycyclohexene, 435.0 K, 0.433; 1-propoxycyclohexene, 455.9 K, 0.608; 1-cyclopentoxycyclopentene, 479.7 K, 1.000. The relative retention times of III and IV were 0.878 and 0.773 leading to normal boiling points of 475.2 and 469.2 K, respectively.

Procedure. The performance of the equilibrations has been described previously.[5] Cyclohexane was used as solvent and iodine as catalyst. At each temperature, the position of equilibrium was approached from both directions. One of the initial mixtures of III and IV consisted of 79 mol % of IV (the crude synthetic product) and the other 55 mol % of IV (obtained by fractionation of the crude synthetic product). As the equilibrium mixtures contained *ca.* 67 to 70 mol % of IV, the position of equilibrium could thus be approached from both directions. The column used in the GLC analyses was a 4 m column containing 10 % Carbowax 1500 on Chromosorb G. The compounds were eluted in the order IV, III. The areas of the peaks were integrated by means of a Hewlett-Packard 3370 B integrator.

RESULTS

The values of the mean equilibrium constant K and its standard error at various temperatures are shown in Table 1. As it turned out that the values of $\Delta H°$ and $\Delta S°$ of the reaction in question do not remain constant over the range of temperature covered by the measurements, the following equation [6] was fitted to the values of K:

$$\ln K = A + B/T + C \ln T \qquad (1)$$

This equation requires that $\Delta H°$ varies linearly as T ($\Delta C_p° = \text{constant}$). The fitting was performed on an IBM 1130 computer. The following results were obtained for the isomerization of III to IV in cyclohexane at 298.15 K:

Table 1. Values of the mean equilibrium constant K and its standard error for the iodine-catalyzed reaction 1,4-dimethoxy-1,4-cyclohexadiene ⇌ 1,4-dimethoxy-1,3-cyclohexadiene in cyclohexane solution at various temperatures. Substrate concentration 0.2 mol dm^{-3}, catalyst concentration 0.008 mol dm^{-3}.

T/K	K
273.2	2.281 ± 0.012
283.2	2.317 ± 0.017
294.5	2.317 ± 0.011
308.2	2.295 ± 0.002
323.7	2.267 ± 0.008
345.2	2.252 ± 0.004
355.2	2.215 ± 0.007
373.2	2.218 ± 0.011
400.2	2.161 ± 0.009
422.2	2.123 ± 0.013

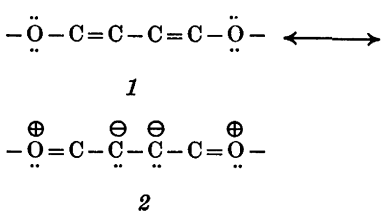
$\Delta G° = -2.061 \pm 0.014$ kJ mol^{-1}
$\Delta H° = -0.17 \pm 0.18$ kJ mol^{-1}
$\Delta S° = 6.4 \pm 0.6$ J K^{-1} mol^{-1}
$\Delta C_p° = -9.2 \pm 4.2$ J K^{-1} mol^{-1}

(the errors are twice the standard errors). The values of $\Delta H°$ and $\Delta S°$ in the ideal gas state at 298.15 K were calculated to be -1.14 ± 0.35 kJ mol^{-1} and 5.5 ± 0.8 J K^{-1} mol^{-1}, respectively, by means of the equations for the standard enthalpy and standard entropy of vaporization at 298.15 K derived in Ref. 5.

DISCUSSION

The enthalpy of 1,3-cyclohexadiene (II) is reported to be *ca.* 1.3 kJ mol^{-1} lower than that of 1,4-cyclohexadiene (I) in acetic acid at 298.15 K, and if the boiling points of I and II are taken as 358.8 and 353.7 K, respectively,[7] the enthalpy of isomerization of I to II is calculated [5] to be about -2.1 kJ mol^{-1} in the ideal gas state at 298.15 K. As the enthalpy change for III→IV is about -1.14 kJ mol^{-1} under the same external conditions, the values of $\Delta H°$ for I→II and III→IV are equal within experimental error. Thus the substitution of methoxy groups for the 1,4-hydrogen atoms of I and II has no observable effect on the enthalpy difference

between the conjugated and nonconjugated diene. This shows that although there is, in principle, a long conjugated system in IV (−O−C=C−C=C−O−), the stabilization brought about by this system is no greater than the stabilization due to the two ordinary vinyl ether functions (O−C=C) in III together. This is understandable, since the resonance structure *2* of the conjugated system in IV shows that an enhanced resonance would lead to a transfer of negative charge at adjacent carbon atoms, which causes structure *2* to be of relatively high energy.



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On the other hand, it was shown in a previous part of this series [8] that electropositive (electron-donating) alkyl groups when attached to the β-carbon of the ethylenic linkage of a vinyl ether tend to oppose the resonance between the lone-pair electrons of the oxygen atom and the π-electrons of the double bond, owing to their inductive effect. In III, there is an alkyl group formed by the ring carbon atoms attached to the β-carbon of each double bond, and thus the extent of resonance stabilization in III is not so pronounced as in vinyl ethers with no β-alkyl substituents. Hence, as the experimental results show, the methoxy groups of III and IV cause a similar stabilization in each compound.

The entropy difference between I and II may be estimated as follows. Assuming that the enthalpy of isomerization of I to II is −1.3 kJ mol^{-1} in the liquid phase at 368 K at which temperature the equilibrium constant for I→II is reported to be 2.22 (see introduction), the entropy change is *ca.* 3.1 J K^{-1} mol^{-1} in the liquid phase. If the value of $\Delta S°$ is assumed to be independent of temperature and if it is corrected to correspond to the ideal gas state,[5] the entropy change in I→II is obtained as about 2.0 J K^{-1} mol^{-1} at 298.15 K. However, II is statistically favored over I by a factor of 2 and thus a term $R \ln 2 = 5.8$ J K^{-1} mol^{-1} should be subtracted from the observed entropy change to eliminate the effect of statistical factors on $\Delta S°$. After this correction, the value of $\Delta S°$ (g, 298.15 K) for I→II is obtained to be about −3.8 J K^{-1} mol^{-1}. This is not far from the value −5.9 J K^{-1} mol^{-1}, calculated from the intrinsic entropies of the above compounds, which have been reported by O'Neal and Benson.[9] Hence there is a difference of *ca.* 9.3 J K^{-1} mol^{-1} in the experimental values of $\Delta S°$ (g, 298.15 K) for the reactions I→II and III→IV, although the entropy changes might be expected to be similar on account of apparently similar changes of structure in both reactions (the statistical factors of III and IV are equal). The reason for this difference is not clear, but it might be imagined that the methoxy groups are free to rotate about the O−C(sp^2) bond in the conjugated diene (IV) because of only a weak conjugation between the methoxy groups and the double bonds. This assumption seems, however, to be in disagreement with the previous discussion concerning the stabilizing effect of methoxy groups on III and IV, which was deduced to be equal in both cases.

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Carbohydrate-dependent Enzymic Quinone Reduction during Lignin Degradation

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Indications for the existence of a new enzyme were obtained when *Polyporus versicolor*, a white-rot fungus, was grown on lignin agar plates supplemented individually with glucose, cellobiose or cellulose. Formation of chromophoric structures occurred through the action of a polyphenol oxidase (laccase) but was effectively reversed when cellulose was present in the agar plates. Under these conditions the fungus produced an extracellular enzyme which, in the presence of cellobiose, reduced the stable quinone tested, namely 3-methoxy-5-*tert*-butylbenzoquinone-(1,2). The enzyme together with cellobiose inhibited phenolic coupling of guaiacol by laccase and although oxygen consumption proceded normally no polymeric products were formed.

It has long been known that the white-rot fungi readily degrade lignin in wood. Isolated lignin in submerged cultures seems, however, to be much more resistant to fungal attack. Under such conditions growth on lignin requires adaptation [1,2] to the polymer and even then it is questionable if depolymerization occurs to any large extent.

It seems likely that submerged cultures do not offer the right growth conditions when lignin is the carbon source, maybe due to insufficient localization of the necessary levels of a combination of several enzymes required for depolymerization. We therefore investigated the use of a solid medium with lignin in which the fungus could produce high local concentrations of enzymes and, hopefully, degrade lignin. To achieve conditions as similar to wood as possible, we also incorporated cellulose in the growth medium to observe if this could affect the lignin degradation. These experiments have led to the discovery of an enzyme, cellobiose: quinone oxidoreductase, which can reduce products of laccase oxidation and different quinones in a reaction in which cellobiose is required and simultaneously oxidized. The phenol-oxidizing enzyme laccase is induced in white-rot fungi both by lignin and simple phenols.[3] The precise role of laccase during lignin degradation is not yet elucidated.

A recent review [4] on lignin degradation concludes that whatever role phenol oxidases play, they can only be a part of the enzyme complex that catalyzes the complete decomposition of lignin.

This paper describes the experiments in which the quinone-reducing enzyme was found and the ability of the enzyme to stop phenolic coupling during laccase oxidation of guaiacol. An accompanying paper [5] describes the induction and some properties of the enzyme.

MATERIALS AND METHODS

Organism. Polyporus versicolor L. ex Fr. isolate Mad. was obtained from Prof. E. B. Cowling, North Carolina State University, Raleigh, North Carolina. USA.

Substrate. The lignin preparation used was a kraft lignin, Indulin AT (Westvaco, Polychemical Div., Charleston, West Virginia, USA). The lignin was dialyzed against a large volume of distilled water (2 × 20 l per 5 g Indulin AT for 20 h) in a dialysis bag with a pore size of 24 Å to remove low molecular weight compounds and impurities. Phosphoric acid swollen cellulose [6] was prepared from Munktell's cellulose powder No. 400.

Cultivation. A basal salt medium [7] was used with the supplement of evenly suspended lignin and different carbon sources and with 1.5 % Noble agar (Difco, Detroit, Mich., USA). Lignin was dissolved in a minimal volume of 0.1 M

KOH and filter-sterilized. The pH of the complete medium was adjusted to 5.6 with phosphoric acid. The plates were incubated at 25°C and 90 % relative humidity.

Enzyme. Laccase (O_2:*p*-diphenol oxidoreductase, E.C. 1.10.3.2) was obtained from *P. versicolor*.[8] The enzyme solution was concentrated to approx. 1/40 of the original volume by ultrafiltration (Amicon UM 10) and the laccase was precipitated with solid ammonium sulfate (90 % of saturation at 25°C). The precipitate was dissolved in a minimum volume of distilled water, dialyzed and partially purified on a Sephadex G-75 chromatography column.

Cellobiose-quinone oxidoreductase was prepared according to methods in the accompanying paper.[5]

Enzyme assays. Laccase activity was measured by following the production of colored material from guaiacol. The incubation mixture contained 25 μmol guaiacol, 50 μmol acetate buffer (pH 5.0) and the enzyme in a total volume of 5 ml. The color production was followed with a Klett colorimeter equipped with a blue filter ($\lambda = 400 - 465$) at 25°C.

The initial rate of formation of colored products was taken as a relative measurement of laccase activity.

Chemical determinations. Guaiacol was determined gas chromatographically with benzyl alcohol as an internal standard. Column: 10 % OV-1 on Chromosorb Q 100 – 120 mesh, 120°C. Retention times: guaiacol 8.9 min and benzyl alcohol 6.6 min.

Oxygen consumption was measured with an oxygen electrode (Rank Brothers, Bottisham, England) at 25°C.

Chemicals. The 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) was kindly supplied by Dr. Augustine E. Opara of this laboratory.

RESULTS

Coloration of lignin agar plates by P. versicolor. *P. versicolor* was cultivated on agar plates containing 1 g/l of lignin individually supplemented with 3 g/l of glucose, cellobiose, or swollen cellulose as indicated. Fig. 1 shows plates (5 day cultivation time), where the surface mycelium has been removed. A few mm in front of the mycelium there was a reddening of the lignin in all plates. However, in those plates which contained cellobiose and cellulose this coloration started to disappear in the area in contact with the outermost edge of the mycelium and reappeared under the oldest mycelium in the center. The plates with lignin and lignin plus glucose had an even red color over the entire surface. The red color most likely originated from phenol oxidase oxidation of lignin or its degradation products. To test for laccase activity, the plates were flooded with a solution of 0.01 M guaiacol which gives an intense red to black color with laccase. The lower series of plates in Fig. 1 shows the plates after this staining. Laccase activity was found in all areas which were visibly red. The decolored areas in plates with lignin and cellulose or cellobiose gave no laccase staining with guaiacol.

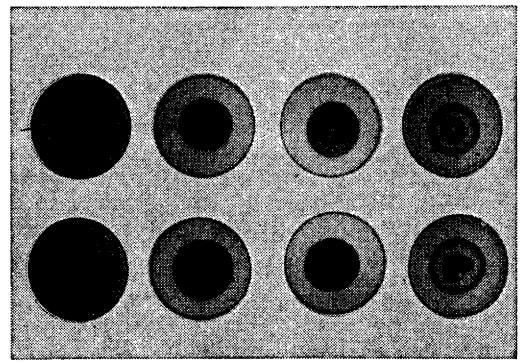

Fig. 1. P. versicolor grown on lignin agar plates supplemented with from left to right: nothing, glucose, cellobiose, and phosphoric acid swollen cellulose. The top row of plates shows the coloration caused by the fungus. The bottom row shows plates which have been flooded with guaiacol to stain for laccase activity.

Extraction of enzymes from lignin – cellulose agar plates. P. versicolor was grown for 5 days on plates containing lignin and cellulose. The surface mycelium was then removed and the plates were separated into three zones according to Fig. 2. Equal amounts of the three zones were extracted with 0.1 M acetate buffer (pH

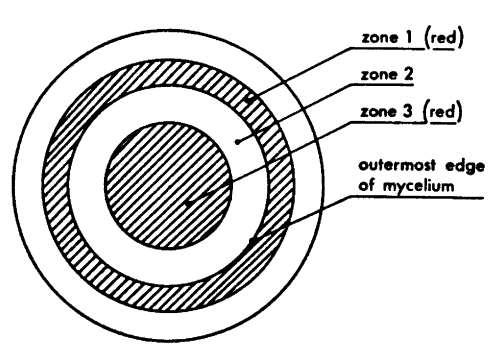

Fig. 2. Pattern of the lignin – cellulose agar plates used for extraction of enzymes from three zones.

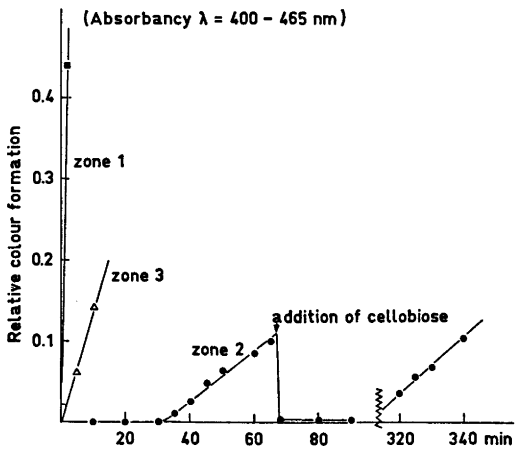

Fig. 3. Relative laccase activity in different zones of lignin – cellulose agar plates.

5.0) and centrifuged. Laccase activity was measured on the supernatants from the different zones. The results are shown in Fig. 3. The extract from zone 2 is of particular interest. Initially no color production from guaiacol was observed but after a while the color formation started and continued at a constant rate. If cellobiose was added, the color formation ceased and the colored products which had been formed disappeared. After a lag period depending upon the amount of cellobiose added, the formation of colored products was observed once more. It is obvious from these experiments that although laccase was present in zone 2, it did not react with guaiacol in the normal way. It is also clear that a reaction with cellobiose occurred in zone 2 and that this reaction inhibited the formation of colored products from laccase oxidation of guaiacol. If cellobiose was added to extracts from zone 3, a small decrease in the formation of colored products from guaiacol was found, but addition of cellobiose did not influence the laccase activity in extracts from zone 1.

Quinone reducing ability of the enzyme extracts from zone 2. The red products from laccase oxidation of both lignin or lignin degradation products and guaiacol are most likely due to quinoid structures.[9] To determine if the extract from zone 2 could reduce quinones, a ten-fold concentrated extract was incubated with a stable quinone, 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2). The quinone has an absorption maximum at 360 nm (acetate buffer, pH 5.0). The reduction of the quinone to the corresponding diphenol, was followed spectrophotometrically at this wave length. The enzyme extract from zone 2 could effectively reduce the quinone while boiled extract had no effect. It was also demonstrated that the reduction of the quinone was coupled to the oxidation of cellobiose. In an accompanying paper,[5] some properties of the enzyme from *C. lignorum* is described, and from these results the name cellobiose:quinone oxidoreductase is suggested. It appears likely that the cellobiose:quinone oxidoreductase caused the disappearence of the red color in zone 2 and also prevented the formation of colored products from guaiacol in this zone. The enzyme cannot use glucose which would explain why no decolored zones occurred in plates with lignin and glucose. The width of the zones may reflect both the amount of enzymes induced and the concentration of the carbohydrate co-substrate.

Inhibition of phenolic coupling by the quinone reducing enzyme. To study the effect of cellobiose:quinone oxidoreductase on the laccase oxidation of guaiacol, the concentration of guaiacol was followed by gas chromatography and laccase activity measured by following oxygen uptake. The reaction mixture contained 25 μmol guaiacol, 90 μmol cellobiose, 1 mmol acetate buffer (pH 5.0), 25 μmol benzyl alcohol (internal standard for gas liquid chromatography), laccase and cellobiose:quinone oxidoreductase in a total volume of 10 ml per sample. The control contained everything except cellobiose:quinone oxidoreductase. Samples and the controls were incubated at 25°C for different time periods after which the reaction mixture was extracted with 5 ml of ethyl acetate and the organic phase was analyzed for guaiacol and benzyl alcohol. Fig. 4 shows that all guaiacol in the control sample was polymerized in about 2 h. Under the same conditions less than 15 % of the guaiacol had been polymerized in the sample containing cellobiose:quinone oxidoreductase. Higher concentrations of oxidoreductase completely prevented the polymerization of guaiacol.

To determine if the laccase was inhibited by the solution of cellobiose:quinone oxidoreductase, the rate of oxygen consumption was

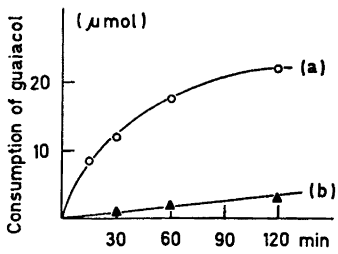

Fig. 4. Influence of cellobiose:quinone oxidoreductase on the consumption of guaiacol by laccase. The amount of guaiacol consumed is shown in (a) the control (laccase alone) and (b) sample containing both laccase and cellobiose: quinone oxidoreductase.

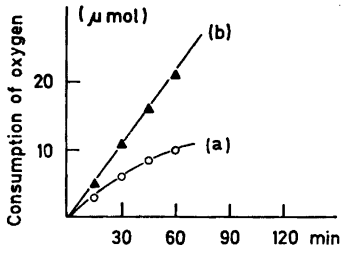

Fig. 5. Consumption of oxygen by laccase in the presence of cellobiose:quinone oxidoreductase. The samples are described in Fig. 4.

measured both in the sample and in the control. Fig. 5 shows that the initial rate of oxygen consumption was the same in both the sample and the control. The oxygen consumption in the control diminished with decreasing guaiacol concentration. In the sample containing cellobiose:quinone oxidoreductase the rate of oxygen consumption was constant. The oxygen consumption before addition of laccase was about the same for both the sample and the control and was subtracted from the readings. It is known that the preliminary product of laccase oxidation of phenols is the aryloxy radical and that the subsequent phenolic coupling is non-enzymatic.[10] Thus, the laccase as usual formed the guaiacoxy radicals but, instead of coupling, the radicals were reduced back to the phenolic state by the cellobiose:quinone oxidoreductase.

It is very likely that this is the same enzyme which reacts with quinones and with the laccase oxidation products. As described in the accompanying paper both of these hydrogen acceptors give the same oxidation product from cellobiose. Preliminary enzyme purification experiments with an ion exchanger and isoelectric focusing have not separated the activities.

DISCUSSION

The wood-degrading fungi are very specialized in the utilization of their substrate wood. In most cases, the white-rot fungi remove lignin and cellulose or hemicellulose simultaneously, perhaps implying that the enzymic degradation of the different wood components is linked. The cellobiose:quinone oxidoreductase requires both a carbohydrate and an oxidized phenol for its activity and both of these substrates are modified during the reaction. Thus, a study of the degradation of pure cellulose and pure lignin would probably not give the same products as from the degradation in wood.

When fungi are cultivated on lignin with or without added glucose they excrete large amounts of phenol oxidases which cause a polymerization of phenolic substances. The results in this paper show that in a more wood-like surrounding, quite different reactions can occur. The laccase functions as normal, but its initial products are effectively reduced back to the phenolic state and polymerization is prevented.

The ability of white-rot fungi to reduce quinones has been observed earlier.[11] In this case the white-rot fungus *Polystictus sanguineus* was cultivated on agar plates containing small amounts of phenols. The phenols were first oxidized to colored products (probably quinones) but this color began to disappear after ten or twelwe days. Agar plates containing preformed quinones were also slowly decolorized. It was suggested that this color change was due to a quinone reduction process.

Protocatechuate-3,4-oxygenase and catechol-1,2-oxygenase are the key enzymes in the catabolism of aromatic substances in most fungi.[12] It is known that these enzymes are inhibited by *o*- and *p*-benzoquinone.[13] If the lignin is degraded to simple phenols or quinones, it must be of importance for the fungus to keep the phenols in a reduced state to ensure the further breakdown of the aromatic nucleus. Cellobiose:quinone oxidoreductase can obviously serve this function.

Acknowledgement. We wish to thank Majlis Jansson for skilful technical assistance and Dr. A. Opara for supplying the quinone. We are also indebted to Dr. B. Lindgren and Dr. J. Gierer of this laboratory for stimulating discussions.

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Cellobiose:Quinone Oxidoreductase, a New Wood-degrading Enzyme from White-rot Fungi

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Production of an extracellular cellobiose: quinone oxidoreductase (cellobiose dehydrogenase) by *Polyporus versicolor* and *Chrysosporium lignorum** grown on cellulosic substrates was investigated. Highest yields of the enzyme occurred with powder cellulose as the carbon source. In *C. lignorum* development of oxidoreductase activity and cellulolytic enzymes were parallel.

The enzyme from *C. lignorum* was selected for further study. It was found to catalyze an oxidation – reduction reaction in which reduction of a quinone to the corresponding phenol was coupled to the conversion of cellobiose to cellobionic acid probably through cellobiono-δ-lactone. The enzyme has a high specificity for the oxidation of cellobiose. Lactose was oxidized at a slower rate but no other mono- or disaccharides tested served as substrate for the oxidoreductase. However, quinone requirement was less specific and the enzyme was able to reduce both *ortho* and *para* quinones. Optimum pH for oxidoreductase activity was approximately 4.5 – 5.0. An extracellular lactonase from *C. lignorum*, able to hydrolyze cellobiono-δ-lactone is also reported.

White-rot fungi utilize a number of extracellular hydrolytic enzymes for the degradation of cellulose.[1-3] In most studies of cellulose degradation, these enzymes are obtained by cultivation of fungi on pure cellulose. With cellulose and cellodextrins as substrates, the cellulolytic enzymes produce glucose and cellobiose as the final products of degradation. Results from these types of experiments are often extrapolated as description of the degradation of cellulose in wood. However, in this and in the accompanying paper,[4] we describe the occurrence of an extracellular enzyme which, in the presence of a suitable hydrogen acceptor, yields acidic sugars as breakdown products of cellulose. The enzyme can utilize reaction products of the phenol-oxidizing enzyme laccase and a suitable phenol, for example of lignin origin, as the hydrogen acceptor.

* Recent investigations have revealed that *C. lignorum* is identical with *Sporotrichum pulverulentum* Novobranova, and the latter name should be used.

EXPERIMENTAL

Organisms. *Chrysosporium lignorum* P 127-1 was obtained from Dr. T. Nilsson, Royal College of Forestry, Stockholm, Sweden. *Polyporus versicolor* L. ex Fr. isolate Madison, was obtained from Prof. E. B. Cowling, North Carolina State University, Raleigh, North Carolina, USA.

Cultivations. A basal salt medium [5] was used with 3 g of carbon source/liter.

To follow enzyme induction the fungi were grown in 250 ml Erlenmeyer flasks containing 80 ml culture solution on the following carbon sources: powder cellulose, wood meal, phosphoric acid-swollen cellulose, cellobiose, and sucrose. The flasks were incubated at 25°C on a reciprocal shaker at 135 rpm and two flasks were harvested every second day. The solid material was removed by filtration and the culture solution was concentrated 5-fold in a collodion tube before analysis.

For enzyme production, *C. lignorum* was cultivated in one-liter Erlenmeyer flasks on powder cellulose in 300 ml culture solution for 8 – 9 days at 25°C reciprocal shaking. The culture solution was filtered and then concentrated by ultrafiltration (membrane Amicon UM-10). The proteins were precipitated by addition of solid ammonium sulfate at room temperature to 90 % of saturation, redissolved and extensively dialyzed against distilled water before use.

Enzyme assays. Cellobiose dehydrogenase was assayed as follows: The reduction of 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) was followed

spectrophotometrically at 360 nm at 25°C. The reaction mixture contained 300 μmol acetate buffer (pH 4.5), 1 μmol of the quinone, 2 μmol cellobiose and the enzyme in a total volume of 3 ml. Absorbancy was usually determined at one minute intervals. The decrease in absorbancy was linear with time until 90 % of the quinone was reduced. One enzyme unit was defined as the amount of enzyme that reduced 1 μmol quinone per min according to the Enzyme Commission.

Lactonase was assayed by measuring the decrease in the amount of cellobiono-δ-lactone. The lactone was determined colorimetrically as its hydroxamic acid derivative.[6] After incubation at 25°C for different times, hydroxylamine solution (pH 5.0) was added and the reaction mixture was allowed to stand at room temperature for 15 min before the ferric chloride solution was added. All assays were compared with a blank incubation mixture lacking enzyme to correct for the spontaneous hydrolysis of the substrate. Aryl-β-glucosidase activity was measured colorimetrically.[7]

Cellulase activity was determined viscosimetrically with CMC as a substrate [8] and colorimetrically with Avicel as a substrate. For the Avicelase measurement, the enzyme solution was incubated in a reaction mixture containing 20 mg of Avicel (American Viscose Co., Marcus Hook, Pennsylvania, USA) and 300 μmol acetate buffer (pH 5.0) in a total reaction volume of 3 ml. The sample was incubated for 2 h at 30°C with continuous shaking, filtered, and analyzed for reducing sugars.[9] Reduction of viscosity of CMC gives only a measurement of randomly-hydrolyzing endo-β-1,4-glucanases, while production of reducing sugars from Avicel, a crystalline cellulose, gives a measurement of both endo- and exo-β-glucanases.

Chemical determinations. The concentrations of quinones were measured spectrophotometrically. 3-Methoxy-5-*tert*-butyl-benzoquinone-(1,2) λ_{max}(nm) = 360 (ε = 2180) in 0.1 M acetate buffer (pH 4.5); 2-methoxybenzoquinone-(1,4) λ_{max}(nm) = 368 (ε = 1238) in 0.1 M acetate buffer (pH 4.5); cerulignone λ_{max}(nm) = 472 (ε = 19 180) in 0.1 M acetate buffer (pH 4.5) containing 50 % dioxan.

Lactones were measured colorimetrically [6] at pH 5.0. The hydroxamic acid derivative of cellobionolactone has an ε = 239 at λ = 500 nm. If a quinoid hydrogen acceptor was present the reaction mixture was extracted with chloroform to remove phenols and quinones before analysis for lactones.

Paper chromatography. Identification of reaction products were made by ascending paper chromatography on Whatman No. 1 chromatography paper. The samples were extracted with chloroform to remove phenols, concentrated by evaporation under vaccum, adjusted to pH 8 with NaOH, boiled for 2 min and finally treated with a strong cation exchanger (Dowex 50WX8).

Cellobionic acid had the following $R_{glucose}$: 0.29 in ethylacetate – pyridine – water (4:2:1 v/v/v), 0.55 in butanol – ethanol – water (10:3:5), and 0.87 in ethylacetate – acetic acid – water (3:1:1).

Gas chromatography. Gas-liquid chromatography was carried out on the trimethylsilyl ethers of cellobionic acid and cellobiono-δ-lactone. Column: QF-1 11 % on Cromosorb W 100 – 120 mesh, 235°. The samples were extracted with chloroform, adjusted to pH 8 and placed on an anion exchange column (Dowex 1-X4) (acetate form). The column was carefully washed with distilled water to remove neutral sugars and the acidic reaction products were eluted with 1 M acetic acid. The acetic acid was then removed by repeated evaporation and the residue was silylated both directly and as the Na-salt. *N,O*-bis(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane were used in the preparation of the silyl ethers. Retention time for the trimethylsilylated cellobionolactone was 12.15 min and 13.4 min for the trimethylsilylated cellobionic acid.

Chemicals. Cellobiono-δ-lactone was synthesized by chlorite oxidation of cellobiose.[10] Cellobionolactone had a m.p. of 181.7 – 182.4°C. No gluconic acid could be detected in the preparation. Hydrolysis of the synthetic cellobionolactone (100°C, 1 M HCl, 4 h) yielded glucose and gluconic acid as identified by paper chromatography. Cellobionic acid was prepared by alkaline hydrolysis at pH 8 of cellobionolactone. The free acid was obtained by cation exchange (Dowex 50W-X8).

Phosphoric acid-swollen cellulose was prepared from Munktell's Cellulose Powder No. 400.[11] The 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) and cerulignone were kindly supplied by Dr. Augustine E. Opara of this department.

RESULTS

Induction of cellobiose dehydrogenase on different carbon sources

The production of cellobiose dehydrogenase from *C. lignorum* and *P. versicolor* was followed during a twenty day cultivation. *C. lignorum* was cultivated on wood meal, cellulose, phosphoric acid-swollen cellulose, cellobiose, and sucrose. *P. versicolor* was cultivated on cellulose and swollen cellulose. Fig. 1 shows the development of enzyme in the culture solution of *C. lignorum*. Cellulose induced the highest cellobiose dehydrogenase activity in the culture solution. The other cellulosic materials, wood meal and swollen cellulose, also induced enzyme activity. Very small amounts of enzyme were

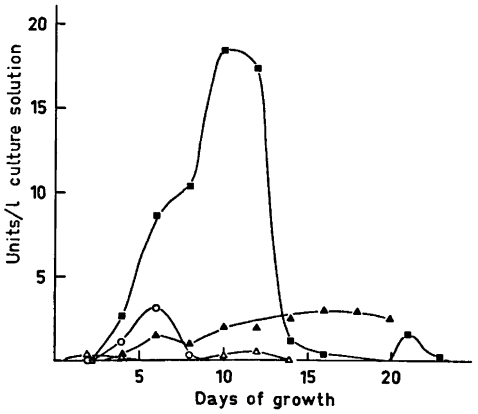


Fig. 1. Production of cellobiose dehydrogenase by *C. lignorum* grown on different carbon sources. ■ Cellulose powder. ○ Phosphoric acid swollen cellulose. ▲ Wood meal. △ Cellobiose.

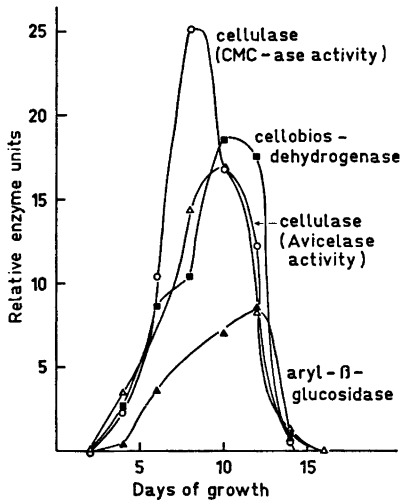


Fig. 2. Development of cellulose-degrading enzymes in a culture solution of *C. lignorum* grown on powder cellulose.

produced with cellobiose and no activity was detected with sucrose. Cultivations of *P. versicolor* gave similar results but the maximal production was about 13 units/l culture solution compared with 18 units/l culture solution for *C. lignorum*. Therefore, the latter fungus was selected for further study.

Fig. 2 shows the development of cellulose-degrading enzymes in a culture solution of *C. lignorum* grown on powder cellulose. The development of cellobiose dehydrogenase was parallel to the cellulose-degrading enzymes. Cellobiose dehydrogenase, aryl-β-glucosidase, and avicelase activity all reached maximum after about ten days. CMC-ase activity reached maximum after about eight days. Without added cellobiose the quinone-reducing activity of the culture solution was very low during the whole cultivation period.

Properties of cellobiose dehydrogenase

(a) Isolation and identification of the reaction products. Quinone reduction. Treatment of 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) with the enzyme in the presence of cellobiose yielded one single aromatic compound. It was identified as the corresponding reduced quinone, 3-methoxy-5-*tert*-butyl-catechol-(1,2). The ultraviolet spectrum of the product of the enzymic reduction was in agreement with the spectrum of the authentic catechol. The identity of the product was further confirmed by thin-layer chromatography using authentic material as a reference compound.

To identify the products of the enzymic oxidation of cellobiose, the following two mixtures were used: (I) 1 mmol cellobiose, 3 mmol acetate buffer (pH 5.0), 25 μmol guaiacol, the phenol-oxidizing enzyme laccase and the cellobiose dehydrogenase in a reaction mixture of 18 ml. The reaction proceeded for 5 h at 25°C with shaking to supply the laccase with oxygen. (II) 50 μmol cellobiose, 50 μmol 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2), 5 mmol acetate buffer (pH 5.0), and cellobiose dehydrogenase in a total volume of 50 ml. The reaction proceeded at 25°C until all the quinone was reduced.

The reaction products were indentified by paper chromatography with glucose, cellobiose gluconic acid, and cellobionic acid as reference compounds. Spots running the same as glucose, cellobiose, and cellobionic acid were identified from the reaction mixture but no gluconic acid was found. Part of the reaction mixture (I) was also analyzed by gas-liquid chromatography. The silylated samples from the reaction mixture gave peaks with the same retention time as the synthetic cellobiono-δ-lactone and cellobionic acid. The aldonic acids are in equilibrium with

Table 1. Stoichiometry of reaction. Reduction of 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) by cellobiose dehydrogenase. The reaction mixture contained 300 μmol acetate buffer (pH 4.5), quinone and cellobiose according to the table, and the enzyme in a total volume of 3 ml.

Quinone μmol	Cellobiose μmol	Quinone reduced μmol
2.0	2.0	2.0
2.0	1.0	1.0
2.0	0.2	0.2
2.0	0	0
2.0	200	2.0

Table 2. Stoichiometry of reaction. Acid formation from the oxidation of cellobiose by cellobiose dehydrogenase. The reaction mixture contained cellobiose and 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) according to the table together with enzyme in 18 ml distilled water. The initial pH was adjusted to 5 with nitric acid and the acid-formation was followed at pH 5 with a pH-stat.

Quinone μmol	Cellobiose μmol	Acid formed μmol
37	75	31
18	75	16
11	75	10
0	75	0
18	0	0

their lactones at acidic pH and the working up procedure did not ascertain whether cellobionic acid or cellobiono-δ-lactone was the primary product of the reaction. Direct analysis of the assay mixture (I) during the reaction failed to detect the presence of lactones. However, the enzyme preparations used contained high lactonase activities (see later).

(b) Stoichiometry of reaction. To evaluate the stoichiometry of the reaction, two series of experiments were run, one with cellobiose and one with quinone as the limiting substrate. Table 1 shows the reduction of the quinone when cellobiose was limiting and Table 2 the production of acid from cellobiose with limiting quinone.

The acid production was followed in a pH-stat at pH 5.0. Both Table 1 and Table 2 suggest that one mol of quinone is reduced when one mol of cellobiose is oxidized.

Table 3. Oxidation of different sugars. The reaction mixture contained 300 μmol acetate buffer (pH 4.5), 1 μmol 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2), 2 μmol of sugar and the enzyme in a total reaction volume of 3 ml. The reaction was measured by following the reduction of the quinone.

Substrate	Relative rate of oxidation
Cellobiose	100
Lactose	44
Maltose	0
Sucrose	0
Xylobiose	0
Glucose	0
Galactose	0
Mannose	0
Gluconolactone	0
Arabinose	0
Xylose	0

(c) Substrate specificity. (a) Carbohydrate oxidation. The relative rates of oxidation for a number of mono- and disaccharides were investigated and the results are presented in Table 3. The enzyme appears to have high specificity for the oxidation of cellobiose. Glucose was not oxidized at all by the enzyme and oligosaccharides could not be tested, since they would rapidly be degraded to cellobiose by contaminating enzymes.

(b) Quinone reduction. The specificity of the quinone-reducing ability of the enzyme appears to be less restricted and quinones used as sub-

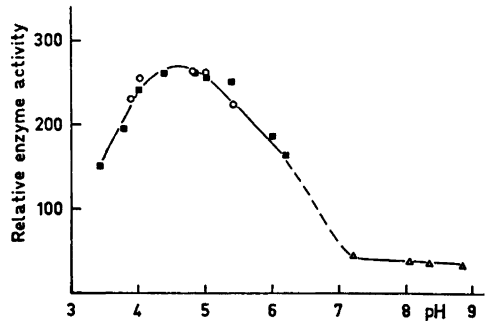


Fig. 3. Effect of pH on the activity of cellobiose dehydrogenase. The reaction mixture contained 300 μmol buffer, 1 μmol 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2), 2 μmol cellobiose and the enzyme in a total volume of 3 ml. ■ Citrate buffer. ○ Acetate buffer. △ Tris buffer.

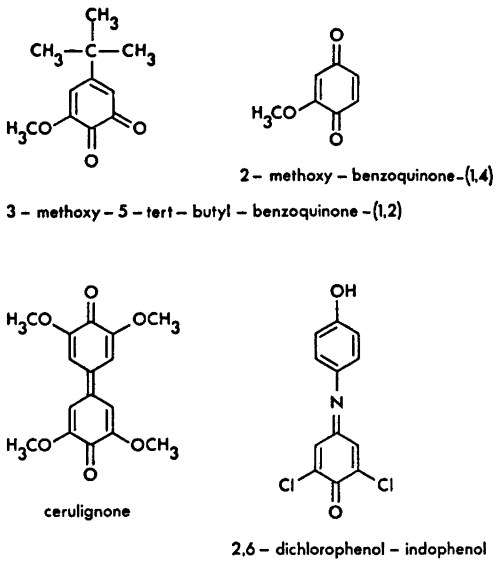


Fig. 4. Quinones which can be reduced by cellobiose dehydrogenase.

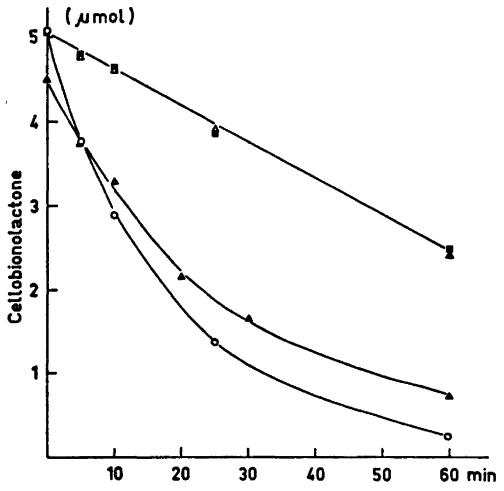


Fig. 5. Hydrolysis of cellobiono-δ-lactone. The reaction mixture contained 100 μmol of acetate buffer (pH 5.0), 5 μmol cellobiono-δ-lactone and the enzyme in a total volume of 1 ml. ○ 0.15 ml enzyme. ▲ 0.1 ml enzyme. △ Boiled enzyme control. ■ No enzyme (spontaneous hydrolysis).

strates for the enzyme are summarized in Fig. 4. The oxidoreductase can reduce both *ortho* and *para* quinones and also the artificial electron acceptor 2,6-dichlorophenolindophenol, but it cannot use molecular oxygen as hydrogen acceptor. What is more interesting from the point of view of wood degradation is that the enzyme can use reaction products of the phenol-oxidizing enzyme laccase and a suitable phenolic compound, for example lignin or lignin-degradation products, as hydrogen acceptor. As discussed in Ref. 4 the phenoxy radical formed from laccase oxidation of phenols is probably the substrate for the enzyme.

(d) pH-Optimum for the enzyme. The effect of pH on the enzymic activity was measured in citrate, acetate, and tris-buffers. The results given in Fig. 3 show that the enzyme has its optimal activity around 4.5 – 5. Phosphate buffer was somewhat inhibitory to the enzyme.

Enzymic hydrolysis of sugar lactones. It was expected that the primary product of cellobiose oxidation should be the cellobiono-δ-lactone if the enzyme reacted with the pyranoside form of cellobiose. However, all experiments to show the presence of the lactone failed. The cellobionolactone is spontaneously hydrolyzed at pH 5.0 (half life of about 58 min) but this could not explain the fact that no lactone could be detected in the reaction mixture after the oxidation of cellobiose.

To determine if enzymatic hydrolysis of lactones occurred, concentrated culture filtrates were assayed for lactonizing activity as described under methods. Fig. 5 shows the rate of hydrolysis of cellobionolactone by the culture solution. The concentrated culture solution also contained activity against glucono-δ-lactone but arabino-γ-lactone was not hydrolyzed by the enzyme solution. Thus, the first product of the cellobiose oxidation may be the cellobiono-δ-lactone which is immediately hydrolyzed by the lactonase, also secreted into the medium by the fungus.

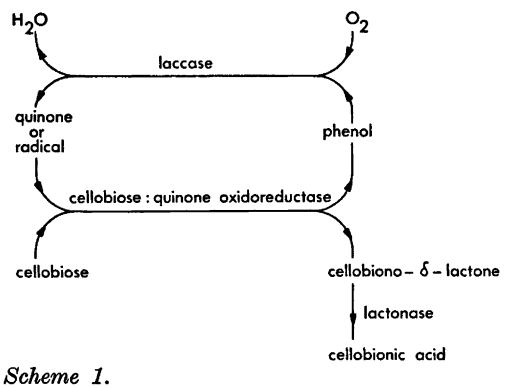
DISCUSSION

Certain bacteria, especially from the genus *Pseudomonas*,[12] can oxidize the disaccharides lactose and maltose to the corresponding aldobionic acids in the presence of a suitable hydrogen acceptor, without prior hydrolysis to monosaccharides. The enzyme responsible for the oxidation of lactose in *Pseudomonas graveolens* has been studied [13] and seems to have a similar mechanism of action as the *C. lignorum*

enzyme. However, the *P. graveolens* enzyme is intracellular, localized in the particulate fraction, while the *C. lignorum* is extracellular. Furthermore, the *P. graveolens* enzyme can oxidize several disaccharides including cellobiose, and also pentoses and hexoses, while the *C. lignorum* enzyme seems to be quite specific for cellobiose and fails to oxidize monosaccharides.

Cellobiose dehydrogenase may be involved in the enzymic degradation of both cellulose and lignin in wood. Several points suggest that carbohydrate oxidation is the important function of the enzyme. First, cellobiose dehydrogenase is induced by cellulose and cellulosic materials and is produced simultaneously with the cellulolytic enzymes in *C. lignorum*. The pH-optimum of the enzyme is in accordance with that of the cellulases from the same fungus.[14] Also, cellobiose dehydrogenase seems to have a high specificity for cellobiose which is an abundant product of cellulose degradation. It was impossible to determine if higher oligosaccharides and cellulose could also be oxidized by the enzyme, since the preparation contained cellulases. Finally, preliminary experiments indicate that cultivation of the fungus on xylan produces a similar enzyme specific for oxidation of xylan degradation products.

The function of laccase in white-rot fungi during wood degradation has been a topic of much discussion. It has been observed that some aromatic compounds stimulate the growth of white-rot fungi[15] and it was suggested that laccase participates in their electron-transport chain. The results in this paper indicate that laccase may, in fact, function as a link in an extracellular "electron-transport chain." A proposed mechanism for cellobiose dehydrogenase is given in Scheme 1.



Scheme 1.

Cellobionolactone could not be detected in the reaction but the presence of an extracellular lactonase which hydrolyzes cellobionolactone to cellobionic acid suggests that the primary product of the oxidation is the lactone. It is known that sugar lactones, especially gluconо-1,5-lactone, inhibit cellulases and β-glucosidases[16] so it is probably important for the fungus to hydrolyze immediately any lactones formed. In a study of the enzymic cleavage of cellobionic acid[17] it was found that enzymes in a commercial cellulase preparation and also an enzyme extract from *Aspergillus niger* could cleave cellobionic acid to glucose and gluconic acid.

Acknowledgements. We want to thank Majlis Jansson for skilful technical assistance.

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Reaction of Diethyl Pyrocarbonate with Nucleophiles

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In order to clarify the kinetic background of the biochemical and biological action pattern of diethyl pyrocarbonate (diethyl oxydiformate) the rates of reaction of this compound with amines, thiols, and compounds with nucleophilic oxygen were studied at 25°. The nucleophilic strength within each series of compounds is correlated with the basicity. The primary amines are approximately five times as reactive as the thiols and thousand times as reactive as the oxygen anions, when compared at equal pK_a values.

Diethyl pyrocarbonate has been widely used as a food preservative because of its bactericidal action.[1] More recently it has been applied as a nuclease inhibitor in the preparation of undegraded high molecular weight RNA.[2-4]

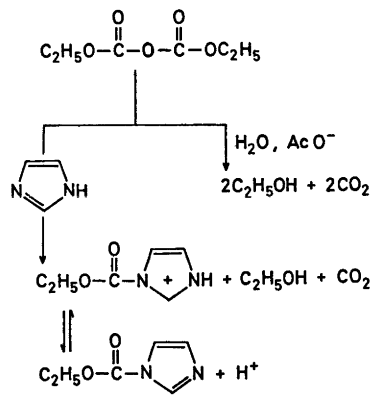
Diethyl pyrocarbonate can be regarded as the anhydride of ethoxyformic acid, and as such, although stabilized by the ethoxy group, it is very susceptible towards nucleophilic attack on a carbonyl carbon. There has been an extensive literature on the carbethoxylation by diethyl pyrocarbonate of amines, [5,6] thiols,[7] phenols,[8] amino acids,[5,9,10] and proteins.[11-13] Data on reaction rates, however, are at present very scanty.[12,14] Such data are needed for the understanding of the biological effects of diethyl pyrocarbonate,[15,16] for the optimization of the nucleic acid extraction method based on the use of diethyl pyrocarbonate, as well as for finding proper conditions for the use of diethyl pyrocarbonate in studies of the secondary structure of nucleic acids.[17]

MATERIALS AND METHODS

Diethyl pyrocarbonate was obtained from Schuchardt, München. Tris(hydroxymethyl)-aminomethane (Tris), glycylglycine, 2-mercaptoethanol, and 3-mercaptopropionic acid were purchased from Sigma, St. Louis, Mo. *p*-Nitrophenyl mercaptan was synthesized according to Augustinsson *et al.*[18] All other chemicals were obtained from Merck, Darmstadt, and were of reagent grade.

The reactions of diethyl pyrocarbonate were with a few exceptions followed in the presence of a large excess of the respective nucleophile to give pseudo first order kinetics. The temperature was maintained at $25 \pm 0.1°$ in a Hetotherm ultrathermostat. The ionic strength was kept constant ($I = 0.1$) by addition of NaCl, except in the study of buffer compounds.

Reaction with imidazole. Diethyl pyrocarbonate reacts rapidly with imidazole to form *N*-carbethoxyimidazole,[12] according to eqn. (1).



(1)

The rate of carbethoxylation was followed spectrophotometrically at 231 nm (λ_{max} for *N*-carbethoxyimidazole; $\varepsilon = 3 \times 10^3$ l mol^{-1} cm^{-1}). To a 10 – 20 mM solution of imidazole in acetate buffer (pH 4.4 – 4.9) diethyl pyrocarbonate dissolved in acetonitrile was added to give an initial concentration of 0.2 mM. The reaction was followed for ten half-lives to give the end-point A_∞. The half-time was determined from a plot of log $(A_\infty - A_t)$ against t, where A_t

is the absorbance at the time t. The observed first order rate constant was calculated from

$$k_{obs} = \ln 2/t_{1/2} \quad (2)$$

The pseudo first order rate constant for the reactions of diethyl pyrocarbonate with imidazole (IM) was obtained by subtracting the constants for hydrolysis and reaction with acetate (AcO^-), determined in separate experiments, from the observed rate constant; see eqn. (3). The second order rate constant was obtained by dividing the pseudo first order constant by the concentration of free base according to eqn. (4).

$$k_{obs} = k'_{H_2O} + k'_{AcO^-} + k'_{IM} \quad (3)$$

$$k_2 = k'_{IM}/[IM] \quad (4)$$

The fraction of base was calculated from

$$pH = pK'_a + \log \frac{[IM]}{[IMH^+]} \quad (5)$$

Reactions with water, CH_3COO^-, HPO_4^{2-}, Tris, and CO_3^{2-}. The rapid reaction with imidazole was used for the quantitative determination of diethyl pyrocarbonate. 0.1 ml samples were taken at appropriate intervals from a 2 – 3 mM solution of diethyl pyrocarbonate in the respective buffer and added to 5.0 ml of 5 mM imidazole in 0.035 M phosphate buffer (pH 8.0). The samples were carefully shaken and the absorption at 231 nm was measured after a few minutes. The errors due to parallel reaction of diethyl pyrocarbonate with water and buffers, change in background absorption of imidazole and hydrolysis of *N*-carbethoxyimidazole [12] were negligible under these conditions.

The reaction rates of the buffer compounds were studied at selected concentrations and pH in the following ranges: 0.05 – 0.2 M acetate buffer pH 4.5 – 5.0, 0.05 – 0.2 M phosphate buffer pH 6.5 – 7.5, 0.02 – 0.05 M Tris buffer pH 7.5 – 8.1, 0.05 – 0.1 M carbonate buffer pH 10.0. The rate constants were calculated from the stoichiometric composition of the buffer.

Reactions with glycylglycine, ammonia, ethanolamine, glycine, and β-alanine. The rate constants were determined by measuring the yield of *N*-carbethoxyimidazole in a solution of imidazole in the presence and absence of the amine under study.

The reactions were carried out in 0.02 M Tris or phosphate buffer at pH 8.0. Diethyl pyrocarbonate was dissolved in acetonitrile and added to the reaction mixture to give an initial concentration of 0.08 mM. The concentration of imidazole was 2 mM (4 mM in the reference experiment) and the amines were studied in the following concentration ranges: glycylglycine 1 – 2 mM, NH_4Cl 60 – 80 mM, ethanolamine 6 – 12 mM, glycine 5 – 20 mM, and β-alanine 15 – 50 mM. The absorption at 231 nm was measured after 5 min. The experiments were repeated for two of the compounds (glycylglycine and glycine) at pH 7.0 without buffer and with various concentrations of the amines (5 – 10 times higher than used at pH 8.0). The rate constants so determined were the same as those obtained at pH 8.0 within the limits of experimental error. The possibility of a general basic catalysis of the buffers or imidazole on the carbethoxylation of the investigated amines in the concentration ranges used could be disregarded. The stability of the *N*-carbethoxyimidazole in the presence of the amines was checked.

The concentration of the amines was large in comparison with the concentration of diethyl pyrocarbonate. Thus, the ratio of the pseudo first order rate constants could be determined directly from the ratio of the concentrations of the final products (*cf.*, *e.g.*, Ref. 19).

$$\frac{k'_{IM}}{k'_{RNH_2}} = \frac{k_{IM}[IM]}{k_{RNH_2}[RNH_2]} = \frac{[N\text{-carbethoxyimidazole}]}{[\text{urethan}]} \quad (6)$$

The second order rate constants refer to the concentrations of the free bases. The pK'_a values at $I = 0.1$ were taken from the literature (see Table 1).

Reaction with p-bromoaniline. The reaction was followed in an equimolar solution (3 – 4 mM) of diethyl pyrocarbonate and *p*-bromoaniline in 0.02 M phosphate buffer (pH 6.6). The amount of unreacted *p*-bromoaniline was determined by diazotation and coupling with histamine.[7] 0.1 ml samples were withdrawn every 10 sec and dispersed in 3.0 ml ice-cold $NaNO_2$-HCl (0.15 % $NaNO_2$, 0.1 M HCl, 1:3). After 15 min in ice-bath 1.0 ml 0.5 M borate buffer (pH 9.5) and 1.0 ml 0.01 M histamine dihydrochloride were added. The samples were carefully shaken after each addition. A yellow colour developed in 30 min at room temperature. Spectrophotometric readings were taken at 436 nm.

The second order rate constant k_2 was calculated from

$$1/c = k_2 t + C \quad (7)$$

where c is the concentration of *p*-bromoaniline (or diethyl pyrocarbonate) at time t.

Reactions with p-nitrophenyl mercaptan, 2-mercaptoethanol, and 3-mercaptopropionic acid. The reactions of diethyl pyrocarbonate with 2-mercaptoethanol and 3-mercaptopropionic acid were studied by three independent methods:

(a) By measuring the decrease of the concentration of diethyl pyrocarbonate. The reaction mixture was 1.5 mM with respect to diethyl pyrocarbonate and 15 mM with

respect to the SH compound in 0.05 M phosphate buffer (pH 7.0). 0.1 ml samples were taken at appropriate intervals and added to 3.0 ml 5 mM imidazole in phosphate buffer (pH 8). The *N*-carbethoxyimidazole was determined spectrophotometrically.

(b) By measuring the decrease of the concentration of the SH compound. The reaction between diethyl pyrocarbonate and the mercaptan in equal concentrations (2 – 4 mM) in 0.035 M phosphate buffer (pH 8.0) was followed by taking 0.1 ml samples every 7 sec and adding them to 5.0 ml of Ellman's reagent, 10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) in 0.035 M phosphate buffer (pH 8).[20] The absorption was determined at 412 nm ($\varepsilon = 13\,600$ l mol^{-1} cm^{-1} for the liberated 4-nitro-3-carboxythiophenolate ion). A disturbing fading of the colour, possibly due to a reaction of the coloured anion with the remaining diethyl pyrocarbonate made it necessary to measure every sample several times and extrapolate the absorption to the time when the sample was taken. The rate constant was calculated from a plot of $1/c$ against time [eqn. (7)].

(c) By measuring the appearance of a peak in UV at 213 nm corresponding to the formation of an ethoxyformic acid thioester.[7] The reactions were carried out at pH 8.0 in 0.035 M phosphate buffer. The initial concentrations of diethyl pyrocarbonate and mercaptan were 0.1 mM and 1.1 mM, respectively. The increase in absorbance was measured directly at 213 nm. The reactions were followed for at least ten half-lives to get an accurate end-point determination. Log $(A_\infty - A_t)$ was plotted against time t to give a straight line from which the half-life was determined.

The reaction between diethyl pyrocarbonate and *p*-nitrophenyl mercaptan was followed by measuring the decrease of the absorption at 412 nm ($\varepsilon = 9000$ l mol^{-1} cm^{-1}; Ref. 18). The reaction conditions were the same as described under (b). The samples were diluted 50 times before reading.

The purity of the SH compounds was determined with the aid of Ellman's reagent.[20]

RESULTS AND DISCUSSION

The rate constants determined for the reaction of diethyl pyrocarbonate with the different nucleophilic reagents at 25° are summarized in Table 1. Good agreement was obtained with previous studies of the reaction rates with three of the nucleophiles, *viz.* the rate of the reaction with water [k (first order) = 0.0246 min^{-1}], determined by adding samples to diethylamine solutions and titrating the diethylamine in excess;[14] the rate of the reaction with imidazole ($k = 3240$ l mol^{-1} min^{-1}), determined spectrophotometrically at 231 nm, λ_{max} for *N*-carbethoxyimidazole;[12] and the rate of the reaction with ammonia ($k = 1600$ l mol^{-1} min^{-1}) determined by quantitative gas chromatographic analysis of the urethan formed (G. Löfroth, *personal communication*).

Table 1. Rate constants for reaction of diethyl pyrocarbonate with different nucleophiles at 25°. The given constants are mean values of 3 – 10 determinations; the error (95 % confidence interval) of the constants is mostly less than ± 10 %.

Nucleophile	pK_a	k l mol^{-1} min^{-1}
Water	−1.74[a]	4.3×10^{-4}
CH_3COO^-	4.8[a]	0.35
HPO_4^{2-}	6.9[a]	0.9
CO_3^{2-}	10.4[a]	0.6×10^2
Tris	8.1[a]	24
p-Bromoaniline	3.9[b]	2.2×10^2
Imidazole	7.0[c]	3.2×10^3
Ammonia	9.29[d]	1.7×10^3
Glycylglycine	8.09[e]	1.0×10^4
Ethanolamine	9.5[f]	2.7×10^4
Glycine	9.60[g]	4.0×10^4
β-Alanine	10.3[h]	5.4×10^4
p-Nitrophenyl mercaptan	5.1[i]	1×10^{2}[k]
2-Mercaptoethanol	9.5[a]	1.0×10^4
3-Mercaptopropionic acid	10.2[j]	1.2×10^4

[a] Ref. 21 and literature quoted; [b] Ref. 30; [c] Ref. 31; [d] Ref. 32; [e] Ref. 33; [f] Ref. 34; [g] Ref. 35; [h] Ref. 36; [i] Ref. 37; [j] Ref. 38; [k] One determination only.

A Brønsted plot of the data (Fig. 1) indicates that primary amines follow the relationship[19]

$$\log k = \beta \, pK_a + C \qquad (8)$$

where $\beta = 0.4$ gives the dependence of reaction rate on the basicity of the amino group. The rate constant for the tertiary amine, imidazole, falls on the same curve; a more detailed study on strictly defined structurally related imidazoles and primary amines is required to show whether imidazole truly belongs to the group of amines in this respect or the position of k_{IM} is the fortuitous resultant of mutually counteracting factors. The strong solvation of the ammonium ion which causes an abnormally high pK_a, and the low polarizability of NH_3 nitrogen, probably account for the relatively low reactivity of ammonia.[19,21] The slow reaction of tris(hydroxymethyl)aminomethane (Tris) as compared to other primary amines of

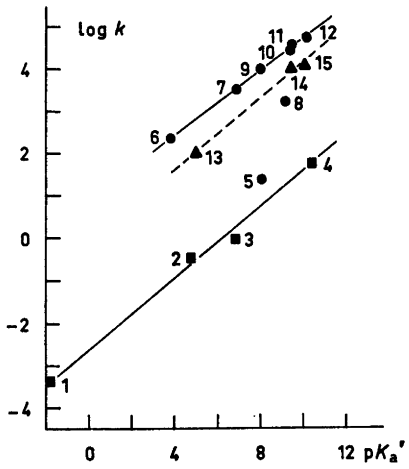
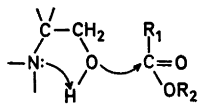


Fig. 1. Log k (k in 1 mol^{-1} min^{-1}), as a function of pK'_a of reactions of diethyl pyrocarbonate with nucleophiles at 25°. 1, water; 2, CH_3COO^-; 3, HPO_4^{2-}; 4, CO_3^{2-}; 5, Tris; 6, *p*-bromoaniline; 7, imidazole; 8, ammonia; 9, glycylglycine; 10, ethanolamine; 11, glycine; 12, β-alanine; 13, *p*-nitrophenyl mercaptan; 14, 2-mercaptoethanol; 15, 3-mercaptopropionic acid.

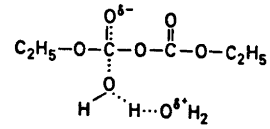
corresponding pK'_a may reflect steric hindrance at the nitrogen. It is probable, however, that this steric hindrance is comparatively large and that the reaction measured proceeds *via* an alternative mechanism, *viz.*, the intramolecular base-catalyzed attack by an oxygen according to formula (9), indicated to operate in the reactions of Tris and hydroxylamine with *p*-nitrophenyl acetate,[21] and suggested to explain the activation of the serine hydroxyl group at the active site of chymotrypsin.[22]



(9)

The three sulfhydryl compounds studied react more slowly with diethyl pyrocarbonate than primary amines with comparable dissociation constants. In this respect diethyl pyrocarbonate differs from the carefully investigated acylating agent, *p*-nitrophenyl acetate which is attacked somewhat more rapidly by thiol anions than by primary amines.[21] The difference in nucleophilic reactivity towards diethyl pyrocarbonate and *p*-nitrophenyl acetate seems to be the high reactivity of the primary amines towards diethyl pyrocarbonate, rather than the low reactivity of the thiols.

As expected, compounds with nucleophilic oxygen react slowly with diethyl pyrocarbonate. The rate constants for reaction of diethyl pyrocarbonate with water, acetate, hydrogen phosphate, and carbonate exhibit a dependence on pK_a which is approximately equal to that of the amines and the thiols. Kivinen [14] concluded that the neutral hydrolysis of diethyl pyrocarbonate in water takes place by a general base-catalyzed mechanism with water functioning as a base, and proposed the structure (10) for the transition state.



(10)

The acetate, hydrogen phosphate, and carbonate may serve either as general base catalysts or as real nucleophilic catalysts.

Preliminary studies of the carbethoxylation of RNA in phosphate buffer indicates that diethyl pyrocarbonate acts *via* a carbethoxy phosphate intermediate (which is certainly very unstable [23]), and accordingly the reaction mechanism would involve a nucleophilic attack of hydrogen phosphate on the carbonyl carbon (data to be published).

In biochemical studies, *e.g.* of enzyme inactivation with diethyl pyrocarbonate, its concentration, [DEP], decreases with time, t, according to

$$[\mathrm{DEP}] = [\mathrm{DEP}]_0 \, e^{-k't} \qquad (11)$$

where $[\mathrm{DEP}]_0$ is the initial concentration of diethyl pyrocarbonate and

$$k' = k_{H_2O}[H_2O] + \sum_i k_i c_i \qquad (12)$$

k_i and c_i being the rate constants and concentrations, respectively, of nucleophiles in the system. The rate constants of buffer components permit the calculation of k' in the reaction media and hence the dose, *i.e.* the time integral of the concentration of diethyl pyrocarbonate.

In reactions with proteins no absolute specificity, *e. g.* towards histidine, occurs. Reaction is expected to occur in a random fashion with various groups (*cf.* Ref. 24), if sterically accessible, and proportionally to the respective rate constants and the degrees of dissociation of these groups at the pH of the treatment. Fig. 2 illustrates the selectivity of the reaction with imidazole groups. It gives the expected total rates of carbethoxylation at pH 7 of amino ($\equiv N + \equiv NH^+$) and thiol ($-S^- + -SH$)

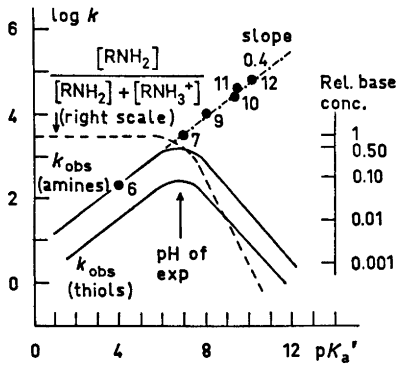


Fig. 2. The total observed reactivities at pH 7 (log k, scale to the left) of diethyl pyrocarbonate towards amines and thiols [solid lines marked k_{obs} (amines) and k_{obs} (thiols), respectively] as a function of pK'_a of the nucleophiles. For nucleophiles with $pK'_a <$ pH of the experiment (in the figure pH 7) the concentration of the reactive species, *i.e.* free base, approaches the total concentration of the nucleophile, whereas the relative concentration of the free base of nucleophiles with higher pK'_a approaches a line with slope -1 (shown for amines in curve – – – marked $[RNH_2]/([RNH_2]+[RNH_3^+])$; right scale). k_{obs} of weak bases ($pK'_a <$ pH) approach the respective Brønsted curve with slope 0.4 (–·–· curve for amines from Fig. 1). k_{obs} of strong bases approach the resultants of the Brønsted slope and the slope -1. *Cf.* Ref. 19 p. 84.

groups as a function of their dissociation constants. The rate of carbethoxylation of (total) imidazole exhibits a maximum aroung pK_a. For similar reasons, enzyme histidines will be carbethoxylated with a certain selectivity at or below pK_a (around 6.5).[12] The rate of inactivation of ribonuclease [13,15] by diethyl pyrocarbonate in the pH range 4 – 7 agrees well with the expected rate of reaction with histidine. The rate constants for inactivation of ATP-creatine phosphotransferase and ATP-L-arginine phosphotransferase [25] in 0.05 M phosphate buffer (pH 6.1) correspond to $k_{his} = 1000$ l mol^{-1} min^{-1} in agreement with expectation.

Lysine ε-amino groups (pK_a about 10) will react about 50 times slower than histidines in neutral solution but their reaction rates will increase rapidly with pH.

The second order rate constant for the reaction of diethyl pyrocarbonate with RNA was found to be about 0.003 l (mol nucleotide)$^{-1}$ min^{-1} in 0.05 M phosphate buffer (pH 6.5 – 7.5) and 25°. Since the reaction rate was found to depend on the concentration of phosphate, indicating a role of transcarbethoxylation *via* carbethoxy phosphate, the value of the constant is preliminary. It agrees acceptably with earlier data for the inactivation of virus RNA [26-28] and transfer RNA[29] by diethyl pyrocarbonate.

An RNA molecule containing 1000 nucleotides (in single-stranded regions [26-29]) will, under the conditions given, be inactivated at a rate of about 3 l (mol RNA)$^{-1}$ min^{-1}, *i.e.* two orders of magnitude more slowly than enzymes inactivated by carbethoxylation of histidine.

Changes in the ultraviolet spectra of diethyl pyrocarbonate-treated nucleic acids indicate [17,39] that diethyl pyrocarbonate reacts with single-stranded regions of RNA and DNA. Isotope studies using ^{3}H-labelled diethyl pyrocarbonate and homopolymers have revealed [40] that in the polynucleotide chain it is adenosine whose reaction rate with diethyl pyrocarbonate is the highest. If we now assume that in *E. coli* rRNA it is also adenosine which reacts fastest and that about 40 %[41] of its adenosine residues are in single-stranded regions, the rate constant at 25° for the reaction with RNA-adenosine will be about 0.03 l mol^{-1} min^{-1} in 0.05 M phosphate buffer and some three times higher in 0.2 M buffer. This rate is at least one order of magnitude lower than the value of 1.1 ($\pm$0.2) l mol^{-1} min^{-1} determined from ultraviolet spectral studies (our unpublished data *) for the reaction of

* Partly in collaboration with Prof. E. I. Budovskii (Shemyakin Institute for the Chemistry of Natural Products, Academy of Sciences, Moscow, U.S.S.R.) whose contribution is greatly acknowledged.

diethyl pyrocarbonate with adenosine in phosphate buffers, independently of buffer concentration. Adenosine residues in RNA are therefore less reactive towards diethyl pyrocarbonate than the free nucleoside and it remains to be shown whether the two reactions proceed by the same mechanism.

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We are indebted to Mrs. Inger Wennberg for skilful technical assistance, and to Mr. Felix Hultén for participation in certain parts of the investigation.

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Studies on the Bacteriolytic Activity of *Streptomyces albus* Culture Filtrates. 3. Affinity for Chitin

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The enzymes of *Streptomyces albus* G culture filtrates were absorbed to chitin and deaminated chitin, and desorbed quantitatively under mild conditions. The affinity was dependent on low ionic strength, whereas variation in pH between 4.5 and 9.0 had no effect. The similarity to the results obtained with carboxymethyl cellulose suggests a binding to substrate-analogous structures.

Earlier studies[1,2] have shown that culture filtrates of *Streptomyces albus* G contain a muramidase, an amidase, and several endopeptidase and caseinolytic activities. The two former activities were isolated in apparently pure states on carboxymethyl cellulose, whereas the latter activities were eluted together without detectable amidase and hexosaminidase activities. Further separation was not obtained either on gel filtration or electrophoresis.

Since the first report by Berger and Weiser[3] on degradation of chitin by egg-white lysozyme, the binding of this and other muramidases (E.C. 3.2.1.17) has been extensively studied using chitin as substrate.[4–8] This property of muramidases, *i.e.* reversible binding to a structural analog of its substrate, has also been utilized in isolation and purification of this type of enzyme.[9]

The intention of the present study was to test chitin as a possible adsorbent for the endo-*N*-acetylmuramidase of *Streptomyces albus* G culture filtrates.

MATERIALS AND METHODS

Chitin, a polymer of essentially non-branched chains of β-(1-4)-*N*-acetyl-D-glucosamine units, was obtained from Sigma Chemical Co., Mo. U.S.A. All other materials and chemicals used were described previously,[1,2] including the test bacteria abbreviated: Sa (*Staphylococcus aureus* Copenhagen), Sp (*Streptococcus pyogenes* A), Pc (*Planococcus* 2389), M-144 (*Micrococcus luteus* 144), M-84 (*M. conglomeratus* 84), Ml (*M. lysodeikticus* NCTC 2665), Sl (*Sarcina lutea*), and Bm (*Bacillus megaterium*).

The methods for testing of lytic activities, dialysis, concentration, determination and quantitation of free groups and *N*-acetylamino sugars, and estimation of enzymatic specificities were those described or referred to earlier.[1,2]

Preparation of chitin. Chitin was prepared and treated according to the description of Jensen and Kleppe[9]. Deamination was performed as described by Cherkasov and Kravchenko,[10] suspending chitin (10 ml) in a mixture of 1.27 ml 12 N HCl and 1 g $NaNO_2$ in 30 ml of water, incubating for 6 h at 0°C with continuous stirring, followed by washing in 1 % NaCl, 1 % acetic acid and finally distilled water.

Column fractionation. Columns (1.2 × 2 cm) of chitin and deaminated chitin, 50 – 100 mesh, were packed and equilibrated with buffer. Enzyme solutions were applied at a rate of about 5 ml/h followed by washing with buffer (100 ml). The columns were eluted with either (a) M acetic acid or (b) 0.01 M Tris-HCl pH 8.0, and a linear NaCl-gradient (200 ml) from 0 to 0.2 M. The elution rate was approximately 5 ml/h. Fractions (2 or 5 ml) were collected, dialyzed against 0.01 M Tris-HCl pH 8.0, and subjected to activity tests. All fractionation experiments were carried out at 4°C.

RESULTS

The various activities of crude enzyme preparation (CEP)[1] were apparently adsorbed to chitin. Only a slight caseinolytic activity was demonstrated in the effluent, but due to low activity there was uncertainty with respect to

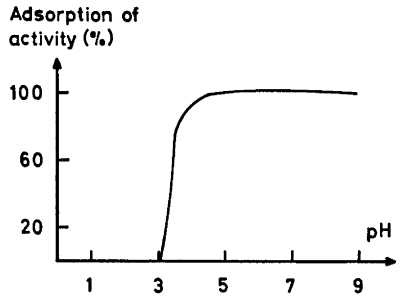

Fig. 1. Percentage adsorption to chitin of bacteriolytic activity (tested on *B. megaterium*) at various pH-values.

the amidase. The effect of pH on the binding to chitin was studied over a pH-range of 2.6–9.0 using the buffer systems: 0.01 M Na-citrate-HCl (pH 2.6–5.0), 0.01 M Na-phosphate (pH 5.0–8.0), and 0.01 M Tris-HCl (pH 8.0–9.0). CEP (10 ml), dialyzed against the appropriate buffer, was applied to the chitin column equilibrated with the same buffer. After washing with buffer, the activity adsorbed was eluted with M acetic acid and dialyzed against 0.01 M Tris-HCl, pH 8.0. Washings and eluates were tested for lytic activity on heat-killed cells of *B. megaterium* and for caseinolytic activity. The percentage of total lytic activity applied to the column which was bound at various pH-values is illustrated in Fig. 1. Above pH 4.2 complete adsorption of bacteriolytic activity was obtained. An abrupt drop in adsorption occurred below pH 4.2 and at approximately pH 3.2 the affinity of lytic enzymes for chitin was zero. Upon digestion of Sa mucopeptide, Bm cell wall, and casein with the eluates, free amino and reducing groups showed an increase in the same pH-range, as did also lysis of casein. The use of deaminated chitin as column material gave the same results as for chitin, and rubbed paper (Whatman No. 1 chromatography paper) at pH 8.0 (0.01 M Tris-HCl) behaved similarly as far as adsorbing capability is concerned.

Elution of enzyme-loaded (15 ml of the CEP) column with the NaCl-gradient and testing of every second fraction for bacteriolytic and caseinolytic activity gave the results shown in Figs. 2 and 3. To some extent the lytic activity of the material applied to the column seems, ac-

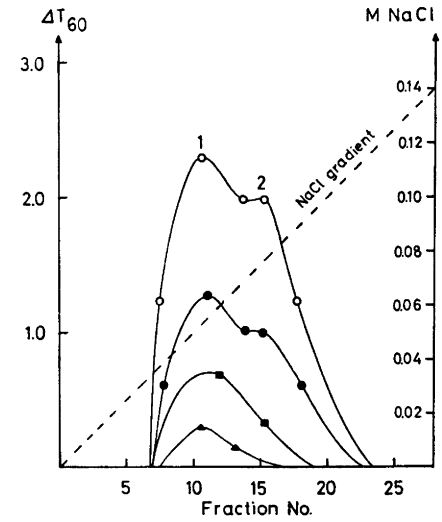

Fig. 2. Lytic activity in fractions (5 ml) from chitin column eluted with a linear NaCl-gradient, as tested on heat-killed Bm (○), Sa (●), Ml and Sl (■), and M-144, M-84 and Sp (▲). ΔT_{60} (reduction in turbidity after 60 min) = (ΔTt/t) × 60.

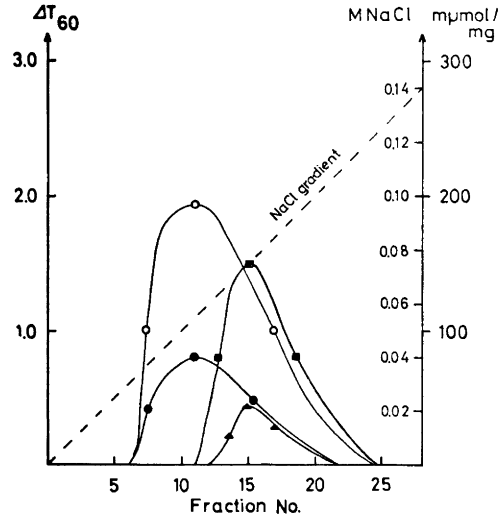

Fig. 3. Caseinolytic activity (●) and the increase in free amino groups on Sa mucopeptide (○), reducing groups (■) and *N*-acetylamino sugars (▲) of Bm cell wall upon digestion with fractions from the chitin column eluted with a linear NaCl-gradient. ΔT_{60}: See legend Fig. 2.

cording to the tests on Bm and Sa cells, to be divided into two peaks (Fig. 2). One of these peaks corresponds to the increase in free amino groups (tested on Sa mucopeptide) and the other to the increase in reducing groups (tested on Bm cell wall) (Fig. 3). The fractions showed no absorbance at 280 nm. Of the two activity peaks indicated, only the first showed lytic activity on all test bacteria and on casein within a digestion period of 90 min. The second peak lysed only Sa and Bm cells and increased the amount of reducing groups and *N*-acetylamino sugar. The recovery of bacteriolytic and caseinolytic activity was calculated to be about 80 % and 30 %, respectively. Deaminated chitin gave a similar elution pattern.

DISCUSSION

All the bacteriolytic enzymes of *Streptomyces albus* cultures seem to bind reversibly to chitin. The binding is apparently dependent on low ionic strength since elution of lytic material starts below 0.04 M NaCl (Fig. 2), but shows high affinity at pH-values above 4.2. The abrupt drop in binding affinity below pH 4.2, decreasing to zero at pH 3.2, is in accord with data reported for lysozymes.[7,9] In these reports the carboxyl groups localized to the "active sites" of the enzymes were supposed to be essential for binding to chitin. No difference between chitin and deaminated chitin was observed in the adsorption and elution pattern. Purified chitin contains some free amino groups revealed by deacetylation. These groups have been shown to exhibit ion-exchange effect,[5] but seem, according to the results with deaminated chitin, to be of no importance for adsorption.

Both endopeptidase and the endo-*N*-acetylmuramidase activity were demonstrated in the eluates from the chitin materials, the elution pattern being very similar to that of the carboxy methyl cellulose columns.[2] The binding of the muramidase to chitin is most probably due to a real enzyme-substrate affinity on account of analogy in the structures of glycan chains of mucopeptide and chitin, a specific binding of the endopeptidases being more doubtful. It has, however, previously been shown[11] that the endopeptidase activities of *Streptomyces albus* G to a certain degree depend on an intact glycan chain, and this may have a connection with a possible binding site of endopeptidases to the glycan chain.

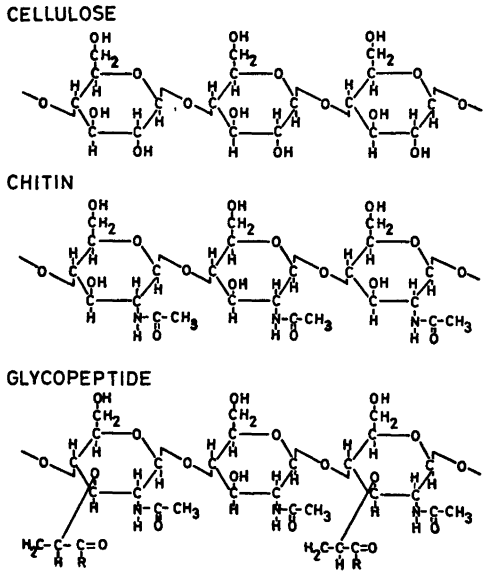

Fig. 4. The glycan chain of cellulose, chitin and mucopeptide (glycopeptide). R = OH or peptide.

The results of the present experiments with chitin are comparable with those obtained with carboxy methyl cellulose, and cellulose also with regard to adsorbing ability. This points to the possibility that binding of the lytic enzymes to the glycan chains is effected similarly in mucopeptides, cellulose, carboxy methyl cellulose and chitin (see Fig. 4 for a structural comparison), and that the introduced carboxyl groups have only a minor influence on this binding.

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3,3-Dialkylindolin-2-ones and 3,3-Dialkylisoindolin-1-ones. 1. Hofmann Hypohalite Degradation of 4,4-Dialkyl-1,3-dioxo-1,2,3,4-tetrahydroisoquinolines (4,4-Dialkylhomophthalimides)

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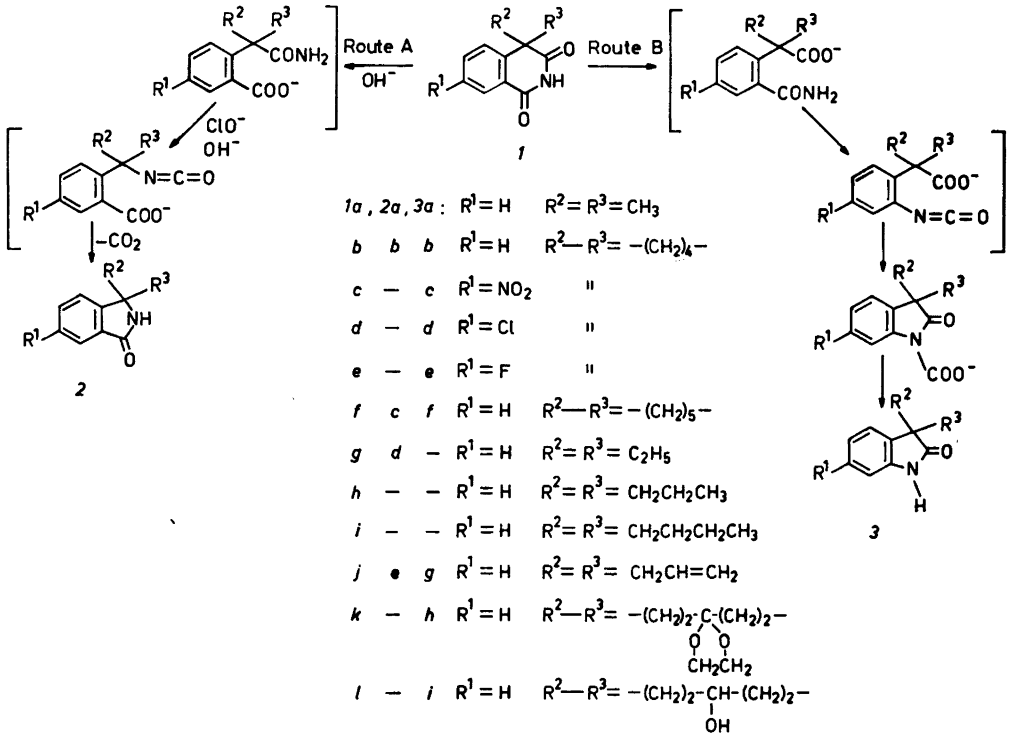
Hofmann hypohalite degradation of certain 4,4-dialkyl substituted homophthalimides has been found to give a high yield of the corresponding 3,3-dialkylindolin-2-ones. If these homophthalimides are subjected to alkaline hydrolysis prior to treatment with hypohalite, isoindolin-1-ones are produced together with the indolin-2-ones.

In the course of an investigation on certain types of biologically active compounds, we required a number of 3,3-dialkylisoindolin-1-ones. Of the limited number of such compounds described in the literature, the 3,3-dimethyl derivative had been prepared by zinc dust distillation of "1,1-dimethyl-3-amino-ψ-isoindole"[1] and its *N*-phenyl derivative was reportedly formed when 3,3-dimethylphthalide was heated with aniline.[2] Hauser *et al.*[3] reported that a number of *o*-(*N*-alkylcarbamoyl)benzyl alcohols, including the α,α-pentamethylene derivatives, undergo ring-closure to isoindolinones when treated with strong acids. A reinvestigation of the structures of these reaction products by Bailey and De Grazia,[4] however, revealed that some of them are iminophthalides rather than isoindolinones. New routes had therefore to be developed for the preparation of our intermediates.

Since isoindolin-1-ones may be regarded as lactams of *o*-aminomethylbenzoic acids, our interest became focussed on the means of preparation of such acids dialkylated at the methylene carbon atom. Of the large number of methods generally available for the preparation of amines, very few are adaptable to the synthesis of derivatives fully alkylated at the α-carbon atom. Methods involving nucleophilic replacement reactions generally fail because eliminations prevail, many other methods fail because of the steric hindrance exerted by the α-substituents. The Hofmann degradation of amides,[5] however, is an exception and permits the synthesis of this sort of amine in high yields from amides of α,α-dialkyl fatty acids. One route to the desired isoindolin-1-ones would therefore use *o*-carboxy-α,α-dialkylphenylacetamides as intermediates. The facile preparation of 4,4-dialkylhomophthalimides from the readily available homophthalimide [6,7] or from suitably substituted homophthalic acids induced us to investigate the possibilities of this type of compound as start material for the acetamide derivatives. Although it has been reported [8] that homophthalimide itself is hydrolysed to *o*-carbamoylphenylacetic acid rather than to *o*-carboxyphenylacetamide (however, *cf.* Ref. 9) it was felt that because of the steric hindrance exerted by the alkyl groups in the *geminally* alkylated homologues the formation of phenylacetamide derivatives from these compounds would be favoured. Hofmann degradation of these acetamide derivatives followed by ring closure would then afford the desired products, according to the sequence outlined in Scheme 1, Route A.

For our investigation of this type of reaction, we used mainly 1′,3′-dioxo-1′,2′,3′,4′-tetrahydrospiro(cyclopentane-1,4′-isoquinoline), (4,4-



Scheme 1.

tetramethylenehomophthalimide*) (*1b*) as our model compound, since this derivative could be readily prepared in good yield [7] and also proved to be eminently suited for the isolation of the products of reaction with little loss.

Hydrolysis experiments with the homophthalimide derivative *1b* soon showed that whereas the unsubstituted homophthalimide could be completely hydrolysed within 3 days at room temp.[8], the 4,4-dialkyl derivatives were apparently very resistant to alkaline hydrolysis even at elevated temperatures. Thus when a solution of *1b* in excess of 2 N sodium hydroxide was kept at room temp. for various times up to 25 days and subsequently treated with hypochlorite solution, the maximum yield of the expected 1′-oxospiro(cyclopentane-1,3′-isoindoline) (*2b*) that could be isolated was about 35 %. An interesting observation was the fact that almost maximum yields were obtained already within the first few days, after which the rise in yield was almost imperceptible (Fig. 1). Using elevated temperatures (60° and 95°) did not improve the yield of *2b*, indeed, it became lower, probably because of the secondary hydrolysis of the amide group, as indicated by a distinct smell of ammonia noticeable at the higher temp.

In order to recover start material supposed to be present in the mother liquor after *2b* had been filtered off, the filtrate was acidified. Vigorous evolution of carbon dioxide ensued and a material precipitated out which was subsequently identified as the known 2′-oxospiro(cyclopentane-1,3′-indoline) (*3b*). When the alkaline reaction mixture was cooled strongly instead of being acidified, a sodium salt precipitated which, according to elemental analysis, was apparently sodium 2′-oxospiro(cyclopentane-1,3′-indoline)-1′-carboxylate. The structure was confirmed by esterification with dimethyl sulphate followed by pyrolysis to the known 1′-methylspiro(cyclopentane-1,3′-indolin)-2′-one.

* For convenience, the compounds are named as derivatives of homophthalimide when this can be done without risk of misunderstanding.

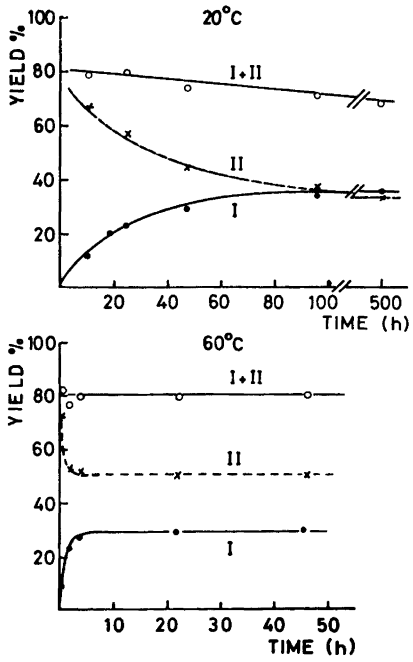


Fig. 1. Semiquantitative determination of the yields of 1′-oxospiro(cyclopentane-1,3′-isoindoline), I, and 2′-oxospiro(cyclopentane-1,3′-indoline), II, as functions of time and temp.

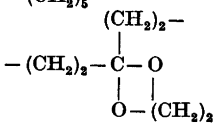
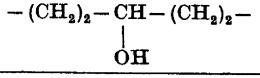
We later found that *3b* could be obtained in 89 % yield if the homophthalimide derivative was dissolved in aqueous alkali and immediately treated with the hypochlorite solution, even if the reaction was allowed to proceed at room temp. for as short a time as 1/2 h; under these conditions, none of the isoindolinone *2b* could be detected. This reaction, when applied to a variety of *geminally* disubstituted homophthalimides, afforded in high yields the disubstituted indolinones listed in Table 1, and thus offers a convenient route to several 3,3-dialkylated indolinones unsubstituted at the nitrogen atom, a class of compounds which has hitherto only been available through lengthy procedures and in unsatisfactory yields.[10–13]

When the reaction was carried out with 4,4-diallylhomophthalimide (*1j*) much longer reaction times were required (of the order of 24 h) and a mixture of the indolinone and the isoindolinone derivatives was isolated together with unreacted start material. 4,4-Diethylhomophthalimide failed to give any 3,3-diethylindolin-2-one but afforded up to 36 % of 3,3-diethylisoindolin-1-one when the homophthalimide derivative was allowed to react with alkaline hypochlorite solution for 3 days at room temp. 4,4-Dipropyl- and 4,4-dibutylhomophthalimide failed to react under any of the conditions tried. It thus appears that increasing steric hindrance prevents progressively first the reaction leading to an indolinone derivative and then that leading to an isoindolinone. Unsubstituted homophthalimide appears to give halogenated products with no indication of any rearrangement when treated with hypohalite solution.

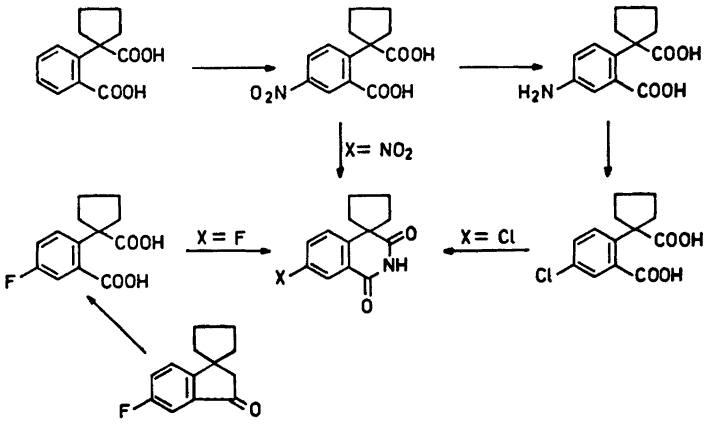
Some comments on the modes of formation of the indolinones seem to be of interest. Although other mechanisms have been suggested [14] it is generally accepted [5,15,16] that the Hofmann degradation of imides is preceded by a hydrolytic opening of the imide ring to produce an "amic" acid intermediate which then rearranges. The formation of the isoindoline and indoline derivatives after pretreatment with alkali seems to be readily explicable by such ring opening mechanisms, Scheme 1. It is interesting that the yield of the isoindolinone *2b* levels off at about 35 % despite the fact that very little further hydrolysis to the dicarboxylic acid occurs, as is evident from Fig. 1. A possible explanation for this would be that the two hydrolysis routes, A and B in Scheme 1, compete with each other, finally resulting in an equilibrium mixture containing approx. 35 % of 1-(2-carboxyphenyl)cyclopentanecarboxamide.

However, it is much more difficult to envisage the mechanism by which the indolinones are formed when the homophthalimides are treated with alkaline hypochlorite solution without prehydrolysis. Since, under these conditions, the indolinones are formed very rapidly in almost quantitative yields, in contrast to the presumably very slow hydrolysis of the homophthalimide derivatives, it is extremely difficult to reconcile the rearrangement reaction with a mechanism involving a conventional ring opening of the homophthalimide derivative to the intermediate *o*-carbamoylphenylacetic acid. Because of this, we undertook a more extensive investigation of the mechanism

Table 1.

No.	R^1	R^2	R^3	Formula	M.p. °C	Yield %	Analyses Found C	H	N	O	Calculated C	H	N	O
3a	H	CH_3	CH_3	$C_{10}H_{11}NO$	150–151 [19]	84.5	74.3	6.9	8.6	10.3	74.5	6.9	8.7	9.9
b	H	$-(CH_2)_4-$		$C_{12}H_{13}NO$	114–113 [11]	89	77.0	7.0	7.5	9.0	77.0	7.0	7.5	8.6
c	NO_2	»		$C_{12}H_{12}N_2O_3$	228–230	84.5	61.7	5.2	11.9	20.8	62.1	5.2	12.1	20.7
d	Cl	»		$C_{12}H_{12}ClNO$	143–145 (128[a])	73	65.2	5.6	6.3	7.4 Cl 15.7	65.0	5.5	6.3	7.2 Cl 16.0
e	F	»		$C_{12}H_{12}FNO$	124	81.5	70.4	5.9	6.9	F 9.3	70.2	6.0	6.8	F 9.3
f	H	$-(CH_2)_5-$		$C_{13}H_{15}NO$	120–124 [11]	52								
h	H			$C_{15}H_{17}NO_3$	211	88	69.3	6.68	5.35	18.9	69.5	6.61	5.40	18.9
i	H			$C_{13}H_{15}NO_2$	191	71	71.4	7.0	6.4	15.2	71.9	7.0	6.5	14.7

*a** Unstable isomorph.



Scheme 2.

of this reaction, which will be the subject of a forthcoming paper.

Several of the substituted homophthalimides used as start material have been described in the literature.[6,7] The syntheses of the new compounds are described in the Experimental Part. The halogenated derivatives of 4,4-tetramethylenehomophthalimide were prepared according to Scheme 2.

The structures of the rearranged products were established by means of IR-, UV-, and NMR-spectroscopy. Especially the UV and NMR spectra were found to be very informative. In methanolic solution, all the indolinones investigated had a strong absorption band at 245 – 250 nm and only weak absorption around 225 nm. The isoindolinones, on the other hand, had a very strong, complex peak around 225 nm, almost no absorption around 250 nm but two small peaks at about 270 and 280 nm (cf. Ref. 20). In the NMR spectra of the indolinones the aromatic hydrogen atom *ortho* to the amino group is clearly distinguishable at τ 2.8 – 3.0 ppm ($CDCl_3$ solution), the corresponding atom appearing at τ 2.0 – 2.2 ppm in the isoindolinones. The IR spectra (in KBr) are less informative but there is a trend towards a shifting of the carbonyl frequences to higher values in the isoindolinones. (Thus the diallylindolinone derivative *3g* has two C=O absorption bands at 1675 and 1715 cm^{-1}, the corresponding bands in the isoindolinone derivative *2e* appearing at 1650 and 1700 cm^{-1}).

EXPERIMENTAL PART

Melting points were taken on a Heraeus Fus-O-Mat melting point apparatus. The structures of the compounds were determined by elemental analyses and by IR-, UV-, and NMR-spectroscopy. The microanalyses were carried out by Prof. K. J. Karrman, University of Lund, Lund, Sweden, and by Dr. Alfred Bernhardt, Mikroanalytisches Laboratorium, Elbach über Engelskirchen, Germany.

A. Preparation of homophthalic acid derivatives

1-(2-Carboxy-4-nitrophenyl)cyclopentanecarboxylic acid. 1-(2-Carboxyphenyl)cyclopentanecarboxylic acid [17] (355 g; 1.53 mol) was added portionwise to fuming nitric acid (sp. gr. 1.52; 1100 ml) under vigorous stirring, the temp. being kept at 5 – 10° by external cooling. After being stirred for 3 h, the mixture was poured onto crushed ice, the product was collected, washed copiously with water and dried, affording 400 g (93.5 %) of white crystals, m.p. *ca.* 200° (rapid heating). A sample crystallized from methanol-petroleum ether had m.p. 210°. (Found: C 55.7; H 4.72; N 5.00; O 34.4. $C_{13}H_{13}NO_6$ requires: C 55.9; H 4.70; N 5.02; O 34.4). If the reaction is carried out at 20°, the corresponding *anhydride* is obtained in 90 % yield, m.p. 132° (from benzene). (Found: C 59.9; H 4.17; N 5.36; O 30.4. $C_{13}H_{11}NO_5$ requires: C 59.8; H 4.24; N 5.36; O 30.6).

1-(4-Amino-2-carboxyphenyl)cyclopentanecarboxylic acid. The foregoing nitro compound (56 g; 0.2 mol) was dissolved in 150 ml of methanol, Raney nickel (20 g wet weight) was added and the mixture shaken in an atmosphere of hydrogen at an initial pressure of about

4 kg/cm². After 4 h, by when the theoretical quantity of hydrogen had been consumed, the grey precipitate was filtered off and sucked dry, the product was dissolved in conc. ammonia (50 ml), the solution filtered, cooled and neutralised with dil. hydrochloric acid. After acidification with acetic acid, the precipitate was collected, washed with cold water and dried, affording 46 g (92 %) of a grey-white powder of indefinite m.p. Recrystallization from water gave a product containing 1 mol of water. (Found: C 57.6; H 6.43; N 5.19; O 30.4. $C_{13}H_{15}NO_4.H_2O$ requires: C 58.4; H 6.41; N 5.24; O 29.9). Recrystallization from acetic acid gave the corresponding anhydride. (Found: C 67.1; H 5.64; N 5.84; O 21.4. $C_{13}H_{13}NO_3$ requires: C 67.4; H 5.67; N 6.06; O 20.8).

Note: It is important to observe that the starting material in this reaction contains no anhydride and that the reaction temp. is not allowed to rise to a level that will cause the diacid to be converted to the anhydride. If this is not rigorously followed, considerable quantities of polymeric products resulting from reaction between the anhydride and amino acid will be obtained.

1-(2-Carboxy-4-chlorophenyl)cyclopentanecarboxylic acid. The foregoing amino acid (31.8 g; 0.15 mol) in a mixture of 35 ml of conc. hydrochloric acid and 200 g of ice was diazotised at 5 – 10° with sodium nitrite (10.5 g; 0.15 mol) in 75 ml of water. After addition, the mixture was stirred for about 15 min and then added portionwise with vigorous stirring to an ice-cold solution of freshly prepared cuprous chloride (from 195 g of $CuSO_4.5H_2O$; 0.75 mol) in conc. hydrochloric acid (400 ml). This reaction should be carried out in a 3 litre beaker since copious effervescence occurs. After standing at ambient temp. for about 2 h the precipitate was collected, washed with dilute hydrochloric acid and water, and dried, giving 28.2 g (69.5 %) of a cream coloured product of indeterminable m.p. A sample warmed with acetic anhydride afforded an orange coloured product which was purified by column chromatography on alumina and subsequently crystallized from benzene-petroleum ether, affording the corresponding *anhydride* as bright orange crystals, m.p. 120 – 122°. (Found: C 62.9; H 4.38; Cl 13.5; O 19.2. $C_{13}H_{11}ClO_3$ requires: C 62.3; H 4.42; Cl 14.1; O 19.1).

1-(2-Carboxy-4-fluorophenyl)cyclopentanecarboxylic acid. 5'-Fluorospiro(cyclopentane-1,1'-indan)-3'-one [18] (41 g; 0.2 mol) in 0.5 N sodium hydroxide solution (800 ml; 0.4 mol) was warmed to 95° and treated portionwise with potassium permanganate (90 g; 0.55 mol) under stirring. After addition, the mixture was stirred on a water-bath for about 15 min by when the permanganate colour had become discharged. The mixture was filtered hot, the manganese dioxide cake was washed with hot water, the filtrate was cooled and acidified with conc. hydrochloric acid till crystals began to appear. Sodium bicarbonate was added, the mixture was warmed, filtered, cooled and acidified. The precipitate was collected, washed and dried giving 34 g (67.5 %) of crude diacid, m.p. 130 – 140°, with conversion to the *anhydride*, m.p. 83°. For analysis, a sample of the diacid was recrystallized from diisopropyl ether-petrol. ether and then melted at *ca.* 140°. (Found: C 61.9; H 5.20; F 7.53. $C_{13}H_{13}FO_4$ requires: C 61.9; H 5.19; F 7.53).

B. Preparation of homophthalimide derivatives

1',3'-Dioxo-7'-nitro-1',2',3',4'-tetrahydrospiro-(cyclopentane-1,4'-isoquinoline) (1c). 1-(2-Carboxy-4-nitrophenyl)cyclopentanecarboxylic acid (28 g; 0.1 mol) was treated with conc. ammonia (30 ml) and the mixture heated in an open flask over an open flame. The mixture darkened and eventually became converted to a high melting solid. On further heating, it melted with effervescence. Heating was continued until a clear dark melt was obtained which, after solidification, was pulverised and crystallized from 100 ml of dioxane, yielding 19.9 g (76.5 %) if the imide *1c*, m.p. 227°. (Found: C 59.9; H 4.65; N 10.7; O 24.6. $C_{13}H_{12}N_2O_4$ requires: C 60.0; H 4.65; N 10.8; O 24.6).

The following two compounds were prepared in a similar manner.

7'-Chloro-1',3'-dioxo-1',2',3',4'-tetrahydrospiro-(cyclopentane-1,4'-isoquinoline) (1d), hair-like needles from ethanol, m.p. 215 – 216°. Yield almost 100 %. (Found: C 62.7; H 4.87; Cl 13.8; N 5.63; O 13.2. $C_{13}H_{12}ClNO_2$ requires: C 62.5; H 4.87; Cl 14.2; N 5.61; O 12.8).

1',3'-Dioxo-7'-fluoro-1',2',3',4'-tetrahydrospiro(cyclopentane-1,4'-isoquinoline) (1e), white hair-like crystals from ethanol, m.p. 213°, yield 87 %. (Found: C 67.0; H 5.19; F 8.82; N 6.10; O 13.6. $C_{13}H_{12}FNO_2$ requires: C 66.9; H 5.19; F 8.15; N 6.01; O 13.7).

Ethylene glycol ketal of 1',3',4-trioxo-1',2',3',4'-tetrahydrospiro(cyclohexane-1,4'-isoquinoline) (1k). 1',3',4-Trioxo-1',2',3',4'-tetrahydrospiro-(cyclohexane-1,4'-isoquinoline) [17] (4.9 g; 0.02 mol) in toluene (250 ml) was treated with ethylene glycol (52 ml) and *p*-toluenesulphonic acid (200 mg). The mixture was distilled slowly from an oil-bath during 20 h, the volume of the reaction mixture being kept approximately constant by the periodic addition of dry toluene. After about 200 ml of distillate had been collected the mixture was cooled, the crystals were collected, washed with petroleum ether and water, and dried, giving 5.5 g (96 %) of stout, pale-yellow crystals, m.p. 220°. Crystallization from ethanol afforded a white product melting at 223 – 224°. (Found: C 66.1; H 6.10; N 4.89;

O 22.3. $C_{16}H_{17}NO_4$ requires: C 66.9; H 5.96; N 4.88; O 22.3).

1',3'-Dioxo-4-hydroxy-1',2',3',4'-tetrahydrospiro(cyclohexane-1,4'-isoquinoline) (11). 1',3',4-Trioxo-1',2',3',4'-tetrahydrospiro(cyclohexane-1,4'-isoquinoline) [17] (7.3 g; 0.03 mol) in abs. ethanol was treated at room temp. with sodium borohydride (0.6 g; 0.015 mol) under vigorous stirring. After 1.5 h, the clear yellow solution was treated with 2.0 ml of acetic acid, the volatile matter was removed under vacuum and the residual syrup was triturated with diisopropyl ether, causing it to become crystalline. The product was collected (8.2 g, m.p. *ca.* 170°) and crystallized from 100 ml of 50 % methanol, affording 4.8 g (64 %) of white crystals, m.p. 189 – 190°. (Found: C 68.6; H 6.16; N 5.70; O 19.7. $C_{14}H_{15}NO_3$ requires: C 68.6; H 6.16; N 5.71; O 19.6). The NMR spectrum in $(CD_3)_2SO$ reveals 1 aromatic H at τ 1.8 – 1.9 ppm, 3 aromatic H centered around τ 2.2 ppm. From the mother liquor there was isolated 0.75 g of white crystals melting at 247° after crystallization from dioxane, which gave a correct analysis for a *dihydro derivative* of compound 11. (Found: C 67.8; H 7.03; N 5.65; O 19.8. $C_{14}H_{17}NO_3$ requires: C 68.0; H 6.93; N 5.66; O 19.4). The IR-spectrum of this compound indicates two different hydroxyl groups. The NMR spectrum in $(CD_3)_2SO$ reveals 4 aromatic H centered around τ 2.9 ppm, indicating reduction of the aromatic carboxyl group. The compound thus is *1',4-dihydroxy-3'-oxo-1',2',3',4'-tetrahydrospiro(cyclohexane-1,4'-isoquinoline)*.

C. Simultaneous preparation of isoindolin-1-one and indolin-2-one derivatives

Spiro(cyclopentane-1,3'-isoindolin)-1'-one (2b) *and spiro(cyclopentane-1,3'-indolin)-2'-one* (3b). A stock solution of 1',3'-dioxo-1',2',3',4'-tetrahydrospiro(cyclopentane-1,4'-isoquinoline) [7] (65 g; 0.3 mol) in 2 N sodium hydroxide solution (600 ml; 1.2 mol) was prepared and stored at room temp. At regular intervals, an aliquot corresponding to 0.03 mol was withdrawn and treated with 1.08 M sodium hypochlorite solution (32 ml; 0.033 mol). After standing for 2 h, the solution was heated on a water bath for 15 min, cooled, and the precipitated spiro(cyclopentane-1,3'-isoindolin)-1'-one filtered off, washed and dried. The compound was analytically pure and melted at 179°. (Found: C 77.2; H 6.98; N 7.34; O 8.85. $C_{12}H_{13}NO$ requires: C 77.0; H 7.00; N 7.48; O 8.55). The filtrate from above was acidified with acetic acid, whereupon evolution of carbon dioxide ensued and a viscous oil fell out which gradually solidified on cooling. Recrystallization from aqueous methanol afforded spiro(cyclopentane-1,3'-indolin)-2'-one as colourless crystals, m.p. 114° (Lit.[11] m.p. 113°). The yields of these lactams varied as a function of time in the manner depicted in Fig. 1. The results of a similar series of experiments performed at 60° are likewise presented in Fig. 1.

3,3-Dimethylisoindolin-1-one (2a), *and 3,3-dimethylindolin-2-one* (3a). When the above reaction was carried out with 4,4-dimethylhomophthalimide at approximately 20°, yields of 3,3-dimethylisoindolin-1-one ranging from 10 % to 17 % and of 3,3-dimethylindolin-2-one ranging from 60 % to 44 % were obtained, the times of reaction with alkali varying from 18 to 67 h. Compound *3a* melted at 150° (Lit.[19] m.p. 150 – 151°), compound *2a* melted at 158 – 162° (Lit.[1] m.p. 162°).

3,3-Diallylisoindolin-1-one (2e) *and 3,3-diallylindolin-2-one* (3i). When a solution of 4,4-diallylhomophthalimide [6] in alkaline sodium hypochlorite solution was allowed to stand at room temp. for about 24 h, *3,3-diallylisoindolin-1-one* precipitated out in yields up to 43 %. Crystallization of the crude product from dilute ethanol gave beige crystals, m.p. 112°. (Found: C 78.3; H 7.06; N 6.57; O 7.79. $C_{14}H_{15}NO$ requires: C 78.8; H 7.09; N 6.57; O 7.50). When the alkaline filtrate was acidified, a crystalline precipitate was obtained which, after washing with dilute sodium hydroxide solution and water, afforded *3,3-diallylindolin-2-one*, m.p. 96° after crystallization from dilute ethanol. Yield 16 – 28 %. (Found: C 78.2; H 7.04; N 6.55; O 7.93. $C_{14}H_{15}NO$ requires: C 78.8; H 7.09; N 6.57; O 7.50).

3,3-Diethylisoindolin-1-one (2d). 4,4-Diethylhomophthalimide [6] was allowed to react with sodium hydroxide solution for 3 days at 20° and then treated with hypochlorite as described. The titel compound separated from the solution and was collected. White crystals from ethanol, m.p. 171 – 172°. Yield 36 %. (Found: C 76.2; H 8.01; N 7.31; O 18.1. $C_{12}H_{15}NO$ requires: C 76.2; H 7.99; N 7.40; O 18.5). When the alkaline filtrate was acidified, only unreacted start material was recovered with no evidence of any formation of the indolinone derivative.

Note: When 4,4-dipropyl- and 4,4-dibutylhomophthalimide were submitted to this treatment only unreacted start material could be recovered from the reaction mixtures even when the temp. in the hydrolysis step was as high as 90°.

D. Preparation of indolin-2-one derivatives unaccompanied by formation of isoindolin-1-one derivatives

The following procedure, used in the preparation of spiro(cyclopentane-1,3'-indolin)-2'-one, illustrates the method that was used for the synthesis of the indolin-2-ones listed in Table 1. 4,4-Dipropyl- and 4,4-dibutylhomophthalimide failed to react under these conditions and the start materials were recovered.

Sodium hypochlorite (1.08 M, 31 ml; 0.033 mol) was added to a cold solution of 1',3'-dioxo-1',2',3',4'-tetrahydrospiro(cyclopentane-1,4'-isoquinoline)[7] (6.5 g; 0.03 mol) in 2 N sodium hydroxide (60 ml; 0.12 mol) and the mixture stood at room temp. for 2 h. The clear solution was then warmed on a water bath for 15 min, cooled, and acidified with acetic acid, whereupon brisk evolution of carbon dioxide ensued and a viscous oily material fell out which became crystalline on cooling and scratching. The crystals were collected, washed with dilute sodium hydroxide and water and dried, affording 5.0 g (89 %) of the title compound *3b*, m.p. 114°. (Lit.[11] m.p. 113°).

Sodium 2'-oxospiro(cyclopentane-1,3'-indoline)-1'-carboxylate and *1'-methylspiro(cyclopentane-1,3'-indolin)-2'-one.* When the reaction mixture prepared as above was cooled strongly with ice-water before acidification with acetic acid, a white crystalline precipitate, approximately analysing for $C_{13}H_{12}NO_3Na$ and apparently consisting of *sodium 2'-oxospiro(cyclopentane-1,3'-indoline)-1'-carboxylate*, was isolated in about 50 % yield. This salt (7.6 g; 0.03 mol) was dissolved in water (40 ml) and the stirred solution treated dropwise with dimethyl sulphate (6.5 ml; 0.06 mol) at room temp. Dilute sodium hydroxide was added simultaneously at such a rate as to maintain an alkaline pH. The mixture was extracted with chloroform, the extract was washed with sodium bicarbonate solution and water, dried and the solvent evaporated, affording 6.1 g (83 %) of an oil assumed to be *methyl 2'-oxospiro(cyclopentane-1,3'-indoline)-1'-carboxylate.* Distillation at 110 – 120°/1.0 Torr caused decomposition and the product isolated was *1'-methylspiro(cyclopentane-1,3'-indolin)-2'-one,* m.p. 57 – 59° after crystallization from hexane (Lit.[21] m.p. 63°). (Found: C 77.1; H 7.50; N 6.96; O 7.72. $C_{13}H_{15}NO$ requires: C 77.6; H 7.51; N 6.96; O 7.95).

2',4-Dioxospiro(cyclohexane-1,3'-indoline). The ketal *3h* (1.04 g; 0.004 mol) was dissolved in a mixture of acetic acid (5 ml) and 2 N hydrochloric acid (5 ml) and the solution heated over a free flame for some minutes. After cooling, the product was collected, affording 0.66 g (78 %) of white crystals, m.p. 200 – 201°. (Found: C 72.2; H 6.11; N 6.45; O 15.1. $C_{13}H_{13}NO_2$ requires: C 72.5; H 6.09; N 6.51; O 14.9).

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Arginase in Chang's Liver Cells

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Arginase activity has been studied in lysates of Chang's liver cells, an established cell line derived from human liver. It was found that the enzyme of Chang cells differed only slightly from human liver arginase with respect to activation by manganese, pH dependence, Michaelis constant, and inhibition by basic amino acids. On the other hand there was a marked difference in electrophoretic mobility – the Chang cell enzyme moved towards the anode while liver arginase moved towards the cathode at pH 8.6. Furthermore, cell fractionation studies revealed, that the localization of the enzyme was different, the bulk of the Chang cell arginase being firmly bound to cytoplasmic particles sedimenting with mitochondria.

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is a wide spread enzyme present in a variety of animal and plant cells.[1] By far the highest enzyme activity is found in the liver of ureotelic animals, where it mediates the final step in the ornithine-citrulline-urea cycle. When liver cells are maintained in cell culture the urea cycle is lost.[2] Thus, Chang's liver cells, an established cell line derived from human liver have no ornithine transcarbamylase activity [2] and consequently citrulline can not be formed from ornithine by these cells. However, arginase is still present but with a specific activity in the range of 0.1 % of the activity reported for intact liver.[3,4] The functional role of arginase in cells and tissues lacking the urea cycle has generally been ascribed to the catabolism of arginine and to the synthesis of proline by way of ornithine.[5]

Four isoenzymes with arginase activity present in varying amounts in different rat organs have been separated by means of chromatography.[6] It has also been shown that arginases isolated from rat kidney and liver differ in many properties.[7]

In human liver Bascur *et al.*[8] found two protein fractions with arginase activity, which could be separated by chromatography. Using immunological methods and electrophoresis Cabello *et al.*[9] compared purified liver arginase with arginase isolated from red blood cells. They found two, possibly identical isoenzymes in both preparations.

The aim of the present investigation was to compare arginase from Chang's liver cells with arginase from human liver. It seemed of interest to know whether the cultured cells contained the liver enzyme in reduced amounts or if they contained an arginase with different properties.

MATERIAL AND METHODS

Chang's liver is an established cell line originally derived from human liver.[10] Chang cells were grown as spinner cultures in Eagle's minimal medium for suspension cultures, containing 10 % horse serum.[11] The cultures were diluted with fresh medium three times a week and were routinely examined for bacterial contamination. (No contamination was found).

Arginase activity was determined according to the micromethod of Schimke,[12] using guanido labelled arginine as substrate followed by determination of $^{14}CO_2$ liberated by urease. Cell samples from the suspension cultures were centrifuged at 700 *g* for 3 min and resuspended in 50 mM Tris-HCl buffer, pH 7.8, at a density of approximately 10^8 cells per ml. The cell suspension was frozen and stored at −17°. As pointed out by Schimke [13] preparations made by freezing and thawing gave the same arginase activity as cell homogenates prepared in other ways. After thawing $MnCl_2$ was added to a concentration of 10 mM. The cell suspension was then heated to 55° for 5 min in order to activate arginase. 100 µl samples of the activated suspension were transferred to test tubes with screw caps. The reaction was started by the

addition of 100 µl 0.2 M guanido-^{14}C-arginine pH 9.7 (sp. act. 0.01 mC/mmol), the pH of the reaction mixture was 9.0. After incubation at 37° for appropriate times (usually 30 or 60 min) the reaction was stopped by the addition of 50 µl 0.4 M HCl. Strips of filterpaper were attached inside the screw caps with one end of the strip inserted under a plastic disc fitting into the bottom of the cap. 25 µl of hyamine hydroxide (1 M in methanol) was placed on the paper strip. Urease was dissolved in 0.2 M potassium phosphate buffer pH 6.3 to a concentration of 5 mg per ml. One ml of urease solution was added to the reaction mixture and the test tubes were immediately closed with the screw caps. The tubes were then placed in a rotating test tube rack at 37° for 45 min. The part of the paper strips containing hyamine was cut off and placed in scintillation vials. Radioactivity was determined, using Omnifluor (NEN) in toluene, in an Intertechnique SL 36 liquid scintillation spectrometer at 4°. The amount of enzyme added and the incubation time during the arginase reaction was modified so that the amount of urea formed did not exceed 0.5 µmol (2.5 % of the substrate). Standard solutions containing 0.02 to 0.6 µmol of ^{14}C labelled urea with a specific activity of 0.01 mC/mmol were used for incubations with urease under identical conditions. 1 µmol urea gave 16 000 cpm. One unit of arginase was defined as the amount of enzyme which produced 1 µmol urea per hour under the above conditions. Arginase activity was determined in the same way in subcellular fractions of Chang cells. For kinetic studies guanido-^{14}C-arginine with a specific activity of 0.02 mC/mmol was used.

Protein content was determined using Biuret reagent [14] or in clear supernatant fractions by the method of Warburg and Christian.[15]

Electrophoresis. Chang cell arginase activity could be removed from particulate cell fractions by a non-ionic detergent. Cells were washed in Hank's balanced salt solution [16] and suspended in 50 mM Tris buffer, pH 7.8. $MnCl_2$ and Lubrol (I.C.I.) were added to final concentrations of 10 mM and 0.5 %, respectively, and the cell suspension was shaken on a Vortex mixer. After adjusting the pH to 7.4 with Tris base the suspension was heated to 55° for 20 min and then centrifuged at 15 000 *g* for 20 min. Before electrophoresis the supernatant was concentrated 6 times by dialysis for 4 h against a 10 mM Tris-HCl buffer, pH 7.8, containing 10 mM $MnCl_2$, 10 mM NaCl, 1.5 mM $MgCl_2$, and 15 % polyethyleneglycol (MW 6000). After dialysis the preparation contained 5 to 10 units of arginase activity per ml.

Human foetal liver from a 20 – 25 week old foetus was washed in Hank's salt solution and homogenized in a Potter-Elvehjem homogenizer in 3 volumes cold 10 mM Tris-HCl buffer, containing 150 mM KCl, 20 mM $MnCl_2$, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.8. The homogenate was then centrifuged at 15 000 *g*. The supernatant containing the entire arginase activity was finally heat activated for 20 min at 55° and centrifuged. Immediately before electrophoresis the liver enzyme was diluted 200 times with 10 mM Tris buffer, containing 10 mM $MnCl_2$, in order to obtain an arginase activity in the same order of magnitude as that of the Chang cell preparation.

Three identical 30 µl samples were applied to each glass slide covered with 1 % buffered agarose gel. Sodium barbital buffer, pH 8.6, ionic strength 0.1, was used. Potential difference at the electrodes was 100 V and the current intensity 3 mA. Electrophoresis was performed during 4 h. The agarose gel was sectioned in 3 stripes longitudinally, each containing one sample well, and in 13 cross sections giving pieces of gel approx. 100 µl in volume. These were transferred to test tubes, minced with a glass-rod, and 10 µl 0.1 M $MnCl_2$ was added. After heating for 5 min at 55° arginase activity was determined as described above.

Cell fractionation. Lysates of Chang cells were prepared according to Holtzman *et al.*[17] After washing the cells with cold Hank's salt solution, the cells were suspended in ice cold hypotonic buffer, 10 mM Tris pH 7.8, containing 10 mM NaCl and 1.5 mM $MgCl_2$, and allowed to swell at 0 – 4° for 20 min. The cells were then homogenized in a tight fitting Dounce homogenizer. Microscopic inspection showed very few intact cells. $MnCl_2$ and KCl were then added to the homogenate to final concentrations of 10 mM and 150 mM, respectively. Homogenate of foetal human liver was prepared as described above. Centrifugation at 15 000 *g* for 15 min gave pellets containing *nuclei and larger cytoplasmic particles*. For arginase activity determination pellets were suspended in 50 mM Tris, pH 7.8.

Separation of nuclei from cytoplasm was performed using the above mentioned hypotonic buffer but containing 0.05 mM spermine. Cells were washed, lysed, and homogenized as above. Nuclei were sedimented at 1600 *g* for 2 min. The nuclear pellet was resuspended in buffer and adhering cytoplasm was removed from the nuclei by pipetting several times with a narrow Pasteur pipett. After centrifugation at 1600 *g* the supernatant was added to the first supernatant. The combined supernatants, which did not contain any visible nuclei on microscopic inspection, were centrifuged at 15 000 *g* for 15 min. The resulting pellet, which contained *cytoplasmic particles, mainly mitochondria*, was suspended in 50 mM Tris, pH 7.8 and used for arginase activity and protein determinations.

The *nuclei* were further purified by suspension in 3.75 ml of 0.34 M sucrose in the above buffer containing spermine and layered over 1.25 ml of 1.35 M sucrose in the same buffer. After centrifugation at 15 000 *g* for 15 min the pellet was suspended in 2.5 ml of 2.1 M sucrose

and layered over 2.5 ml of the same sucrose solution. After centrifugation for 45 min at 75 000 *g* the nuclear pellet was washed with 5 ml of 0.34 M sucrose and sedimented at 15 000 *g* for 15 min. The pellet was suspended in 50 mM Tris buffer, pH 7.8. The majority of the nuclei looked intact, but some were severely fragmented. The suspension was used for arginase activity and protein determinations.

L-Arginine-(guanido)-^{14}C was obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England. Hyamine hydroxide from the Packard Instrument Company Inc., Ill. USA. Omnifluor and urea-^{14}C from NEN Chemicals GmbH, Frankfurt, West Germany, urease (Jack bean type III) from Sigma Chemical Company, St. Louis, USA and Eagle's medium and horse serum from Statens Bakteriologiska Laboratorium, Solna, Sweden.

RESULTS AND DISCUSSION

Kinetic properties. Arginase is activated by bivalent cations.[1] Preincubation of Chang cell lysates at 55° for 5 min in the presence of 10 mM $MnCl_2$ increased the arginase activity by 70 %. Longer preincubation times did not increase the activity further. Fig. 1 shows the pH dependence of the reaction. Maximum activity was above pH 9, which is in agreement with results using arginase from ureotelic liver. On the other hand, Gasiorowska *et al.* obtained two pH optima at 7.5 and 9.5 for arginase from kidney, submaxillary gland and brain of the rat.[6]

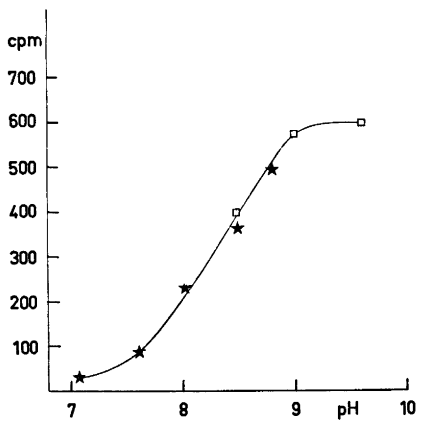

Fig. 1. pH dependence. Cell lysate in 5 mM Tris-HCl, pH 7.8, was activated by heating in the presence of 10 mM $MnCl_2$ prior to determination of arginase activities. Incubation mixtures contained 5 mM $MnCl_2$, 0.1 M ^{14}C-(guanido)-arginine (0.01 mC/mmol) and 50 mM Tris-HCl (pH 7.0 – 8.8 ★) or 50 mM glycine-NaOH (pH 8.5 – 9.6 □). Ordinate: pH, abscissa: arginase activity (cpm in $^{14}CO_2$, liberated by urease).

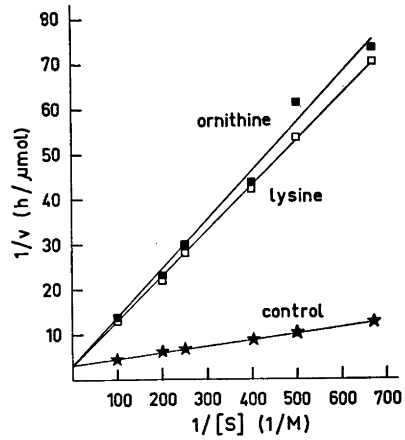

Fig. 2. Michaelis constant and inhibition by lysine and ornithine. Cell lysates were prepared as in Material and Methods, and arginase activated by heating in the presence of 10 mM $MnCl_2$. Incubation mixtures contained 2×10^7 lysed cells, 50 mM glycine-NaOH (pH 9.7), 25 mM Tris-HCl, 5 mM $MnCl_2$, 1.5 – 10 mM ^{14}C-(guanido)-arginine (0.02 mC/mmol). No addition or 10 mM lysine or 10 mM ornithine, respectively. Final pH in the reaction mixtures was 9.0.

K_m and $1/V$ were determined graphically. K_i's for lysine and ornithine were determined using the formula: slope $= (K_m/V)(1 + I/K_i)$ (Ref. 20).

The K_m for arginase as measured in crude Chang cell preparations varied in different experiments between 4 and 7 mM. Lysine and ornithine were both competitive inhibitors. A typical experiment is shown in Fig. 2 where the K_m was 4.5 mM, and the K_i values for lysine and ornithine were 1.6 mM and 1.5 mM, as calculated from the experimental results. With respect to substrate affinity and competitive inhibition by lysine, Chang cell arginase does not differ significantly from human liver arginase, as determined by Bascur *et al.*[8] These authors separated arginase, from human liver, by chromatography, into two components which differed with respect to their inhibition by ornithine. The larger component, corresponding to more than 90 % of the total activity, showed a mixed type of inhibition, while the

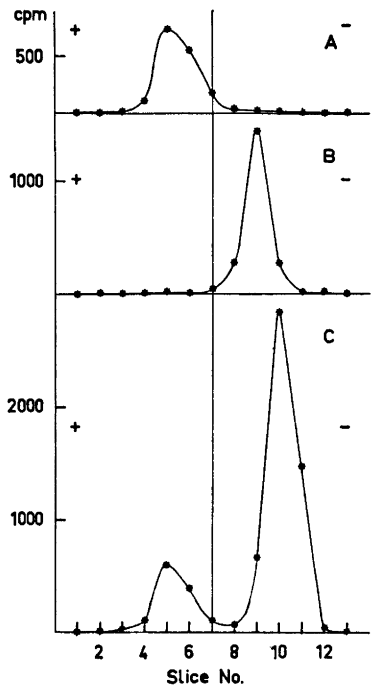

Fig. 3. Agar gel electrophoresis of arginase. A, 30 µl of Chang cell lysate prepared as in Material and Methods (1 mg protein/ml). B, 30 µl liver enzyme (0.03 mg protein/ml). C, Mixture of approx. 15 µl Chang cell arginase and 15 µl liver arginase. Ordinate: agar section, abscissa: arginase activity, adjusted to the total of 15 µl Chang cell enzyme (A), 15 µl liver enzyme (B), and 15 µl Chang cell enzyme + 15 µl liver enzyme (C).

smaller component was competitively inhibited, with a K_i of 3.1. Evidently the Chang cell arginase resembles the minor component of the liver enzyme with respect to inhibition by ornithine.

Electrophoretic mobility. Electrophoresis of Chang cell and foetal human liver preparations were carried out according to Material and Methods. The liver enzyme moved towards the cathode (Fig. 3B), which is in accordance with the results of Cabello *et al.*[9] using arginase from human liver and erythrocytes, where arginase from both sources moved towards the cathode at pH 8.6. Chang cell arginase on the other hand moved towards the anode (Fig. 3A). The activity from Chang cells recovered in the peak (Fig. 3A) corresponded to 100 % of the activity added. (Since Chang cell enzyme was prepared in the presence of 0.5 % Lubrol, a control experiment with human liver in the presence of Lubrol was performed which indicated that the electrophoretic mobility was unaffected). A mixture of the enzyme from human liver and Chang cells was separated into two peaks with maximum activities on the anodal side, corresponding to Chang cell arginase, and on the cathodal, corresponding to human liver arginase (Fig. 3C).

Therefore, the electrophoretic mobility of Chang cell arginase differs from that of human liver. Further studies using purified enzyme preparations may indicate whether this difference is due to the association of Chang cell arginase with some other molecule, thus altering the electrophoretic mobility, or if there is a true difference in the enzyme proteins.

Localization of the enzyme. Using standard procedures for extraction of arginase from liver, in the presence of 10 mM $MnCl_2$ and 150 mM KCl, the activity was found exclusively in the supernatant after centrifugation at 15 000 *g* for 15 min (Table 1). In order to homogenise Chang cells it was first necessary to swell the cells in hypotonic buffer. Immediately after homo-

Table 1. Arginase activity in subcellular fractions (cpm in $^{14}CO_2$).

Fraction	Chang cells	Liver
Whole homogenate	2800 [a]	45 000 [b]
15 000 *g* pellet	2500	–
15 000 *g* supernatant	300	46 000

[a] 100 µl, incubation time 60 min. [b] 10 µl, incubation time 10 min.

Table 2. Release of arginase from the particulate fraction by Lubrol treatment for 3 min at 37°.

Fraction	Arginase activity (cpm in $^{14}CO_2$)
15 000 *g* pellet before Lubrol treatment	3150
15 000 *g* supernatant after Lubrol treatment	
0.1 % Lubrol	3280
0.2 % Lubrol	3200
0.5 % Lubrol	3010

Table 3. Arginase activity in cytoplasmic particles and purified nuclei.

Fraction	Protein [a] (mg)	Arginase [a] (cpm in CO_2)	Arginase [a] (μmol urea per hour)	Arginase specific activity
A. 15 000 *g* pellet	0.68	1690	0.106	0.156
B_1. "mitochondrial fraction"	0.24	1180	0.073	0.310
B_2. purified nuclei	0.25	80	0.005	0.020

[a] per 100 μl sample.

genisation KCl was added to 150 mM and $MnCl_2$ to 10 mM, *i.e.* ion concentrations that completely remove arginase from the particulate fractions of liver.[18] After centrifugation 90 % of the arginase activity was consistently found in the pellet (Table 1). This was also true when the $MnCl_2$ concentration was increased to 50 mM. The enzyme could be released from the particulate fraction by treatment with a non-ionic detergent, *e.g.* Lubrol (Table 2).

In order to further clarify, whether arginase was bound to nuclei or to cytoplasmic particles, cells were lysed in a hypotonic buffer containing spermine, which maintains the integrity of the nuclear membrane.[19] One part of the lysate was centrifuged at 15 000 *g* yielding nuclei together with cytoplasmic particles. The other part was fractionated as described in Material and Methods, separating the cytoplasmic particles from the nuclei. 70 % of the arginase activity of the combined nuclear and mitochondrial pellet was found in the cytoplasmic particle fraction (Table 3). Attempts to purify the nuclei by centrifugation in hypotonic sucrose, yielded only 3 % activity in the pure nuclear fraction, while the remaining 25 % was lost during the purification procedure.

In summary, unlike liver, most of the arginase activity in Chang cells was firmly bound to cytoplasmic particles sedimenting with mitochondria. The activity cannot be removed from these particles by salt, but it can be released by non-ionic detergents. A similar localization of arginase activity in the mitochondrial fraction has been reported by Kaysen [7] for rat kidney.

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Studies on Orchidaceae Alkaloids. XXXIX.* Isolation of (−)-Cryptostyline I, II, III and two Quaternary Salts from *Cryptostylis erythroglossa* Hayata. Biosynthetic Studies of (−)-Cryptostyline I

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(−)-Cryptostyline I, II and III, together with 1-(3,4-methylenedioxyphenyl)-6,7-dimethoxy-2-methyl-3,4-dihydroisoquinolinium iodide and 1-(3,4-methylenedioxyphenyl)-6,7-dimethoxy-2-methylisoquinolinium chloride have been isolated from *Cryptostylis erythroglossa* Hayata.

The biosynthesis of (−)-cryptostyline I has been studied using radioactive precursors and the position of the radio-label determined by degradation. The biosynthetic results show that tyrosine and 3,4-dihydroxyphenylalanine as well as tyramine and dopamine are specifically incorporated. The finding that 3-hydroxy-4-methoxyphenethylamine is better incorporated than the isomeric 4-hydroxy-3-methoxyphenethylamine suggests that the ring closure to the tetrahydroisoquinoline skeleton is facilitated by a *para*-hydroxy group.

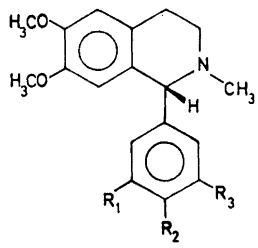
(+)-Cryptostyline I, II and III have been isolated by Leander *et al.*[2] from *Cryptostylis fulva* Schltr. The absolute configuration of these alkaloids has been established by two X-ray diffraction investigations.[3,4] In this paper we report the isolation of (−)-cryptostyline I, II and III from *C. erythroglossa* Hayata together with the immonium salt IV and the isoquinolinium salt V. Biosynthetic studies of (−)-cryptostyline I are also reported.

The structure of IV was established by comparing its iodide with an authentic sample of 1-(3,4-methylenedioxyphenyl)-6,7-dimethoxy-

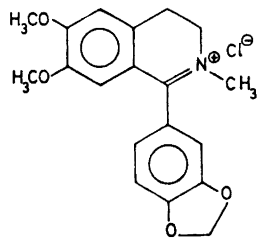
* For number XXXVIII, see Ref. 1.

Table 1.

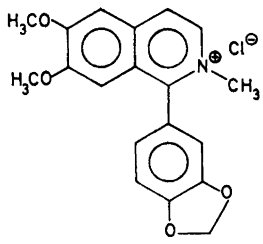
Precursor introduced	Amount fed mg	Amount fed μCi	Cryptostyline I Isolated mg	Cryptostyline I Dpm/mmol	Radioactivity % I	VIII	IX	X	XI
(±)-Tyrosine-α-^{14}C	0.18	50	100	68 300	100	84	7	88	
Tyramine-α-^{14}C	5.7	150	30	12 800	100	87	3	91	
(±)-3,4-Dihydroxyphenylalanine-α-^{14}C	0.12	50	30	13 500	100	94	5	91	
Dopamine-α-^{14}C	1.9	50	30	7 230	100	77	16	74	
3-Hydroxy-4-methoxyphenethylamine-α,β-^{3}H	3.9	70	25	6 870	100	69			25
4-Hydroxy-3-methoxyphenethylamine-α,β-^{3}	3.7	38	30	523					
4-Hydroxy-3-methoxyphenethylamine-5-^{3}H	0.003	250	30	1 470					



R(-)-Cryptostyline
I R_1=H, R_2R_3= O—CH_2—O
II R_1=H, R_2=R_3=OCH_3
III R_1=R_2=R_3=OCH_3



1-(3,4-Methylenedioxyphenyl)-
-6,7-dimethoxy-2-methyl-3,4-
-dihydroisoquinolinium chloride
(IV)



1-(3,4-Methylenedioxyphenyl)-
-6,7-dimethoxy-2-methyl-
-isoquinolinium chloride
(V)

2-methyl-3,4-dihydroisoquinolinium iodide.[2] Identification of V was accomplished by comparison with a synthetic sample, obtained by dehydrogenation of 1-(3,4-methylenedioxyphenyl)-6,7-dimethoxy-3,4-dihydroisoquinoline[2] with selenium, followed by methylation.

The cryptostyline alkaloids are interesting from a biogenetic point of view since they are the first 1-phenyl-tetrahydroisoquinolines isolated from Nature. For this reason the biosynthesis of (−)-cryptostyline I in *C. erythroglossa* has been studied using radioactive precursors.

The potential precursors shown in Table 1 were administered to the plant and (−)-cryptostyline I was isolated. The results show that tyrosine and 3,4-dihydroxyphenylalanine as well as the corresponding amines, tyramine and dopamine, were specifically incorporated, but in low yields. The results of feeding experiments with the two isomeric compounds 3-hydroxy-4-methoxyphenethylamine and 4-hydroxy-3-methoxyphenethylamine suggest that only the former compound (which occurs in, *e. g.*, *Pachycereus pecten-aboriginum* (Eng.) Br&R.[5]) is a precursor of (−)-cryptostyline I.

The early steps in the formation of 1-benzyltetrahydroisoquinoline alkaloids have so far received scant attention,[6] whereas the formation of the tetrahydroisoquinoline skeleton of cactus alkaloids has been extensively studied.[7–9] The present results indicating alternative paths (Fig. 2) to dopamine from tyrosine *via* tyramine or from 3,4-dihydroxyphenylalanine are analogous to previous results on the biosynthesis of anhalamine and anhalonidine in the cactus *Lophophora williamsii* (Lem.) Coult.[9]

In the biosynthesis of, *e.g.*, anhalamine, which is a 6,7,8-trisubstituted tetrahydroisoquinoline, 3-hydroxy-4,5-dimethoxyphenethylamine is the immediate progenitor of the tetrahydroisoquinoline skeleton, thus providing an *ortho*-activation suitable for ring-closure.[9] The present results with (−)-cryptostyline I suggest that the ring-closure is facilitated by a *para*-hydroxy group (Fig. 2). The origin of the remaining C_6-C_1 moiety of (−)-cryptostyline I remains to be elucidated. It may possibly be derived from protocatechualdehyde or partially *O*-methylated derivates thereof as has been shown for some Amaryllidaceae alkaloids.[6]

The specificity in the incorporation of the precursors into (−)-cryptostyline I was established by degradation. The ^{14}C-labelled precursors would, if incorporated without extensive break-down, label (−)-cryptostyline I at C-3. This carbon atom was isolated as the dimedone derivative of formaldehyde (X), obtained as shown in Fig. 1. The extensive presence of radioactivity in this position (Table

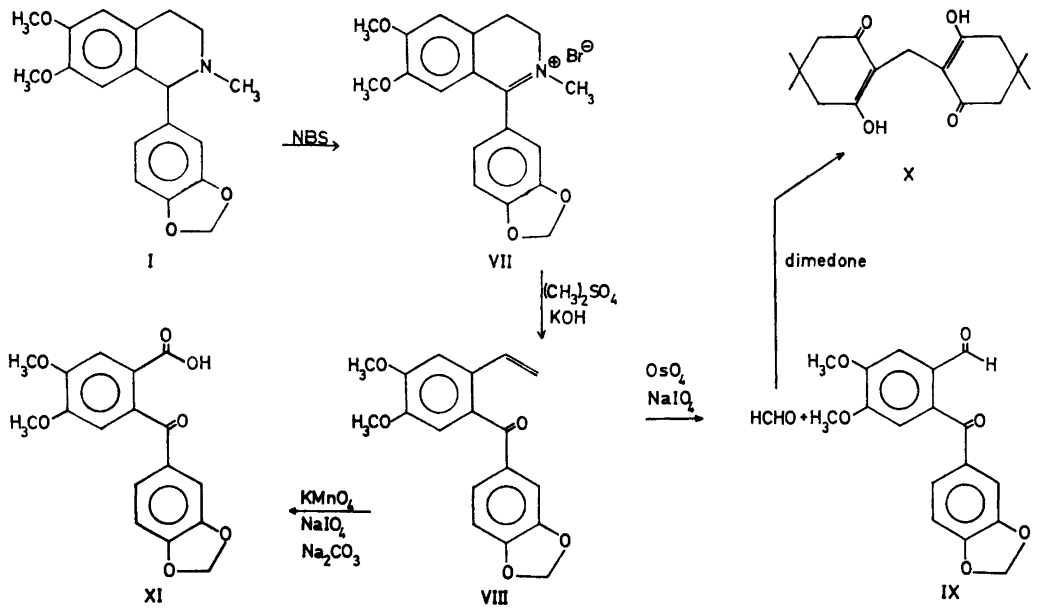


Fig. 1. Degradation of cryptostyline I.

1) indicates direct incorporation. The ^{3}H-labelled precursors would be expected to label the C_3 and C_4 positions in (−)-cryptostyline I. Degradation to compound XI showed predominant labelling in the expected positions (Table 1).

EXPERIMENTAL

Melting points are corrected. Mass spectra were measured on a Perkin-Elmer 270 instrument, the IR spectra on a Perkin-Elmer 257 instrument, the UV spectra on a Beckman DK 2 instrument and the NMR spectra on a Varian A-60A spectrometer. Elemental analyses were carried out at Alfred Bernhardt, Mikroanalytisches Laboratorium, Elbach über Engelskirchen, Germany, and Mikroanalyslaboratoriet, Lantbrukshögskolan, Uppsala, Sweden. Radioactivities were measured with a Packard Tri-Carb Model 3375 liquid scintillation spectrometer, in a solvent system consisting of 2 ml absolute ethanol and 10 ml Instagel® (Packard Instrument Corp.). External standardization was used for efficiency determination. Preparative thin-layer chromatography was carried out on alumina (1.5 mm) F-254 Type T (Merck) or silica gel (2 mm) 60F-254 (Merck).

(±)-Tyrosine-α-^{14}C, (±)-3,4-dihydroxyphenylalanine-α-^{14}C, tyramine-α-^{14}C, dopamine-α-^{14}C and 4-hydroxy-3-methoxy-5-^{3}H-phenethylamine were obtained from the Radiochemical Centre, Amersham, UK and New England Nuclear Corp., Boston, USA. The preparation of 4-hydroxy-3-methoxy-α,β-^{3}H-phenethylamine and 3-hydroxy-4-methoxy-α,β-^{3}H-phenethylamine has already been described.[10]

The plants were purchased from Chow Cheng Orchids, 194 Litoh-St. Taichung, Taiwan.

Feeding experiments. Each labelled precursor was dissolved in a minute quantity of water and injected into the stems of two plants of *C. erythroglossa*. After three weeks the alkaloid fraction was isolated as described below. Cryptostyline I ($R_F = 0.5$) was separated from the other alkaloids by preparative thin-layer chromatography on alumina plates with ether as eluent. The isolated alkaloid was diluted with 100 mg non-labelled (±)-cryptostyline I and recrystallized from ether to constant specific activity.

Isolation of the alkaloids. Fresh plants of *C. erythroglossa* (0.3 kg) were extracted with methanol (5 l). The extract was concentrated to 1 l, acidified (pH 3) with hydrochloric acid and washed with carbon tetrachloride (6 × 50 ml). The aqueous layer was made alkaline (pH 8) with sodium hydrogen carbonate and extracted with ether (3 × 50 ml). The combined ether solutions were treated as described earlier,[2] giving (−)-cryptostyline I (m.p. 101−102°; $[\alpha]_D^{22}$ −56°, *c* 0.4, chloroform), (−)-cryptostyline II (m.p. 116−117°; $[\alpha]_D^{22}$ −58°, *c* 0.4, chloroform) and (−)-cryptostyline III (m.p.

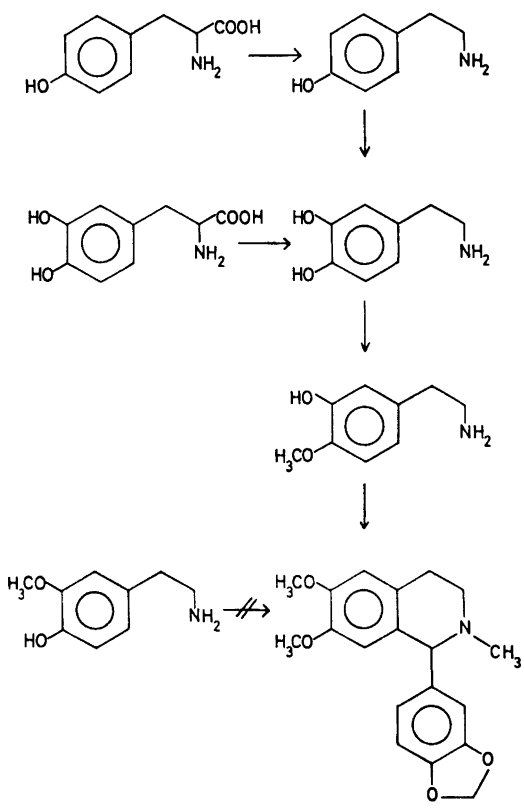


Fig. 2. Suggested pathway for the biosynthesis of (−)-cryptostyline I.

128 − 130°; $[\alpha]_D^{22}$ −52°, *c* 0.3, chloroform). The aqueous layer was then extracted with chloroform (8 × 50 ml). The chloroform extract was dried (Na_2SO_4) and evaporated to dryness. The residue was dissolved in water and filtered through a column of IRA-400 (Cl^-, 1 × 20 cm) irrigated with water, and the filtrate evaporated to dryness. This residue was separated into the crude chlorides IV ($R_F = 0.35$) and V ($R_F = 0.1$) by preparative thin-layer chromatography on alumina using chloroform/methanol (19:1) as eluent. The iodide corresponding to IV (5 mg), obtained by filtering the chloride through a column of IRA-400 (I^-, 1 × 15 cm) irrigated with water, was indistinguishable (m.p., TLC, IR) from an authentic sample of 1-(3,4-methylenedioxyphenyl-6,7-dimethoxy-2-methyl-3,4-dihydroisoquinolinium iodide.[2] Purification of V by preparative thin-layer chromatography on silica gel using chloroform/methanol (2:1) as eluent yielded V as an amorphous solid (25 mg) indistinguishable (TLC, UV, NMR, m.p. of the picrate) from an authentic sample of 1-(3,4-methylenedioxyphenyl)-6,7-dimethoxy-2-methylisoquinolinium chloride (*vide infra*).

1-(3,4-Methylenedioxyphenyl)-6,7-dimethoxyisoquinoline (VI). 1-(3,4-Methylenedioxyphenyl)- 6,7- dimethoxy- 3,4-dihydroisoquinoline [2] (0.40 g) and selenium (0.48 g) were heated under nitrogen at 220 − 230° for 15 min. The reaction mixture was dissolved in methanol and filtered through a column of silica gel. The eluate was chromatographed on neutral alumina using chloroform as eluent. The first fraction contained VI and unreacted imine, which were separated by preparative thin-layer chromatography on silica gel plates developed with ether. Recrystallization from methanol gave VI (0.10 g), m.p. 129 − 132°. (Found: C 69.8; H 5.1; N 4.4 Calc. for $C_{18}H_{15}NO_4$: C 69.9; H 4.9; N 4.5). UV spectrum, nm (ε): λ_{max} (ethanol) 329 (8000), 292 (8000), 238 (46 000); $\lambda_{shoulder}$ 245 (43 000). NMR spectrum ($CDCl_3$): τ 1.53 (d, 1 H, $J = 5$ Hz), 2.43 − 3.20 (m, 6 H); 3.95 (s, 2 H), 5.98 (s, 3 H), 6.12 (s, 3 H).

1-(3,4-Methylenedioxyphenyl)-6,7-dimethoxy-2-methylisoquinolinium chloride. An excess of methyl iodide was added to a solution of VI in acetone. The mixture was refluxed for 1.5 h, cooled and evaporated to dryness. The residue was filtered through a column of IRA-400 (Cl^-, 1 × 20 cm) irrigated with water. Evaporation of the eluate to dryness and purification of the residue by preparative thin-layer chromatography on silica gel using chloroform/methanol (2:1) as eluent gave the title compound as an amorphous solid. UV spectrum, nm (ε): λ_{max} (ethanol) 319 (4500), 290 (3500), 258 (25 100); $\lambda_{shoulder}$ 348 (2900). NMR spectrum ($CDCl_3$): τ 1.36 and 1.61 (AB spectrum, 2 H, $J = 6.5$ Hz), 2.22 (s, 1 H), 2.78 (s, 3 H), 3.00 (s, 1 H), 3.77 (s, 2 H), 5.77 (s, 3 H), 5.87 (s, 3 H), 6.17 (s, 3 H).

The *picrate*, m.p. 218 − 223°, was recrystallized from methanol-water. (Found: C 54.2; H 3.6; N 9.8. Calc. for $C_{25}H_{20}N_4O_{11}$: C 54.4; H 3.7; N 10.1).

1-(3,4-Methylenedioxyphenyl)-6,7- dimethoxy-2-methyl-3,4-dihydroisoquinolinium bromide (VII). *N*-Bromosuccinimide (75 mg) was added with stirring to a solution of cryptostyline I (100 mg) in absolute ether (20 ml). During the addition, a yellow precipitate appeared. After 1 h acetone (3 ml) was added and the reaction mixture was refluxed for 4 h to give the crude immonium salt (VII) as a light yellow crystalline precipitate. The precipitate was washed with a mixture of ether-acetone (7:1) to remove succinimide, giving VII (118 mg), m.p. 207 − 208°. (Found: C 56.0; H 4.8; Br 19.9; N 3.5; O 15.8. Calc. for $C_{19}H_{20}BrNO_4$: C 56.2; H 4.9; Br 19.7; N 3.5; O 15.8). IR spectrum: σ_{max} (KBr) 1640 (m) cm^{-1}. UV spectrum, nm (ε): λ_{max} (ethanol) 367 (13 400), 315 (11 500), 252 (22 000). NMR spectrum (CD_3OD): τ 2.78 − 2.95 (m, 4 H), 3.42 (s, 1 H), 3.85 (s, 2 H), 5.60 − 6.05 (m, 2 H), 6.50 − 6.85 (m, 2 H), 6.02 (s, 3 H), 6.38 (s, 6 H).

2-(3,4-Methylenedioxybenzoyl)-4,5-dimethoxystyrene (VIII). A mixture of VII (118 mg), dimethyl sulphate (0.06 ml), ethanol (0.12 ml) and aqueous potassium hydroxide (0.40 ml, 20 %) was refluxed for 3 h. Water (10 ml) was added and the cold solution was extracted with chloroform (5 × 10 ml). The combined chloroform solutions were dried (Na_2SO_4) and evaporated to dryness. The residue was purified by preparative thin-layer chromatography on silica gel using ethanol as eluent ($R_F = 0.9$). Recrystallization from ethanol gave VIII (77 mg), m.p. 98–99°. (Found: C 69.4; H 5.2; O 25.6 Calc. for $C_{18}H_{16}O_5$: C 69.2; H 5.1; O 25.6). IR spectrum: σ_{max} (KBr) 1620 (m), 1655 (s) cm^{-1}. UV spectrum, nm (ε): λ_{max} (ethanol) 314 (13 800), 258 (22 900), 237 (28 200). NMR spectrum ($CDCl_3$): τ 2.60–3.53 (m, 6 H), 3.95 (s, 2 H), 4,45 (q, 1 H, $J_1 = 1$ Hz, $J_2 = 17$ Hz), 4.85 (q, 1 H, $J_1 = 1$ Hz, $J_2 = 11$ Hz), 6.05 (s, 3 H), 6.15 (s, 3 H). MS: M^+ 312.

2-(3,4-Methylenedioxybenzoyl)-4,5-dimethoxybenzaldehyde (IX). A catalytic amount of osmium tetroxide (2 mg) was added to a solution of the alkene VIII (77 mg) in water-dioxane (1:4, 5 ml). After 0.5 h sodium periodate (145 mg) was added and the mixture was heated at 80° for 2 h. The solvent was evaporated and the residue was suspended in water and extracted with chloroform (5 × 25 ml). The combined chloroform solutions were dried (Na_2SO_4) and evaporated to dryness. The residue was purified by preparative thin-layer chromatography on silica gel using ethanol as eluent ($R_F = 0.7$). Crystallization from chloroform-ether gave IX (55 mg), m.p. 162–163°. (Found: C 64.8; H 4.5; O 30.4. Calc. for $C_{17}H_{14}O_6$: C 65.0; H 4.5; O 30.6). IR spectrum: σ_{max} (KBr) 1635 (m), 1685 (s) cm^{-1}. UV spectrum, nm (ε): λ_{max} (ethanol) 316 (12 600), 268 (10 800), 237 (21 700). NMR spectrum ($CDCl_3$): τ 1.23 (s, 1 H), 2.40–3.32 (m, 5 H), 3.92 (s, 2 H), 6.03 (s, 3 H), 6.06 (s, 3 H). MS: M^+ 314.

The formaldehyde formed in the reaction was trapped in a solution of dimedone (100 mg) in water (40 ml). The precipitate was recrystallized twice from ethanol giving methylenebisdimedone (X, 30 mg), m.p. 191° (Lit.[11] m.p. 191°).

2-(3,4-Methylenedioxybenzoyl)4,5-dimethoxybenzoic acid (XI). *t*-Butanol (4 ml) and 2-(3,4-methylenedioxybenzoyl)-4,5-dimethoxystyrene (VII, 55 mg) were added to a solution of sodium periodate (0.70 g) and potassium permanganate (0.35 g) in water (12 ml). The pH of the solution was adjusted to 8.5 by the addition of solid sodium carbonate. The mixture was stirred for 15 h at 25°, and then acidified (pH 4) with aqueous sulphuric acid. The excess of permanganate was destroyed with sodium sulphite and the solution extracted with chloroform (5 × 25 ml). The combined chloroform solutions were dried and evaporated to dryness. The residue was chromatographed on silica gel (3 × 10 cm, 70–230 mesh). Unreacted alkene (VII) was eluted with chloroform. Exchange of the solvent to ethanol eluted the acid (XI), which was further purified by preparative thin-layer chromatography on silica gel using ethanol as eluent ($R_F = 0.6$). Recrystallization from ethanol gave XI, m.p. 206–207°. (Found: C 61.8; H 4.4; O 33.8. Calc. for $C_{17}H_{14}O_7$: C 61.8; H 4.2; O 33.9). IR spectrum: σ_{max} (KBr) 1655 (m), 1665 (m), 3300–2500 (m) cm^{-1}. UV spectrum, nm (ε): λ_{max} (ethanol) 312 (9200), 275 (8400), 232 (24 300). NMR spectrum (CD_3OD): τ 2.16–3.27 (m, 5 H), 3.94 (s, 2 H), 6.02 (s, 3 H), 6.12 (s, 3 H).

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C_{50}-Carotenoids. 12.* Steric Effects on the Intensity Ratios of the (M − 92)/(M − 106) Ions in the Mass Spectra of Carotenoids

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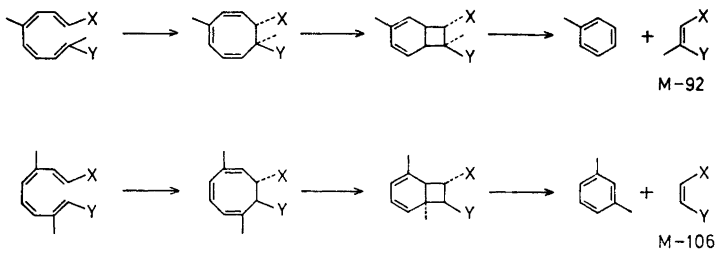
Steric factors are shown to allow rationalisation of hitherto unexplained variations of the ratio (R) of the (M − 92)/(M − 106) ions in the mass spectra of C_{50}-carotenoids. It is suggested that this ratio may be used to provide information about the position of the extra prenyl groups relative to the chromophoric system in acyclic C_{50}-carotenoids.

Characteristic ions at M − 92 and M − 106 mass units are observed in the mass spectra of virtually all carotenoids.[1-4] These ions are attributed to species where six consecutive carbon atoms of the conjugated chain and the substituents carried by these atoms have been extruded. The mechanism shown in Scheme 1 has been proposed for the formation of both M − 92 and M − 106 mass unit species and may be applied to their genesis by either direct electron bombardment or through the intermediacy of prior thermal decomposition.[5,6]

The values obtained for the intensity ratio (R) of the (M − 92)/(M − 106) ions can be related to the chromophoric system present in most cases.[2,6] In particular, in the case of C_{40}-carotenoids lacking substituents directly conjugated to the acyclic chromophore, the R-values found decrease as the number of double bonds in the acyclic chromophore (DB) increases from 9 to 13; representative examples and the observed limits for R are cited in Scheme 2.[2] The R-values found when carbonyl or aromatic functions are directly conjugated to the acyclic chromophore vary from those found in the above case, but may be rationalised in terms of the type of substitution present.[6] The ratio R may thus be used to obtain information about the chromophore of C_{40}-carotenoids.

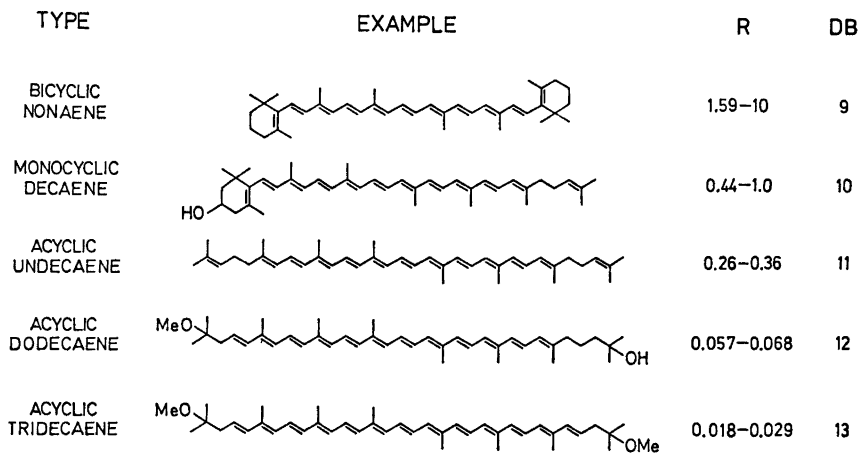
RESULTS AND DISCUSSION

The R-values found with C_{45}- and C_{50}-carotenoids are often markedly different from those obtained with C_{40}-carotenoids having the same chromophore.[7] Consideration of work [8] on the responsibility of steric factors, particularly



Scheme 1. Proposed mechanism for the formation of M − 92 and M − 106 mass unit species found in the mass spectra of carotenoids.[5,6]

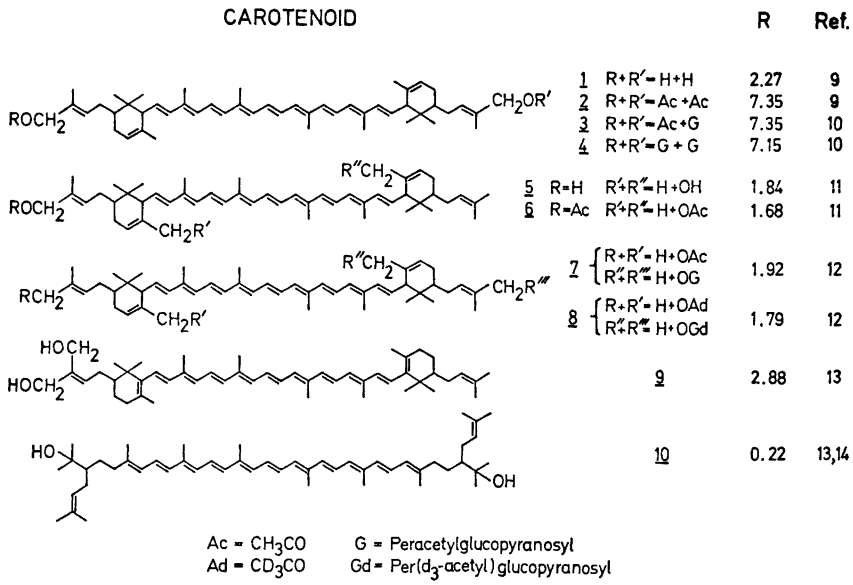
* Part 11, *Acta Chem. Scand. 27* (1973) 2321.



Scheme 2. Range of values for the intensity ratio (R) of the $(M-92)/(M-106)$ ions in the mass spectra of C_{40}-carotenoids having various numbers of double bonds (DB) in the acyclic chromophore. A typical example of each structural type is shown.[2]

end group bulk (see Fig. 1), for variations in this intensity ratio in the case of C_{40}-carotenoids suggested that steric effects might also be of decisive importance in the present case.

The end groups found in carotenoids with supernumerary carbon atoms may be categorised according to whether the additional prenyl unit is immediately adjacent to the acyclic chromophore or not. Those compounds (*1–10*) shown in Scheme 3 have only end



Scheme 3. Observed values for the intensity ratio (R) for C_{50}-carotenoids showing R-values similar to those found in C_{40}-carotenoids. The compounds *1–9* have nine double bonds in the acyclic chromophore ($DB=9$), while the compound *10* has $DB=11$.

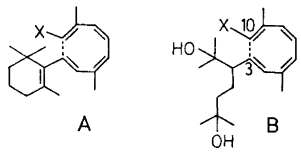
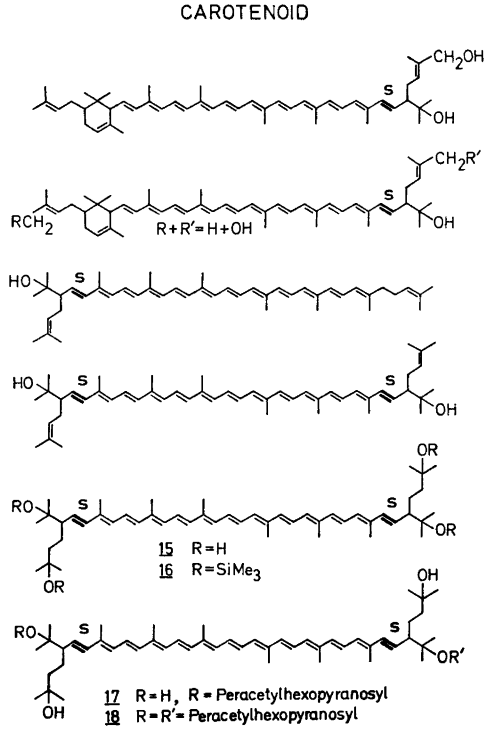


Fig. 1. Steric conflict in the cyclisation process required to provide M − 106 mass unit species by rearrangement of the terminal double bond of the acyclic chromophore in the presence of (*A*) cyclic end groups,[8] and (*B*) acyclic end groups carrying an additional prenyl group.

groups belonging to the latter category and all show values [9–14] of the ratio (*R*) close to those found with C_{40}-carotenoids having the same *DB*-values, *cf.* Scheme 2. The *R*-values are thus normal and in agreement with the structural classification of these compounds (*1 – 10*) as remotely-substituted C_{40}-carotenoids.

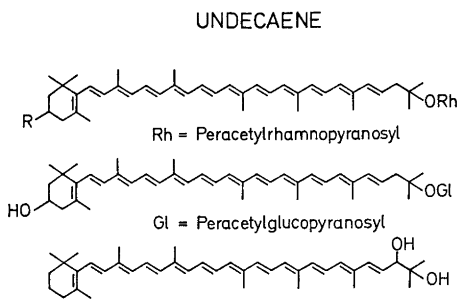
The compounds *11 – 18*, shown in Scheme 4, all contain at least one end group where the extra prenyl group is carried by the carbon atom immediately adjacent to the terminus of the conjugated chain. Before examining the observed *R*-values [7,13–15] for these compounds it should be noted that the position of the methyl groups carried by the conjugated chain is known to be critical in determining these values.[8,16] The compounds *11* and *12* [7,18] are thus not directly comparable to the usual C_{40}-carotenoid undecaenes which have symmetric chromophoric systems carrying six methyl groups, and must be compared with monocyclic undecaenes carrying five methyl groups on the aliphatic polyene chain. Available data [17,18] for monocyclic C_{40}-carotenoids with $DB = 11$ are compiled in Scheme 5 with *R*-values (0.052 – 0.081) considerably lower than for the acyclic undecaenes (0.26 – 0.36).

Examination of the *R*-values [7,13–15] for the compounds *11 – 18* reveals that in all cases the observed values are considerably higher than



CAROTENOID	DB	R	RA	RB	Ref.
11	11	0.39	0.052–0.081	0.44–1.0	7,13
12	11	0.23	0.052–0.081	0.44–1.0	7,13
13	12	0.25	0.057–0.068	0.26–0.36	7,13
14	13	0.11	0.018–0.029	0.26–0.36	7,13
15	13	0.23	0.018–0.029	0.26–0.36	14,15
16	13	0.25	0.018–0.029	0.26–0.36	7,14
17	13	0.30	0.018–0.029	0.26–0.36	15
18	13	0.26	0.018–0.029	0.26–0.36	15

Scheme 4. *R*-values for C_{45} and C_{50}-carotenoids having at least one acyclic end group with a sterically hindered double bond (*s*). *DB* indicates the length of the acyclic chromophore and *RA* the range of *R*-values found in C_{40}-carotenoids having this chromophoric system. *RB* gives the range of values expected for the ratio (*R*) in compounds lacking the sterically hindered double bond(s).



	R	Ref.
R = H	0.065	17
R = OH	0.081	17
	0.052	17
	0.057 *	18

[* Average Value]

Scheme 5. *R*-values for some monocyclic carotenoid undecaenes ($DB = 11$). The *R*-value for the diol was redetermined for this investigation.

those found for C_{40}-carotenoids with the same chromophore. Thus, the formation of the M − 106 species is less favourable than would be expected by analogy with C_{40}-carotenoids with the same chromophoric system.

The critical factor in extrusion reactions leading to M − 92 and M − 106 mass unit species is believed to be the initial new bond formation.[6] Where the C-2 carbon atom in an acyclic end group carries an extra prenyl group, as with the compounds in Scheme 4, the approach of C-3 and C-10 will be more difficult than in the absence of such substituents. The extrusion modes at more distant sites would be little affected. Thus, the only mode affected by this cause of steric hindrance is one leading to an M − 106 mass unit species and this steric factor may thus be applied to explain the observation that the *R*-values for the compounds *11* − *18* are only slightly lower than those expected for C_{40}-carotenoids lacking the 3,4-double bond. The somewhat lower values may in fact be the result of the higher temperatures needed for volatilisation of C_{45} and C_{50}-carotenoids as compared with those for the C_{40}-compounds: the contribution of thermal genesis is less for the M − 92 than for the M − 106 mass unit species.[16]

The above argument allows rationalisation of the recorded *R*-values for carotenoids with extra prenyl groups. It should thus be possible to use *R*-values for acyclic carotenoids with extra prenyl groups to indicate the position of these extra groups relative to the chromophore. The *R*-values used in this study were obtained from spectra previously published or recorded during previous work on C_{45} and C_{50}-carotenoids.[7,9–15]

EXPERIMENTAL

The spectra of the C_{45} and C_{50}-carotenoids were recorded on an AEI MS 902 mass spectrometer using the direct insertion probe. All spectra were recorded at 70 eV, 4 or 8 KV, and with the ion source at the minimum temperature required to achieve volatilisation (190 − 290 C). The time elapsing between insertion of the probe into the ion source and recording of the spectra was kept to a minimum in order to reduce thermal decomposition as much as possible.

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The Crystal Structure of L-Mimosine Sulphate Hydrate

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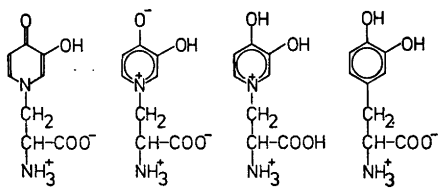
The structure of L-mimosine sulphate hydrate, $C_5H_3(OH)_2NHC_2CH(NH_3)COOH.SO_4.(H_2O)_{1.5}$ has been determined by X-ray methods using 2451 observed reflections collected by counter methods. The crystals are orthorhombic, space group $P2_12_12_1$, with unit cell dimensions $a = 6.42_3$ Å; $b = 13.88_6$ Å; $c = 14.48_7$ Å. The refinements yielded a conventional R-factor of 0.051. The crystal structure is discussed and the bond lengths and angles are compared to those found in the un-protonized mimosine molecules.[1] The conformation of the amino acid is similar to that of L-tyrosine, the α hydrogen atom being in *trans* position relative to the aromatic part.

A previous structure determination [1] showed the anhydrous form of L-mimosine to exist in the crystals as an intermediate between the structures I and II of Fig. 1.



I II III DOPA

Fig. 1.

L-Mimosine forms a dibasic salt with sulphuric acid.[2] The protonized form is assumed to have the structure indicated by III which closely corresponds to the structure of L-DOPA. In order to investigate a molecule of this kind we have studied the sulphate of L-mimosine which is reported to crystallize with 1.5 molecule H_2O per formula unit and to have a melting point nearly 100°C below that of mimosine itself.[2]

A sample of L-mimosine was kindly given to us by Dr. M. P. Hegarty (The Cunningham Laboratory, CSIRO, Australia).

EXPERIMENTAL

Single crystals of the compound were formed by slow evaporation of a solution of L-mimosine in diluted sulphuric acid. A single crystal of approximate dimensions $0.3 \times 0.3 \times 0.17$ mm³ was used in all the X-ray experiments.

Weissenberg photographs indicated orthorhombic symmetry; systematically absent reflections proved the space group to be $P2_12_12_1$. Unit cell dimensions were determined from diffractometer measurements on 15 general reflections using Mo$K(\alpha_1 + \alpha_2)$ radiation ($\lambda = 0.71069$ Å).

The intensity data were recorded with the use of a SYNTEX $P\bar{1}$ diffractometer with graphite crystal monochromated MoK radiation. 3269 independent reflections with $2\theta < 70°$ were measured using the $\omega - 2\theta$ scanning mode with the 2θ scan speed varying from 2 to 12° min^{-1} depending on the intensity. The scan range was from 1° below $2\theta(\alpha_1)$ to 1° above $2\theta(\alpha_2)$ and background counts were taken for half the scan time at each of the scan range limits. Three standard reflections were measured after every 50 reflections; they showed no systematic variation during the experiment.

The estimation of the standard deviation of the intensities was based on counting statistics with 2.5 % addition in order to account for other errors. The 2451 reflections with intensity larger than $2.5\sigma(I)$ were considered to be observed; the remaining data were excluded from the structure refinement procedure.

The structure was determined by the use of the program MULTAN written by P. Main, M. M. Woolfsen and G. Germain. All other computer programs applied are described in Ref. 3. Atomic form factors applied were those of Doyle and Turner [4] for sulphur, oxygen, nitrogen, and carbon atoms and of Stewart, Davidson and Simpson [5] for hydrogen.

CRYSTAL DATA

L-Mimosine sulphate hydrate, $C_8H_{19}N_2O_4.H_2SO_4.1.5H_2O$, m.p. 143 – 143.5°C, with decom-

position.[3] Orthorhombic; $a = 6.423(0.001)$ Å, $b = 13.886(3)$ Å, $c = 14.487(3)$ Å. Figures in parentheses are estimated standard deviations. $V = 1292.1$ Å^3; formula weight 323.29; $F(000) = 676$; $Z = 4$; $D_{calc} = 1.662$ g cm^{-3}. Absent reflections: (h00) for h odd, (0k0) for k odd, (00l) for l odd; space group $P2_12_12_1$.

STRUCTURE DETERMINATION

After being corrected for Lorentz and polarization effects the data were put on an approximate absolute scale by Wilson's statistical method and normalized structure amplitudes were calculated. 472 reflections with E-values greater than 1.25 were used as input in the program assembly MULTAN.[6] Of the solutions obtained the one with the highest absolute figure of merit was used as the basis for an E-map in which all except three of the non-hydrogen atoms were localized. After one Fourier refinement cycle two of the missing heavy atoms appeared in the electron density map. The trial structure was refined by a few least-squares cycles and the approximate positions of the hydrogen atoms bonded to carbon were calculated. A couple of cycles of alternating least-squares refinements and difference Fourier syntheses yielded the positions of the oxygen atom of the "half" water molecule as well as the remaining hydrogen atoms of the mimosine ion and the other water molecule.

In the subsequent full-matrix least-squares refinement the 2451 observed reflections were included; the function minimized was $\sum w(F_o - F_c)^2$, the weight assigned to each reflection was

Table 1. Fractional coordinates and thermal parameters ($\times 10^5$) with estimated standard deviations (in parentheses). The temperature factor is given by exp $-(B11h^2 + B22k^2 + B33l^2 + B12hk + B13hl + B23kl)$.

ATOM	X	Y	Z	B11	B22	B33	B12	B13	B23
S	50974(14)	29430(4)	19360(4)	1603(17)	215(3)	161(2)	-201(15)	-62(13)	18(4)
OS1	48321(60)	39828(16)	24693(17)	4236(100)	238(10)	488(12)	3(67)	-1391(75)	-93(18)
OS2	51624(50)	27049(16)	9461(12)	2686(72)	464(12)	169(7)	-632(64)	154(48)	13(15)
OS3	70037(52)	26244(24)	23968(17)	2359(81)	685(19)	276(11)	886(65)	-91(52)	58(23)
OS4	32973(54)	24479(24)	23313(19)	2748(87)	711(19)	272(18)	-1473(70)	656(56)	-278(24)
O1	19476(46)	22596(15)	39970(14)	2768(75)	226(10)	257(9)	-22(48)	281(46)	-20(15)
O2	18889(41)	22291(16)	58139(14)	2040(62)	327(11)	234(9)	222(48)	184(43)	124(16)
O3	-4467(47)	52435(21)	22453(15)	2516(82)	653(18)	253(9)	-260(61)	377(48)	-295(21)
O4	-31974(41)	51018(17)	31652(15)	2044(63)	482(12)	293(10)	-566(60)	25(46)	-218(18)
OV1	919(99)	33975(32)	9902(29)	2126(126)	381(21)	348(19)	56(121)	125(108)	58(34)
OV2	-15228(50)	52981(20)	2626(19)	2418(78)	465(14)	498(13)	-243(59)	-475(59)	-298(23)
N1	16592(41)	48089(17)	44808(15)	914(52)	211(10)	212(9)	88(42)	-23(39)	22(16)
N2	-18714(48)	62987(17)	44189(16)	1487(65)	232(11)	222(10)	-28(46)	-218(46)	-102(17)
C1	17777(52)	39776(20)	39950(17)	1566(72)	245(12)	178(9)	-63(56)	162(48)	-1(19)
C2	18351(49)	31037(20)	44409(18)	1182(66)	255(13)	229(11)	98(52)	122(48)	-8(19)
C3	17692(49)	30803(21)	54163(18)	1091(64)	301(14)	238(11)	80(55)	102(47)	28(20)
C4	16138(55)	39523(23)	58812(18)	1601(77)	377(16)	193(10)	178(63)	57(51)	-112(22)
C5	15609(56)	47926(22)	54170(19)	1523(75)	286(14)	227(11)	142(57)	-9(51)	-111(21)
C6	17131(56)	57553(20)	39866(20)	1509(76)	222(12)	296(12)	-182(55)	-14(56)	83(21)
C7	-3993(48)	60934(19)	36516(17)	1596(80)	196(11)	211(10)	-50(51)	87(48)	10(18)
C8	-13865(55)	54257(20)	29409(20)	1812(81)	237(12)	235(12)	95(53)	-232(54)	9(20)

Table 2. Fractional coordinates ($\times 10^4$) and the isotropic thermal parameter B for hydrogen atoms. Figures in parentheses are standard deviations.

ATOM	X	Y	Z	B
HO1	2340(65)	2434(29)	3348(31)	3.3(.8)
HO2	1608(62)	2282(42)	6371(42)	4.9(1.4)
HO4	-3770(84)	4655(39)	2746(36)	4.2(1.0)
H1V2	-1582(87)	5814(40)	259(34)	5.7(1.0)
H2V2	-1127(74)	5205(32)	874(33)	6.0(.9)
H1N2	-2475(58)	5864(27)	4620(22)	2.7(.6)
H2N2	-1435(53)	6546(23)	4746(19)	1.6(.6)
H3N2	-2817(92)	6775(41)	4279(34)	6.3(1.1)
HC1	1887(60)	4051(26)	3330(26)	3.0(.6)
HC4	1582(51)	3888(21)	6592(21)	2.3(.5)
HC5	1496(78)	5446(32)	5686(29)	3.2(.8)
H1C6	2382(31)	6246(14)	4351(13)	1.0(.3)
H2C6	2914(72)	5544(30)	3565(28)	2.5(.8)
HC7	-265(59)	6756(26)	3418(24)	1.2(.7)

Table 3. Observed and calculated structure factors (× 10).

H=	0,K=	0	3	427	448	16	91	95	15	47	43	14	115	123	1	234	218	4	150	148	H=	1,K=	19
2	88	82	4	72	70	18	100	113	H=	0,K=	18	16	87	89	2	35	42	5	198	202	3	136	130
4	151	171	5	249	241	19	49	53	1	158	148	17	82	89	3	430	427	6	132	140	4	48	52
6	1078	1054	6	38	19	20	74	74	2	44	40	18	86	92	5	307	301	7	131	149	5	115	114
8	624	624	7	106	137	H=	0,K=	11	4	138	133	19	123	118	6	204	201	8	82	82	6	50	43
10	411	444	8	85	86	1	264	255	6	49	35	21	75	63	7	236	250	9	182	186	9	56	50
12	145	145	9	164	147	2	123	126	7	63	64	23	54	45	8	176	178	11	99	106	11	41	36
14	191	212	10	244	240	3	399	394	8	98	92	H=	1,K=	3	9	143	146	12	90	85	12	46	43
16	32	24	11	71	71	4	30	28	9	100	105	0	120	113	10	42	48	13	86	89	H=	1,K=	20
18	124	123	12	280	279	5	333	356	10	86	85	1	391	422	11	212	218	14	94	90	0	40	44
20	223	215	14	83	80	6	176	178	11	84	88	2	615	602	12	70	72	15	91	88	1	80	82
22	142	135	16	91	84	7	31	46	12	79	79	3	442	460	13	180	187	16	41	40	2	65	56
H=	0,K=	1	20	120	114	9	57	46	H=	0,K=	19	4	172	156	14	101	96	17	73	74	3	44	45
1	130	168	21	59	56	11	157	162	2	62	61	5	633	606	15	77	70	18	53	50	7	73	67
2	322	384	22	114	118	12	127	134	3	51	54	6	343	329	16	80	78	19	93	98	9	69	63
3	1015	960	H=	0,K=	6	13	151	155	4	50	46	7	303	306	17	98	97	H=	1,K=	13	H=	1,K=	21
4	64	64	0	307	346	14	55	54	5	58	60	8	193	222	18	102	97	0	163	166	2	43	43
5	825	816	1	133	143	15	283	293	6	110	109	9	57	59	19	41	39	1	127	138	4	88	81
6	327	336	2	410	371	16	83	92	7	85	87	10	140	134	21	94	98	2	73	77	5	37	21
7	303	311	3	319	309	19	66	78	8	55	58	11	69	68	H=	1,K=	8	3	144	146	6	101	105
8	119	118	4	331	306	H=	0,K=	12	10	78	77	12	144	148	0	495	492	4	124	124	H=	1,K=	22
9	277	294	6	473	481	0	256	260	H=	0,K=	20	13	135	128	1	183	168	5	85	85	0	43	36
10	272	253	7	97	75	2	34	37	1	64	57	14	71	77	2	192	185	6	121	119	1	99	84
12	65	71	8	395	357	3	45	34	2	139	140	15	91	94	3	40	71	7	88	88	2	44	34
13	127	119	9	298	305	4	65	64	3	43	25	16	50	51	4	226	226	8	38	45	3	44	54
14	149	157	10	270	278	6	110	113	4	45	30	17	63	65	5	121	130	9	32	26	H=	2,K=	0
15	148	148	11	211	212	8	243	248	6	86	90	19	70	76	6	46	47	10	73	73	0	451	482
16	55	46	12	218	220	9	106	113	7	68	55	20	114	111	7	235	229	12	146	146	1	1260	1226
18	41	28	13	103	102	10	120	114	8	49	39	21	79	76	8	227	235	13	82	75	2	893	888
19	99	102	15	119	110	12	148	148	9	64	72	22	103	94	9	74	71	14	97	101	3	221	218
20	77	75	16	124	123	13	70	60	10	70	73	23	47	51	10	245	238	15	85	79	4	117	90
21	117	120	17	242	246	14	67	65	H=	0,K=	21	H=	1,K=	4	11	71	68	16	62	66	5	179	169
22	47	57	18	64	64	15	89	84	3	119	109	0	29	59	12	70	75	H=	1,K=	14	6	451	460
23	117	120	19	74	78	16	97	103	4	51	45	1	328	325	13	107	101	0	104	100	7	365	351
H=	0,K=	2	H=	0,K=	7	17	42	34	5	80	80	2	104	117	14	74	72	1	157	154	8	44	87
0	450	416	1	332	330	18	88	86	6	47	34	3	457	434	15	96	94	2	84	82	10	440	435
1	136	79	2	341	343	19	60	40	H=	0,K=	22	4	453	446	16	95	100	4	56	54	11	99	1
2	349	349	3	395	386	H=	0,K=	13	0	52	63	5	235	234	17	88	88	5	92	100	12	175	169
3	490	555	4	338	341	1	208	210	1	39	24	6	113	118	18	127	127	6	98	98	14	110	107
4	357	337	5	309	293	4	107	110	2	48	38	7	132	121	19	67	60	7	114	118	15	229	239
5	131	144	6	311	273	5	104	97	H=	1,K=	0	8	284	279	20	87	95	8	139	139	16	76	80
6	720	672	7	98	89	6	164	164	1	508	538	9	115	113	H=	1,K=	9	9	65	68	18	168	168
7	35	25	8	26	22	7	134	146	2	43	58	10	217	219	0	266	261	10	64	68	21	65	63
8	88	67	9	84	68	9	97	111	3	578	563	11	149	164	1	305	276	11	68	70	23	51	50
9	349	376	10	165	167	10	56	47	4	280	259	12	106	107	2	101	92	12	66	69	H=	2,K=	1
10	223	199	11	534	544	12	88	87	6	396	394	13	97	102	3	235	217	16	91	88	0	1019	1017
11	105	103	12	203	206	13	158	156	7	284	279	14	102	98	4	261	256	17	84	83	1	957	899
12	244	254	13	77	80	19	51	40	7	284	279	15	106	102	5	127	117	18	56	61	2	733	696
13	39	47	14	237	229	H=	0,K=	14	8	57	60	16	127	125	6	186	185	H=	1,K=	15	3	512	508
14	68	80	15	63	62	1	70	67	9	508	507	17	112	113	7	143	146	0	70	61	4	324	322
15	49	60	17	133	134	2	43	29	10	155	160	18	138	139	9	67	74	1	48	47	5	371	345
16	94	89	20	66	64	3	131	127	11	106	102	19	96	99	10	125	126	2	109	101	6	462	455
17	66	56	22	104	110	4	80	81	12	123	128	20	84	84	11	100	97	3	34	33	7	313	309
18	149	158	H=	0,K=	8	5	73	73	13	115	123	22	46	42	12	147	156	4	114	111	8	356	355
19	39	43	0	414	422	7	96	94	14	55	50	23	51	46	13	112	115	5	115	110	9	191	181
20	101	93	1	599	601	8	131	126	15	90	95	H=	1,K=	5	14	215	211	6	69	73	10	90	87
21	72	67	2	82	71	9	61	59	16	106	114	0	571	561	15	71	70	7	63	63	11	410	409
H=	0,K=	3	3	357	335	10	96	110	17	172	184	1	130	133	16	112	115	8	58	62	12	54	56
1	173	180	5	209	213	11	80	85	19	200	196	2	597	595	17	67	68	9	40	33	13	279	283
2	445	382	6	290	273	12	70	69	20	85	76	3	237	225	21	76	78	11	41	54	14	61	73
3	35	10	7	333	333	13	36	37	22	112	109	4	364	338	H=	1,K=	10	12	51	45	15	164	164
4	754	783	8	173	168	14	69	72	H=	1,K=	1	5	257	258	0	348	348	13	90	86	17	167	171
5	415	432	9	297	290	15	50	45	0	44	48	6	224	229	1	306	302	14	67	74	19	35	18
6	68	87	10	162	158	16	74	82	1	485	504	7	143	137	2	49	55	15	49	49	20	79	65
7	137	129	11	208	211	17	52	62	2	629	636	8	192	179	3	179	183	H=	1,K=	16	21	138	138
8	285	277	12	130	137	H=	0,K=	15	3	242	248	9	221	229	4	48	37	0	33	30	22	74	74
9	176	172	14	62	64	3	77	75	4	678	664	10	125	132	5	83	93	1	44	64	23	60	51
10	231	237	17	101	99	4	196	191	5	84	93	11	203	203	6	77	83	2	94	94	H=	2,K=	2
11	55	60	18	46	56	5	61	49	6	606	584	12	265	279	7	285	279	3	67	60	0	101	98
12	393	403	19	75	72	6	34	38	7	170	172	13	246	257	8	81	92	5	45	37	1	537	515
14	171	170	20	57	54	11	147	158	8	365	363	14	89	95	9	317	319	8	91	89	2	199	163
15	136	141	H=	0,K=	9	12	64	61	9	109	79	15	139	131	10	118	122	9	124	115	3	153	141
16	59	51	1	80	78	13	67	77	10	100	90	16	107	107	11	220	218	10	106	108	4	314	296
18	73	69	2	124	136	14	93	100	11	82	83	18	91	92	12	55	45	12	48	43	5	311	312
19	101	90	3	132	134	15	48	18	12	243	246	20	53	50	13	67	68	13	55	43	6	482	474
20	91	87	4	240	217	16	65	73	13	179	178	21	97	91	14	64	58	14	44	40	7	198	199
21	124	119	5	261	253	H=	0,K=	16	14	120	127	22	51	49	15	41	41	16	72	67	8	409	389
22	81	83	6	174	181	0	138	141	15	127	130	H=	1,K=	6	17	96	108	H=	1,K=	17	9	319	314
23	50	53	8	247	250	1	97	92	16	92	84	0	548	552	18	42	45	0	61	63	10	324	323
H=	0,K=	4	9	59	53	2	42	32	17	95	100	1	448	433	19	89	89	3	103	98	11	182	180
0	1279	1313	11	94	86	3	84	80	18	38	50	2	345	354	20	70	51	5	148	142	12	167	173
1	916	883	12	175	170	4	48	43	19	88	85	3	59	59	H=	1,K=	11	6	38	42	13	102	111
2	450	460	13	175	180	5	79	85	20	107	106	4	122	118	0	471	477	7	111	115	14	164	164
3	87	60	15	57	60	7	97	109	21	102	98	5	538	523	1	117	125	9	39	38	15	84	81
4	688	629	17	79	74	9	65	69	22	141	142	6	147	126	2	259	256	11	137	133	16	101	105
5	116	108	18	85	81	10	166	162	23	69	64	7	153	168	3	75	79	12	44	46	18	146	136
6	256	246	H=	0,K=	10	11	55	52	H=	1,K=	2	8	167	175	4	258	255	13	112	111	19	96	88
7	117	125	0	487	483	13	83	84	0	750	786	9	152	163	6	186	186	15	51	57	20	131	130
8	103	118	2	66	63	16	57	53	2	570	551	10	465	471	8	140	151	H=	1,K=	18	21	53	58
9	362	358	4	99	101	H=	0,K=	17	3	242	230	11	195	199	10	74	74	0	137	140	H=	2,K=	3
10	40	38	5	61	51	2	96	96	4	520	521	12	192	212	11	47	49	2	122	122	0	195	193
11	199	196	6	174	158	4	149	146	5	399	379	13	114	118	12	186	191	3	74	72	1	397	405
12	35	43	7	71	78	5	152	140	6	228	198	14	54	58	14	120	116	4	81	74	2	257	241
14	179	178	8	327	342	6	138	139	7	204	202	15	91	90	16	63	56	5	82	74	3	145	150
17	170	164	9	153	148	8	39	37	8	158	156	16	79	78	20	83	68	6	98	104	4	258	264
18	69	57	10	143	137	9	61	65	9	278	252	17	57	63	H=	1,K=	12	8	125	129	5	266	260
19	109	108	11	37	28	11	161	159	10	214	217	18	140	137	0	37	41	9	103	100	6	196	205
H=	0,K=	5	12	126	124	12	57	56	11	186	192	20	71	66	1	336	349	10	64	67	7	237	227
1	488	489	13	84	73	13	54	54	12	206	206	H=	1,K=	7	2	247	250	11	93	84	8	107	105
2	507	492	14	86	79	14	84	98	13	56	59	0	60	60	3	34	16	12	89	85	9	97	92

Table 3. Continued.

10	181	184	18	54	53	9	60	42	5	398	388	4	228	213	14	58	53	3	93	93	18	88	83
11	296	306	19	74	74	10	61	57	6	337	318	5	209	193	15	131	125	6	46	36	19	81	70
12	183	189	Hk	2,Kk	9	13	56	55	7	274	242	6	150	138	16	45	51	7	42	53	Hk	4,Kk	5
13	153	151	0	194	180	14	58	58	8	100	108	7	107	104	Hk	3,Kk	12	Hk	3,Kk	21	1	61	57
14	144	151	1	151	145	15	44	45	9	131	131	8	372	381	0	473	489	4	68	75	2	258	250
15	100	96	2	183	170	16	40	37	10	249	263	9	159	162	1	250	253	Hk	4,Kk	0	3	137	136
16	99	100	3	283	285	17	60	53	11	160	170	10	104	106	2	62	64	0	189	208	4	195	192
17	80	56	4	184	174	Hk	2,Kk	15	12	228	235	11	210	215	3	143	152	1	787	759	5	141	142
21	64	61	5	351	355	1	69	69	13	38	47	12	169	170	4	53	56	2	383	370	6	114	117
22	87	79	6	281	286	2	108	106	14	171	179	13	133	142	5	66	31	3	65	67	7	82	85
Hk	2,Kk	4	7	278	266	3	35	30	15	120	123	14	91	82	6	128	133	4	145	136	8	150	154
0	379	340	8	75	75	4	98	98	16	178	186	16	178	176	7	188	183	5	286	280	9	91	93
1	608	606	9	73	72	5	45	47	18	83	66	17	113	111	8	133	144	6	46	46	10	106	100
2	172	167	10	137	142	6	73	79	19	140	136	19	47	46	9	137	140	7	125	128	11	213	213
3	162	170	12	126	124	7	82	84	20	90	83	20	86	86	11	81	85	8	116	118	12	79	74
4	223	229	13	85	83	8	33	34	Hk	3,Kk	2	Hk	3,Kk	7	12	88	77	9	79	76	13	61	64
5	204	180	14	63	71	9	35	26	0	397	394	1	284	250	15	79	80	10	242	253	14	88	86
7	107	78	15	123	120	10	64	58	1	112	130	2	62	52	17	87	87	11	171	163	16	62	63
8	137	129	16	59	57	11	88	82	2	261	263	3	108	131	Hk	3,Kk	13	13	152	160	17	59	49
9	286	273	17	40	46	13	43	41	3	134	122	4	305	282	0	113	110	15	101	100	18	72	63
10	147	153	21	63	60	14	105	99	4	35	39	5	37	37	1	107	113	16	89	85	19	81	79
11	253	265	Hk	2,Kk	10	Hk	2,Kk	16	5	416	403	6	93	94	2	202	204	17	48	52	20	86	79
13	38	36	0	213	220	0	96	96	6	111	97	7	264	259	3	88	73	18	108	107	Hk	4,Kk	6
14	65	65	1	214	214	1	139	144	7	222	230	8	115	114	4	57	62	21	123	119	0	87	74
15	79	78	2	361	369	2	72	74	8	164	169	9	68	67	5	111	114	Hk	4,Kk	1	1	291	297
17	108	101	3	73	67	3	56	50	9	358	361	10	182	189	6	148	145	0	379	341	2	98	97
18	47	43	4	168	171	4	83	82	10	113	112	11	118	116	7	101	99	1	387	379	3	159	154
19	114	107	5	34	51	5	86	83	11	165	166	12	41	47	9	49	58	2	331	303	4	138	135
20	53	49	6	140	149	6	46	35	12	111	112	13	81	81	10	74	70	3	256	261	5	251	245
21	37	22	7	205	203	7	95	96	13	30	26	14	33	29	12	60	57	4	230	249	6	49	55
Hk	2,Kk	5	8	84	80	8	76	65	14	66	67	15	96	97	13	40	40	5	144	149	7	165	162
0	297	318	9	134	134	9	63	66	15	97	100	16	53	58	14	114	113	6	291	281	8	108	108
1	299	313	10	145	140	10	53	38	16	59	65	17	58	64	15	65	61	7	213	211	9	253	257
2	340	337	11	71	69	11	66	61	17	81	85	20	58	55	Hk	3,Kk	14	8	239	235	10	61	69
3	177	177	12	131	131	12	72	72	18	94	93	21	83	81	1	108	111	9	73	62	11	214	216
4	320	323	13	38	38	15	64	56	19	85	83	Hk	3,Kk	8	2	88	75	10	95	101	12	102	98
5	229	216	14	68	67	Hk	2,Kk	17	20	64	68	0	267	278	3	43	37	11	143	144	13	85	96
6	150	153	15	74	71	0	57	52	22	66	57	1	350	349	4	53	56	12	40	52	15	85	88
7	68	56	16	122	123	1	84	81	Hk	3,Kk	3	2	155	154	5	64	65	13	149	161	16	107	100
8	103	99	17	37	25	2	122	122	0	108	115	3	277	273	6	50	41	14	72	70	17	111	109
9	210	209	18	110	109	3	94	94	1	169	167	4	27	24	9	54	48	15	81	83	19	110	102
10	205	198	19	70	63	4	176	172	2	127	123	5	77	74	10	77	88	16	35	27	Hk	4,Kk	7
11	250	260	20	41	41	5	80	81	3	318	307	6	132	141	14	50	45	17	69	68	1	80	86
12	112	112	Hk	2,Kk	11	6	73	75	4	105	103	8	90	92	15	47	41	19	56	47	2	257	261
13	118	117	0	374	377	7	50	43	5	181	171	9	104	100	16	39	25	20	47	46	3	172	170
14	147	145	1	234	232	8	73	69	6	163	150	10	244	246	Hk	3,Kk	15	21	86	85	4	194	188
15	43	48	2	184	185	9	88	92	7	191	197	11	76	78	1	71	60	Hk	4,Kk	2	5	127	127
16	152	144	3	194	199	10	180	164	8	187	185	12	67	61	2	45	50	0	549	536	6	195	193
17	130	139	4	96	97	11	66	67	9	140	141	13	33	39	3	140	130	1	287	290	7	80	103
18	95	86	5	170	169	12	98	84	10	165	160	15	37	32	4	41	37	2	31	20	8	109	114
20	114	110	6	98	108	13	41	29	11	118	124	16	39	33	5	130	134	3	111	105	9	105	99
22	73	63	7	147	148	14	85	75	12	211	215	17	122	112	7	38	54	4	66	40	10	183	184
Hk	2,Kk	6	8	66	70	Hk	2,Kk	18	13	98	91	18	117	123	8	38	42	5	108	102	11	82	80
0	122	117	9	60	67	1	105	107	14	124	123	19	54	47	11	109	96	6	119	115	12	188	195
1	218	218	10	64	67	2	60	57	15	111	112	20	69	63	13	56	61	7	192	197	13	79	68
2	241	227	11	68	71	3	123	111	16	128	133	Hk	3,Kk	9	15	57	40	8	240	243	14	84	80
3	142	138	12	87	91	4	46	39	17	82	68	0	58	65	16	38	28	9	246	249	16	68	67
5	450	405	13	160	157	5	58	50	20	89	73	1	191	191	Hk	3,Kk	16	10	261	252	20	66	60
6	271	286	14	50	36	7	99	93	21	45	55	2	174	176	0	125	129	11	139	138	Hk	4,Kk	8
7	151	159	15	92	95	8	58	51	Hk	3,Kk	4	3	329	327	1	82	77	12	80	85	0	71	73
8	240	232	16	54	62	9	86	87	0	194	193	4	194	194	2	75	76	13	82	85	1	206	202
9	298	302	17	48	56	10	95	88	1	238	236	5	70	79	3	70	66	14	96	97	2	84	57
10	187	186	19	48	43	11	96	90	2	160	163	6	187	192	4	114	110	16	37	33	3	176	182
11	331	336	Hk	2,Kk	12	12	60	61	3	420	388	7	143	145	5	50	46	17	54	52	4	41	38
12	161	167	0	178	180	13	55	50	4	198	203	8	54	56	6	108	95	18	106	94	5	39	32
13	143	148	2	244	251	Hk	2,Kk	19	5	268	259	9	80	81	8	156	158	19	77	80	6	76	73
15	100	108	3	230	232	2	40	46	6	236	223	11	151	155	10	38	32	20	112	109	7	64	60
16	107	108	5	57	62	3	37	38	7	73	75	12	175	173	11	91	85	Hk	4,Kk	3	8	126	124
17	119	117	6	173	172	4	125	119	8	223	215	13	87	99	12	81	79	0	32	57	9	177	179
19	171	169	7	162	166	5	101	98	9	138	132	14	38	54	13	51	52	1	166	147	10	89	95
22	46	35	8	125	126	6	84	84	10	123	116	15	79	84	14	46	38	2	158	165	11	84	86
Hk	2,Kk	7	9	44	38	7	61	50	11	167	175	16	43	39	Hk	3,Kk	17	3	175	182	12	100	103
0	169	191	10	142	144	Hk	2,Kk	20	12	143	145	17	54	55	2	40	31	4	130	118	14	55	47
1	82	90	12	63	53	0	119	121	14	63	72	19	40	39	3	51	61	5	113	112	15	44	50
2	188	205	13	48	40	1	50	55	15	34	33	20	90	88	4	64	67	6	136	140	16	60	60
3	294	269	14	45	47	2	40	34	16	114	106	Hk	3,Kk	10	5	130	136	7	160	151	17	60	72
4	480	451	15	45	28	3	50	56	17	57	45	0	100	104	6	84	94	8	105	109	18	59	45
5	252	241	16	93	89	6	50	35	18	81	82	1	223	232	7	56	42	9	103	103	19	71	66
6	139	142	17	57	63	8	79	68	19	88	90	2	137	134	10	66	59	10	54	48	Hk	4,Kk	9
7	96	90	18	80	81	9	66	62	20	87	84	3	181	177	11	58	51	11	120	117	1	72	77
8	138	138	Hk	2,Kk	13	Hk	2,Kk	21	21	44	27	4	84	77	12	44	41	12	171	174	2	97	108
9	215	211	0	50	51	1	53	51	Hk	3,Kk	5	5	73	78	13	78	79	13	93	90	3	235	241
10	178	184	1	145	147	3	58	65	0	29	45	6	71	68	Hk	3,Kk	18	14	44	52	4	114	117
11	119	125	2	123	128	5	81	68	1	276	274	7	33	29	0	77	83	15	89	86	5	200	199
12	283	283	3	152	153	Hk	2,Kk	22	2	142	166	9	158	156	1	53	39	16	48	46	6	119	117
13	79	86	4	129	128	0	95	95	3	439	418	10	97	94	2	81	80	17	39	35	7	126	123
14	110	115	5	172	179	Hk	3,Kk	0	4	201	191	11	94	100	3	60	51	18	36	20	8	69	65
16	149	142	6	140	147	1	96	116	5	164	155	12	48	60	4	57	47	20	62	53	9	60	60
17	100	94	7	67	67	2	39	38	6	152	126	13	40	36	5	47	42	21	42	34	10	38	44
18	62	68	8	65	62	3	414	411	7	161	166	14	61	71	7	43	36	Hk	4,Kk	4	11	94	94
20	64	71	9	47	47	5	37	24	8	179	178	15	87	86	8	41	36	0	32	22	12	49	58
Hk	2,Kk	8	10	56	67	6	161	154	9	118	123	17	48	45	9	44	41	1	342	314	13	77	68
0	244	222	11	119	118	7	316	282	10	90	87	19	43	46	10	121	110	2	158	154	15	104	102
1	273	272	13	39	49	9	432	440	11	528	525	Hk	3,Kk	11	Hk	3,Kk	19	3	116	105	16	45	44
2	190	216	14	98	94	10	67	70	12	125	129	0	36	26	0	39	26	4	128	124	17	47	40
3	219	220	15	56	49	11	352	361	13	100	97	1	213	212	1	79	82	5	80	73	Hk	4,Kk	10
4	78	82	16	46	49	12	48	50	15	34	21	2	117	126	2	49	47	6	70	71	0	177	183
5	128	120	Hk	2,Kk	14	13	81	81	16	95	85	3	87	98	3	41	46	7	178	181	1	222	225
6	47	50	0	40	45	15	113	122	17	88	86	4	228	229	4	97	87	8	37	31	2	235	243
7	128	120	1	94	93	19	82	87	18	92	87	5	233	239	5	87	85	9	203	210	3	69	72
8	200	204	2	84	84	21	232	225	19	77	73	6	250	256	6	59	62	10	174	176	4	106	110
9	265	269	3	130	126	Hk	3,Kk	1	21	89	80	7	59	59	7	64	68	11	48	53	5	42	37
10	113	113	4	75	71	0	212	190	Hk	3,Kk	6	8	122	121	9	78	71	12	114	110	6	148	153
11	181	181	5	47	55	1	340	320	0	193	142	9	78	76	Hk	3,Kk	20	13	79	80	7	88	90
14	71	66	6	58	55	2	322	330	1	108	108	10	64	62	0	104	102	15	91	87	8	138	137
15	56	58	7	114	117	3	320	319	2	102	134	11	75	81	1	53	49	16	59	58	9	111	110
16	87	81	8	83	94	4	90	92	3	165	181	12	60	58	2	46	52	17	81	83	10	143	142

Table 3. Continued.

11	47	50	9	91	93	10	114	115	0	174	173	5	138	135	4	65	74	7	87	89	7	55	61
12	110	102	Hm	4,Km	19	11	65	68	1	145	150	6	46	54	5	68	70	9	57	54	8	41	52
13	90	83	3	41	45	12	110	104	2	131	138	7	66	66	6	62	67	10	49	45	11	76	73
14	36	31	4	71	64	13	123	119	3	42	32	8	54	47	7	39	39	12	67	59	14	45	43
15	94	93	5	61	37	15	80	82	4	49	43	9	104	108	8	68	64	13	80	71	Hm	7,Km	10
16	83	81	6	75	78	16	59	55	5	45	53	10	82	86	9	52	66	14	81	79	0	57	56
Hm	4,Km	11	Hm	4,Km	20	17	40	24	6	150	152	11	49	49	11	43	46	16	59	62	1	111	112
0	165	163	0	61	64	18	44	47	7	123	124	12	61	66	12	76	78	17	42	36	3	51	45
1	195	203	2	43	33	Hm	5,Km	6	8	60	63	18	66	54	13	106	107	Hm	7,Km	2	6	65	58
2	228	223	Hm	5,Km	0	0	186	184	9	119	117	Hm	6,Km	3	15	41	35	1	145	155	7	49	53
3	120	128	1	318	312	1	60	53	11	95	90	0	157	149	16	46	44	2	61	66	9	79	77
5	134	146	2	67	68	2	177	171	12	44	48	1	41	38	Hm	6,Km	10	4	103	99	10	70	66
6	153	156	3	352	346	4	113	103	13	39	36	2	130	129	0	186	193	6	72	70	11	70	62
7	95	102	4	151	160	5	154	157	14	73	62	3	78	69	1	32	24	7	86	87	13	48	43
8	69	71	5	208	215	6	33	41	15	46	47	4	167	164	3	58	64	8	47	48	Hm	7,Km	11
9	65	66	7	159	162	7	60	63	Hm	5,Km	13	5	76	76	4	69	78	9	118	111	0	137	135
10	44	46	8	106	113	9	55	53	0	38	40	6	109	108	5	45	43	10	84	85	2	91	101
11	64	52	9	179	192	10	255	254	1	113	117	7	52	60	6	43	40	11	76	75	3	64	58
13	140	134	10	68	67	11	175	185	3	61	61	8	84	79	8	158	158	12	78	78	4	75	69
14	59	54	11	110	109	12	93	90	4	123	116	9	54	58	9	63	54	13	44	36	6	102	97
15	82	86	17	140	139	13	48	42	5	35	24	10	91	88	10	71	72	14	46	41	7	40	37
16	75	69	19	105	97	14	79	69	7	58	61	11	54	46	12	59	59	15	41	44	8	39	52
17	44	45	20	77	71	18	113	113	9	43	46	12	125	120	14	44	42	17	48	41	12	49	48
18	56	62	Hm	5,Km	1	19	39	27	10	57	55	13	39	26	Hm	6,Km	11	Hm	7,Km	3	Hm	7,Km	12
Hm	4,Km	12	0	161	166	Hm	5,Km	7	12	93	90	14	80	84	0	68	68	1	79	77	1	137	144
0	90	98	1	177	173	0	71	73	13	66	62	16	40	24	1	111	111	2	77	71	2	40	45
2	98	103	2	180	175	1	71	65	Hm	5,Km	14	18	41	43	2	34	41	3	69	68	4	95	87
4	31	25	3	84	84	3	200	206	0	34	32	Hm	6,Km	4	3	154	152	4	63	63	5	59	57
6	75	66	4	291	287	4	60	56	1	51	52	0	161	165	4	34	27	5	59	50	7	52	40
7	121	126	5	48	55	5	183	188	2	44	47	1	171	173	5	163	159	6	42	41	9	65	63
8	104	104	6	204	211	6	88	90	5	54	51	2	97	56	6	54	54	7	49	47	Hm	7,Km	13
9	35	43	7	96	91	7	95	95	6	70	72	3	48	47	11	88	93	8	73	73	0	37	40
10	59	73	8	159	155	8	84	80	7	67	68	4	105	115	12	51	48	11	100	101	1	71	69
11	63	50	9	58	62	9	43	48	8	84	87	5	55	49	13	52	50	12	64	58	2	46	45
14	45	38	10	68	68	10	104	105	9	66	61	6	57	43	15	117	113	13	41	38	3	45	44
16	102	96	12	122	126	11	99	102	Hm	5,Km	15	7	50	58	Hm	6,Km	12	15	43	34	5	67	60
Hm	4,Km	13	13	58	59	13	127	130	1	81	73	8	86	85	0	84	81	16	47	51	6	42	46
0	44	36	14	74	72	14	120	117	2	43	43	9	168	166	1	44	43	17	42	43	8	51	42
1	51	47	15	62	67	15	38	33	4	75	70	10	45	44	2	66	65	Hm	7,Km	4	Hm	7,Km	14
2	41	43	16	39	30	16	52	53	5	56	49	11	78	75	3	41	26	0	56	62	0	88	91
3	115	117	20	51	53	17	62	69	6	39	41	12	71	79	4	37	31	1	49	42	1	51	40
4	132	141	Hm	5,Km	2	Hm	5,Km	8	7	79	78	13	42	36	6	61	64	2	73	68	2	45	46
5	120	118	0	134	136	0	311	320	12	52	48	14	42	50	8	107	110	5	82	81	4	47	47
6	103	103	1	251	244	1	88	91	13	66	60	16	38	40	9	37	41	6	65	60	8	39	43
7	61	65	2	151	144	2	111	112	Hm	5,Km	16	17	76	76	10	49	51	7	44	44	Hm	7,Km	15
10	36	30	3	75	83	3	97	102	0	37	39	Hm	6,Km	5	12	61	57	8	96	100	2	42	30
11	81	81	4	126	125	4	58	49	2	65	62	1	101	104	13	58	49	9	40	35	3	43	43
12	52	45	5	130	136	5	58	58	3	55	48	2	106	108	Hm	6,Km	13	11	44	49	5	53	51
13	73	61	6	37	38	7	141	134	7	45	46	3	110	113	0	48	41	12	65	63	6	42	37
14	103	102	7	158	152	8	125	119	8	65	58	4	58	59	1	109	107	13	50	49	Hm	7,Km	16
15	47	47	8	81	90	9	57	57	9	79	70	6	82	71	2	40	36	14	56	48	0	45	41
16	47	47	9	77	76	10	154	155	10	73	77	7	100	94	4	49	46	16	52	43	2	52	51
Hm	4,Km	14	10	63	69	11	64	65	11	73	64	8	39	42	6	44	48	Hm	7,Km	5	Hm	8,Km	0
0	116	125	11	41	37	13	96	91	Hm	5,Km	17	9	77	75	7	65	67	0	58	45	0	105	111
1	45	60	12	42	47	14	60	68	1	37	30	10	51	55	8	58	56	1	67	68	1	74	86
2	43	53	14	33	21	15	67	66	3	78	78	11	44	41	9	46	52	2	128	127	2	120	120
3	94	98	16	63	72	16	49	49	5	73	67	12	159	164	12	40	42	3	93	94	3	86	97
5	64	64	17	46	55	17	43	32	7	42	40	13	54	48	13	66	73	4	69	68	6	116	118
6	51	45	18	53	52	18	78	73	Hm	5,Km	18	14	51	59	Hm	6,Km	14	5	86	88	7	47	44
7	86	87	19	96	90	Hm	5,Km	9	0	33	38	17	54	57	1	50	52	7	68	68	10	104	108
8	82	88	20	39	31	0	129	126	2	52	48	18	56	51	2	49	38	9	47	50	11	62	66
9	77	75	Hm	5,Km	3	1	66	72	5	56	49	Hm	6,Km	6	3	71	68	10	196	193	Hm	8,Km	1
10	82	80	0	60	60	2	64	63	6	78	75	1	97	99	4	42	40	12	97	93	0	72	85
11	79	81	1	186	190	3	109	109	8	84	78	2	139	139	5	68	68	13	82	79	1	95	102
13	54	50	2	160	162	4	83	88	Hm	5,Km	19	3	30	32	6	42	40	15	49	47	2	87	83
14	46	42	3	159	159	5	111	102	1	39	23	4	115	109	7	73	66	16	84	37	3	46	45
Hm	4,Km	15	4	80	76	6	83	83	2	45	40	6	115	117	8	56	40	Hm	7,Km	6	4	48	38
1	56	51	5	241	229	7	68	66	3	75	64	7	75	63	9	42	37	0	100	98	7	79	80
2	59	59	6	133	137	10	94	96	5	40	42	8	139	140	10	48	48	2	80	85	8	48	52
3	34	29	7	176	183	12	69	70	Hm	6,Km	0	9	160	160	Hm	6,Km	15	3	46	37	10	51	49
4	64	62	8	92	100	13	57	61	0	724	752	10	119	112	2	51	53	5	119	111	11	76	62
5	81	81	9	74	76	14	114	112	2	54	53	11	43	49	3	61	59	6	70	72	12	47	37
6	50	47	10	92	95	15	37	33	6	130	128	12	93	92	4	98	90	8	82	80	13	65	69
7	52	60	12	51	46	16	55	58	7	112	103	13	50	53	6	59	49	9	79	74	Hm	8,Km	2
8	51	49	13	111	118	18	43	44	8	242	251	14	52	45	10	63	56	10	107	103	0	36	30
10	62	57	14	44	41	Hm	5,Km	10	9	64	49	16	46	51	11	69	68	12	108	104	1	95	104
12	56	48	15	75	73	0	191	192	10	114	106	17	81	83	Hm	6,Km	16	13	66	75	2	60	65
13	85	74	18	75	74	1	110	115	12	69	72	Hm	6,Km	7	1	46	44	16	46	41	6	65	65
14	45	62	Hm	5,Km	4	3	38	39	13	36	40	0	85	86	3	68	50	Hm	7,Km	7	7	47	54
Hm	4,Km	16	0	209	210	5	50	55	14	116	118	1	101	92	4	39	42	1	57	59	8	55	51
0	36	19	1	252	255	6	94	99	15	43	43	2	88	86	5	38	35	3	87	87	10	79	72
1	71	74	2	229	223	7	177	181	18	47	49	3	160	161	7	49	51	5	52	45	12	52	42
2	45	40	3	144	139	8	76	68	19	59	47	4	141	129	9	45	43	7	60	57	13	45	45
5	77	81	4	182	182	9	138	144	Hm	6,Km	1	6	134	136	Hm	6,Km	17	9	45	43	Hm	8,Km	3
7	96	84	5	104	104	10	56	52	0	252	266	8	57	45	2	50	30	10	40	44	1	70	73
9	46	37	6	29	26	11	94	100	2	50	50	9	100	106	4	67	71	11	101	96	2	67	62
10	39	33	7	126	126	12	69	64	3	199	205	10	65	62	6	59	58	13	66	59	3	39	38
11	81	74	8	155	150	15	55	54	4	46	47	11	165	170	Hm	6,Km	18	Hm	7,Km	8	6	54	56
12	71	66	9	94	96	17	77	76	5	169	174	12	64	64	0	46	51	0	39	52	8	67	69
Hm	4,Km	17	10	195	201	Hm	5,Km	11	6	86	92	14	110	102	1	71	66	1	39	37	9	45	45
1	40	33	11	43	43	0	123	130	7	92	92	15	48	28	Hm	7,Km	0	2	70	67	10	53	45
2	90	96	13	47	39	1	80	86	8	56	56	17	56	62	1	90	94	3	71	73	11	41	45
3	44	48	16	45	46	2	144	144	9	89	97	Hm	6,Km	8	7	73	75	6	52	46	13	49	40
4	118	116	17	56	44	4	117	114	10	42	39	1	134	127	8	76	72	7	50	52	14	43	36
6	84	75	18	99	93	5	60	61	11	89	89	3	65	71	9	98	97	8	91	91	Hm	8,Km	4
8	65	69	20	57	55	6	103	102	12	35	44	5	37	42	11	46	42	9	47	52	1	59	65
10	149	145	Hm	5,Km	5	7	68	76	13	87	81	6	66	72	12	72	61	10	80	41	3	45	55
12	100	97	0	45	25	8	71	71	14	48	53	7	111	117	15	43	32	12	82	49	4	49	48
Hm	4,Km	18	1	93	90	9	75	73	15	63	66	8	74	71	16	49	40	14	48	35	7	69	59
0	37	44	2	125	129	10	55	66	16	57	57	9	103	101	Hm	7,Km	1	Hm	7,Km	9	9	61	61
1	83	85	3	37	23	12	130	128	19	56	44	10	80	73	0	33	35	0	92	92	11	107	115
2	52	52	4	189	185	13	37	26	Hm	6,Km	2	11	101	104	1	48	59	1	87	92	14	48	44
3	93	86	5	236	230	14	90	88	0	97	96	12	87	83	2	111	117	2	34	46	Hm	8,Km	5
4	60	61	6	93	89	15	61	66	1	86	85	17	64	61	3	52	58	3	35	33	1	61	50
5	57	45	7	66	67	16	42	31	2	145	146	Hm	6,Km	9	4	88	96	4	120	118	4	79	70
7	71	74	8	144	144	17	45	45	3	161	158	2	42	40	5	44	37	5	40	45	6	83	78
8	49	35	9	92	85	Hm	5,Km	12	4	71	77	3	49	48	6	108	113	6	63	56	7	37	34

Table 3. Continued.

9	60	58	H=	8,K=	7	10	59	56	5	44	34	5	74	68	H=	9,K=	5	1	48	40	4	46	24
10	66	64	1	59	64	11	48	44	7	70	67	6	45	40	0	43	34	H=	9,K=	9	5	42	35
12	46	49	4	102	94	H=	8,K=	9	H=	8,K=	12	7	38	34	3	99	99	2	51	54	H=	10,K=	2
13	39	28	5	64	61	1	59	62	0	60	58	9	41	50	4	42	42	3	43	38	0	99	92
14	40	44	7	51	53	2	69	74	2	75	75	H=	9,K=	2	8	60	47	5	39	31	1	51	38
H=	8,K=	6	8	65	58	5	61	55	3	64	70	0	41	53	9	53	60	6	53	51	H=	10,K=	3
1	54	58	9	44	50	6	51	54	H=	8,K=	13	2	51	32	H=	9,K=	6	H=	9,K=	10	1	42	36
2	52	49	10	40	45	7	68	64	2	60	58	3	64	62	0	93	91	0	58	44	3	45	39
3	42	53	11	75	77	9	49	48	3	61	57	7	40	31	5	44	33	1	50	54	4	41	39
4	45	52	12	67	58	H=	8,K=	10	5	65	58	8	49	49	7	46	49	4	40	43	H=	10,K=	4
5	40	55	H=	8,K=	8	1	39	36	H=	9,K=	0	9	52	56	8	52	65	H=	9,K=	11	2	49	42
6	53	45	0	80	75	2	78	76	1	68	68	H=	9,K=	3	H=	9,K=	7	1	47	45	H=	10,K=	6
8	61	62	2	79	79	4	49	42	6	52	45	6	55	53	0	66	59	2	48	51	1	43	39
9	72	72	3	36	17	6	57	39	10	42	31	11	40	40	2	41	22	H=	10,K=	0	H=	1,K=	2
10	51	61	5	50	42	7	40	39	H=	9,K=	1	H=	9,K=	4	5	49	41	1	86	98	1	1105	1104
11	90	87	6	42	30	H=	8,K=	11	1	72	76	0	70	65	8	48	45	H=	10,K=	1	H=	9,K=	11
12	40	47	7	51	56	0	110	120	2	55	53	2	44	45	H=	9,K=	8	2	55	58	1	47	45
13	51	50	8	53	41	1	46	54	3	44	41	9	38	34									

Table 4. R.m.s. amplitudes of vibration $(\overline{u^2})^{1/2}$ and B-values (Å^2) along the principal axes of vibration given by the components of a unit vector **e** in fractional coordinates ($\times 10^4$).

	$(\overline{u^2})^{1/2}$	B	e_x	e_y	e_z		$(\overline{u^2})^{1/2}$	B	e_x	e_y	e_z
	,187	2,77	-1474	224	65		,152	1,82	187	352	597
S	,140	1,54	501	674	103	N1	,147	1,71	933	475	- 315
	,130	1,33	64	- 123	680		,132	1,38	1233	- 413	148
	,328	8,51	1341	22	- 351		,184	2,68	1308	144	- 349
OS1	,185	2,70	755	- 253	554	N2	,163	2,10	736	- 524	345
	,147	1,71	240	675	218		,130	1,33	416	474	487
	,257	5,22	1283	- 407	38		,184	2,67	1499	- 102	161
OS2	,189	2,83	861	592	99	C1	,154	1,87	200	713	48
	,132	1,38	195	64	- 683		,131	1,35	375	27	- 670
	,283	6,32	850	604	14		,168	2,22	1143	317	358
OS3	,194	2,96	-1220	362	254	C2	,159	1,99	58	- 567	425
	,168	2,22	465	- 155	642		,144	1,64	-1057	312	410
	,321	8,14	974	- 528	187		,175	2,42	480	644	227
OS4	,175	2,41	1025	491	223	C3	,158	1,98	717	- 318	532
	,149	1,75	654	17	- 627		,145	1,66	-1297	63	378
	,243	4,66	1524	- 15	142		,201	3,18	695	625	- 155
O1	,162	2,06	318	159	- 659	C4	,180	2,55	1370	- 273	199
	,148	1,73	- 42	703	152		,135	1,45	- 257	234	643
	,216	3,68	1336	304	203		,186	2,75	1095	446	- 242
O2	,178	2,51	- 795	555	266	C5	,171	2,32	1086	- 359	357
	,145	1,67	101	346	- 605		,141	1,56	- 227	438	540
	,270	5,75	624	- 609	245		,186	2,73	- 992	301	448
O3	,224	3,96	1392	321	37	C6	,177	2,46	1112	2	484
	,142	1,59	- 315	213	645		,138	1,50	454	655	- 207
	,238	4,46	1015	- 497	220		,184	2,67	1530	- 52	121
O4	,190	2,84	1041	233	- 463	C7	,149	1,75	- 242	157	666
	,144	1,63	560	468	463		,137	1,49	167	701	- 141
	,214	3,62	1408	180	240		,200	3,17	1456	80	- 233
OV1	,196	3,03	- 625	559	338	C8	,155	1,90	256	511	474
	,183	2,64	230	419	- 553		,148	1,72	490	- 503	445
	,258	5,26	611	337	- 547						
OV2	,232	4,26	1248	- 430	31						
	,166	2,17	704	470	421						

taken as the inverse of the variance of the observed structure factor. The parameters refined were the scale factor, 105 positional parameters, 189 anisotropic thermal parameters for the heavy atoms, and 14 isotropic thermal parameters for the hydrogen atoms.

L-Mimosine sulphate was reported to contain 1.5 molecules of water per formula unit.[3] The X-ray crystallographic study revealed the position of one water molecule which is firmly hydrogen bonded to mimosine ions and for which the hydrogen positions were determined. Another position was found for an additional water molecule where only weak interactions with the surrounding molecules are possible; the occupancy for this water molecule is probably less than one per position. When allowed to vary during the least-squares refinement the occupancy factor for the oxygen atom of this water molecule (OV1) refined to 0.6. The thermal parameters associated with the atom were rather large, however, and highly correlated with the occupancy factor. When fixing the occupancy factor at 0.5 the thermal parameters refined to values comparable to those of the other water molecule (*cf.* Table 4). Because of the correlation we cannot decide whether this is correct or if the molecule has larger fractional population and thermal parameters.

The refinement converged with a final conventional R-factor of 0.051 ($R_w = 0.050$). The corresponding atomic parameters are listed in Tables 1 and 2. A comparison of observed and calculated structure factors is given in Table 3.

Magnitudes and directions of the principal axes of vibration for the heavy atoms are given in Table 4. As may be seen from these values the amplitudes of thermal vibration along the main axes for the oxygen atoms of the sulphate ion are quite large. A rigid-body analysis showed that the thermal motion of the ion could to a good approximation be interpreted in terms of translational and librational oscillations. A corresponding analysis for the entire mimosine ion was far less satisfactory; omitting the alanine part of the ion in the analysis seemed to give adequate agreement between observed and calculated U-values.

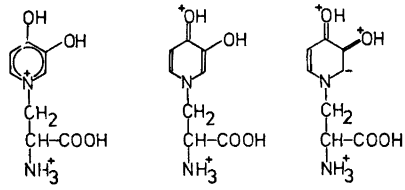
Bond lengths and angles, and some intermolecular distances are given in Table 5. Bond lengths corrected for thermal librations are given where such corrections seem to be justified.

Standard deviations in distances and angles were calculated from the correlation matrix.

DISCUSSION OF THE STRUCTURE

A drawing of the protonized form of L-mimosine as found in the crystals of the sulphate is presented in Fig. 2 where also the numbering of the atoms is indicated. The bond lengths and angles arrived at in the present analysis are indicated in this figure and may also be found in Table 5. The standard deviations in the bond lengths are 0.003 Å for S–O bonds, 0.004–0.005 for bonds between C, N, and O atoms, and 0.04–0.05 Å for bonds involving hydrogen atoms. The standard deviations in angles are 0.2–0.3°.

The six-membered ring of the hydroxypyridyl part of the molecule is planar. The distances from various atoms to a least-squares plane through the ring atoms are indicated in Fig. 2c. O1 and O2 are situated slightly out of the plane and the N1–C6 bond is bent out from the C3–N1 direction. The two C–N bonds in the pyridyl ring are of equal lengths (1.354 Å and 1.358 Å) and comparable to the mean value (1.356 Å) of the corresponding bonds in mimosine.[1] For the latter structure it was concluded that the contributions from the two resonance structures I and II of Fig. 1 are about equal. If an analogous simple valence bond picture is applied to the doubly protonized form the contributions from the structures IV–VI seem to be of importance.

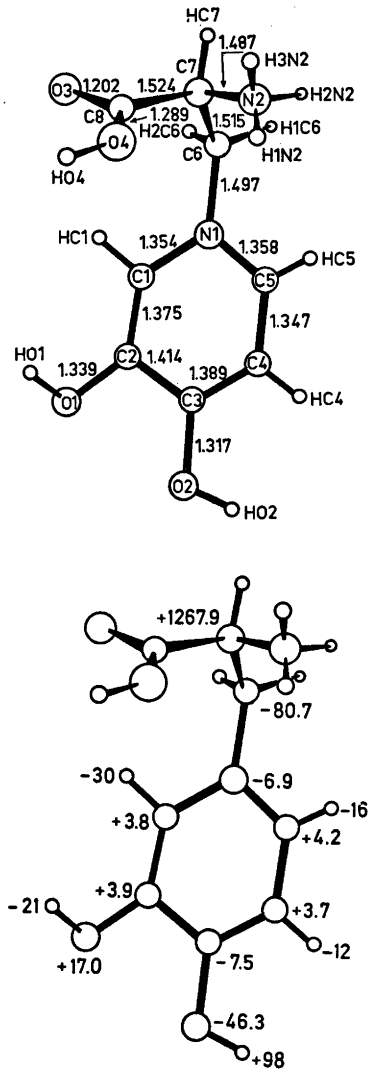


IV V VI

The contribution from VI explains the rather short C–O bonds (1.339 Å and 1.317 Å, as compared to the C–O bonds in phenols, 1.36–1.38 Å) and also that C4–C5 (1.347 Å) is shorther than C1–C2 (1.375 Å). The difference between the C–O bond lengths may be explained by the contribution from V, which also

Table 5. Bond lengths (Å), bond angles (°) and some intermolecular distances in mimosine sulphate hydrate. Distances given in parentheses are corrected for thermal libration.

Bond		Corr.	Angle		Hydrogen bonds		
S–OS1	1.467	(1.485)	OS1–S–OS2	110.7	OS1···HO4–O4	(1+x, y, z)	2.557
S–OS2	1.472	(1.483)	OS1–S–OS3	109.6	OS2···H2N2–N2	(½−x, 1−y, −½+z)	2.831
S–OS3	1.463	(1.479)	OS1–S–OS4	108.6	OS2···H3N2–N2	(−x, −½+y, ½−z)	2.926
S–OS4	1.462	(1.480)	OS2–S–OS3	110.7	OS3···HO2–O2	(½+x, ½−y, 1−z)	2.601
N1–C1	1.354	(1.359)	OS2–S–OS4	107.4	OS4···HO1–O1	(x, y, z)	2.577
C1–C2	1.375	(1.378)	OS3–S–OS4	109.9	O1···H1V2–OV2	(−x, −½+y, ½−z)	2.940
C2–C3	1.414	(1.421)	N1–C1–C2	120.6	O3···H2V2–OV2	(x, y, z)	2.955
C3–C4	1.389	(1.393)	C1–C2–C3	119.3	OV2···H1N2–N2	(−½−x, 1−y, −½+z)	2.734
C4–C5	1.347	(1.350)	C2–C3–C4	117.8			
C5–N1	1.358	(1.364)	C3–C4–C5	121.0	Distances involving the half		
C2–O1	1.339	(1.342)	C4–C5–N1	120·8	water molecule (less than 3.2 Å)		
C3–O2	1.317	(1.319)	C5–N1–C1	120.5	OV1···OS3	(−1+x, y, z)	3.040
N1–C6	1.497	(1.499)	C1–C2–O1	123.3	OV1···OS4	(x, y, z)	3.123
C6–C7	1.515		O1–C2–C3	117.5	OV1···O3	(x, y, z)	3.162
C7–N2	1.487		C2–C3–O2	117.1	OV1···OV2	(x, y, z)	3.025
C7–C8	1.524		O2–C3–C4	125.1	OV1···OS2	(−½+x, ½−y, −z)	3.196
C8–O3	1.202		C5–N1–C6	119.6	OV1···N2	(−½−x, 1−y, −½+z)	3.104
C8–O4	1.289		C1–N1–C6	119.9	OV1···N2	(−x, −½+y, ½−z)	3.186
O1–HO1	1.00		N1–C6–C7	113.9	OV1···HC7	(−x, −½+y, ½−z)	2.438
O2–HO2	0.83		C6–C7–N2	1129			
O4–HO4	0.94		C6–C7–C8	113.6			
OV2–H1V2	0.72		C8–C7–N2	110.9			
OV2–H2V2	0.93		C7–C8–O3	119.1			
N2–H1N2	0.77		C7–C8–O4	114.7			
N2–H2N2	0.65		O3–C8–O4	126.3			
N2–H3N2	0.92						
C1–HC1	0.97						
C4–HC4	1.03						
C5–HC5	0.99						
C6–H1C6	0.96						
C6–H2C6	1.03						
C7–HC7	0.98						



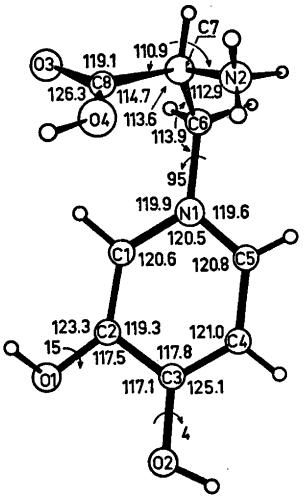


Fig. 2. a. Bond lengths (Å) and *b.* bond angles (°) in the mimosine ion. *c.* Deviations ($\times 10^3$ Å) from the best plane through the pyridine ring.

accounts for the shortness of the C1 – C2 and the C4 – C5 bonds relative to the aromatic C – C bond as found in pyridine. In accordance with the resulting charge distribution the two phenolic oxygen atoms act as hydrogen donors in fairly short hydrogen bonds (2.601 Å and 2.577 Å to oxygen atoms of the sulphate ion).

The external angles at the carbon atoms carrying the hydroxyl groups exhibit the characteristics commonly found in hydrogen bonded phenols and catechols: the angles "*cis*" to the hydrogen atoms are greater than 120° (123.3° and 125.1° for O1 and O2, respectively) whereas the angles "*trans*" are less than 120° (117.5° and 117.1°), even if the consequence is a rather small O – O nonbonded separation. The phenolic hydrogen atoms are close to the plane of the pyridyl ring, the dihedral angles C – C – O – H being 15° (O1) and 4° (O2).

The N1 – C6 bond connecting the hydroxypyridyl ring to the alanine moiety is found to be somewhat longer (1.497 Å) than in mimosine (1.478 Å). The bond lengths found within the alanine part of the ion correspond closely to

those given by Sundaralingam and Putkey [7] as the mean values for a number of protonized amino acids. As for the bond angles the agreement is also good with the exception of the two C – C – O angles which show a significant difference. Their figures are based on amino acids having the normal conformation, however, with the nitrogen atom situated close to plane of the carboxyl group and in the *anti* position relative to the hydroxyl oxygen atom. In the present structure the conformation is *syn*, resulting in a 3.5° opening of the C – C – OH angle and a corresponding decrease in the C – C = O angle relative to the values given in the paper referred to; the O – C – O angle remains unchanged. The nitrogen atom is situated close to the plane through $C_\alpha - CO_2$ (0.13 Å); the dihedral angle O(H) – C – C – N is 5.6°. The conformation about the C – N bond is staggered.

The conformation of the mimosine ion with respect to the $C_\alpha - C_\beta$ bond is different from that found for L-mimosine but is the same as that reported for L-tyrosine.[8] The carboxyl and the amino groups are both in *gauche* positions relative to the pyridyl-ring with torsional angles N1 – C6 – C7 – N2 and N1 – C6 – C7 – C8 equal to 64.6° and – 62.8°, respectively. The angle between the ring plane and the plane through N1, C6, and C7 is 85.0° in good agreement with the corresponding angles in several phenylalanine derivatives with this specific conformation (83 – 86°).

The oxygen atoms of the sulphate ion are tetrahedrally arranged around the sulphur atom with O – S – O bond angles in the range 107.4° to 110.7°. The S – O bond lengths are equal, mean value 1.466 Å (1.482 Å corrected for thermal motion). This agrees well with the average S – O bond length of 1.473 Å (1.486 Å corrected) as given by Taesler and Olovsson from a number of structure determinations of the sulphate ion.[9]

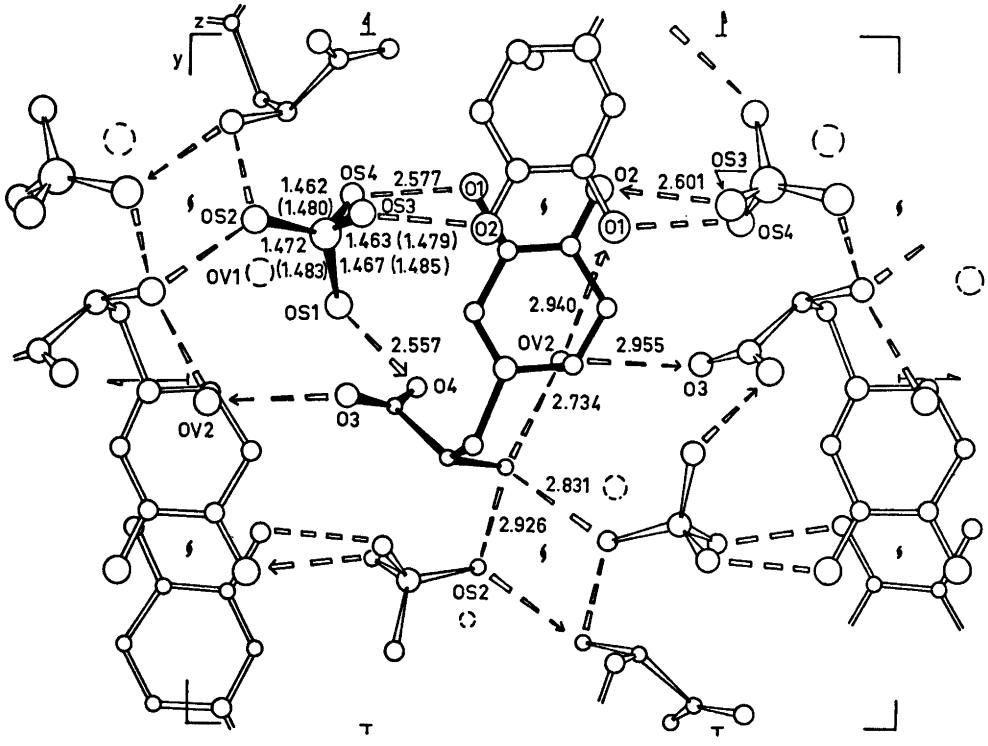


Fig. 3. The crystal structure of mimosine sulphate hydrate as seen down the *a* axis. Hydrogen bonds are indicated by broken lines, arrows indicate hydrogen bonds to atoms in another unit cell.

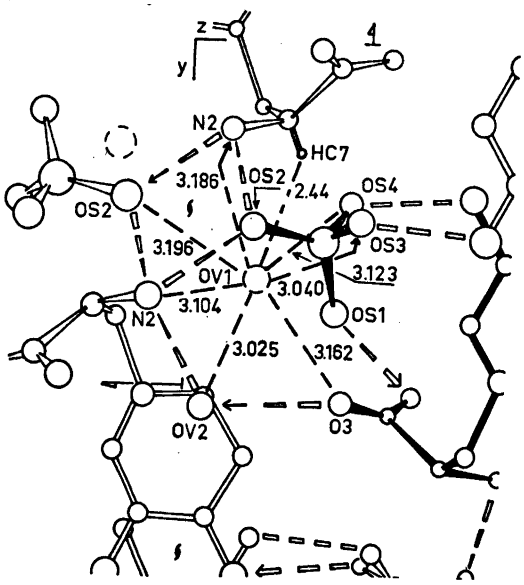


Fig. 4. The cavity containing the "half" water molecule.

The positions of the water molecules may be seen from Fig. 3. OV2 is involved in three hydrogen bonds; two as a hydrogen donor and one (to an ammonium nitrogen atom) as an acceptor. Fig. 4 illustrates the cavity containing the "half" water molecule. The molecule is surrounded by three sulphate ions, two ammonium groups, one water molecule and a carboxyl oxygen atom; there are eight oxygen or nitrogen atoms in distances between 3.0 and 3.2 Å from the OV1 atom. Four of these have lone pairs available for hydrogen bond formation. Since we were not able to localize the hydrogen atoms in this water molecule we are unable to determine which of the hydrogen bonds are established, and there may indeed exist different bonds in different unit cells in a statistical disordered way.

The crystal structure, packing and hydrogen bonding is illustrated in Fig. 3. From this drawing it may be seen that each mimosine ion is coordinated to five sulphate ions in a distorted trigonal bipyramidal arrangement. Each sulphate ion is surrounded by and hydrogen bonded to five mimosine residues. There is no direct contact between mimosine ions.

The molecular units are tied together in the crystal in an extensive three-dimensional hydrogen bond network, with the possible exception of the "half" water molecule. All hydrogen atoms bonded to hetero atoms are engaged in hydrogen bonds. The strongest of these occur between the oxygen atoms of the sulphate ion and the three hydroxyl groups in the mimosine ion, 2.557 Å (OS1 – O4), 2.577 Å (OS4 – O1) and 2.601 Å (OS3 – O2). The carbonyl oxygen atom is rather weakly hydrogen bonded to the water molecule (2.955 Å). The other hydrogen atom of this water molecule is engaged in a weak hydrogen bond to the phenolic oxygen atom O1 (2.940 Å). The N2 atom is involved as a hydrogen donor in three hydrogen bonds, one of 2.940 Å to one sulphate oxygen atom, one of 2.831 Å to another and a third of 2.734 Å to the oxygen atom of the water molecule.

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Short Communications

Synthesis of 6-Deoxy-D-*galacto*-heptose

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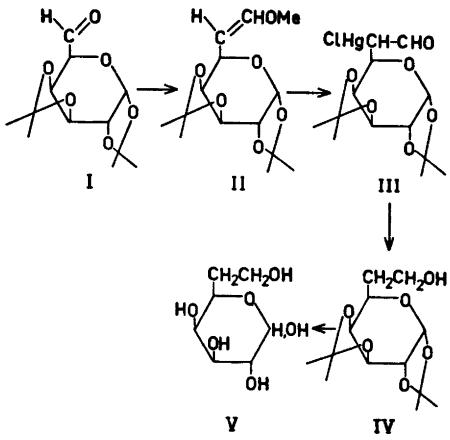
We recently established that 6-deoxy-D-*manno*-heptose is a component sugar of the *Yersinia (Pasteurella) pseudotuberculosis* group IIa lipopolysaccharide.[1] The identification of this sugar was confirmed by synthesis.[2] Several isomeric 6-deoxyhexoses have been found in Nature,[3] and are synthesized from nucleoside hexoses *via* the corresponding 6-deoxyhexos-4-ulose derivatives. It seems most probable that the 6-deoxy-D-*manno*-heptose is likewise synthesized from a nucleoside heptose. Other 6-deoxy-heptoses may also be found in Nature and the syntheses of these sugars is therefore a matter of some interest. 6-Deoxy-D-*gluco*-heptose, as the 1,2-*O*-isopropylidene derivative, was recently synthesized by Rosenthal and Kan [5] *via* the hydroformylation of 5,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose. We now report the synthesis of 6-deoxy-D-*galacto*-heptose.

The 6-deoxy-D-*galacto*-heptose was synthesized by a Wittig reaction, analogous to that used for the synthesis of the corresponding D-*manno* derivative.[2] The various synthetic transformations are outlined in Scheme 1. 1,2:3,4-Di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose [6] was treated with methoxymethylenetriphenylphosphorane to afford the vinylic derivative II, which was transformed into the 7-aldehydo sugar III by treatment with mercuric chloride and mercuric oxide.[7] Reduction of III with sodium borohydride afforded the heptose derivative IV in an over-all yield of 10 % from I. Acid hydrolysis of IV produced 6-deoxy-D-*galacto*-heptose.



Scheme 1.

Experimental. General methods were the same as those described in a previous paper [2] NMR spectra, recorded for all compounds, except for III, were in accordance with the presumed structures.

*6-Deoxy-1,2:3,4-di-*O*-isopropylidene-α-*D*-galacto-heptopyranose (IV).* Methoxymethyltriphenylphosphonium chloride (6.7 g) in dry diethyl ether (44 ml) was enclosed in a serum bottle with a magnetic stirrer under nitrogen and cooled to −10°. Butyllithium (9.2 ml, 30 % in hexane) was added by syringe. The mixture was allowed to stand with stirring for 20 min. 1,2:3,4-Di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose (I) [6] (2.5 g) in dry diethyl ether (25 ml) at −10° was added by syringe and the mixture was allowed to stand with stirring under nitrogen for 1 h at −10° and then at room temperature for 18 h. Partitioning between diethyl ether and water, drying the diethyl ether solution over sodium sulphate, filtration and concentration afforded a crude syrup (II).

Crude, syrupy II was dissolved in acetone-water (10:1, 12 ml). Mercuric oxide (360 mg) was added with magnetic stirring and then, dropwise, with stirring, mercuric chloride (360 mg) in acetone-water (10:1, 4.0 ml).[7] The reaction was monitored by TLC and interrupted after about 5 min at room temperature. The mixture was filtered, concentrated to dryness to yield syrupy, crude III which immediately was dissolved in ethanol (15 ml); the ethanol solution was diluted with water to near turbidity. Exess sodium borohydride was added and the solution was allowed to stand at room temperature overnight. Sodium cations were removed with Dowex 50 (H^+ form). Filtration, concentration and repeated concentrations with methanol in order to remove boric acid as methyl borate afforded a syrup which after purification on silica gel [diethyl ether-light petroleum (40 – 60°)] gave chromatographically pure IV (260 mg), $[\alpha]_D$ −44° (*c* 0.6, $CHCl_3$). (Found: C 56.8; H 8.28, $C_{13}H_{22}O_6$ requires: C 56.9; H 8.08).

6-Deoxy-D-galacto-*heptose (V)*. The above di-*O*-isopropylidene derivative IV (39 mg) was treated with trifluoracetic acid-water [8] (9:1, 10 ml) at room temperature for 5 min. The solution was concentrated and the product dissolved in water, filtered and concentrated to a syrup (27 mg) $[\alpha]_D + 75°$ (*c* 0.9, H_2O). An aliquot of the material was transformed into 6-deoxy-D-*galacto*-heptitol hexaacetate by reduction with sodium borohydride followed by acetylation.[9] The GLC retention time relative to that of D-glucitol hexa-acetate (3 % ECNSS-M on Gas-Chrom Q at 180° and a flow rate of 30 ml/min) was 1.35. Another aliquot was reduced with sodium borodeuteride and then converted into the hexamethyl ether. The MS of the resulting 6-deoxy-1-deuterio-1,2,3,4,5,7-hexa-*O*-methyl-D *galacto*-heptitol showed the following (primary) fragments: *m/e* 235, 191, 178, 147, 134, 103, 90, 46, and 45. The remainder of the spectrum was in accordance with the presumed structure. The above 6-deoxy-D-*galacto*-heptitol hexaacetate crystallized, m.p. 123–125°, $[\alpha]_D$ −9° (*c* 0.4, $CHCl_3$). (Found: C 50.7; H 6.29. $C_{19}H_{28}O_{12}$ requires: C 50.9; H 6.29).

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The Structure of Methyl β-D-Ribopyranoside

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X-Ray crystallographic studies on the pyranose form of aldopentoses [1–6] have shown that the structures agree with those predicted by Reeves.[7] Thus, β-arabinose,[1–3] β-lyxose,[4] and α-xylose,[5,6] are found to have the conversion forms 1*a*2*e*3*e*4*a*, 1*e*2*a*3*e*4*e*, and 1*a*2*e*3*e*4*e*, respectively.

As regards the fourth aldopentose, ribose, preliminary crystallographic data only have been reported.[8] However, a structure study of 2-deoxy-β-ribose has been carried out.[9]

The conversion form 1*a*3*e*4*a* which 2-deoxy-β-ribose has in the crystalline state, does not agree with Reeves' predictions, and it has been proposed in this connection that an axial substituent on C(1) should be regarded as an element of stability rather than an element of instability.[9] An X-ray structure study of methyl β-ribopyranoside which may occur as 1*a*2*a*3*e*4*a* or 1*e*2*e*3*a*4*e*, was therefore thought of interest.

The results from this study show, *cf.* Fig. 1, that methyl β-D-ribopyranoside in the crystalline state has the conversion form 1*a*2*a*3*e*4*a*, which from Reeves' stability scheme [7] is supposed to be the least stable one.

One may query, however, whether the assumed stabilizing effect of the 1*a* substituent has been decisive for the structure in this case. There is namely an intramolecular hydrogen bond between O(2) and O(4) which at least to some degree stabilizes the molecule. Similar intramolecular hydrogen bonds occur in methyl 1-thio-β-D-ribopyranoside and methyl 1,5-dithio-β-D-ribopyranoside,[10] and the possibility for such bonding should therefore be taken into account when judging about the stability of the conversion forms of pyranoses. It should be mentioned in this connection that methyl 5-thio-β-D-ribopyranoside occurs as 1*e*2*e*3*a*4*e* in the crystalline state.[11]

The C–O and C–C bond lengths in methyl β-D-ribopyranoside, as derived from the coordinates in Table 1, are shown in Fig. 1. The values agree with those reported by James and Stevens from an independent X-ray study of the compound,[12] and also with those previously reported for glycoside structures.[13,14]

The three hydroxyl hydrogens participate in hydrogen bonds. They are, O(2)···O(4) = 2.77 Å with H(O2)···O(4) = 2.09 Å, O(3)···O(2)′ = 2.85 Å with H(O3)···O(2)′ = 2.03 Å, and O(4)···O(3)′ = 2.87 Å with H(O4)···O(3)′ = 2.22 Å. Thus each of the hydroxyl oxygens donates as well as accepts a hydrogen bond.

Methyl β-D-ribopyranoside crystallizes from ethyl acetate,[15] as orthorhombic prisms elon-

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Methyl β-D-ribopyranoside crystallizes from ethyl acetate,[15] as orthorhombic prisms elon-

Table 1. Atomic coordinates in fractions of corresponding cell edges. Standard deviations, referring to last digits, are given in parentheses.

	x	*y*	*z*
C(1)	−0.2423(11)	0.35556(31)	0.5762(11)
C(2)	−0.2066(10)	0.42624(30)	0.4870(11)
C(3)	0.0068(11)	0.45093(32)	0.5691(13)
C(4)	0.1735(11)	0.40168(34)	0.5023(14)
C(5)	0.1177(14)	0.33432(38)	0.5942(18)
C(6)	−0.3515(18)	0.30015(18)	0.9149(18)
O(1)	−0.2778(9)	0.35935(23)	0.8146(8)
O(2)	−0.2255(9)	0.42778(24)	0.2445(9)
O(3)	0.0569(7)	0.51497(20)	0.4822(9)
O(4)	0.1937(9)	0.39510(27)	0.2554(11)
O(5)	−0.0808(7)	0.31181(19)	0.5150(8)
H(11)	−0.358(11)	0.334(3)	0.491(11)
H(21)	−0.317(9)	0.455(3)	0.578(11)
H(31)	−0.018(8)	0.452(2)	0.733(10)
H(41)	0.295(9)	0.419(3)	0.579(10)
H(51)	0.123(13)	0.338(3)	0.754(13)
H(52)	0.217(10)	0.299(3)	0.544(11)
H(61)	−0.453(13)	0.310(3)	1.025(14)
H(62)	−0.254(15)	0.279(4)	1.005(20)
H(63)	−0.408(16)	0.269(5)	0.858(17)
H(O2)	−0.126(13)	0.407(4)	0.204(15)
H(O3)	−0.037(10)	0.542(3)	0.559(11)
H(O4)	0.222(11)	0.426(3)	0.196(13)

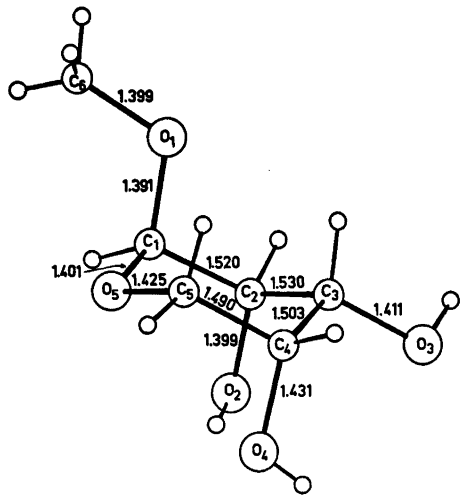

Fig. 1. The methyl β-D-ribopyranoside molecule with C−C and C−O bond lengths. The standard deviations are 0.009−0.011 Å for the C−C bonds and 0.008−0.011 Å for the C−O bonds.

gated along the *c*-axis. The crystals belong to the space group $P2_12_12_1$ with $Z=4$.

Unit cell dimensions were determined by measuring the 2θ settings for eight reflections on a Picker four-angle automatic diffractometer, with Cu$K\beta$ radiation ($\lambda=1.39217$ Å). From a least squares treatment, the cell dimensions are, $a=6.415(2)$ Å, $b=19.994(3)$ Å, and $c=5.747(2)$ Å. The calculated density is 1.479 g/cm³, as compared with the density 1.48 g/cm³ found by flotation.

Intensity data were collected on the diffractometer, using Cu$K\alpha$ radiation and omega scan. 660 reflections, measured within $\sin\theta=0.865$, were reduced to structure factors in the usual way. The crystal used for data collection had the dimensions $0.2\times0.2\times0.4$ mm in the three axial directions. Absorption corrections were not applied.

The structure was solved by direct methods [16] and refined by full matrix least squares to a final *R* factor of 0.07. The scattering factors used for oxygen and carbon were those given in *International Tables*.[17] For hydrogen the scattering factor curve given by Stewart *et al.*[18] was used.

Final atomic coordinates are listed in Table 1. The final list of structure factors is available on request.

This work has been carried out at the University of Pittsburgh (1968) and at the University of Bergen. The author wishes to thank Professor George A. Jeffrey and his staff members at the Crystallography Laboratory, University of Pittsburgh, for great hospitality.

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Electroörganic Preparations. XXXVI. Stepwise Reduction of Benzotrifluoride

HENNING LUND and NIELS J. JENSEN

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Electrochemical reduction of trifluoromethyl compounds has been observed in some instances.[1–4] In most cases reported the reduction has been performed in protic solvents, and under these conditions a complete reduction involving 6 F/mol to a methyl group is generally observed;[1–3] the only exception seems to be the reduction of 2,2,2-trifluoroacetophenone to acetophenone in 80 % aqueous ethanol during which monofluoroacetophenone was detected as an intermediate.[3]

In *N*,*N*-dimethylformamide (DMF) some trifluoromethylbenzenesulphonamides have been investigated polarographically [4] and a stepwise reduction was found in some cases; the electrode reaction corresponded to a reductive cleavage of the carbon-sulphur bonds, and the resulting benzotrifluoride was reported to give a single polarographic wave.

In the present work the electrochemical behaviour of benzotrifluoride and some other α-halogenated toluenes have been investigated by means of cyclic voltammetry (CV) and controlled potential electrolysis (CPE).

Results and discussion. In Table 1 are given the peak potentials of benzotrifluoride (I), α,α-difluorotoluene (II), α-fluorotoluene (III), benzotrichloride (IV), α,α-dichloro-α-fluorotoluene (V), α-chloro-α,α-difluorotoluene (VI), benzal chloride (VII), α-chloro-α-fluorotoluene (VIII), and benzyl chloride (IX). None of the electrode reactions are reversible on CV.

Whereas the peak potentials of II and III are very nearly equal, the potential of I is more than 200 mV less negative than that of II, and a reduction of I to II should be possible with a reasonable selectivity. The half-peak potential of II is about 0.05 V less negative than that of III which might indicate that the signal from II is a composite wave due to poorly separated peaks of the reduction of II to III and III to toluene. A reduction of II to III would thus be expected to be less selective than a reduction of I to II.

Cyclic voltammograms of I in DMF with added water showed that the peak potentials of the first and second peak were not affected by addition of up to 2 % of water. The background current raised and at a water content higher than about 2 % the waves merged with the background current.

The difference in reduction potential between that of the carbon-chlorine bond and that of the carbon-fluorine bonds in VI is about 0.7 V, sufficiently for a selective reduction; this was confirmed by a preparative reduction of VI to II in good yield. The potential difference between the reduction of the first and second carbon-chlorine bond in V is about 0.4 V; a selective reduction of V to VIII was shown to be possible.

The reduction of I was investigated in more detail; in Fig. 1 is shown the dependence of the concentrations of I, II, III, and toluene (X) on the electron consumption. The samples with-

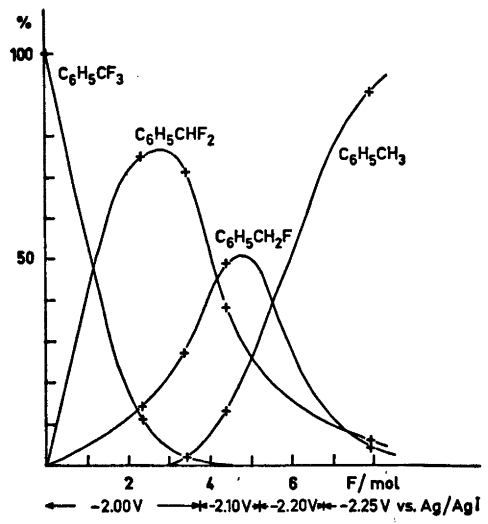

Fig. 1. Dependence on concentration of benzotrifluoride (I), α,α-difluorotoluene (II), α-fluorotoluene (III), and toluene (X) on electron consumption during electrolytic reduction of I in DMF containing tetrabutylammonium iodide.

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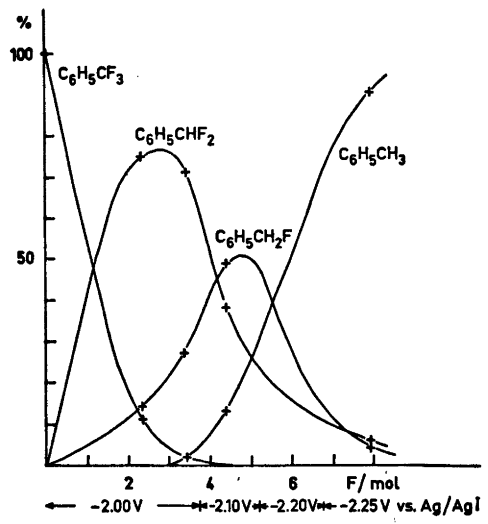

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Table 1. Peak potentials (V *vs.* aq. SCE) at the hanging mercury drop electrode of α-halogen substituted toluenes in DMF containing 0.1 m tetrabutylammonium iodide. Sweep rate: 400 mV/sec.

Compound		E_p (1)	E_p (2)	E_p (3)	V *vs.* aq. SCE
I	$C_6H_5CF_3$	2.61	2.84		
II	$C_6H_5CHF_2$	2.83_5			
III	$C_6H_5CH_2F$	2.84			
IV	$C_6H_5CCl_3$	1.77	2.13	2.35	
V	$C_6H_5CCl_2F$	1.90	2.34	2.84	
VI	$C_6H_5CClF_2$	2.11	2.84		
VII	$C_6H_5CHCl_2$	2.12	2.36		
VIII	C_6H_5CHClF	2.36	2.84		
IX	$C_6H_5CH_2Cl$	2.35			

drawn during the electrolysis were analyzed by GLC; the retention times of the compounds were I < X < II < III. The electron consumption is somewhat higher (~20 %) than the theoretical one reflecting a certain reduction of background components (*e.g.* supporting electrolyte) due to the very negative potential.

The cyclic voltammetry indicated the possibility of a reasonably selective reduction of I to II, but it seems somewhat more surprising that III is found in a rather high concentration during the latter part of the reduction, although the half-peak potential of III is only 0.05 V more negative than that of II. From the peak potentials a reduction of II to X could be expected.

Trifluoromethylbenzene (I) is thus in DMF reduced stepwise to toluene in a manner similar to other α,α,α-trihalotoluenes, although the differences in potential of the three steps are very small for I compared with the other trihalotoluenes. In protic media the trifluoromethyl compounds, when reducible, have mostly been found to be reduced to the toluenes without detection of any partly dehalogenated intermediates. The reason for this difference is not apparent, possibly the difference in ability of the fluoride ion to act as a leaving group in protic solvents, where it is highly solvated, and in DMF, where fluoride ions are less solvated, may play a role.

Reduction of I in protic solvents has not been attempted, as no voltammetric waves were observed; reduction of methyl *p*-trifluoromethylbenzoate in methanol produced the methyl toluate;[2] cyclic voltammetric and coulometric studies in DMF pointed, however, to a primary two-electron reduction.[5]

Cyclic voltammetric data in DMF of other substituted trifluoromethyl benzenes point to a stepwise reduction of these compounds; further studies with controlled potential reduction may show whether a stepwise reduction of the trifluoromethyl group is the general reduction mode in aprotic media.

Experimental. Apparatus. A Juul-electronics potentiostat, a F&M Research Chromatograph Model 810 with a Hewlett-Packard 3370B electronic integrator, and a CEC 21 – 104 Mass Spectrometer were used.

Materials. Benzotrifluoride (I), benzyl fluoride (III), benzotrichloride (IV), benzal chloride (VII), and benzyl chloride (IX) were commercially available. α,α-Dichloro-α-fluorotoluene (V) and α-chloro-α,α-difluorotoluene (VI) were prepared according to Swarts,[6] V (b.p. 178 – 180°C) was purified by preparative GLC on a 25 % SE 52 column, $t = 80$°C; $n_D^{23} = 1.5109$.

α,α-Difluorotoluene (II). 5 ml of α-chloro-α,α-difluorotoluene (VI) were reduced in 150 ml of DMF containing 5 % of water and 0.1 m tetrabutylammonium iodide (TBAI) at −1.80 V *vs.* Ag/AgI (DMF), $n = 1.87$. The reduction completed the DMF was diluted with 1 l of water and extracted 3 times with 50 ml petrol ether. The petrol ether was washed 5 times with water to remove DMF and dried on "Sikkon Fluka". After removal of the petrol ether through a short column the residue, 3.6 g, was purified by preparative GLC on a 10 % FFAP column at 50°C, 1.24 g. $n_D^{22} = 1.4550$. NMR-spectrum (neat): $\delta = 6.42$, 1 H, triplet, $J_{HF_2} = 57$ Hz; $\delta = 7.1 - 7.5$, 5 H, multiplet. Mass spectrum, *m/e* (%): 39 (3), 41 (3), 43 (3), 50 (7), 51 (14), 55 (6), 57 (5), 63 (3), 69 (3), 74 (4), 75 (3), 77 (10), 78 (32), 79 (3), 81 (4), 83 (7), 101 (3), 107 (5), 109 (23), 126 (3), 127 (100), 128 (59), 129 (5).

α-Chloro-α-fluorotoluene (VIII). α,α-Dichloro-α-fluorotoluene (V) (2.7 g) was reduced in 150 ml of DMF containing 5 ml of water and 0.1 m TBAI at −1.3 V *vs.* Ag/AgI (DMF), $n = 2.03$. The catholyte was treated as described above for II, and the residue purified on a 10 % FFAP-column at 70°C; 1.34 g. NMR (CCl_4): $\delta = 5.30$, 1 H, doublet, $J_{HF} = 48$Hz. $\delta = 7.28$, 5 H, singlet. Mass spectrum, *m/e* (%): 27 (7), 29 (9), 39 (7), 41 (21), 42 (16), 43 (27), 50 (8), 51 (8), 56 (23), 57 (38), 63 (8), 83 (17), 89 (6), 107 (9), 109 (100), 110 (13), 125 (2.4), 127 (0.9), 144 (29), 146 (11).

Reduction of benzotrifluoride (I). A. I (34 mg) was reduced in 45 ml DMF containing 0.1 m TBAI at −2.0 – −2.3 V *vs.* Ag/AgI. During the reduction samples were withdrawn, the electricity consumption noted, and the samples

analyzed by GLC on a 2.5 % diisodecylphthalate (DIDP)-column, programmed 4°/min from 40° to 150°, injection temperature 140°. The retention times (sec) were: I 470, toluene 603, II 900, III 967. The results are presented in Fig. 1.

B. I (2.0 ml) was reduced in 235 ml DMF containing 0.1 m TBAI at −1.95 Ag/AgI (DMF). After an electron consumption of 1.86 F/mol the reduction was stopped. Analysis by GLC gave I (11 %), II (82 %), and III (7 %). The catholyte was divided in two parts; one was treated as described above for the reduction of VI. Analysis of the residue showed a relative content of I, II, and III of 8:87:5.

The other part of the catholyte was reduced further at −2.1 V (the foot of the wave of II); after further 2 F/mol the catholyte was analyzed by GLC; II 13 %, III 72 %, and toluene 14 %.

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Fungal Extractives. VII.* A Formal Synthesis of (±)-Lactaral

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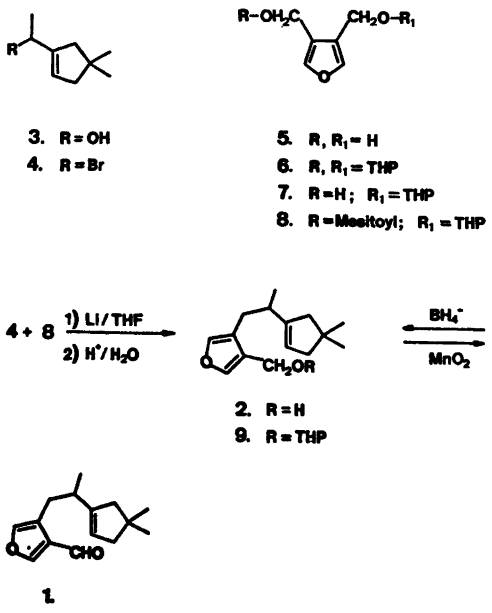
The structure of lactaral (*1*), a new sesquiterpene furan-3-aldehyde from *Lactarius vellereus* and *L. pergamenus* (Russulaceae), has been described.[1] We now report a formal synthesis of lactaral confirming structure *1*.

A direct reductive cross-coupling of the allylic alcohol *3* and the 3-furyl alcohol *7* with $TiCl_4$/butyllithium (or methyllithium) in monoglyme [2] was unsuccessful. However, a lithium-promoted coupling reaction between the mesitoate *8* of the furyl alcohol *7* and the allylic bromide *4* [3] gave the tetrahydropyranyl ether (THP) *9* of lactarol in low yield. Hydrolysis of compound *9* afforded racemic (±)-lactarol (*2*). This synthetic alcohol was spectroscopically identical with an authentic sample prepared from native lactaral by borohydride reduction (MS, IR and NMR). (−)Lactarol was reoxidized to lactaral with active manganese dioxide thus formally completing the total synthesis.



Experimental. The NMR spectra were recorded on a Varian T-60 spectrometer. Mass spectra were recorded on an LKB 1100 instrument.

4,4-Dimethyl-1-(1-bromo)ethylcyclopentene(4). The allylic alcohol *3* [1] was brominated with triphenylphosphine-carbon tetrabromide in ether.[4] After reflux for 24 h the reaction mixture was worked up to give the *bromide 4* in 73 % yield. B.p.$_{20}$ 78−81°; n_D^{26} 1.5250; ν_{max}(neat) 3050, 1640, 1380, 1370, 820 cm^{-1}; NMR: δ_{TMS}($CDCl_3$) 5.62 (1 H, s broad), 4.83 (1 H, q, J=7 Hz), 2.25 2.15 (2 H each, s broad), 1.80 (3 H, d, J=7 Hz), 1.10 (6 H, s) ppm; MS: *m/e* 122 (24 %) (M^+−HBr), 107 (100 %), 91 (31 %), 79 (19 %).

3,4-Bis(hydroxymethyl)furan (5). The diol *5* was prepared by lithium aluminium hydride reduction of the corresponding commercially available diethyl ester according to the literature.[5] Yield 84 % (lit. 83 %). B.p. 100−102°/0.2 mmHg (lit. 129−130°/2 mmHg); n_D^{22} 1.5103 (lit. n_D^{20} 1.5080).

Reaction of diol 5 with 3,4-dihydro-2H-pyran. A mixture of diol *5* (1.28 g, 0.0100 mol), 3,4-

* Part VI see Ref. 1.

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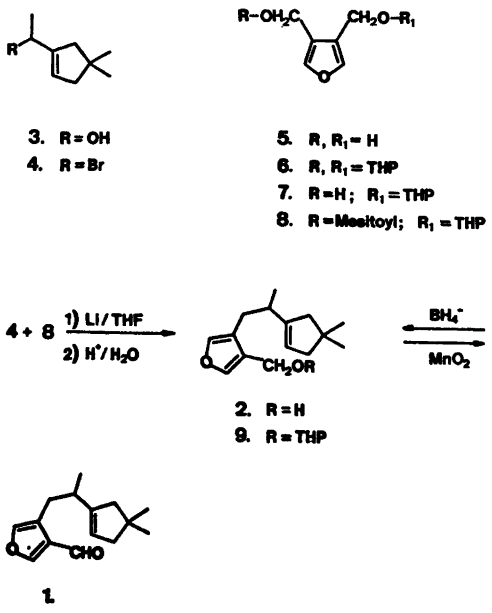

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dihydro-2*H*-pyran (1.00 ml, 0.0109 mol) and a few crystals of *p*-toluenesulfonic acid was stirred at room temperature for 24 h. The products were then separated on a silica gel (100 g) column with methylene chloride-ethyl acetate (3 : 1) as eluent. 15 ml fractions were collected. From fr. 9–18.

3,4-Bis[(2-tetrahydropyranyl)oxymethylene]-furan (6) (0.81 g, 28 %) was obtained as a colourless oil which had: n_D^{21} 1.4911; IR (neat): ν_{max} 1550, 1120, 1025, 880, 870 cm^{-1}; NMR ($CDCl_3$, TMS): δ 7.40 (2 H, s; two fur-*H*), 4.70, 4.40 (2 H each, d, $J = 12$ Hz; two fur–*HCH*–O), 4.70 (2 H, s broad; two O–C*H*–O), 3.25–4.15 (4 H, m; two O–CH_2–CH_2) ppm; MS *m/e*: 296 (M^+, 1 %) ($C_{16}H_{24}O_5$), 111 (18 %), 95 (20 %), 94 (81 %), 85 (100 %; base peak). It was distilled *in vacuo* for analysis. (Found: C 64.9; H 8.3. $C_{16}H_{24}O_5$ requires: C 64.8; H 8.2). From fr. 20–41 *3-hydroxymethyl-4-(2-tetrahydropyranyl)-oxymethylene-furan (7)* (1.06 g, 50 %) was obtained. The compound, a colourless oil, had: n_D^{21} 1.4957; IR (neat): ν_{max} 3400–3600 (OH), 3150 3120 1555 (furan), 1120, 1020, 905, 875 (furan), 805 cm^{-1}; NMR ($CDCl_3$, TMS): δ 7.40 (2 H, s; two fur–*H*), 4.73 4.43 (1 H each, d, $J = 12$ Hz; fur–*HCH*–OTHP), 4.72 (1 H, s broad; O–C*H*–O), 4.51 (2 H, s; fur-CH_2–OH), 3.25–4.15 (2 H, m; O–CH_2–CH_2) ppm; MS *m/e*: 212 (M^+; 5 %) ($C_{11}H_{16}O_4$), 128 (11 %), 112 (54 %), 111 (100 %; base peak), 110 (47 %), 85 (60 %). It was distilled *in vacuo* for analysis. (Found: C 62.3; H 7.7. $C_{11}H_{16}O_4$ requires: C 62.3; H 7.6). The terminal fractions yielded smaller amounts of unreacted *diol 5* (0.05 g).

3-(2-Tetrahydropyranyl)oxymethylene-4-(2,4,6-trimethylbenzoyl)oxymethylenefuran (8). The 3-furyl alcohol *7* was esterified with mesitoyl chloride [6] in ethanol-free chloroform.[3] Column chromatography of the crude product on silica gel with methylene chloride-ethyl acetate (4 : 1) as eluent gave the *mesitoate 8* in 82 % yield. It had: ν_{max}(neat) 3150, 3120, 1730, 1265, 1080, 875 cm^{-1}; NMR: δ_{TMS}($CDCl_3$) 7.54 (1 H, d, $J = 2$ Hz; fur–*H*), 7.40 (1 H, s broad; fur–*H*), 6.84 (3 H, s broad; ϕ–*H*), 5.14 (2 H, s; fur–CH_2–OCO–), 4.74 4.42 (1 H each, d, $J = 12$ Hz; fur–*HCH*–OTHP), 4.65 (1 H, s broad; O–C*H*–O–), 3.28–4.10 (2 H, m; –O–CH_2–CH_2–), 2.27 (9 H, s; three ϕ–CH_3); MS: *m/e* 358 (1 %) (M^+; $C_{21}H_{26}O_5$), 279 (6 %), 256 (5 %), 167 (20 %), 149 (63 %), 147 (100 %), 146 (25 %). It was distilled *in vacuo* for analysis. (Found: C 70.5; H 7.4. Calc. for $C_{21}H_{26}O_5$: C 70.4; H 7.3).

Reaction between 4 and 8 to give (±)-lactarol (2). Lithium (345 mg; 50 mmol) was added to a stirred, ice-cold solution of the mesitoate *8* (1790 mg; 5 mmol) and the bromide *4* (1015 mg; 5 mmol) in dry tetrahydrofuran (25 ml) under nitrogen.[3] When the solution turned deep brown-red in colour (15 min) the reaction was quenched with water (0.5 ml). Work-up of the reaction mixture gave mesitoic acid (315 mg). The neutral fraction was chromatographed on a silica gel (100 g) column with ethyl acetate (2 %) in methylene chloride as eluent. A small amount of the crude *tetrahydropyranyl ether 9* (IR, NMR) (230 mg) was obtained. Hydrolysis of the crude ether *9* (115 mg) was accomplished with 1 M sulphuric acid (1 ml) in dimethoxyethane (5 ml). After 48 h the partially hydrolyzed mixture was worked up. Column chromatography on silica gel (10 g) with ethyl acetate (2 %) in methylene chloride as eluent gave (R,S)-*lactarol (2)* (22 mg). This compound gave spectra (IR, NMR, MS) identical with those of an authentic sample of lactarol (*vide infra*). *(–)-Lactarol (2)*. Lactaral (*1*) was reduced with potassium borohydride in water-ethanol to give an almost quantitative yield of *lactarol (2)*. It had: $[\alpha]_D^{22} - 3.5°$ (*c* 0.9 in chloroform); ν_{max}(neat) 3350 (broad), 1610, 1545, 1385, 1380, 1055, 885, 795 cm^{-1}; NMR: δ_{TMS}($CDCl_3$) 7.36, 7.20 (1 H each, s broad; two fur–*H*), 5.28 (1 H, s broad; –C=C*H*–CH_2–), 4.55 (2 H, s; fur–CH_2–OH), 2.20–2.70 (3 H, m; allylic protons), 2.12 (4 H, s; two –CH_2–C=C–), 1.08 (6 H, s; two *gem*-CH_3), 1.05 (3 H, d, $J = 7$ Hz; –CH–CH_3); MS: *m/e* 234 (18 %) (M^+; $C_{15}H_{22}O_2$), 217 (20 %), 201 (18 %), 160 (13 %), 123 (100 %), 81 (60 %).

(–)-Lactarol was reoxidized with active manganese dioxide [7] in methylene chloride-pentane (1 : 1) to lactaral which was identical in all respects with the original aldehyde.

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Intramolecular Coupling of Diarylpropanes. Evidence for the Phenoxonium Ion Mechanism

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Spirodienones (cyclohexa-2,5-dienone derivatives like V) are important in biosynthesis and occur widely in nature.[1] Biosynthetically they are believed to be formed *via* coupling of a diphenolic precursor.[1] However, attempts to make spirodienones *in vitro* by oxidation of diphenols with conventional reagents have generally been unsuccessful,[1] probably due to the inability of these reagents to oxidize both phenolic moieties of the diphenol simultaneously and because free phenoxy radicals are involved in these reactions. Recently, spirodienones have been obtained from diphenolic precursors by using a vanadium oxytrichloride reagent[2] or a ferric chloride dimethylformamide complex,[3] which are able to oxidize both phenolic moieties simultaneously.

In a previous communication [4] we demonstrated that phenoxonium ions derived from hindered phenols by anodic oxidation react with anisole to give 4-(*p*-anisyl)-cyclohexa-2,5-dienones in high yield. We now report an application of the same principle to intramolecular cyclization of diarylpropanes, which gives spirodienones in high yield, and present evidence for the involvement of phenoxonium ions.

Phenols are more easily oxidized than the corresponding methyl ethers. Oxidation at the potential of the first oxidation stage of I (R = H) therefore occurs in the phenolic group without competition from oxidation of the other ring. The initial electron transfer produces the acidic cation radical (II) which deprotonates instantaneously in neutral or basic media.

In principle the cation radical (II) could also dimerize or cyclize (*via* electrophilic attack on the ether moiety or I). An investigation of the anodic oxidation of the methyl ether of I (R = OMe) has shown [5] that the stereochemistry of the diarylpropanes is in fact very favourable for the latter kind of reaction. However, on the basis of the following argument we believe that reactions other than deprotonation of the cation radical II can be ruled out. Electronically, 3-methylanisole and *p*-cresol are very similar and therefore would be expected to have similar oxidation potentials. Thus the observed difference in peak potentials (= 0.4 V)[4,8] must depend on a very rapid deprotonation of the *p*-cresol cation radical (the pK of cation radicals derived from monohydric phenols is about −5 (Ref. 6)). The deprotonation rate can be estimated [7] to be at least 10^7 times faster than dimerization of the *p*-cresol or 3-methylanisole cation radical (assuming that these two species have similar rates of dimerization). The potential of the first oxidation stage of I (R = H) is 1.30 V (standard calomel electrode) just as for *p*-cresol. This means that if cyclization is the preferred reaction for II it should be 10^7 times faster than dimerization of the *p*-cresol cation radical which is improbable (by analogy with phenol ethers,[8] we believe that the most likely reaction (next to deprotonation) of cation radicals derived from phenols is dimerization).

Any chemical reaction following electron transfer must then involve either the radical III or the phenoxonium ion IV. Reactions in which phenoxy radicals participate generally result in a mixture of products due to the delocalisation of the unshared electron over the oxygen atom and the carbon atoms of the ring. On the other hand, the positive charge of the phenoxonium ion is concentrated in the ring,[9] favouring reactions with bond formation to carbon. Thus, controlled anodic conditions ensure that the phenolic part of the molecule undergoes oxidation and an analysis of the products formed will distinguish between the phenoxonium ion and the phenoxy radical mechanisms.

The anodic oxidation of I (R = H) (0.1 mmol) in acetonitrile (50 ml) containing sodium perchlorate (0.1 M) and sodium carbonate (1 g) in a three compartment cell was monitored by peak voltammetry during constant current coulometry.[10] Precisely 2.0 Faradays per mol of I (R = H) were consumed during exhaustive electrolysis. When the oxidation was complete, the sodium carbonate was filtered off. For analysis of the product, trifluoroacetic acid (TFA) (5 ml) was added to the electrolysis mixture to convert the electro-inactive spirodienone (V, R = H) to the corresponding phenol (VII, R = H). The reaction was monitored by the appearance of an oxidation peak at 0.95 V and was complete in less than 30 min at room temperature. The height of the oxidation peak at 0.95 V indicated that the overall result of the reaction was quantitative conversion of V (R = H) to VII (R = H).

A series of preparative experiments were carried out, and the results are shown in Table 1. The formation of the compound VI represents an interesting electrochemical analogy (the reduction step in this transformation takes place at the cathode of the undivided cell) to the dienone-benzene rearrangement which is frequently encountered in alkaloid biosynthesis.[12]

While it is clear that only the phenolic ring undergoes oxidation in the case of I (R = H)

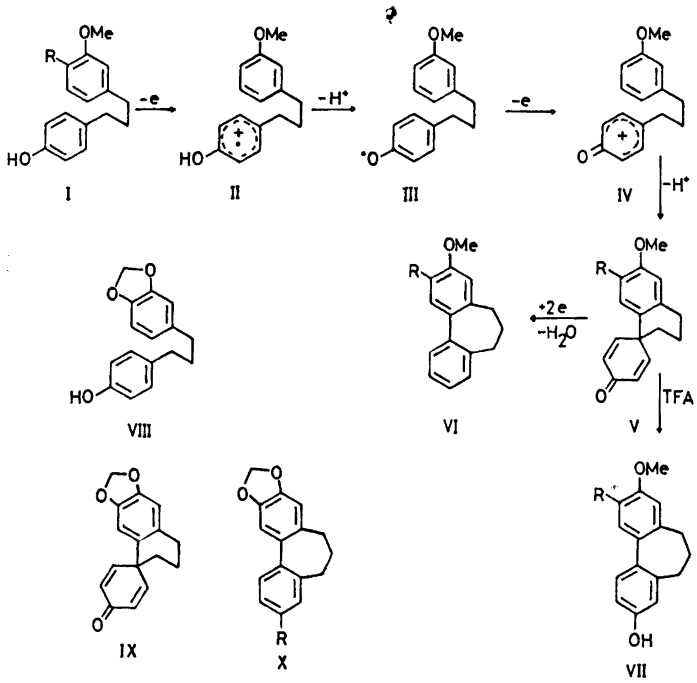

Table 1. Anodic oxidation of various diarylpropanes on a platinum anode in acetonitrile.[a]

Substrate	Products, yield (%)	TFA[b] → Phenol
I (R=H)	VI (R=H) (17): V (R=H) (68)	VII (R=H)[c]
I (R=OMe)	VI (R=OMe) (20); V (R=OMe) (73)	VII (R=OMe)[c]
VIII[d]	X (R=H) (21); IX (75)	X (R=OH)[c]

[a] These oxidations were carried out in an undivided cell (nickel cathode) on 2 mmol [0.2 mmol in case of I (R=H)] of substrate in acetonitrile (100 ml) containing sodium perchlorate (0.1 M). The potential was controlled at 1.25 V and the electrolysis was interrupted when 2 Faradays per mol of substrate had been passed. The products were isolated by chromatography on alumina and characterized by their NMR, mass spectrum and elemental analysis. [b] Compound obtained by treatment of the spirodienone with TFA. [c] Identified as its methyl ether by comparison with an authentic sample obtained from anodic coupling of the corresponding methoxylated diarylpropane.[5] [d] This compound has been oxidized to IX in 87 % yield with thallium trifluoroacetate.[11]

the same cannot be said with certainty for the oxidation of I (R=OMe) or of VIII since the additional ether group in these compounds lowers the oxidation potential of the second ring and it is expected that both aryl groups should undergo oxidation at about the same potential. Thus a dication diradical mechanism similar to that demonstrated for the anodic cyclization of methoxybibenzyls [13] cannot be ruled out for these reactions.

The absence of intermolecular coupling products and products involving carbon-oxygen bond formation from the oxidation of I (R=H) shows that the radical III is oxidized anodically as soon as it is formed. The nature of the products obtained (V and VI) strongly suggests that the phenoxonium ion IV is the result of this oxidation.

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Molecular Structure of Methyl 6-*O*-Acetyl-β-D-glucopyranoside

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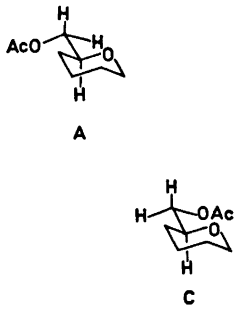
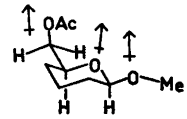
In our attempts to correlate the circular dichroism of glycoside acetates with molecular geometry, we find that the single, negative CD band observed for methyl 6-*O*-acetyl-β-D-glucopyranoside is best explained by assuming that rotamer C predominates in ethanol solution.

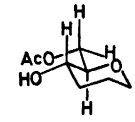


This contrasts with the results obtained for the corresponding α-anomer, for which a double CD band was observed, and which led to the suggestion that both rotamers B and C contributed to the observed CD. We suggested that for the β-anomer an unfavourable dipolar interaction for rotamer B would lead to the presence of

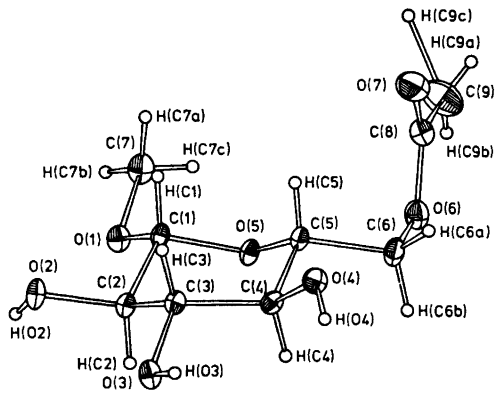


rotamer C only. Since this interaction is absent in the corresponding α-anomer, both rotamers B and C can be present.[1] Lemieux and coworkers have suggested, on the basis of NMR studies, as well as from considerations of optical rotation, that for D-*erythro*-hexopyranosides, rotamer C predominates over A and B.[2,3]

We have previously reported an X-ray crystallographic study on methyl 6-*O*-acetyl-β-D-galactopyranoside.[4] This showed that in the crystalline state rotamer A predominated. In the D-*gluco* series, however, this rotamer is unimportant, due to an unfavourable 1,3 interaction.



The present study, summarized in Fig. 1 and Table 1 establishes that for methyl 6-*O*-acetyl-β-D-glucopyranoside the conformation in the crystalline state corresponds to rotamer C, in agreement with the interpretation of the results from the CD investigation. The compound crystallized in space group $P2_1$, $a = 10.201$, $b = 7.239$, $c = 7.863$, $Z = 2$. The X-ray data were obtained on a Philips PW 1100 computer-controlled single-crystal diffractometer with graphite monochromatized Cu$K\alpha$ radiation. The phase



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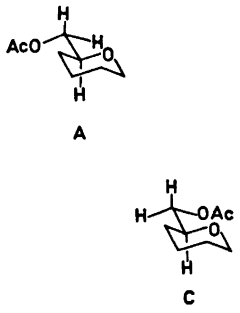

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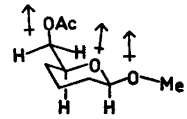

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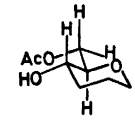

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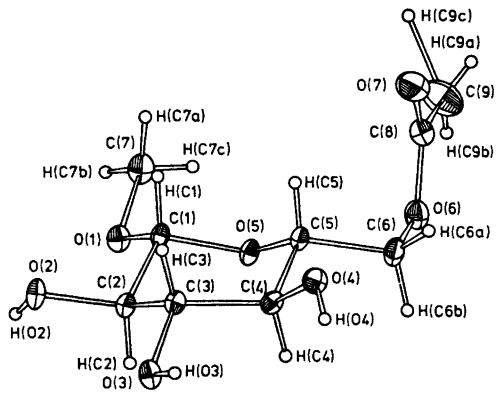

Fig. 1. Molecular structure of methyl 6-*O*-acetyl-β-D-glucopyranoside.

Table 1. Intramolecular nonhydrogen bond distances (Å) and angles(°). Estimated standard deviations are given in parentheses.

C(1)–C(2)	1.522(4)	C(1)–C(2)–C(3)	108.2(3)
C(2)–C(3)	1.522(5)	C(2)–C(3)–C(4)	112.4(3)
C(3)–C(4)	1.514(5)	C(3)–C(4)–C(5)	111.7(3)
C(4)–C(5)	1.534(4)	C(4)–C(5)–O(5)	109.4(3)
C(5)–C(6)	1.522(6)	C(5)–O(5)–C(1)	110.8(2)
C(8)–C(9)	1.538(8)	O(5)–C(1)–C(2)	107.9(3)
C(1)–O(1)	1.384(4)	C(4)–C(5)–C(6)	110.8(3)
C(7)–O(1)	1.428(5)	O(5)–C(5)–C(6)	107.0(3)
C(2)–O(2)	1.425(5)	C(5)–C(6)–O(6)	113.2(3)
C(3)–O(3)	1.423(4)	C(1)–O(1)–C(7)	112.4(3)
C(4)–O(4)	1.421(4)	C(6)–O(6)–C(8)	115.9(4)
C(1)–O(5)	1.429(4)	O(6)–C(8)–C(9)	111.5(4)
C(5)–O(5)	1.429(4)	O(6)–C(8)–O(7)	126.2(5)
C(6)–O(6)	1.423(5)	C(9)–C(8)–O(7)	122.3(5)
C(8)–O(6)	1.295(5)	O(1)–C(1)–O(5)	106.9(3)
C(8)–O(7)	1.184(6)	O(1)–C(1)–C(2)	109.4(3)
		C(1)–C(2)–O(2)	111.2(3)
		C(3)–C(2)–O(2)	108.1(3)
		C(2)–C(3)–O(3)	107.8(3)
		C(4)–C(3)–O(3)	110.2(3)
		C(3)–C(4)–O(4)	110.6(3)
		C(5)–C(4)–O(4)	108.0(3)

determinations were carried out by a computerized application of direct methods using the weighted phase-sum formula described by Norrestam.[6] Several cycles of full-matrix least-squares refinement (anisotropic nonhydrogen and fixed isotropic hydrogen temperature parameters) gave an R-value of 0.046. The molecular structure is shown in Fig. 1. Intramolecular distances and angles are listed in Table 1. Full details of the X-ray diffraction investigation will be published elsewhere.[7]

Acknowledgements. We are indebted to Professor Peder Kierkegaard and to Professor Bengt Lindberg for their interest. The work received financial support from Riksbankens Jubileumsfond, Hierta-Retzius Stipendiefond and Statens Naturvetenskapliga Forskningsråd.

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Synthesis of Asterosterol, a Novel C_{26} Marine Sterol

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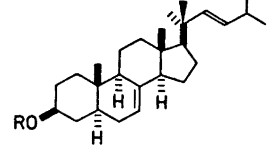
Recently Kobayashi *et al.*[1,2] suggested structure *1a* for a new marine C_{26} sterol, asterosterol, isolated from several asteroids [3] and stated [1] that they had synthesized a 22-*cis* and -*trans* mixture of 24-*nor*-cholesta-7,22-dien-3β-ol, resistant to separation. Through the investigation of the sterol components of the marine sponge *Halicondria panicea* we have now found the same sterol present as a minor component.



1a : R = H
1b : R = Ac

Due to the uncertainties associated with the stereochemistry at C-20 as well as the biogenetic novelty of the sidechain structure a final proof

Table 1. Intramolecular nonhydrogen bond distances (Å) and angles(°). Estimated standard deviations are given in parentheses.

C(1)–C(2)	1.522(4)	C(1)–C(2)–C(3)	108.2(3)
C(2)–C(3)	1.522(5)	C(2)–C(3)–C(4)	112.4(3)
C(3)–C(4)	1.514(5)	C(3)–C(4)–C(5)	111.7(3)
C(4)–C(5)	1.534(4)	C(4)–C(5)–O(5)	109.4(3)
C(5)–C(6)	1.522(6)	C(5)–O(5)–C(1)	110.8(2)
C(8)–C(9)	1.538(8)	O(5)–C(1)–C(2)	107.9(3)
C(1)–O(1)	1.384(4)	C(4)–C(5)–C(6)	110.8(3)
C(7)–O(1)	1.428(5)	O(5)–C(5)–C(6)	107.0(3)
C(2)–O(2)	1.425(5)	C(5)–C(6)–O(6)	113.2(3)
C(3)–O(3)	1.423(4)	C(1)–O(1)–C(7)	112.4(3)
C(4)–O(4)	1.421(4)	C(6)–O(6)–C(8)	115.9(4)
C(1)–O(5)	1.429(4)	O(6)–C(8)–C(9)	111.5(4)
C(5)–O(5)	1.429(4)	O(6)–C(8)–O(7)	126.2(5)
C(6)–O(6)	1.423(5)	C(9)–C(8)–O(7)	122.3(5)
C(8)–O(6)	1.295(5)	O(1)–C(1)–O(5)	106.9(3)
C(8)–O(7)	1.184(6)	O(1)–C(1)–C(2)	109.4(3)
		C(1)–C(2)–O(2)	111.2(3)
		C(3)–C(2)–O(2)	108.1(3)
		C(2)–C(3)–O(3)	107.8(3)
		C(4)–C(3)–O(3)	110.2(3)
		C(3)–C(4)–O(4)	110.6(3)
		C(5)–C(4)–O(4)	108.0(3)

determinations were carried out by a computerized application of direct methods using the weighted phase-sum formula described by Norrestam.[6] Several cycles of full-matrix least-squares refinement (anisotropic nonhydrogen and fixed isotropic hydrogen temperature parameters) gave an R-value of 0.046. The molecular structure is shown in Fig. 1. Intramolecular distances and angles are listed in Table 1. Full details of the X-ray diffraction investigation will be published elsewhere.[7]

Acknowledgements. We are indebted to Professor Peder Kierkegaard and to Professor Bengt Lindberg for their interest. The work received financial support from Riksbankens Jubileumsfond, Hierta-Retzius Stipendiefond and Statens Naturvetenskapliga Forskningsråd.

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Synthesis of Asterosterol, a Novel C_{26} Marine Sterol

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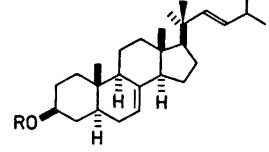
Recently Kobayashi *et al.*[1,2] suggested structure *1a* for a new marine C_{26} sterol, asterosterol, isolated from several asteroids [3] and stated [1] that they had synthesized a 22-*cis* and -*trans* mixture of 24-*nor*-cholesta-7,22-dien-3β-ol, resistant to separation. Through the investigation of the sterol components of the marine sponge *Halicondria panicea* we have now found the same sterol present as a minor component.

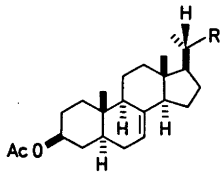


1a : R = H
1b : R = Ac

Due to the uncertainties associated with the stereochemistry at C-20 as well as the biogenetic novelty of the sidechain structure a final proof

of structure was desirable and a partial synthesis of asterosterol from 5α,6-dihydroergosterol has been developed.

5α,6-Dihydroergosterol acetate (*2b*) was ozonized at −70° in methylene chloride in the presence of pyridine followed by reductive work-up (Zn-AcOH). Preparative layer chromatography (hexane-ethyl acetate 9:1) gave 19 % unchanged starting material (*2b*) and 28 % of the pure aldehyde (*2a*) which showed, in the



2a : R = CHO
2b : R = trans-CH:CH·CHEt·CHMe$_2$

NMR spectrum,* the aldehyde proton as a doublet at 9.54 ppm. Acid work-up gives according to Barton *et al.*[4] an epimerized product with the aldehyde proton as two doublets at 9.55 and 9.60 ppm in the ratio of 4:1. Wittig reaction of the (20*S*)-aldehyde with isobutyltriphenylphosphonium bromide (butyllithium, heptane-ether 3:1) gave 46 % of the acetates of 22-*trans*-24-*nor*-5α-cholesta-7,22-dien-3β-ol (*1b*) and its 22-*cis*-isomer in the proportions 1:5. The separation of the two isomers was accomplished by TLC in hexane-benzene (3:1) on SiO_2-20 % $AgNO_3$.

The NMR spectrum of the synthetic 22-*trans*-isomer (*1b*), m.p. 171 − 173°, $[\alpha]_D^{25}$ −21.0° (lit.[2] m.p. 134 − 136.5°, $[\alpha]_D$ −2.8°), showed signals of 18-H_3 (δ 0.533, s), 19-H_3 (0.803, s), 26,27-H_6 (0.903, d, *J* 6.7), 21-H_3 (1.01, d, *J* 6.7), acetyl (2.00), 3-H (4.67, m) and 7-H, 22-H and 23-H (5.14, ill-defined). All NMR values were within the experimental error of those reported by Kobayashi *et al.*[2] The mass spectrum of the synthetic compound (*1b*) was identical with that published [2] and the IR spectrum, also as reported, was superimposable on that of 5α,6-dihydroergosterol acetate including the finger print region and showed absorption at 965 cm^{-1} (*trans*-disubstituted sidechain double bond). Hydrolysis of *1b* gave *1a* as needles from MeOH, m.p. 171 − 172°, $[\alpha]_D^{25}$ −23.9°, which displayed physical constants distinctly different from those reported (lit.[2] 129 − 130°, $[\alpha]_D \pm 0°$). Even taken into consideration the discrepancies in m.p. and specific rotation, the synthesis just reported clearly indicates that the structure assigned to asterosterol by Kobayashi *et al.*[2] is correct.

* Chloroform was used for NMR and optical rotation measurements. IR spectra were measured in KBr. All new compounds gave correct analyses.

The synthetic sterol acetate (*1b*) was identical with the acetylated natural material isolated from *Halicondria panicea*.

The mass spectrum of the 22-*cis* C_{26} sterol acetate, m.p. 145 − 145.5°, $[\alpha]_D^{25}$ −31.3°, showed the expected molecular ion at *m/e* 412. The fragmentation was the same as that of *1b*, also with respect to the side chain-fragments. The IR spectrum is in agreement with the *cis*-structure (766 cm^{-1}). The only differences observed between the NMR spectra of *1b* and its *cis*-isomer are related to 18-H_3 (0.559), 21-H_3 (0.969, d, *J* 6.4), 26, 27-H_6 (0.927, d, *J* 6.4), 22-H and 23-H (AB, 4.95, 5.07, *J* 2.1). Hydrolysis gave the free sterol, m.p. 137 − 138.5°, $[\alpha]_D^{25}$ −35.9°.

Reduction (H_2-Pd) of *1b* or its isomer leads to the same compound, the corresponding stanol, m.p. 129 − 134°, $[\alpha]_D^{25}$ +8.6° (lit.[5] 128 − 135°). The observed differences in the rotatory powers of *1b* and its isomer are thus related to the stereochemistry at C-22 and C-23.

Acknowledgement. The author is grateful to Dr. E. Caspi for helpful discussions during this work, which was carried out at the Worcester Foundation of Experimental Biology, Shrewsbury, Mass. He is also thankful to the Worcester Foundation and the Danish Science Research Council for support.

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Synthesis of 11,11′-Deuterated ε-Carotene and Lycopene

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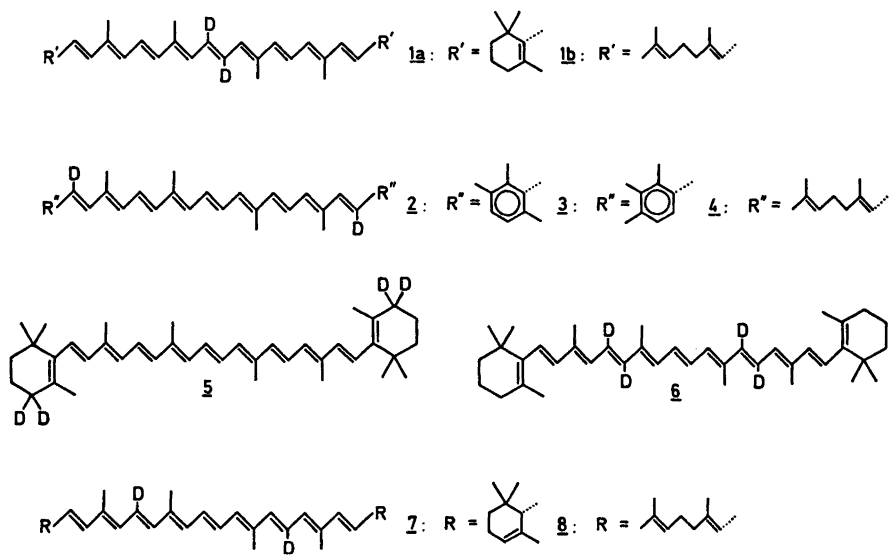
The synthesis of 11,11′-deuterated ε-carotene (*7*) and 11,11′-deuterated lycopene (*8*) by a combination of routes previously used for the unlabelled carotenes, are reported.

The deuterium was introduced by lithium aluminium deuteride reduction of the intermediate C_{15}-esters (*10* and *15*).

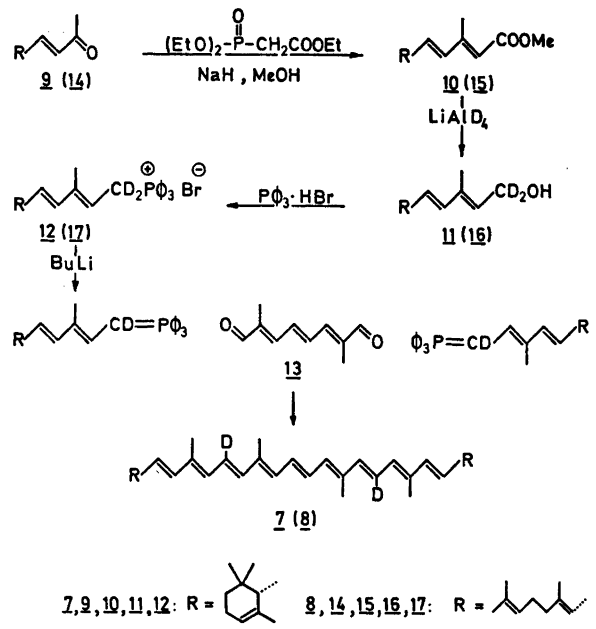
Specifically deuterated carotenes are of special interest for a systematic study of the electron impact induced fragmentation of carotenoids.

The preparation of only few specifically deuterated carotenes has been reported. Schwieter *et al.*[1] have synthesized 15,15′-dideuterio-β,β-carotene (*1a*, Scheme 1) and 15,15′-dideuterio-lycopene (*1b*) by selective deuteration of the acetylenic analogues. Some 7,7′-deuterated carotenes (*2,3,4*) have been previously prepared by our group,[2] the deuterium atoms being introduced by lithium aluminium deuteride reduction of intermediate C_{10}-carboxylic acids. More recently the partial synthesis of 4,4′-tetradeuterio-β-carotene (*5*) from canthaxanthin by reduction with lithium aluminium deuteride in the presence of aluminium chloride has been described by Brzezinka.[3] The same author also prepared 11,12,11′,12′-tetradeuterio-β-carotene (*6*) by total synthesis; in principle by selective deuteration of an acetylenic intermediate.

We now report the synthesis of 11,11′-deuterated ε-carotene (*7*) and 11,11′-deuterated lycopene (*8*).



Scheme 1.



Scheme 2.

RESULTS AND DISCUSSION

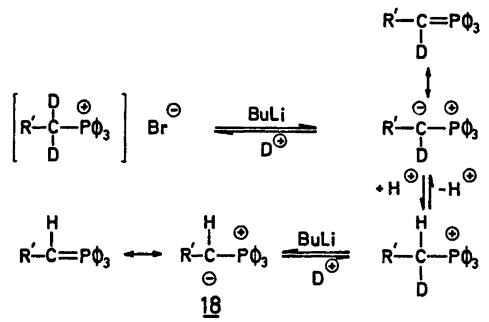
The route employed, given in Scheme 2, was based on a combination of the routes previously described by Weedon and co-workers [4] for undeuterated ε-carotene by a $C_{15}+C_{10}+C_{15}=C_{40}$ approach.

Racemic α-ionone (*9*) was condensed in a Horner reaction with the carbanion of ethyl diethylphosphonoacetate, obtained with sodium hydride, to the C_{15}-ester (*10*). The C_{15}-ester (*10*) was reduced with lithium aluminium deuteride to the allylic alcohol (*11*), which was converted to the corresponding phosphonium salt (*12*) with triphenylphosphine hydrobromide. In the final Wittig reaction the ylid of the phosphonium salt (*12*) was reacted with C_{10}-dial (*13*) to give 11,11′-deuterated ε-carotene (*7*).

Pseudo-ionone (*14*) gave 11,11′-deuterated lycopene (*8*) by the same route *via* *15*, *16* and *17*.

On first attempt the deuterium incorporation was low in ε-carotene. According to the PMR-spectra the allylic alcohol (*11*) as well as the phosphonium salt (*12*) had complete deuterium incorporation. The ylid formation and/or subsequent condensation consequently represented the crucial steps.

According to the accepted mechanism of ylid formation,[5a] given in Scheme 3, deuterium-hydrogen exchange of the ylid is expected to occur in protic media, resulting in unlabelled ylid (*18*).



Scheme 3.

Using diethyl ether, dried over metallic sodium and distilled over lithium aluminium hydride, the following incorporation was obtained: 11,11′-deuterated ε-carotene (*7*), 87 % deuterium incorporation, $D_0:D_1:D_2=3:23:76$ and for 11,11′-deuterated lycopene (*8*), 80 % deuterium incorporation, $D_0:D_1:D_2=8:25:67$, judged by the intensity of the molecular ions observed on electron impact. Percentage deuteration

reflects average number of deuterium atoms incorporated relative to the theoretically possible incorporation. The ratio of deuterated species is calculated as in previous work,[2] taking into account ^{13}C isotope contributions, *cf.* Ref. 5b.

The deuterated carotenes were characterized by means of electronic spectra, IR-spectra, and mass spectra (*7* and *8*) and PMR-spectrum (*7*), melting points and chromatographic behaviour. Undeuterated ε-carotene was prepared for comparison. Mixed melting points of the deuterated carotenes (*7* and *8*) with the corresponding undeuterated carotenes gave no depression. No chromatographic separation from undeuterated analogs was obtained. The deuterium incorporation, judged from mass spectra, was retained on chromatography and recrystallization. Extinction coefficients lower than predicted[4,6] are ascribed to sterically inhomogeneous products (*cis* and *trans*).

The mass-spectrometric results will be discussed separately in a communication also comprising other deuterated carotenes.[7]

EXPERIMENTAL PART

Materials and methods were as generally used in this laboratory.[2] For column chromatography neutral alumina activity grade 2 was used. Only diagnostically useful spectroscopic properties of the intermediates are quoted.

Methyl α-ionylideneacetate (*10*) was prepared from α-ionone (*9*, 4 g) and ethyl diethylphosphonoacetate (4.5 g) by the procedure of Manchand *et al.*[4] Column chromatography on alumina (eluent pet.ether) gave a mixture of *10* (*ca.* 80 %) and the ethyl ester (*ca.* 20 %); yield 6.2 g (70 %); τ ($CDCl_3$) 9.17 s and 9.08 s (*gem.* dimethyl), 8.42 s (ring methyl), 7.71 s (on-chain methyl), 6.33 s (ester methyl), 8.72 t and 5.8 q (ester ethyl) and olefinic signals; used without further purification.

Deuterated α-ionylidene-ethanol (*11*) was prepared by adding dropwise a suspension of $LiAlD_4$ (0.5 g) in anhydrous ether (25 ml) to *10* (4.0 g) in anhydrous ether at 0°C. After 1 h excess reagent was destroyed with ethyl acetate (2 ml) and the reaction mixture poured on ice and 1 N H_2SO_4. *11* was extracted with ether in the usual manner; yield 3.0 g (90 %) *11*; τ ($CDCl_3$) 9.17 s and 9.08 s (*gem.* dimethyl), 8.42 s (ring methyl), 8.20 s (on-chain methyl), and 4.8 – 3.8 (olefinic protons), but no signal at τ 5.73; used without further purification.

The undeuterated analogue was similarly prepared using $LiAlH_4$; yield 3.1 g (90 %); τ ($CDCl_3$) as for *11* above and 5.73 d ($J = 7$ cps, CH_2OH).

Deuterated α-ionylidene-ethyltriphenylphosphonium bromide (*12*). *11* (2.0 g) and triphenylphosphonium bromide (2.3 g) in dry methanol (30 ml) was kept at room temperature for 56 h. The solvent was removed under vacuum, and the residue washed with water. The crude, colourless solid *12*, yield 3.5 g (81 %); τ ($CDCl_3$) 9.23 s and 9.13 s (*gem.* dimethyl), 2.5 – 2 (aromatic protons), no signals at τ 6 – 5 for CH_2P; was used without further purification.

The undeuterated phosphonium salt was similarly prepared from undeuterated α-ionylidene-ethanol; yield 3.8 g (88 %); τ ($CDCl_3$) 9.23 s and 9.13 s (*gem.* dimethyl), 6 – 5 m (CH_2P), 2.5 – 2 (aromatic protons).

2,7-Dimethylocta-2,4,6-trienedial[8] (*13*). 2,7-Dimethylocta-2,6-dien-4-ynedial (1.0 g) in ethyl acetate (50 ml) was hydrogenated in the presence of Lindlar catalyst (0.7 g) until one equivalent of hydrogen was absorbed. The catalyst was removed by filtration and the solvent evaporated. Recrystallization from methanol gave *cis* and *trans* *13* (0.5 g, 50 %); λ_{max} (methanol) 323 nm; τ ($CDCl_3$) 8.05 s (6H, methyl), 3.3 – 2.5 m (4H, olefinic), 0.35 s (2H, aldehydic).

11,11'-Deuterated ε-carotene (*7*). To a suspension of *12* (920 mg) in anhydrous ether (75 ml, dried over Na and distilled over $LiAlH_4$) was added dropwise a 1 N solution of BuLi in anhydrous ether until the red phosphorane was prepared. Excess BuLi was destroyed by addition of CH_2Cl_2 (3 ml). C_{10}-dial (*13*, 124 mg) in CH_2Cl_2 (10 ml) was added dropwise and the reaction mixture left at room temperature over night. The solvent was removed and the residue dissolved in 90 % aqueous methanol. The pigments were transferred to pet. ether in the usual manner, chromatographed on alumina (eluent pet. ether) and *7* crystallized and recrystallized from benzene-methanol; yield 25 mg (10 %); m.p. 194 – 196°C, undepressed on admixture with undeuterated ε-carotene of m.p. 189 – 190°C; $R_F = 0.83$ (alumina paper, petroleum ether, no separation from undeuterated ε-carotene; λ_{max} (pet. ether) 413, 437 ($E_{1\%,1cm} = 2600$) and 467 nm, % III/II[9] = 93; ν_{max} (KBr) 3030, 2960, 2920, and 2860 (CH), 1450 and 1435 (CH_2), 1385, 1375 and 1365 (methyl, *gem.* dimethyl) 1270, 1215, 1080, 1030, 1015, 965 (*trans* disubst. double bonds), 920 (medium, *trans*-CD=CH–, see Ref. 10), 825 (trisubst. double bonds), 800 and 730 cm^{-1}; τ ($CDCl_3$) 9.17 s and 9.08 s (12 H, *gem.* dimethyl), 8.42 s (6 H, 18,18'-methyl), 8.10 s (6 H, 19,19'-methyl), 8.05 s (6 H, 20,20'-methyl) and 4.6 – 3.0 m (olefinic protons); *m/e* 538 (MD_2), 537 (MD_1), 536 (MD_0), 482 ($MD_2 - 56$), 446 ($MD_2 - 92$), 445 ($MD_2 - 93$), 431 ($MD_2 - 107$), 379 ($MD_2 - 159$) with intensity ratios ($MD_2 - 92$):($MD_2 - 93$):($MD_2 - 94$) = 37:63:0, ($MD_2 - 106$):($MD_2 - 107$):($MD_2 - 108$) = 0:100:0, ($MD_2 - 158$):($MD_2 - 159$):($MD_2 - 160$) = 0:100:0; MD_0:MD_1:MD_2 = 3:23:74; 86 % deuterium incorporation.

Undeuterated ε-carotene was prepared in the

corresponding manner from the undeuterated phosphonium salt; yield 60 mg (17 %); m.p. 189 – 190°C; λ_{max} (pet. ether) 413, 437 ($E_{1\%,1cm}$ = 2400) and 467 nm, % III/II = 87, spectrum conform with that of *7*; ν_{max} (KBr) close to those of *7*, but band at 920 cm^{-1} absent; τ ($CDCl_3$) 9.17 s and 9.08 s (12H, *gem*.dimethyl), 8.42 s (6 H, 18,18′-methyl), 8.08 s (19,19′-methyl), 8.04 s (20,20′-methyl) and 4.6 – 3.0 m (olefinic protons); *m/e* 536 (M), 480 (M – 56), 444 (M – 92), 430 (M – 106), 388 (M – 56 – 92), 378 (M – 158) with intensity ratio (M – 92):(M – 106) = 8.9.

Methyl ψ-ionylideneacetate (*15*) was prepared from *14* (4 g) by the same procedure as for *9*; yield 6.5 g (87 %) of a mixture of the methyl ester *15* (*ca.* 75 %) and the ethyl ester (*ca.* 25 %); τ ($CDCl_3$) 8.39 s and 8.32 s (isopropylidene methyl), 8.19 s (end-of-chain methyl), 7.68 s (in-chain methyl), 6.34 s (ester methyl), 8.72 t and 5.85 q (ester ethyl), 5 – 2 m (olefinic protons); used without further purification.

Deuterated ψ-ionylidene-ethanol (*16*) was prepared from *14* (4 g) by the same procedure as *11* above, avoiding work-up in the presence of H_2SO_4; yield 2.5 g (74 %); τ ($CDCl_3$) 8.40 s and 8.32 s (isopropylidene methyl), 8.20 s and 8.12 s (on-chain methyl) and 5.0 – 2.5 (olefinic protons), but no signal at τ 5.7 for CH_2OH.

Deuterated ψ-ionylidene-ethyltriphenylphosphonium bromide (*17*) was prepared by the same procedure as *12* above. *16* (2.0 g) gave *17* (3.7 g) as a solid; τ ($CDCl_3$) 8.40 s and 8.32 s (isopropylidene methyl), 8.26 (end-of-chain methyl), 4.9 (isopropylidene H), 4.5 – 3.8 (olefinic protons) and 2.5 – 2 (aromatic protons); used without further purification.

11,11′-Deuterated lycopene (*8*). The same procedure as for *7* was used. *13* (40 mg) and *17* (273 mg) gave *8* (*cis*+*trans*, 5 mg, yield 4 %); m.p. 167 – 169°C, undepressed on admixture with undeuterated lycopene of m.p. 172°C; R_F = 0.50 (alumina paper, 1 % acetone in pet.ether); λ_{max} (pet.ether) 440, 468 and 500 nm; *m/e* 538 (MD_2), 537 (MD_1), 536 (MD_0), 469 ($MD_2 - 69$), 446 ($MD_2 - 92$), 445 ($MD_2 - 93$), 431 ($MD_2 - 107$), 379 ($MD_2 - 159$) with intensity ratios ($MD_2 - 92$):($MD_2 - 93$):($MD_2 - 94$) = 38:62:0, ($MD_2 - 106$):($MD_2 - 107$):($MD_2 - 108$) = 0:100:0 and ($MD_2 - 158$):($MD_2 - 159$):($MD_2 - 160$) = 0:100:0; MD_0:MD_1: MD_2 = 8:25:67, 80 % deuterium incorporation. Synthetic undeuterated lycopene (Roche) recorded for comparison had λ_{max} (pet.ether) 462, 470, and 502 nm; *m/e* 536 (M), 467 (M – 69), 444 (M – 92), 430 (M – 106), 399 (M – 137), 378 (M – 158).

Acknowledgements. Undeuterated lycopene, 2,7-dimethylocta-2,6-dien-4-ynedial and Lindlar catalyst were gifts from Hoffmann-La Roche, Basel and Nutley. Å. E. was partly supported by a grant from the Norwegian Research Council of Science and Humatities to S.L.J.

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Intestinal Glycoproteins of Germfree Rats. III. Characterization of a Water-soluble Glycoprotein Fraction*

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A water-soluble glycoprotein fraction was obtained by fractionation of crude intestinal or fecal extracts from germfree rats. This procedure involved proteolytic digestion with pronase, precipitation of the acidic glycoprotein with cetyltrimethylammonium bromide (CTAB), and chromatography on DEAE-Sephadex and on Sepharose 4 B. The purified fractions obtained from intestinal and fecal material, respectively, were very similar in chemical composition and properties. The mucin-type glycoprotein contained 23 % of galactose, 8.6 % of fucose, 18.6 % of *N*-acetylgalactosamine, 18.2 % of *N*-acetylglucosamine, 13.5 % of sialic acid, 1.3 % of sulphate groups, and *ca.* 15 % of amino acids of which threonine, serine, and proline were the predominant ones. The results of gel electrophoresis and of sedimentation equilibrium indicated some polydispersity, consistent with previous findings for mucin glycoproteins. Incubation of the glycoprotein with a neuraminidase from *Clostridium perfringens* removed 57 % of the sialic acid, and an α-(1→2)-L-fucosidase isolated from the same bacterium released 71 % of the fucose residues. The glycoprotein was shown to possess blood-group A and H activity but was virtually free of B activity.

The epithelial surface of the mammalian intestinal tract is protected by a flowing layer of mucus continuously produced by secretory cells of the intestinal mucosa. This viscous layer not only serves as a mechanical protective barrier, but also contributes to maintain a relatively constant pH and ion concentration in the environment of the tender microvilli.

The intestinal mucin secretions are extensively degraded by the microflora of the digestive tract,[1,2] a fact which complicates the isolation and the study of the native product. Furthermore, mucin-containing extracts from mucosal scrapings and especially from intestinal contents are contaminated with other high-molecular-weight components. But, despite the difficulty in obtaining pure and undegraded mucin preparations, it has been demonstrated that mammalian intestinal mucins are composed of glycoproteins [3,4] that contain, like other glycoproteins obtained from mammalian mucins, carbohydrate residues linked by an *N*-acetylgalactosamine residue to serine and threonine residues in the polypeptide chain.[5]

By the use of animals devoid of any microflora in the digestive tract it is possible to obtain the intestinal glycoprotein in a yield better than that obtained from conventional animals. In addition, feeding the animals an entirely synthetic diet [6] consisting only of low-molecular-weight substances reduces the amount of exogenous macromolecules in the intestinal contents, *e.g.* from food components, to an insignificant level. Under germfree conditions one might expect the intestinal mucin to pass essentially unaltered through the digestive tract, which results in an excretory fecal mucin of a composition similar to the intestinal one. The present report describes the purification and characterization of a water-soluble, intestinal glycoprotein fraction and a corresponding fecal glycoprotein fraction, obtained from germfree rats fed a chemically defined diet.

* Part II. See Ref. 7.

RESULTS

The successive steps of the purification procedure are given in Fig. 1. The water-soluble, non-dialysable extract initially obtained,[6] still contained lipid material that was removed by exhaustive extraction with chloroform-methanol mixtures. This treatment was found to facilitate the subsequent fractionation steps. Incubation with pronase resulted in the degradation of contaminating protein, whereas the mucin appeared unaffected by the proteolysis since no retardation of the major carbohydrate-containing material was observed by gel chromatography on Sepharose 4 B, as compared with the non-proteolysed material. Addition of cetyltrimethylammonium bromide (CTAB) to a solution of the pronase-treated, non-dialysable fraction precipitated a large proportion of the mucin glycoprotein, leaving less acidic material in solution. This step removed most of the pentose-containing glycan which is thought to stem from previously given diet.[7] After dissociation of the methanol-soluble, CTAB-glycoprotein complex, the recovered material was subjected to chromatography on DEAE-Sephadex A 25 and, finally, on Sepharose 4 B (Figs. 2 and 3).

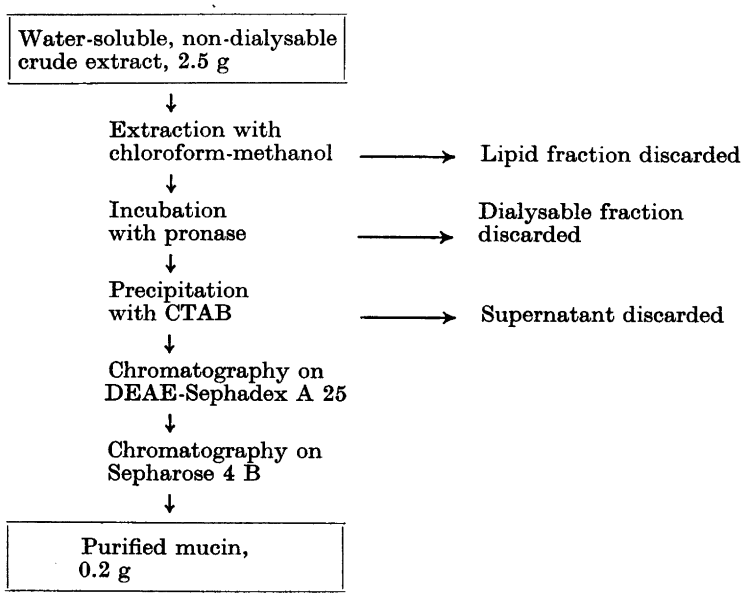

Fig. 1. Schematic diagram of the purification of germfree rat intestinal mucin.

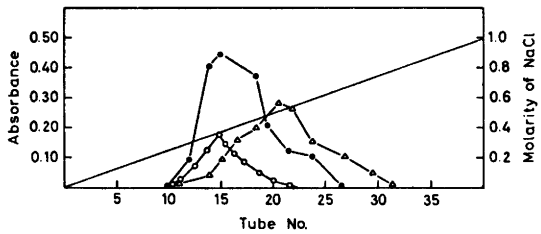

Fig. 2. Chromatography on DEAE-Sephadex A 25 of the material precipitated by CTAB. Effluent fractions were analysed for neutral sugar (●), sialic acid(○), and protein (△). Fractions 11 to 19 were pooled, concentrated, and subjected to gel chromatography on Sepharose 4 B. Experimental details are given in the text.

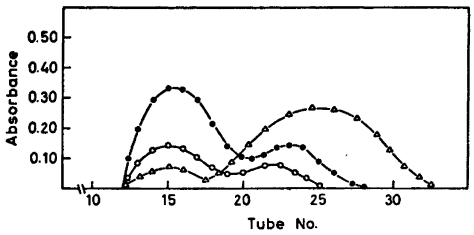

Fig. 3. Chromatography on Sepharose 4 B of fractions 11 to 19 from the DEAE-Sephadex column (Fig. 2). The effluent was analysed for neutral sugar (●), sialic acid (○), and protein (△). Fractions 13 to 17 were pooled, dialysed and lyophilized. Experimental details are given in the text.

The front peak eluted from the Sepharose 4 B column (Fractions 13 – 17) contained the purified glycoprotein; its carbohydrate composition and sulphate content are given in Table 1 and its amino acid composition in Table 2. The second peak obtained from the Sepharose column (Fractions 20 – 25) contained, in addition to the sugars present in the first peak (Table 1), mannose *ca.* 1 %, and smaller amounts of arabinose and xylose. The supernatant solution left after the precipitation with CTAB also contained glycoprotein material that could be recovered by chromatography on Sepharose 4 B, the glycoprotein fraction appearing in the front peak. It had a carbohydrate and amino acid composition similar to that of the purified glycoprotein, but was less acidic and contained some mannose, arabinose, and xylose (Table 3).

The infrared spectrum of the purified glycoprotein showed absorption bands at 3400 cm^{-1} (broad, O – H stretching), at 1640 and 1540

Table 1. Carbohydrate and sulphate content of purified glycoprotein from intestinal and fecal extract.

Residues	Intestinal glycoprotein			Fecal glycoprotein		
	%	molar ratio	μmol/100 mg	%	molar ratio	μmol/100 mg
Galactose	23.0	2.9	128	22.6	2.8	126
Fucose	8.6	1.2	53	8.8	1.2	54
N-Acetylgalactosamine	18.8	1.9	85	19.0	1.9	86
N-Acetylglucosamine	18.2	1.9	82	18.2	1.8	82
N-Acetylneuraminic acid	6.3	1.0	20	6.6	1.0	21
N-Glycolylneuraminic acid	7.2		22	7.2		22
Sulphate	1.3	0.3	13	1.4	0.3	14

Table 2. Amino acid composition of purified glycoprotein from intestinal and fecal extract.

Amino acid	Intestinal glycoprotein		Fecal glycoprotein	
	%	μmol/100 mg	%	μmol/100 mg
Aspartic acid	0.60	4.6	0.67	5.1
Threonine	6.82	57.0	6.73	56.3
Serine	2.20	21.0	2.18	20.8
Glutamic acid	0.73	5.0	0.82	5.6
Proline	2.43	21.1	2.31	20.2
Glycine	0.32	4.3	0.40	5.3
Alanine	0.31	3.5	0.37	4.1
Valine	0.72	6.2	0.75	6.4
Isoleucine	0.45	3.5	0.51	3.9
Leucine	0.12	0.9	0.17	1.3
Lysine	0.23	1.6	0.19	1.3

Table 3. Carbohydrate and amino acid composition of the glycoprotein fraction soluble in the presence of CTAB; the material was eluted in the front fraction by chromatography on Sepharose 4 B.

Carbohydrate		Amino acid	
Residues	%	Residues	%
Galactose	28.6	Aspartic acid	0.70
Mannose	0.3	Threonine	5.45
Fucose	11.8	Serine	2.30
Arabinose	0.4	Glutamic acid	0.51
Xylose	0.9	Proline	2.20
N-Acetylgalactosamine	19.7	Glycine	0.25
N-Acetylglucosamine	19.6	Alanine	0.60
Sialic acid	5.0	Valine	0.23
Sulphate	0.0	Isoleucine	0.21
		Leucine	0.19
		Lysine	0.32

cm^{-1} (acetamido group), at 1240 cm^{-1} (S=O stretching in sulphate group), and at 820 cm^{-1} (C–O–S vibration), indicative of primary alcohol sulphate ester groups.

Gel electrophoresis of the purified glycoprotein in 2 % polyacrylamide – 0.5 % agarose or in 1 % agarose, either in the presence or the absence of sodium dodecyl sulphate, resulted in a single, broad band. At a polyacrylamide concentration of 7.5 %, the whole glycoprotein sample remained at the sample-running gel interphase, whereas a more porous gel containing 5 % polyacrylamide allowed a partial penetration of the glycoprotein into the running gel. The glycoprotein gave a strong reaction for carbohydrate with the periodic acid-Schiff reagent; it could also be detected with Toluidine Blue, owing to its content of sialic acid and sulphate residues, but no bands were revealed with Coomassie Brilliant Blue or with Amido Black under standard conditions for protein staining; this would preclude any significant protein contamination. The failure of carbohydrate-rich glycoproteins to react with protein-stain reagents in polyacrylamide and agarose gels has been observed previously.[8] Treatment of the glycoprotein with dithioerythritol in a 0.05 M Tris chloride buffer of pH 7.8 and subsequent Sepharose gel chromatography in the same buffer, with or without 7 M urea, gave no change in the usual chromatographic elution pattern. Thus, the possibility of polypeptide chains linked by disulphide bridges seems to be ruled out; this is consistent with the absence of cysteine residues in the glycoprotein. The molecular weight was estimated by determination of the sedimentation equilibrium. After the sample had been for 24 h in the ultracentrifuge, an almost straight line was obtained when log c was plotted against r^2; the extrapolation of the curve indicates a molecular weight of 550 000 dalton. After a 48 h run, the curve showed a sharp deflection towards the bottom of the cell, corresponding to a M. W. value in the 1.5 to 2 million range; however, this part of the curve is bound to indicate inaccurate molecular weights. The lower M. W.-limit value calculated after the 48 h run was 390 000. A glycoprotein sample not treated with pronase was subjected to the same procedure, and the curves obtained for the proteolysed and non-proteolysed material were very similar, both in 24 and 48 h ultracentrifuge runs, revealing no essential difference in molecular weight values.

Incubation of the glycoprotein with a *Clostridium perfringens* neuraminidase decreased the sialic acid content from 13.5 to 5.8 %; repeated treatment with the same enzyme failed to liberate any additional sialic acid. Paper chromatographic examination of the sialic acid released showed the presence of approximately equal proportions of *N*-acetyl- and *N*-glycolyl-neuraminic acid. Incubation with trypsin or renewed incubation with pronase of the neuraminidase-treated glycoprotein was followed by chromatography on Sepharose 6 B. No change of the

chromatographic elution curve was observed after proteolysis.

Treatment of the glycoprotein with a *Clostridium perfringens* α(1→2)-L-fucosidase and subsequent fractionation of the digest on Sepharose 6 B yielded a glycoprotein fraction having a fucose content of 2.5 %, a reduction from the value of 8.6 % observed for the original compound. The low-molecular-weight fraction eluted from the Sepharose column contained fucose and a minute proportion of galactose, as shown by GLC.

The glycoprotein was tested for blood-group activity by the hemagglutination-inhibition technique. At a concentration of 1 mg/ml, the glycoprotein inhibited the agglutination of human red A cells by human anti-A serum and the agglutination of human red O cells by human anti-H serum or by *Ulex europeus* H-lectin. Thus A- and H-blood-group activity was clearly demonstrated. On the other hand, the mucin had almost no inhibitory effect in the human red B cell – anti-B cell system.

DISCUSSION

Preparation and characterization of glycoproteins presents a major problem in view of the known microheterogeneity of these polymers.[9] All mucin-type glycoproteins studied so far appear to possess microheterogeneity, both with respect to molecular weight and charge, and the purification of these compounds leads to fractions comprising closely related but mostly nonidentical molecules.

The purified glycoprotein obtained (*ca.* 8 %) is only a part of the total mucin content of the original crude extract, namely the final product of the particular fractionation scheme employed. The use of a different purification procedure would probably have given fractions with compositions different from those observed. Like other mucin-type glycoproteins studied,[5,8,10] the germfree-rat intestinal glycoprotein was clearly polydisperse. It gave a broad band on gel electrophoresis and a broad peak on Sepharose 4 B gel chromatography (Fig. 3). The result of the 48 h sedimentation equilibrium experiment indicated a polydispersity greater than expected. However, aggregation may occur to some extent during prolonged periods of centrifugation and it is difficult to ascertain the effect of molecular aggregation in evaluating the polydispersity.[11]

The present study demonstrates a close similarity between the purified glycoprotein preparations of the intestinal and the fecal extracts. No difference could be observed in the chromatographic elution curves from columns of DEAE-Sephadex or Sepharose, and identical bands were obtained on gel electrophoresis. The carbohydrate and amino acid composition of the two products were strikingly similar (Table 1 and 2). These data suggest that the soluble or brush border-bound enzymes which may degrade or modify the glycoprotein during its passage through the intestinal tract had no effect, in agreement with the observation that the glycoprotein is resistant to trypsin and pronase, even after partial removal of sialic acid residues. In addition to the content of carbohydrate and protein the purified glycoprotein fractions invariably contained *ca.* 1 % of sulphate groups. Since uronic acid residues were absent, contamination with sulphate-containing polysaccharides was precluded. The infrared spectrum of the glycoprotein showed absorption bands characteristic of sulphate ester groups. The presence of a band at 820 cm^{-1} and the absence of the band at 850 cm^{-1} suggested [12] that the sulphate groups were most probably located at C-6 of the galactose or hexosamine residues. Bella and Kim [5] have recently suggested that nearly 40 % of the sulphate groups present in an acidic glycoprotein of the small intestine of the rat were *N*-linked, since they were released by mild acid hydrolysis. When the glycoprotein of the germfree rat was analysed for *N*-sulphate groups by the nitrous acid procedure [13] no such groups could be detected, in agreement with a similar observation made on a glycoprotein of dog gastric mucosa by Pamer *et al.*[14]

Repeated neuraminidase treatment released only 57 % of the total sialic acid present in the glycoprotein. The resistance to neuraminidase may possibly be due to *O*-acetyl groups at C-4 of the sialic acid residues, since Schauer and Faillard [15] have shown that the *N*-acetyl-4-*O*-acetylneuraminic acid groups of horse submaxillary glycoprotein were not split off by *Clostridium perfringens* neuraminidase. In contrast, the authors found that *N*-acetyl-7-*O*-acetyl-, *N*-acetyl-8-*O*-acetyl-, and *N*-acetyl-7,8-

di-*O*-acetylneuraminic acid residues of bovine submaxillary glycoprotein were all released by the same enzyme.

One incubation of the glycoprotein with a specific α-(1→2)-L-fucosidase [16] removed 71 % of the total fucose content. Studies on hog submaxillary [17] and gastric [18] glycoproteins have established that an α-L-fucopyranosyl residue is linked to C-2 of a β-D-galactose residue. Since a terminal *O*-α-L-fucopyranosyl-(1→2)-β-D-galactose moiety is a prerequisite for blood-group H activity, this disaccharide unit probably constitutes the nonreducing end group of, at least, some of the carbohydrate chains in the intestinal glycoprotein of the germfree rat. Further, the serological demonstration of blood-group A activity of the glycoprotein suggests also the presence of chains terminated with the sequence *O*-2-acetamido-2-deoxy-α-D-galactopyranosyl-(1→3)-[*O*-α-L-fucopyranosyl-(1→2)]-β-D-galactose.

EXPERIMENTAL

Prior to analysis, samples were dried in the presence of phosphorus pentaoxide *in vacuo;* solutions were concentrated under reduced pressure with an outside bath temperature of 35 – 40°.

Analytical methods. Quantitative carbohydrate analysis was performed by gas-liquid chromatography (GLC) on a Perkin-Elmer 900 gas chromatograph. After methanolysis the methyl glycosides were converted into the per-(trimethyl)-silyl ethers and separated on a column (150 × 0.3 cm) packed with 0.1 % OV-17 on glass beads (No. GLC 110, 120/140 mesh), as described by Reinhold.[19] Methanolysis of the mucin sample (1 mg) with *myo*-inositol (40 μg) as the internal standard was effected with 1 M hydrogen chloride in methanol (1 ml) at 85° for 20 h. For simultaneous estimation of *N*-acetyl- and *N*-glycolylneuraminic acid, methanolysis was performed with 0.5 M hydrogen chloride in methanol at 65° for 1 h. Sialic acid, free or glycosidically bound, was also estimated by the periodate-resorcinol method of Jourdian *et al.*[20] with the following modification: After the oxidation step, 0.2 ml of 0.1 M sodium arsenite was added and the solution kept for 2 – 3 min at room temperature until the brown colour had disappeared. Then the resorcinol reagent was added and the assay completed as described. The original procedure [20] led to coloured blanks and unsatisfactory reproducibility; however, conversion of the excess of periodate into iodate prior to the hydrochloric acid-resorcinol treatment resulted in colourless blanks and reproducible results.

Amino acids were estimated by GLC of the derived *N*-trifluoroacetyl 1-butyl esters on a column of 0.325 % EGA on Chromosorb W;[21] 1-butyl stearate was used as the internal standard. The glycoprotein sample (2 mg) was hydrolysed with 6 M hydrochloric acid (1 ml) at 110° for 18 – 20 h under nitrogen. After evaporation of the hydrolysate under a stream of nitrogen, the residue was dissolved in water and the insoluble material centrifuged off. The clear supernatant was concentrated to dryness, and the amino acid hydrochlorides converted into the *N*-trifluoroacetyl 1-butyl esters.[21]

Total sulphate groups were determined by the barium chloroanilate method of Spencer,[22] and *N*-sulphate groups by the method of Lagunoff and Warren.[13]

The elution of columns of DEAE-Sephadex and Sepharose was monitored by the phenol-sulphuric acid test,[23] by the method of Lowry *et al.*,[24] and by the periodate-resorcinol test [20] for neutral sugars, protein, and sialic acid, respectively.

Gel electrophoresis of glycoprotein samples (0.1 mg) was performed in 0.09 M Tris-borate buffer, pH 8.2, in 0.5 % agarose – 2 % polyacrylamide or in 1 % agarose (Agarose for electrophoresis, General Biochemicals), as described by Holden *et al.*,[8] some experiments being done in gels containing 0.1 % sodium dodecyl sulphate. In addition gels were stained with 0.2 % Toluidine Blue in 0.1 M acetic acid.[25]

Infrared spectra were recorded for glycoprotein samples (1.5 – 2 mg) in potassium bromide discs with a Perkin-Elmer Model 237 spectrophotometer.

Incubation with neuraminidase (Type V, Sigma, purified from *Clostridium perfringens*) was performed by adding 0.02 units of the enzyme to the sample (20 mg) in 0.05 M sodium acetate buffer, (5 ml) pH 5.4, and keeping the digest at 35° for 24 h under toluene. The same amount of enzyme was added and the incubation continued for 24 h. The digest was then concentrated to 1 ml, applied to a column (1.2 × 45 cm) of Sepharose 6 B and eluted with 0.01 M pyridine – acetate buffer, pH 4.8, 2 ml fractions being collected. The glycoprotein fraction was eluted in the front peak and isolated by lyophilization; the following low-molecular-weight fraction obtained was deionized by passage through a column of Dowex 50 (H^+), and the acidic eluate concentrated at room temperature. The resulting syrup was analysed by paper chromatography in the solvent system 1-butanol-1-propanol-0.1 M hydrochloric acid, 1:2:1 (v/v), and the spots were stained with the thiobarbituric acid spray reagent.[26]

Incubation with α-L-fucosidase purified from *Clostridium perfringens* was kindly performed by Dr. D. Aminoff according to the conditions of Aminoff and Furukawa.[16] The lyophilized enzyme digest (2 mg) was dissolved in 0.01 M pyridine-acetate buffer, pH 4.8, (0.3 ml), applied on a Sepharose 6 B column (1.2 × 29 cm) and

eluted with the same buffer, fractions of 1.5 ml being collected. The glycoprotein-containing front peak was dialysed and lyophilized. The material of the final, phenol-sulphuric acid-positive peak was deionized on columns of Dowex 50 (H^+) and Dowex 1 formate. The carbohydrate composition of the two products was analysed by GLC.

Incubation with trypsin (Type I, Sigma, 2 × crystallized) was performed by dissolving the sample (10 mg) in 0.05 M Tris-chloride buffer containing 0.01 M calcium chloride, pH 7.9, (2 ml) and incubating with the enzyme (0.5 mg) at 35° for 8 h. The digest was immersed briefly in a boiling water bath, applied to a Sepharose 6 B column (1.2 × 45 cm) and eluted with 0.025 M Tris-chloride buffer, pH 7.3, 2 ml fractions being collected.

For the determination of blood group activity the glycoprotein was serologically typed by its ability at a concentration of 1 mg/ml, to inhibit the agglutination of human red cells in the presence of the appropriate antiserum. In addition to human anti-H serum, *Ulex europeus* H-lectin was used as anti-H substance.

The molecular weight determination was performed by sedimentation equilibrium. The sample (4 mg), dissolved in 1 ml of 0.1 M acetate buffer, pH 5.7, was dialysed against the same buffer at 4° for 18 h. The solution was analysed by low-speed equilibrium at 3000 rpm for 48 h at 20° in a Spinco Model E analytical ultracentrifuge. Optical registration was done by a photoelectric scanner at 280 nm. The partial specific volume ($\bar{V}$, 0.64) was calculated from the carbohydrate and amino acid composition of the glycoprotein sample.

Preparation of purified glycoprotein fraction. The crude, nondialysable intestinal or fecal extract was isolated as described previously.[6] The extract (2.5 g) was stirred at room temperature for 24 h with chloroform – methanol, 2:1 (v/v), and then 1:2 (v/v), 300 ml each time. After filtration the remaining material (2.4 g) was dissolved in 0.05 M Tris – chloride buffer, pH 7.9 (300 ml) containing 0.01 M calcium chloride, and incubated with pronase (B grade, Calbiochem.) (25 mg) at 35° for 24 h under toluene. The same amount of pronase was again added and the incubation continued for a further 24 h. The resulting proteolysate was dialysed exhaustively against distilled water, concentrated, and lyophilized to yield 1.8 g. The pronase-resistant material (1.8 g) was dissolved in water (360 ml) to which an aqueous 1 % solution of CTAB (180 ml) was added slowly with stirring. The precipitate was allowed to settle for 4 h at 4°, and was then centrifuged off and dissolved in methanol (400 ml) by stirring at room temperature overnight. A small amount of undissolved dark material was centrifuged off and discarded. To dissociate the CTAB-glycoprotein complex, a solution of equimolar proportions of sodium acetate trihydrate (5.0 g) and acetic acid (2.2 g) in 50 ml of methanol was added with stirring to the clear supernatant.

The precipitated glycoprotein was centrifuged off, dissolved in water, dialysed, and recovered by lyophilization to yield 0.9 g. A portion of this material (0.45 g) dissolved in 0.05 M Tris-chloride buffer, pH 7.3, was applied to a column (2.5 × 30 cm) of DEAE-Sephadex A 25 equilibrated with the same buffer. The column was eluted with a linear gradient of sodium chloride in 0.05 M Tris buffer pH 7.3, from 0 to 1.0 M sodium chloride in a total volume of 400 ml. Fractions of 10 ml were collected and aliquots analysed for hexose, protein, and sialic acid. The fractions containing the glycoprotein (Fractions 11 to 19, Fig. 2) were pooled, dialysed, and lyophilized to afford 0.25 g. The combined fractions obtained from two separations on the DEAE-Sephadex column (0.5 g) were dissolved in 0.025 M Tris-chloride buffer, pH 7.3, and the solution was applied to a 2.5 × 80 cm column of Sepharose 4 B, and eluted with the same buffer; 8 ml fractions were collected and analysed as described for the DEAE-Sephadex column. The fractions containing the glycoprotein (Fractions 13 to 17, Fig. 3) were pooled, dialysed, and lyophilized to yield 0.2 g.

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Tobacco Chemistry. 24. (9*R*)-9-Hydroxy-4-megastigmen-3-one, a New Tobacco Constituent

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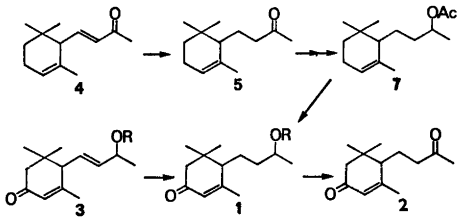
(9*R*)-9-Hydroxy-4-megastigmen-3-one has been isolated from Greek tobacco. Its absolute configuration was determined by correlation with derivatives of (9*R*)-9-hydroxy-4,7*E*-megastigmadien-3-one.

Some time ago we reported on the presence in Greek tobacco, *Nicotiana tabacum* L., of (9*R*)-9-hydroxy-4,7*E*-megastigmadien-3-one** (*8*, R=H, 3-oxo-α-ionol),[1] the absolute configuration of which has recently been established.[2] In the present communication we discuss the absolute configuration and synthesis of a closely related compound not previously encountered in tobacco.

The new compound, $C_{13}H_{22}O_2$, was isolated as its acetate from a complex, medium volatile fraction [3] of an extract [4] of Greek tobacco. The presence of a secondary hydroxyl group was apparent from an NMR deacylation shift [5] (1.1 ppm) observed on hydrolysis, and the formation of a methyl ketone when subjecting the resulting alcohol to oxidation. The second oxygen atom is part of a β,β-disubstituted, conjugated ketone grouping judging from its UV (237 nm), IR (1667 cm^{-1}) and NMR (one-proton multiplet at δ 5.84) spectra. Spin-spin decoupling experiments revealed that the single olefinic proton is coupled to a vinylic methyl group (δ 1.99, *J* 1.4 Hz) thereby unveiling one of the β-substituents. An AB-system at δ 2.02 and 2.36 (*J* 17 Hz) ascribed to a methylene group flanked by a carbonyl group and a fully substituted carbon atom, revealed the partial structure $-C\text{-}CH_2\text{-}CO\text{-}CH=C(CH_3)-$. The above evidence, and the fact that the NMR spectrum also disclosed the presence of two non-coupled methyl groups (singlets at δ 1.03 and 1.06) made structure *1* (R=H) appear likely for this new tobacco constituent.***

Its gross structure was confirmed synthetically by comparison of the acetate *9* (R=Ac) with corresponding racemic material obtained both on selective hydrogenation of (±)-9-acetoxy-4,7*E*-megastigmadien-3-one [2] (*3*, R=Ac), and on selective hydrogenation of (±)-*trans*-α-ionone (*4*), followed by hydride reduction, acetylation, and allylic oxidation.



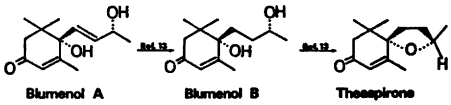
Galbraith and Horn [6] have recently isolated from *Podocarpus blumei* Endl. three structurally related compounds designated blumenol A, B, and C, the first one probably being identical to vomifoliol [9] (*Rauwolfia vomitoria*), and the last being identical to or stereoisomeric with the new tobacco compound. The structures of the blumenols were deduced from spectral data

** Nomenclature and stereochemistry as defined in Ref. 6.

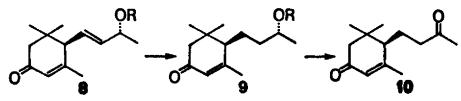
*** Dr. D. L. Roberts, R. J. Reynolds Tobacco Co., Winston-Salem, has simultaneously and independently identified *1* (R=H) as a new tobacco compound (private communication). Racemic *1* (R=H) has previously been reported as an intermediate in a synthesis of a tobacco additive, however without characterization.[7]

(molecular weight given for blumenol C, 208, is a misprint for 210; private communication), and in the cases of blumenol A and B, confirmed synthetically. Based on a comparison of the ORD curve of blumenol C with those of (+)-α-ionone [10] and lutein [11] the absolute configuration at C-6 was proposed as *R* which is consistent with the C-6(*S*) configurations of blumenols A and B very recently established chemically.[12,13]

Weiss *et al.*[13] also demonstrated chemically that the configuration at C-9 of blumenol A and B is *R* by converting (with stereospecific inversion at C-9) blumenol B into theaspirone of known absolute configuration (9*S*). However, contradictory to these results, Galbraith and Horn [12] have simultaneously suggested, on the basis of an assumed biogenetic relationship between the blumenols and theaspirone, that the absolute configuration of all the blumenols is *S* at C-9. It was therefore considered necessary to elucidate the absolute configurations of the new tobacco compound through correlation with synthetic material of known absolute stereochemistry.



The natural compound and its acetate displayed optical rotations somewhat higher than those of the corresponding material which could be prepared from (9*R*)-9-hydroxy-4,7*E*-megastigmadien-3-one (*8*, R = H, *cf.* Table 1) and it was therefore imperative to examine the two chiral centres, C-6 and C-9, separately. The absolute configuration at C-6 was established as *R* by comparison of two samples of the diketone *10* derived on the one hand from the natural product *9* (R = H) and on the other from (9*R*)-9-hydroxy-4,7*E*-megastigmadien-3-one (*8*, R = H), which both exhibited rotations of the same sign and similar magnitude (*cf.* Table 1). The somewhat lower optical activity of the latter material is presumably due to partial epimerization at C-6 during isolation which required basic conditions.



The absolute configuration at C-9 was disclosed by comparing the optical activities of the epimeric mixtures obtained after subjecting the natural alcohol *9* (R = H) and the correponding synthetic specimen obtained on hydrogenation of (9*R*)-9-hydroxy-4,7*E*-megastigmadien-3-one (*8*, R = H) to alkali treatment. Depending on the configuration at C-9, the resulting equilibrium mixtures of epimers would comprise either the 6*R*,9*R* and 6*S*,9*R* or the 6*R*,9*S* and 6*S*,9*S* isomers. Since the components of one mixture would be the enantiomers of those in the other, the equilibrium constants have to be the same in both cases, and hence, the signs of the resulting rotations would reveal whether the configuration at C-9 is *R* or *S*. The two mixtures were found to exhibit optical activities of the same sign and similar magnitude thereby establishing the absolute configuration at C-9 of the new compound as *R*, which is the same as that found by Weiss *et al.*[13] for blumenol A and B. If, as assumed previously,[12] the blumenols are biogenetically interrelated it is considered likely that blumenol C has 9*R* configuration rather

Table 1. Optical activities, [α], of specimens of the natural product (9*R*)-9-hydroxy-4-megastigmen-3-one (*9*, R = H) and its derivatives, and of corresponding authentic compounds derived from (9*R*)-9-hydroxy-4,7*E*-megastigmadien-3-one (*8*, R = H).

Natural [α](nm)	9 (R = H)	9 (R = Ac)	10	Authentic 9 (R = H)	9 (R = Ac)	10
365	+514°	+621°	+456°	+356°	+420°	+382°
436	+147.1°	+219°	+138°	+101.7°	+144.7°	+115°
546	+ 66.4°	+107	+ 64.6°	+ 46.4°	+ 72°	+ 53.6°
578	+ 55.9°	+ 92.2°	+ 56°	+ 39.4°	+ 61.8°	+ 45.5°
589	+ 54°	+ 87.5°	+ 52.6°	+ 37.2°	+ 57.8°	+ 44°
c($CHCl_3$)	0.22	0.31	0.34	0.33	0.55	0.44

than 9*S* as suggested by Galbraith and Horn.[12]

The structural relationship, including absolute configurations, between the two tobacco compounds (9*R*)-9-hydroxy-4-megastigmen-3-one (*9*, R=H) and (9*R*)-9-hydroxy-4,7*E*-megastigmadien-3-one (*8*, R=H) indicates that they are biogenetically interrelated and likely to be derived from a common carotenoid precursor, *e.g.* lutein and/or α-carotene which are known tobacco carotenoids [14] possessing the same absolute configuration in the position corresponding to C-6.[10,11] The new compound *9* (R=H) might in turn be the precursor of two stereomers of 1,3,7,7-tetramethyl-2-oxa-bicyclo[4.4.0]-decan-9-one which were isolated recently from tobacco.[15] The conversion of *1* (R=H) to the latter compounds has been achieved chemically.[7]

EXPERIMENTAL

NMR, IR, UV, and mass spectra were recorded on Varian XL-100, Digilab FTS-14, Beckmann DK-2A, and LKB 9000 (70 eV) instruments, respectively. Optical activities were recorded on a Perkin-Elmer 141 polarimeter. Accurate mass determinations were carried out at the Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm.

Isolation. (9*R*)-9-Hydroxy-4-megastigmen-3-one (9, R=H) was isolated as its acetate (*9*, R=Ac, 25 mg) from a medium-volatile fraction of an extract from 295 kg Greek tobacco, *Nicotiana tabacum* L., using liquid chromatography. The sub-fractionation of this medium-volatile material will be described later.[3]

(9R)-9-Acetoxy-4-megastigmen-3-one (9, R=Ac). $[\alpha]_{20}$: see Table 1; λ_{max} (EtOH): 237 nm (ε 6940); ν_{max} (film): 2965 (s), 2940 (s), 2873 (m), 1737 (s), 1667 (s), 1376 (m), 1245 (s), 1134 (w), 1074 (w), 1023 (w), 949 (w), 896 (w), 844 (w), 671 (w), 611 (w) cm^{-1}; MS: *m/e* 252 (M^+, 30), 177 (26), 150 (35, 138 (36), 136 (33), 135 (50), 123 (24), 121 (29), 109 (29), 108 (62), 93 (42), 55 (29), 43 (100), 41 (45); accurate mass determination: $C_{15}H_{24}O_3$: Found: 252.1732, Calc.: 252.1725; δ ($CDCl_3$): 1.03 (3 H, s), 1.06 (3 H, s), 1.23 (3 H, d, *J* 6.3 Hz), 1.99 (3 H, d, *J* 1.4 Hz), 2.04 (3 H, s), 2.02 and 2.36 (2 H, AB-system, *J* 17 Hz), 4.86 (1 H, m), 5.84 (1 H, m); irradiation at δ 5.84 collapsed the doublet at δ 1.99 to a singlet.

(9R)-9-Hydroxy-4-megastigmen-3-one (9, R=H). The acetate (*9*, R=Ac, 12 mg) was dissolved in 1 % KOH/MeOH (6 ml) and left at room temperature for 2 h. The mixture was diluted with water and extracted with ether. Removal of the solvent left a colourless, TLC-pure oil (9 mg). $[\alpha]_{20}$: see Table 1; ν_{max} (film): 3420 (broad), 2967 (s), 2937 (s), 2872 (m), 1660 (s), 1419 (w), 1379 (m), 1325 (w), 1300 (m), 1258 (m), 1180 (w), 1122 (m), 1081 (w), 989 (w), 945 (w), 898 (w), 840 (w) cm^{-1}; MS: *m/e* 210 (M^+, 41), 150 (48), 135 (76), 123 (47), 109 (70), 108 (86), 95 (62), 93 (59), 69 (55), 55 (51), 45 (49), 43 (100), 41 (88); δ ($CDCl_3$): 1.02 (3 H, s), 1.07 (3 H, s), 1.20 (3 H, d, *J* 6.2 Hz), 2.0 (3 H, d, *J* 1.2 Hz), 2.01 and 2.40 (2 H, AB-system, *J* 17 Hz), 2.75 (OH), 3.76 (1 H, m), 5.83 (1 H, m).

4-Megastigmen-3,9-dione (10). The alcohol (*9*, R=H, 9 mg) was oxidised with CrO_3 to the diketone (*10*, 8 mg) employing the two-phase system described by Brown *et al.*[16] The conversion to the ketone was complete in 30 min, after which the aqueous phase was extracted with ether, washed with $NaHCO_3$, and concentrated leaving a colourless, TLC-pure oil. $[\alpha]^{20}$, see Table 1; ν_{max} (film): 2967 (s), 2938 (m), 2876 (m), 1718 (s), 1662 (s), 1441 (m), 1418 (m), 1379 (m), 1370 (m), 1291 (w), 1252 (m), 1180 (w), 1163 (w), 1121 (w), 1070 (w), 1024 (w), 971 (w), 953 (w), 869 (w), 838 (w) cm^{-1}; MS: *m/e* 208 (M^+, 40), 151 (73), 138 (25), 136 (71), 123 (31), 109 (67), 108 (31), 107 (21), 95 (40), 81 (23), 67 (23), 55 (23), 43 (100), 41 (40); δ ($CDCl_3$): 1.04 (3 H, s), 1.08 (3 H, s), 2.02 (3 H, d, *J* 1.1 Hz), 2.16 (3 H, s), *ca.* 2.28–2.57 (4 H, m), 5.85 (1 H, m), irradiation at δ 5.85 simplified the doublet at δ 2.02 to a singlet.

Preparation of (±)-9-acetoxy-4-megastigmen-3-one (1, R=Ac). (a): (±)-9-Acetoxy-4,7*E*-megastigmadien-3-one [3] (*3*, R=Ac, 60 mg) in dry dioxane (0.25 ml) was added to a suspension of Pd/C (10 %, 14 mg) in dioxane [17] (10 ml) which had been saturated with H_2. The theoretical amount of H_2 was consumed at atmospheric pressure in 40 min, after which the mixture was diluted with water, extracted with ether and concentrated. The colourless oil was chromatographed on silica gel furnishing pure acetate (*1*, R=Ac, 29 mg); its spectral data were identical to those of the acetate derived from the natural alcohol (*9*, R=H). (b): (±)-*trans*-α-Ionone (*4*, 12 g) dissolved in 0.25 N NaOH/EtOH (100 ml) was hydrogenated [18] at atmospheric pressure and room temperature using Pd/C (10 %, 250 mg) as catalyst. One equivalent of H_2 was taken up in 2.5 h after which water was added and the product (*5*, 12 g) extracted with pentane; δ ($CDCl_3$): 0.87 (3 H, s), 0.92 (3 H, s), 1.67 (3 H, d, *J* 1.0 Hz), 2.11 (3 H, s), 2.46 (2 H, t, *J* *ca.* 7 Hz), 5.33 (1 H, m). The dihydroionone (*5*, 1 g) was treated with $NaBH_4$ (300 mg) in EtOH (25 ml) for 1 h. The mixture was diluted with water and extracted with ether. Removal of the solvent left a colourless oil (6, 950 mg); δ ($CDCl_3$): 0.83 (3 H, s), 0.88 (3 H, s), 1.14 (3 H, d, *J* 6 Hz), 1.62 (3 H, d, *J* 1 Hz), *ca.* 1.9 (2 H, m), 2.6 (OH), 3.62 (1 H, m), 5.24 (1 H, m). The alcohol (*6*, 950 mg) was acetylated using acetic anhydride (1 g) in pyridine (10 ml). After 3 h at ambient temperature excess anhydride was destroyed with MeOH, diluted with aqueous sulphuric acid and extracted with ether. Removal of the solvent left the acetate (*7*, 980 mg) as a colourless oil:

δ ($CDCl_3$): 0.86 (3 H, s), 0.90 (3 H, s), 1.19 (3 H, d, J 6 Hz), 1.66 (3 H, d, J 1 Hz), 2.0 (3 H, s), 4.84 (1 H, m), 5.30 (1 H, m). The acetate (*7*, 950 mg) dissolved in acetic acid (5 ml) was added to a solution of CrO_3 (1 g) in acetic acid (15 ml) and stirred at room temperature for 3 h. Water was added and the mixture extracted with ether which was washed with $NaHCO_3$ and water. The residue obtained after distillation of the solvent was chromatogaphed on silica gel furnishing starting material (169 mg) and the ketoacetate (*1*, R=Ac, 177 mg), respectively. The spectral data of the product were indistinguishable from those of the acetate derived from the natural alcohol (9, R=H).

Preparation of (±)-9-hydroxy-4-megastigmen-3-one (1, R=H) and (±)-4-megastigmen-3,9-dione (2). The racemic acetate, prepared above, was hydrolyzed to the corresponding alcohol (*1*, R=H) which subsequently was oxidised to the diketone (*2*) as described above for the natural compound. The physical properties except for optical activity of the products *1* (R=H) and *2* were identical to those of the tobacco-isolate and its oxidation product.

Partial syntheses of (9R)-9-acetoxy-4-megastigmen-3-one (9, R=Ac), (9R)-9-hydroxy-4-megastigmen-3-one (9, R=H) and 4-megastigmen-3,9-dione (10). (9*R*)-9-hydroxy-4,7*E*-megastigmadien-3-one [3] (*8*, R=H, 93 mg) was acetylated, selectively hydrogenated, hydrolyzed, and oxidized as described above. The products were indistinguishable from the natural alcohol, its acetate and oxidation product except for having somewhat lower specific rotations; see Table 1.

Epimerization of authentic (9R)-9-hydroxy-4-megastigmen-3-one (9, R=H). The authentic ketol *9* (R=H, 6.3 mg) was dissolved in MeOH, a small amount of $NaOCH_3$ (2 drops of a 3 % solution in MeOH) was added and the change in optical acitivity was followed. After about 10 h no further change in the activity could be observed; 436 nm: −5.2°, 456 nm: −3.8°, 578 nm: −3.5°, 589 nm: −3.2°.

Epimerization of natural (9R)-9-hydroxy-4-megastigmen-3-one (9, R=H): The natural ketol *9* (R=H, 5.7 mg) was treated as described above for the authentic ketol. Optical activity of the equilibrium mixture: 436 nm: −7.7°, 546 nm: −5.4°, 578 nm: −4.7°, 589 nm: −4.7°. Dilution with water and extraction with ether yielded 4.3 mg which were dissolved in $CHCl_3$ (1 ml): 436 nm: −7.7°, 546 nm: −6.0°, 578 nm: −5.1°, 589 nm: −3.3°.

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Added in proof: Comparison of the optical properties of the new tobacco constituent with those of blumenol C leaves no doubt about their identity (M. N. Galbraith, private communication). Dr. A. Demole, Firmenich SA, Geneva, has recently independently identified the title compound as a burley tobacco constituent (private communication).

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Tobacco Chemistry. 25. Two New Drimane Sesquiterpene Alcohols from Greek *Nicotiana tabacum* L.

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Two new sesquiterpene alcohols, isolated from Greek tobacco, were shown to be driman-8-ol (*3*) and driman-8,11-diol (*5*) on the basis of spectral data and confirmatory synthesis from drim-7-en-11-ol.**

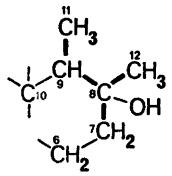
Our continued investigation of the neutral fractions from the volatile extracts of Greek tobacco [1,2] has led to the isolation of two new bicyclic sesquiterpene alcohols.

The first compound, $C_{15}H_{28}O$, was isolated by a combination of liquid and preparative gas chromatography from a fraction of medium polarity (B-5).[2] The presence of a tertiary methyl carbinol group ($>C(OH)CH_3$) followed from IR (3400 cm^{-1}) and NMR data, *i.e.* a methyl singlet at 1.11 ppm and absence of signals downfield of 2.0 ppm. Singlets for three methyl groups at 0.82, 0.82, and 0.88 ppm and a doublet for a methyl group at 0.89 ppm (J 7 Hz) were also observed and with no evidence for unsaturation, these results indicated a bicyclic structure.

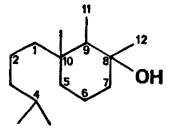
On addition of $Eu(dpm)_3$-d_{27}, the three-proton singlet due to the methyl on the hydroxylated carbon suffered the strongest downfield shift. Three one-proton multiplets also moved downfield strongly and must correspond to three protons on the two carbons, vicinal to the hydroxylated carbon. One of these appeared as a distinct quartet (J 7 Hz) and, as shown by spin decoupling, was attached to the same carbon as the methyl group which gave rise to the doublet (7 Hz). The absence of any other measurable couplings to this proton suggested that both carbons adjacent to the carbon carrying it were quaternary. The other two strongly downfield-shifted one-proton multiplets were assigned to the protons of a methylene group. These were mutually coupled (J_{gem} 12 Hz) and since they were both further coupled to two other vicinal protons, there must be an ethylene group linked to the methyl carbinol group. These results furnished the partial structure *1*.

Available evidence, including the fact that the C_5-unit shown in *1* by heavy lines corresponds to a terminal isoprene unit, indicated that this C_{15}-compound was derived from a normal, head-to-tail linked sesquiterpenoid precursor and hence suggested the partial structure *2*. The ring closure between C(9) and C(10) in *2* (numbering as in the final structure *3*) to incorporate the partial structure *1* is directed by

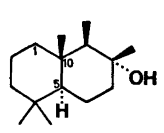
** Nomenclature and stereochemistry as defined in Ref. 3.



1



2

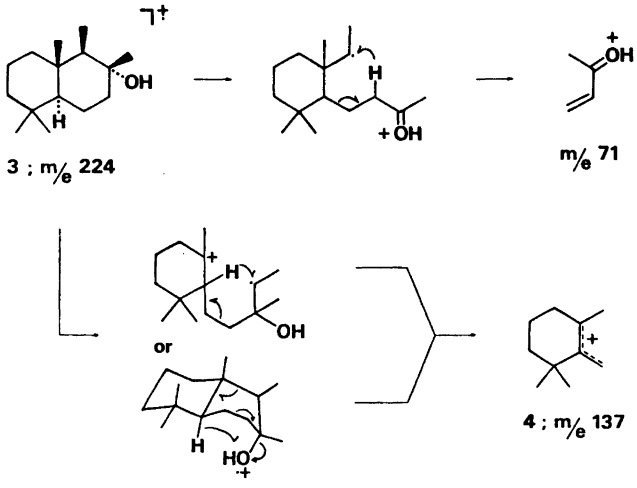


3

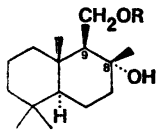
the NMR spectral requirements of (i) a secondary carbon atom at C(9) adjacent to a quaternary carbon atom at C(10), and (ii) that the remaining methyl groups not characterized in the partial structure *1* are at quaternary carbon atoms. The location of the methyl at C(10) is supported by the relative LIS (1.0) observed for a methyl singlet in the NMR spectrum. Cyclisation of *2* at C(4)–C(5) to yield the drimane [3] sesquiterpenoid *3* (no stereochemistry implied) maintains the necessary quaternary centre at C(4) and is preferred to the two remaining alternatives, *i.e.* the bicyclic structures derived by cyclisation of C(4) to C(1) or C(2), because of the mass spectrometric fragmentation pattern. Thus readily explained on the basis of structure *3* are the base peak at *m/e* 71, mainly due to a $C_4H_7O^+$ fragment evidently formed as shown [4] in Scheme 1 through initial α-cleavage of the C(8)–C(9) bond, and the prominent peak at *m/e* 137, characteristic [5] for structurally related labdane diterpenoids (ring A fragment 4). Fragments expected to be prominent on the basis of the alternative structures are insignificant or absent, *e. g.* ions derived by C(1)–C(10) cleavage or formed by reactions initiated by α-cleavage of the C(7)–C(8) bond.

The stereochemical assignments shown in *3* were derived from an analysis of the NMR spectrum after the addition of $Eu(dpm)_3$-d_{27}. C(9)H is coupled only to $C(11)H_3$ and the absence of any long-range W-coupling [6] to the equatorial proton at C(7) indicates that C(9)H is axial. The observed LIS for the methyl groups at C(8), C(9), and C(10) are in the order 2.6:1.8:1.0 and this can be rationalized only by co-ordination of the lanthanide complex to an equatorial C(8)OH group. The relatively weak LIS observed for the methyl groups at C(4) (0.4 axial; 0.2 equatorial) relative to the LIS (1.0) measured for the $C(10)CH_3$ support a *trans*-fused geometry of the rings. The synthesis of *3* (*vide infra*) from drim-7-en-11-ol (*9*) confirms the structure *3*, with stereochemistry as shown, for this tobacco compound.

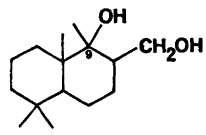
The second compound was isolated by liquid chromatography from a less volatile, neutral fraction of the Greek tobacco extract and purified as the acetate. The latter was the monoacetate of a diol, $C_{15}H_{28}O_2$, as shown by IR absorption at 3470, 1740, and 1243 cm^{-1} and NMR signals at 2.04 ppm (3 H, s) and 5.4 ppm (1 H, broad s, removed by D_2O). Resonances at 4.26 ppm (1 H, dd, *J* 5.5 and 12 Hz) and 4.36 ppm (1 H, dd, *J* 4.5 and 12 Hz), shifted upfield to 3.90 ppm (2 H, m) in the diol, showed the presence of a primary acetate [7] attached to a secondary carbon ($>CH\text{-}CH_2OCOCH_3$). The NMR spectrum of the diol also exhibited singlets for three methyl groups at 0.81, 0.81, and 0.88 ppm and a downfield singlet at 1.33 ppm for a methyl attached to a tertiary carbinol group. With no evidence of unsaturation, these data implied a C_{15} compound having five C_1-substituents on a bicyclic C_{10}-nucleus and hence a drimane skeleton, which made only two alter-



Scheme 1.



5 R = H
6 R = $COCH_3$



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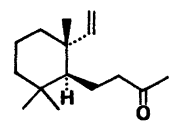

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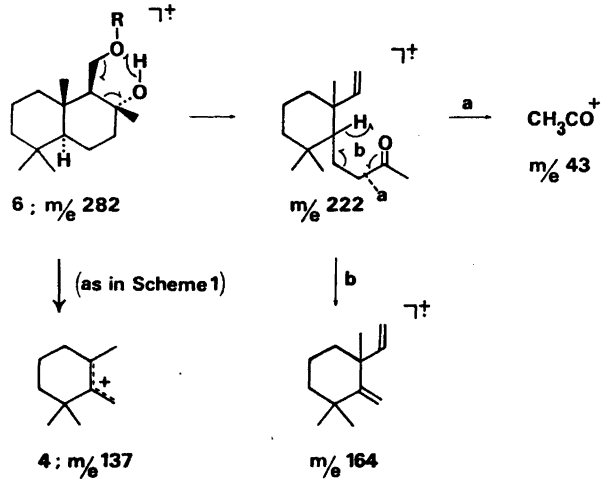
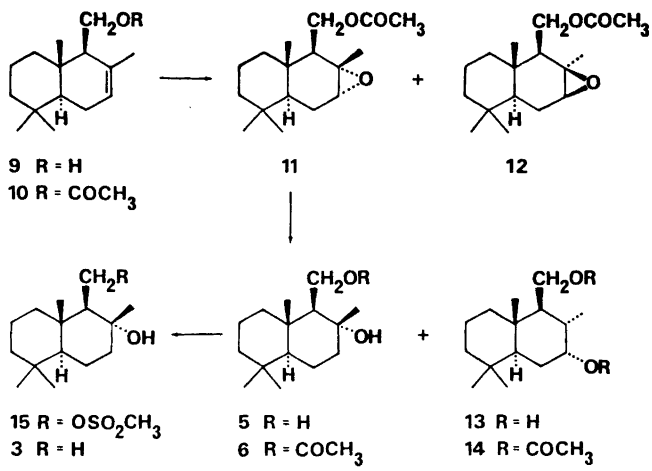
native structures, neglecting stereochemistry, worthy of consideration, namely driman-8,11-diol (*5*) and driman-9,12-diol (*7*). The mass spectra of the diol and the acetate – both displaying the expected *m/e* 137 ion ($C_{10}H_{17}$) corresponding to the drimane ring A fragment 4 – allowed these to be distinguished. Thus the *m/e* 222 ion, due to a $C_{15}H_{26}O$ fragment formed by loss of CH_3COOH from the acetate and water from the diol, underwent loss of acetone to give a *m/e* 164 ion ($C_{12}H_{20}$, m*), a reaction only readily explained in terms of structure *5* (Scheme 2). Moreover, the structures given for the *m/e* 222 and 164 species in Scheme 2 are supported by the fact that the spectra of the diol and the acetate below *m/e* 222 are very similar, except for the *m/e* 137 peak, to that of the ketone *8*. The structure 5, with stereochemistry as shown, was established by synthesis.

It was envisaged that a successful synthesis of the diol *5* would in turn permit the convenient preparation of the alcohol *3* and the synthetic routes to these compounds from drim-7-en-11-ol (9) are outlined in Scheme 3. 11-Acetoxydrim-7-ene (*10*) was treated with *m*-chloroperbenzoic acid in methylene chloride at $-30°$ for three days to give an excellent yield of the desired α-epoxide *11*, formed by preferential attack from the sterically less hindered α-side, and minor amounts of the less polar, and chromatographically readily removed, β-epoxide *12*. Reductive opening of the α-epoxide *11* with excess $LiAlH_4$ in refluxing ether (36 h) gave the diol *5*, identical in all respects with the natural product. However, the yield of the diol *5* was low and the major product was the previously prepared [3] diol *13*, the result of preferred *trans*-diaxial opening of the oxirane ring. Since Ohloff *et al.*[11] have recently shown that in the corresponding 11-nor compound steric hindrance by the $C(10)CH_3$ leads preferentially to abnormal ring opening (nucleophilic attack at C(7) giving the equatorial 8 α-alcohol), it seems that in the acetoxyepoxide



Scheme 2.



Scheme 3.

11 this steric effect is outweighed by a directing influence of the primary oxygen function on the nucleophile. An anchimeric assistance by the hydroxyl group in the ring cleavage reaction such as recently observed by Barton *et al.*[12] for 4α,5α-epoxy-7α-hydroxy steroids, which would also favour the abnormal opening, is unlikely to be of importance in the present case.

Overnight reaction of the diol *5* with excess methanesulphonyl chloride in pyridine at 0° gave a quantitative yield of the monomesylate *15*. Subsequent reduction with $LiAlH_4$ furnished in good yield the alcohol *3*, identical in all respects with the corresponding natural product.

We have previously isolated from Greek tobacco a number of compounds which may be degradation and/or rearrangement products of terpenoid precursors.[8,9,13] The two new compounds (*3* and *5*) are of interest in this context as they both represent potential precursors of the vinylketone *8*, previously also obtained by us from Greek tobacco;[8] *i.e.* from driman-8,11-diol (*5*) by 1,3-diol-rearrangement, and/or from driman-8-ol (*3*) by oxidative fragmentation. Attempts to prepare the vinylketone *8* by oxidation of driman-8-ol (*3*) in refluxing cyclohexane in the presence of calcium carbonate gave, however, only a small quantity of *8*, as detected by GC-MS and TLC.

EXPERIMENTAL

NMR, IR, and mass spectra were recorded on Varian HA100D and XL-100, Digilab FTS-14 and Perkin-Elmer 257, and LKB 9000 (70 eV) instruments, respectively. Rotations were measured on a Perkin-Elmer 141 instrument and accurate mass determinations were carried out at the Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm.

Isolations. Driman-8-ol (*3*) (12 mg) was isolated by column chromatography on silica and silica impregnated with 20 % $AgNO_3$ followed by preparative gas chromatography (3 m × 3.2 mm glass column packed with 5 % Carbowax 20 M on Chromosorb G) from the volatile neutral fraction B-5 of an extract of the leaves of sun-cured Greek *Nicotiana tabacum* L.[1] Driman-8,11-diol (*5*) was isolated as the acetate derivative (*6*) (11 mg) (after acetylation of a complex sub-fraction with excess acetic anhydride in pyridine for 24 h at room temperature) by liquid chromatography on silica from a medium-volatile neutral fraction of the leaves of sun-cured Greek *N. tabacum L.* The fractionation of this medium-volatile material will be described later.[2]

Driman-8-ol (*3*). Colourless needles m.p.73–75° (early softening). MS: 224 (M^+, 8 %), 71 (100), 69 (70), 83 (70), 97 (66), 43 (63), 109 (62), 55 (61), 41 (60), 95 (56); accurate mass measurement: $C_{15}H_{28}O$, found 224.2140, calc. 224.2140; $C_{10}H_{17}$, found 137.1320, calc. 137.1330; C_4H_7O, found 71.0499, calc. 71.0497; C_5H_{11}, found 71.0863, calc. 71.0861, *m/e* 71.0499 and 71.0863 peaks are of approximately equal intensities; $\delta(CDCl_3)$ 0.82 (6 H, s, C(4β)CH_3 and C(10β)CH_3), 0.88 [3 H, s, C(4α)CH_3], 0.89 [3 H, d, J ~7 Hz, C(9β)CH_3], 1.11 [3 H, s, C(8β)CH_3]; on addition of Eu(dpm)$_3$-d_{27}, r (relative induced shift ra-

tio) = 0.2 [3 H, s, C(4α)CH_3], 0.4 (3 H, s, C(4β)CH_3), 1.0 [3 H, s, C(10β)CH_3], 1.8 (3 H, d, J 7 Hz, C(9β)CH_3), 2.6 (3 H, s, C(8β)CH_3, 3.6 (1 H, q, J 7 Hz, C(9α)H), 3.4 (1 H, d of t, J ~3, 12 Hz, C(7β)H), ~4.0 (1 H, broad t of d, J ~4, 12 Hz, C(7α)H); ν_{max}(KBr) 3400 (broad), 1078 (m), 1058 (w), 940 (w) cm^{-1}; $[\alpha]_D^{20}$ −18.2° (c 0.56, $CHCl_3$).

11-Acetoxydriman-8-ol (*6*). M.p. 77 – 79° (early softening): MS: 282 (M^+, 3 %), 43 (100), 41 (30), 95 (28), 109 (28), 69 (26), 55 (24), 82 (23), 81 (22), 123 (18); accurate mass measurement: $C_{17}H_{30}O_3$, found 282.2202, calc. 282.2195; $C_{15}H_{26}O$, found 222.1986, calc. 222.1984; $C_{12}H_{20}$, found 164.1568, calc. 164.1565; C_5H_6O, found 82.0415, calc. 82.0419; C_6H_{10}, found 82.0785, calc. 82.0782; δ ($CDCl_3$) 0.82 (3, H, s), 0.87 (3 H, s), 0.89 (3 H, s), 1.18 (3 H, s), 2.04 (3 H, s), 4.26 (1 H, dd, J 5.5, 12 Hz), 4.36 (1 H, dd, J 4.5, 12 Hz), 5.4 (1 H, broad); ν_{max}(film) 3470 (broad), 1740 (s), 1243 (s), 1030 (w) cm^{-1}; $[\alpha]_D^{20}$ −9° (c 0.53, $CHCl_3$).

Driman-8,11-diol (*5*). Colourless crystals m.p. 119 – 120°. MS: 240 (M^+, 0.5 %), 43 (100), 95 (84), 109 (79), 69 (72), 82 (72), 123 (60), 164 (60), 41 (58), 81 (56), 55 (54); δ($CDCl_3$) 0.81 (6 H, s), 0.88 (3 H, s), 1.33 (3 H, s), 3.9 (2 H, m); ν_{max} (KBr) 3360 (broad), 1076 (w), 1053 (w), 1023 (m), 1015 (w), 994 (w), 940 (w), 913 (w) cm^{-1}; $[\alpha]_D^{20}$ 1.6° (c 0.63, $CHCl_3$).

7α,8-Epoxy-11-acetoxydrimane (*11*). 11-Acetoxydrim-7-ene (*10*) was prepared by overnight reaction of drim-7-en-11-ol (*9*) (1.1 g) with excess acetic anhydride (2.4 g) in pyridine solution (7 ml) at room temperature. TLC showed the crude acetylated product *10* (1.27 g) to be essentially pure and it was used in the next step without further purification. A solution of *m*-chloroperbenzoic acid (1.1 g) in methylene chloride (20 ml) was added dropwise over 2 h to a cold (−30°) stirred solution of *10* (1.27 g) in methylene chloride (50 ml). After 3 days at −30° TLC showed the reaction mixture contained no unreacted *10* and the reaction mixture was diluted with ether, washed with saturated $NaHCO_3$ solution, water and dried (Na_2SO_4). Evaporation of the solvent gave a colourless oil (1.36 g) which crystallized slowly on standing and TLC (SiO_2; 40 % ether/hexane) revealed a mixture of two products (R_F 0.33 and 0.36) in the ratio 9:1. The more polar major product, the α-epoxide *11*, was purified by chromatography on silica: m.p. 55 – 57°; MS: 280 (M^+, 5 %), 99 (100), 43 (91), 123 (42), 69 (42), 41 (41), 85 (38), 124 (38), 71 (36), 55 (32), 73 (28); δ($CDCl_3$) 0.83 (3 H, s), 0.87 (3 H, s), 0.89 (3 H, s), 1.36 (3 H, s), 2.07 (3 H, s), 3.00 (1 H, m, $w_{1/2}$ 4 Hz), 4.0 (1 H, dd, J 9.5, 12 Hz), 4.4 (1 H, dd, J 3, 12 Hz); ν_{max} (KBr) 1740 (s), 1240 (s), 1045 (m), 1036 (m) cm^{-1}; $[\alpha]_D^{20}$ 29.3° (c 0.41, $CHCl_3$).

11-Acetoxydriman-8-ol (*6*) and *7α,11-diacetoxy-8α-drimane* (*14*). Excess $LiAlH_4$ (0.6 g) was added to a solution of the α-epoxide *11* (1.3 g) in dry ether (100 ml) and the mixture was heated under reflux for 30 h. The cooled reaction mixture was treated with ethyl acetate to decompose unreacted $LiAlH_4$, diluted with ether and washed with 1 M HCl, saturated $NaHCO_3$ solution, brine and dried (Na_2SO_4). Evaporation of the solvent gave a crystalline residue (1.1 g) which was treated overnight at room temperature with acetic anhydride (3 g) in pyridine solution (6 ml). Methanol (0.5 ml) was added (at 0°) to decompose excess acetic anhydride (15 min) and the reaction mixture was diluted with ether and washed with 1 M HCl, saturated $NaHCO_3$ solution, brine and dried (Na_2SO_4). Evaporation of the ether gave a colourless oil (1.3 g) which TLC (SiO_2; 60 % ether/hexane) indicated contained some unreacted starting material (*11*) and two products R_F 0.59 and 0.35). Column chromatography on silica (110 g) recovered unreacted *11* (190 mg) and separated the reaction products. The less polar major product (800 mg) was the diacetate *14* isolated as a low-melting crystalline solid. MS: M^+ not visible, 43 (100 %), 204 (84), 189 (61), 119 (38), 69 (34), 121 (30), 55 (28), 41 (26), 81 (26), 109 (16); δ($CDCl_3$) 0.80 (3 H, s), 0.82 (3 H, s), 0.89 (3 H, d, J 6.5 Hz), 0.90 (3 H, s), 2.02 (3 H, s), 2.06 (3 H, s), 4.06 (1 H, dd, J 3, 12 Hz), 4.18 (1 H, dd, J 3, 12 Hz), 5.04 (1 H, m, $w_{1/2}$ 6 Hz); ν_{max} (KBr) 1740 (s), 1240 (s), 1025 (m) cm^{-1}; $[\alpha]_D^{20}$ −37.6° (c 0.51, $CHCl_3$).

The more polar product (97 mg) was the acetate *6*, isolated as a colourless oil that crystallised on standing and identical in all respects to the acetate *6* of the tobacco compound *5*.

Driman-8,11-diol (*5*). Hydrolysis of the synthetic acetate *6* in 10 % methanolic KOH (10 min) and work-up as described above gave synthetic *5* indistinguishable from the diol *5* isolated from the tobacco.

Driman-8-ol-11-yl methane sulphonate (*15*). A cold (0°) solution of the diol *5* (18 mg) in dry pyridine (1 ml) was treated with methanesulphonyl chloride (25 mg) and after 18 h at 0 – 5° excess methanesulphonyl chloride was decomposed with a few drops of water, the reaction mixture was diluted with ether and washed with 1 M HCl, saturated $NaHCO_3$ solution, brine, dried (Na_2SO_4) and the solvent evaporated to leave a pale yellow oil (24 mg). This product showed a single spot on TLC (SiO_2; 80 % ether/hexane) and was used in the next step without further purification. δ($CDCl_3$) 0.82 (3 H, s), 0.90 (6 H, s), 1.15 (3 H, s), 2.1 (1 H, broad s), 3.02 (3 H, s), 4.32 (2 H, dd, J 6, 11 Hz), 4.55 (2 H, dd, J 3, 11 Hz); ν_{max} (film) 3500 (broad), 1355 (s), 1175 (s), 945 (s) cm^{-1}.

Driman-8-ol (*3*). The crude mesylate *15* (24 mg) in dry ether solution (5 ml) was treated with $LiAlH_4$ (40 mg) and the mixture refluxed for 18 h. Ethyl acetate was added to the cooled reaction mixture to decompose excess $LiAlH_4$ and the mixture was diluted with ether and washed with 1 M HCl, saturated $NaHCO_3$ solution, brine and dried (Na_2SO_4). The solvent was evaporated to leave a crystalline residue (18 mg) which was chromatographed on silica to recover

diol *5* (7 mg), and isolate synthetic *3* (11 mg) which was identical in all respects to the tobacco compound *3*.

Oxidation of driman-8-ol (3). Driman-8-ol (4 mg) was added to a mixture of $CaCO_3$ (5 mg) and $Pb(OAc)_4$ (20 mg) in cyclohexane (2 ml) and the mixture heated under reflux for 24 h. Direct analysis of the reaction mixture by GLC-MS revealed about 70 % unreacted *3* and a minor component (~5 %) with a mass spectrum and retention time (50 m × 0.3 mm glass column coated with HB 5000) identical to that of authentic vinylketone *8*.

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Alkylation of Indenyllithium and Alkylindenyllithium Compounds

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The reactions between indenyllithium and various halides (methyl, ethyl, isopropyl, *t*-butyl, benzyl, benzhydryl, and triphenylmethyl) gave almost exclusively the corresponding 1-alkylindene (Ia) in good yields. Methylindenyllithium gave a mixture of 1-alkyl-3-methylindene (IIb) and 1-alkyl-1-methylindene (IIc) when treated with alkyl halide in ether. Methylation of alkylindenyllithium compounds methyl-, ethyl-, *t*-butyl- and benzyl-) with methyl iodide gave the corresponding 1-methyl-3-alkylindenes (IIa) in good yields and only slightly contaminated with 1-alkyl-1-methylindene (IIc). The reactions offer a convenient synthetic route from indene to 1,3-dialkylindenes and especially to the least stable tautomer (IIa).

As a part of investigations on the tautomerism of indene and indene derivatives,[1] a good preparative method for the synthesis of 1-, and especially, 1,3-disubstituted, indenes was required. The fact that the methylene moiety of indene is slightly acidic ($pK_a = 21$) has enabled several workers to prepare metal compounds of indene.[2–5] These compounds could then be used to prepare alkylated indenes.[5,6] However, in most cases in the earlier literature, there is some doubt as to whether a 1- or a 3-substituted indene has been formed. The reaction conditions have sometimes been too vigorous and led to a mixture of products. High temperature or prolonged heating leads to the more stable 3-substituted isomer. However, high temperature is often needed with sluggish reacting reagents such as sodium, sodamide, and alkali-hydroxides in the first step and secondary and tertiary halides in the second step. 1-Substituted indenes have also been prepared in an essentially isomeric pure state from indenylmagnesium bromide and an alkyl halide.[7] This reaction is, however, restricted to the more reactive halides such as benzyl chloride and gives rather poor yields.

With ethyllithium in ether in an inert atmosphere, indene forms a compound, which can be isolated as yellowish crystals. Schlenk and Bergmann [4] used this compound to prepare benzhydrylindene (m.p. 158°C) by reacting it with benzhydryl chloride. When this indene-derivative was treated with methanolic potassium hydroxide, a compound with a melting point of 108 – 109°C was found. They concluded that the compound first isolated was the 1-substituted isomer, which was rearranged to 3-benzhydrylindene in the base-catalyzed reaction. This finding has been confirmed in the present paper by means of NMR data. The metalation and alkylation procedures used by these authors were thus mild enough to permit isolation of the least stable isomer.

In these laboratories preparations of pure 1-substituted and 1,3-disubstituted derivatives have been achieved. Thus, 1,3-dideuterio-indene has been prepared by heating 1,1,3-trideuterio-indene with a sodium dispersion, followed by quenching the reaction mixture with H_2O.[9] Ahlberg *et al.* prepared 1-α-hydroxyisopropyl-indene by reacting indenyllithium with acetone.[6] Isomeric pure 1-deuterio-2-ethylindene has also been prepared and these results will be published elsewhere.[10]

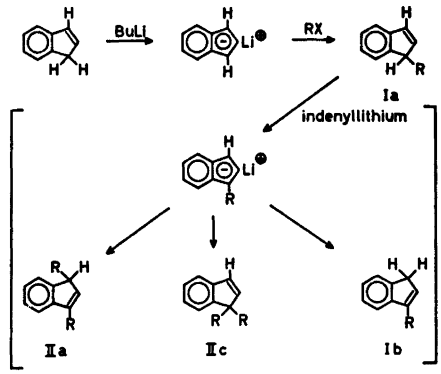
A few years ago, Meth-Cohn and Gronowitz prepared indene-1-carboxylic acid from carbon dioxide and indenyllithium.[11] Quite recently Cedheim and Eberson have reported the preparation of several 1-alkylindenes from indenyl-

lithium.[12] As quoted in their paper indenyllithium had earlier been used for the preparation of 1-benzyl-, 1-benzhydryl-, and 1-triphenylmethylindene. This paper describes the use of indenyllithium compounds for the preparation of both 1- and 1,3-alkylsubstituted indenes. Contrary to the findings of others,[12] no need was found for using the method of reversed addition of reagents.

RESULTS AND DISCUSSION

The metalation of indene was performed by treating indene with a 50 % excess of butyllithium in pentane or ether. The reaction was exothermic and was controlled by external cooling. The metalation was complete within 15 min, since quenching with D_2O, followed by rapid acidification, showed more than 98 % monodeuteration in the indene isolated, as judged from the NMR-spectrum. In one experiment the ether was removed after adding a two-fold excess of butyllithium, xylene was added and the reaction mixture heated for 12 h. After quenching with D_2O and conventional work-up, the NMR-spectrum of the product was examined. The spectrum showed only monodeuteration (*i.e.* one equivalent of butyllithium had reacted) which is opposite to the results obtained by others under similar conditions.[13]

The alkylation was performed by adding an excess of alkyl halide to a cooled ethereal



Scheme 1.

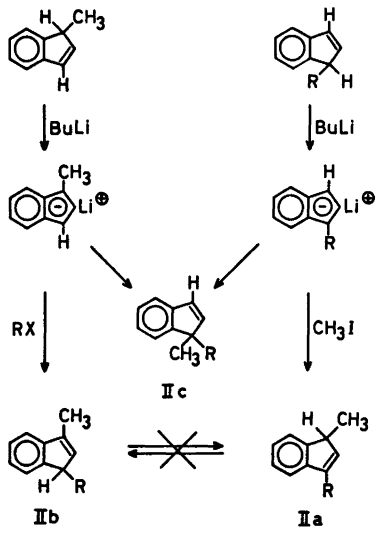
solution of indenyllithium in a nitrogen atmosphere. After standing for the appropriate time and temperature (see Table 1) with stirring, the reaction mixture was poured into ice-cooled, 2 M hydrochloric acid. After work-up and preparative GLC, the product was analyzed with NMR and analytical GLC. This showed that the reaction mixture consisted of the starting material and isomeric pure 1-alkylindene in different proportions, depending on the alkyl halide used. A complication in these syntheses is that excessively high temperature and/or prolonged reaction time lead to 3-alkylindenes or dialkylation (1,3-dialkylation and 1,1-dialkylation) presumably according to scheme 1. Thus when indenyllithium was

Table 1. Alkylation of indenyllithium in ether with various alkylhalides (*cf.* Scheme 1). Retention times are given for the analytical column described in the experimental part. Retention time for indene: 9.0 min.

Alkylating agent	Reaction temp.°C	Reaction time h.	Yield of Ia (%)	Retention time of Ia (min)	Retention time of rearranged prod. Ib (min)
CH_3I	−20	18	93	10.4	15.4
C_2H_5Br	+ 5	18	74	16.2	20.5
i-C_3H_7Br	+25	11	69	20.1	27.9
t-C_4H_9Br	+25	18	35	27.6	36.4
t-C_4H_9Br	+25[a]	12	48	27.6	36.4
t-C_4H_9Br	+35	8	45	27.6	36.4
$C_6H_5CH_2Cl$	+25	4	84	–	–
$(C_6H_5)_2CHCl$	+25	4	73	–	–
$(C_6H_5)_3CCl$	+25	2	81	–	–

[a] Equimolar amount of DMSO added.

methylated at room temperature and the solution left for 18 h, the product consisted of 78 % 1-methylindene, 12 % 3-methylindene, and 10 % dimethylated indenes. Bosch and Brown [5] reported similar results except that they found a larger portion of 3-methylindene. However, if the temperature is kept below −20°C in the alkylation step, 93 % 1-methylindene, about 0.5 % 3-methylindene, and 1.5 % dimethylated products are obtained. Even if a low reaction temperature is necessary, it was found that heating can increase the yield of the 1-substituted indene. Thus heating a mixture of indenyllithium and *t*-butyl bromide increased the yield of 1-*t*-butylindene from 35 to 45 % without isomerization.



Scheme 2.

Alkylation of alkyl-indenyllithium compounds is supposed to occur in both the 1- and the 3-position and result in the formation of 1,1- and 1,3-dialkylindenes (Scheme 2). However, the distribution and yields of these two species vary considerably depending on the substituent in the indene nucleus and the type of alkyl halide used. Methylation of methylindenyllithium thus gave about 10 % 1,1-disubstitution while the same reaction with *t*-butylindenyllithium gave no 1,1-disubstituted product. Ethyl- and isopropylindenyllithium gave yields between these two extremes, while 1-benzylindenyllithium gave about the same amount of the 1,1-disubstituted products as methylindenyllithium (Table 2). However, when methylindenyllithium was alkylated with various alkyl halides (Table 3), the amount of 1,1-disubstitution increased with the bulkiness of the alkylating agent. Thus, *t*-butylbromide gave 55 % 1,1-disubstitution (calculated on the total amount of the disubstituted product), while methyl iodide gave only 10 %. When dimethoxyethane was used as solvent, the relative amount of 1,1-disubstitution was diminished to about 45 %. Benzyl chloride gave about the same amount of 1,1-disubstitution as *t*-butyl bromide. The varying amounts of 1,1-disubstitution is, of course, a disadvantage but, since separation is fairly easy with preparative GLC, the method can be of synthetic value since the previous routes to 1,3-dialkylindenes have been long and tedious. It should also be mentioned that the synthesis of IIa, the least stable isomer, gives the desired product in high yield, only slightly contaminated with 1,1-dialkylated product. No tautomeric mixture (IIa+IIb) is formed in either case (Tables 2 and 3).

For comparison it may be mentioned that quenching of methylindenyllithium with water under controlled conditions, *i.e.* so that no rearrangement can occur, gave exclusively 3-methylindene.

The different trends observed in the proportion of 1,1-dialkylated product (IIc in Scheme 2) obtained in the methylation procedure (Table 2) compared to the alkylation procedure (Table 3) are difficult to account for. In the methylation experiments, the benzyl substituent gives a result which resembles that found for the methyl substituent rather than for the higher alkyls (Table 2). Thus, a simple steric effect cannot account for the results. In the alkylation of methylindenyllithium, benzyl chloride shows, however, more similarity to the higher alkyl halides than to methyl iodide (Table 3). The entire trend in these experiments is thus opposite to that expected if simple steric hindrance between methyl and alkyl is considered. A more detailed study, especially regarding the structure of the indenyllithium compounds, is probably needed before any safe explanation can be given for the above-mentioned facts. This is beyond the scope of the present paper although a few further comments seem appropriate.

Table 2. Methylation of various alkylindenyllithium compounds in ether with methyl iodide (*cf.* Scheme 2). Reaction time 18 h. Reaction temperature 25°C. Retention times were obtained under the conditions given in the experimental part.

Indenyllithium compound	Yield of IIa (%)	Retention time of IIa (min)	Yield of IIc (%)	Retention time of IIc (min)
Methyl-	82	17.5	8	9.5
Ethyl-	76	23.5	5	14.2
Isopropyl-	78	27.5	2	20.5
t-Butyl-	72	34.1	0	30.5
Benzyl-	70	–[a]	12	–

[a] Not isolated. Identification and determination of yield *via* NMR-spectroscopy.

Table 3. Alkylation of methylindenyllithium in ether with various alkyl halides (*cf.* Scheme 2). Reaction time 18 h. Reaction temperature 25°C. Retention times were obtained under the conditions given in the experimental part.

Alkylating agent	Yield of IIb (%)	Retention time of IIb (min)	Yield of IIc (%)	Retention time of IIc (min)
CH_3I	82	17.5	8	9.5
C_2H_5Br	65	22.5	16	14.2
i-C_3H_7Br	35	29.0	23	20.5
t-C_4H_9Br	14	40.0	17	30.5
t-C_4H_9Br[a]	15	40.0	21	30.5
$C_6H_5CH_2Cl$[b]	35	–	40	–
$C_6H_5CH_2Cl$[b,c]	43	–	32	–

[a] Equimolar amount of DMSO added. [b] Not isolated. Composition and yields determined with NMR spectroscopy. [c] Solvent: Dimethoxyethane.

The fact that tautomeric mixtures (IIa + IIb) are not formed in any of the alkylation procedures (Scheme 2, Tables 2 and 3) and that formation of 3-alkylindenes (Ib, Scheme 1 and Table 1) can be avoided, indicates that the indenyllithium compounds must be present in the form of covalent species or contact ion pairs. Solvent separated ion pairs or indenyl anions would bring about a fast isomerization of the alkylindenes formed.[1]

The bonding in delocalized organolithiums varies considerably depending on the stability of the carbanion and the nature of the solvent.[14] Fluorenyllithiums in dioxane occur in the form of contact ion pairs exclusively and in tetrahydrofurane as a mixture of contact and solvent-separated ion pairs.[15] In cyclohexylamine-diethylamine indenyllithium exists as 65 % solvent-separated ion pairs and 35 % contact ion pairs at room temperature.[16] Compared to the fluorenyl anion, the indenyl anion has a more concentrated negative charge and forms a relatively more stable contact ion pair.[16] These data suggest that indenyllithium in ether should exist mainly as contact ion pairs (*cf.* Ref. 17). Alkyl substituted fluorenyllithiums show a greater proportion of solvent separated ion pairs as compared to the fluorenyllithium itself.[14,16] Whether this is true for the indenyllithiums cannot be decided since in this allylic system there may be an equilibrium between two contact ion pairs, with the lithium ion situated above either the 1- or the 3-position. Indenyllithium in tetrahydrofuran has an NMR-spectrum in which H_1 and H_3 are magnetically equivalent. This has been explained as the result of a rapid equilibrium between two covalent structures.[16] The spectrum can, however, also be accounted for as a result of a contact ion pair or an equilibrium between two

such ion pairs in view of the facts mentioned above and of other studies of allyllithium compounds.[14]

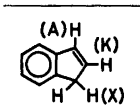
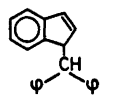
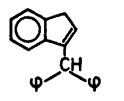
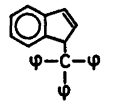
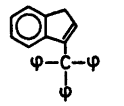
The situation in the relatively concentrated solutions used in the preparative procedures is certainly rather complex, since it is known that allyllithium is highly aggregrated in concentrated ether solution.[14] However, if we may use the simple hypothesis that contact ion pairs and high polarity of the solvent are favourable for the desired outcome of the alkylation reactions, the use of a larger cation than lithium should be preferred,[15] although the use of such cations will present larger preparative problems.

EXPERIMENTAL

General. Preparative GLC was performed on a Varian Aerograph 90-P, equipped with a 4 m × 3/8″ column filled with 15 % Apiezon L on Chromosorb 80/100 mesh with a gas-flow of about 100 ml/min. The column temperature was held constant at a temperature between 120 and 150°C depending on the indene being examined. Tests of indene purity were made on a Perkin-Elmer 900 gas-chromatograph with a flame ionization detector on a 8 m × 1/8″ column filled with 8 % Apiezon L on Varaport 30, 100/120 mesh. The column temperature was 150°C and the initial pressure 1 kg/cm². The NMR-spectra were run on a Varian A 60 D NMR-spectrometer. The samples were about 20 % in CCl_4 and run at 35°C with tetramethylsilane as an internal standard. The aralkylindenes were run in benzene-d_6.

Alkylation procedure. Butyllithium[19] (15 mmol) in 10 ml of pentane was added to a cooled solution of indene (or 1-alkylindene) (10 mmol) in 50 ml of dry ether in an atmosphere of dry nitrogen, purified over a solution of triphenylmethanide anion in pyridine.[20] The resulting solution was stirred at room temperature for about 30 min. The appropriate alkyl halide, bromide or iodide, (20 mmol) in 10 ml of ether was added with cooling to the reaction mixture. Samples were withdrawn and analyzed on the gas chromatograph. After standing at the appropriate temperature for the time scheduled in Tables 1 – 3, the brown-coloured solution was poured on to 100 ml of ice-cooled 2 M hydrochloric acid. The ether phase was separated, the aqueous phase extracted with ether and the combined ether extracts washed with aqueous NaCl sat. and dried over $MgSO_4$. The ether extracts were decolourized with charcoal and the ether evaporated. The residue was purified with preparative GLC in 250 μl portions. In most cases sufficient purity, ≤1 % impurities, was

Table 4. NMR-data for benzhydrylindene and triphenylmethylindene in benzene-d_6 at 35°C. Concentration: 0.3 M.

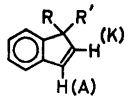
	Coupling constants cps	Shifts ppm(δ) and relative intensities( ) A	K	X	Other protons	M.p. °C
	$J_{CH-X}=11.1$ $J_{A-X}=1.65$ $J_{A-K}=5.75$ $J_{K-X}=1.65$	6.61 (1)	6.22 (1)	4.21 (1)	C – H 3.72 (1)	160 – 160.5
	$J_{CH-X}=1.9$ $J_{K-X}=1.8$ $J_{CH-K}=2.1$		5.80 (1)	3.11 (2)	C – H 5.33 (1)	109 – 110
	$J_{A-K}=5.0$ $J_{A-X}=1.8$ $J_{K-X}=1.9$	6.52 (1)	6.34 (1)	5.05 (1)		[a] 216 – 217
	$J_{K-X}=1.8$		6.25 (1)	3.17 (2)		158 – 159

[a] Anal. Found: C 93.71; H 6.12. Calc. for $C_{28}H_{22}$: C 93.85; H 6.15.

obtained with a spinning band column, if the reactions were run in larger batches.

1-Benzylindene was prepared from 100 mmol of indenyllithium and 200 mmol of benzyl chloride and the product subjected to fractional destillation. Since none of the aralkylindenes can be purified *via* GLC, 1-benzhydrylindene and 1-triphenylmethylindene were recrystallized from isopropanol. 3-Aralkylindene was isolated from equilibrium mixtures after isomerization experiments, the results of which will be published separately.[21] The equilibrium constant was larger than 50 and the 3-aralkylindenes were purified by destillation (3-benzylindene) or recrystallization from isopropanol. Melting points and NMR-data are shown in Table 4. NMR-data for 1,1-dialkylindenes are given in Table 5.

Table 5. NMR-data for 1,1-dialkylindenes in carbontetrachloride at 35°C. Concentration: 1 M. Shifts (δ) relative TMS. $J_{A-K}=5.50$ cps.



R	R′	K	A
$R=R'=CH_3-$			
1.32	1.32	6.21	6.49
3 H	3 H	1 H	1 H
$R=CH_3-$, $R'=CH_3CH_2-$			
1.29	0.61	6.11	6.55
3 H	3 H	1 H	1 H
	1.78		
	2 H		
$R=CH_3-$, $R'=(CH_3)_2CH-$			
1.24	1.02, 0.60	6.18	6.59
3 H	6 H	1 H	1 H
	2.42		
	1 H		
$R=CH_3-$, $R'=(CH_3)_3C-$			
1.27	0.95	6.25	6.52
3 H	9 H	1 H	1 H

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Synthesis of 7,7′-Deuterated Lycopene, 7,7′-Deuterated Rhodopin, and 7-Deuterated Apo-8′-lycopenal

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The synthesis of 7,7′-deuterated lycopene (*8*), 7,7′-deuterated rhodopin (*9*), and 7-deuterated apo-8′-lycopenal (*7*) is described. Deuterium was incorporated into geranyltriphenylphosphonium bromide (*2*) and (7-hydroxy-3,7-dimethyloct-2-en-1-yl) triphenylphosphonium bromide (*3*) through a base catalyzed exchange reaction.

As a model for the exchange reaction was studied the synthesis of undeuterated and deuterated 4,8-dimethyl-1-phenylnona-1,3,7-triene (*14* and *15*), followed by GLC-, PMR-, and IR characterization of the *cis-trans* isomeric products (*14A*, *14C*, *15A* and *15C*).

Spectroscopic and other physical properties of intermediates, products and model compounds are reported.

IR absorption at 920 and 730 cm^{-1} in deuterated carotenoids is on an empirical basis ascribed to out-of-plane deformation of *trans*-CH=CD.

A number of deuterium labelled carotenoids have been prepared recently by partial or total synthesis.[1–5] Furthermore, fully deuterated carotenes have been isolated from algae grown in heavy water.[6] Spectrometric studies on deuterated carotenoids have provided useful information especially on mass spectrometric fragmentations.[1–8]

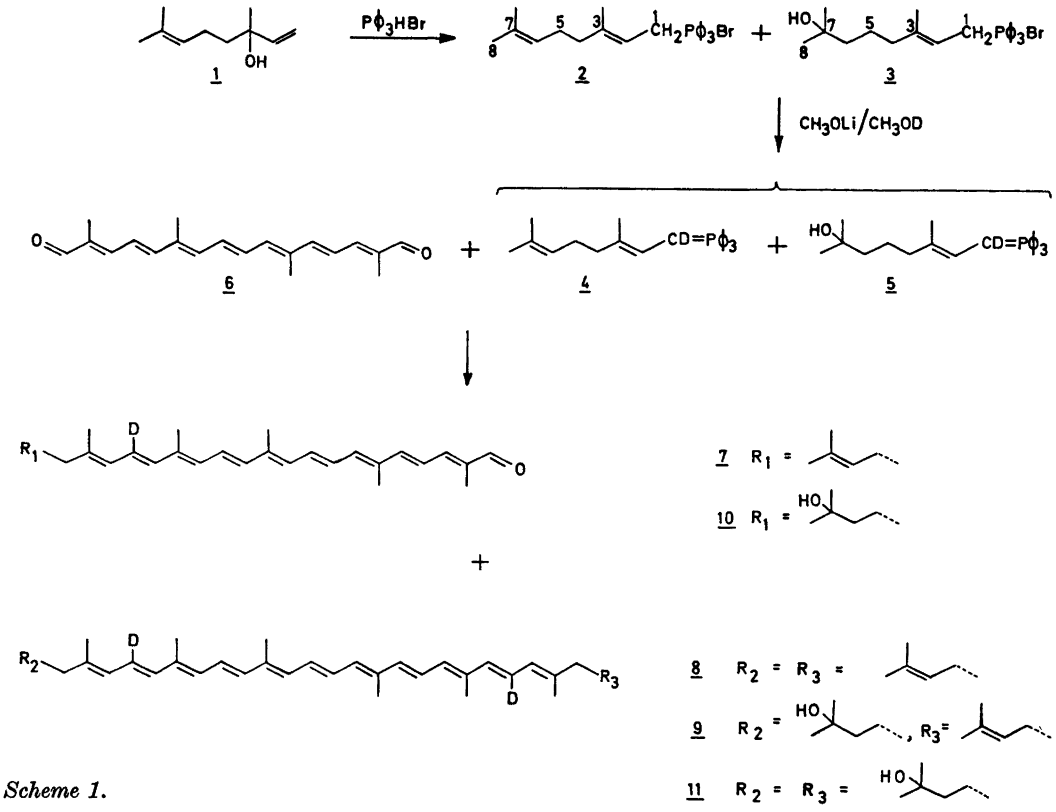
Generally two approaches are used for chemical synthesis of deuterium labelled organic compounds: reduction or hydrogen-deuterium exchange.[9a] Reduction reactions have been used in all previous preparations of deuterium labelled carotenoids. In the present paper we report the first syntheses of deuterium labelled carotenoids using the exchange technique, in principle as discussed in the previous paper.[5] The same type of exchange reaction has recently independently been used by Bestmann *et al.* for the preparation of a tritium labelled phosphorane.[9b] The following paper [10] deals with a related exchange reaction of enolizable hydrogen.

RESULTS AND DISCUSSION

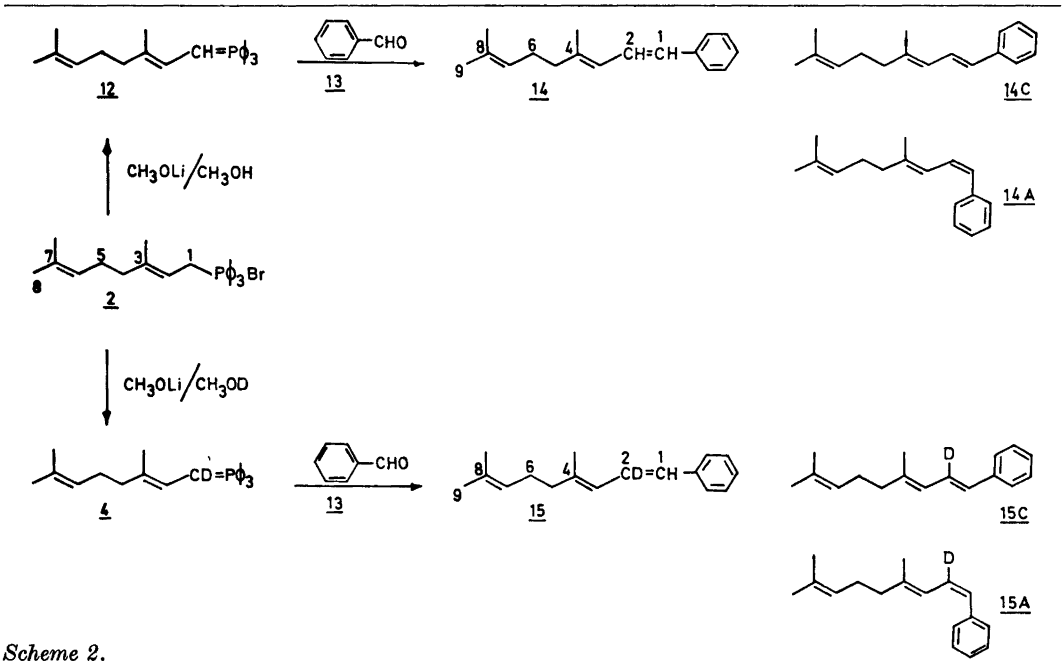
The synthesis of 7,7′-deuterated lycopene (*8*), 7,7′-deuterated rhodopin (*9*), and 7-deuterated apo-8′-lycopenal (*7*) was accomplished [11] by the route shown in Scheme 1 from linalool (*1*).

Hydrogen bromide catalyzed addition of methanol to the isopropylidene group of linalool (*1*) or geraniol has been reported.[12] Chloroform was therefore used as solvent in the first step. However, it is also known that geranyltriphenylphosphonium bromide (*2*) readily adds water.[13] Since water is produced on formation of geranyltriphenylphosphonium bromide (*2*) from linalool (*1*) it is conceivable that water was subsequently added to the phosphonium salt *2*, resulting in a mixture of geranyltriphenylphosphonium bromide (*2*, 76 %) and 7-hydroxy-3,7-dimethyloct-2-enyl)triphenylphosphonium bromide (*3*, 24 %), judged by the PMR spectrum.

Suitable conditions for the following exchange reaction of the mixed phosphonium salts (*2* and *3*) with deuteriomethanol and lithium methylate was studied by a model reaction (Scheme 2). Only products obtained from geranyltriphenylphosphonium bromide (*2*) were examined. In undeuterated methanol/lithium methylate the phosphorane *12* was readily obtained, judged by its further reaction with benzaldehyde (*13*) to the condensation product *14*. Hydrogen-deuterium exchange during phosphorane (*4*)



Scheme 1.



Scheme 2.

formation from *2*, followed by condensation with benzaldehyde (*13*) provided the deuterated hydrocarbon *15*. Under the conditions used 80 % deuterium incorporation was achieved after 5 min. The stereochemistry of the model compounds prepared is discussed below.

The mixed deuterated phosphoranes *4* and *5* (Scheme 1) gave with crocetindial (*6*) the monocondensation product *7* (15 % of carotenoid products) and 7,7′-deuterated lycopene (*8*, 59 %) and 7,7′-deuterated rhodopin (*9*, 26 %) as dicondensation products. The predicted minor condensation products 7-deuterated apo-8′-rhodopinal (*10*) and the diol *11* were not isolated. Large excess of phosphorane was required to obtain high yield of dicondensation products (*8* and *9*) relative to the monocondensation product (*7*). Physical properties of the products *7*, *8*, and *9* are given in the Experimental Part.

Judged by electronic spectra crystalline *8* was the all-*trans* compound. *cis*-Bond formation during the Wittig reaction [14] is in this case of 7(7′) double bond(s) less favourable due to steric hindrance. Prior to crystallization two *cis* isomers of 7,7′-deuterated lycopene (*8*) were isolated. However, these *cis* isomers were identical, juded by R_F-values and electronic spectra, to those present in the iodine catalyzed stereomutation mixture of all-*trans* *8*, known to contain no isomers with sterically hindered double bonds.[15] It consequently appears that the Wittig reactions leading to *7*, *8*, and *9* had *trans* stereospecificity.

7,7′-Deuterated lycopene (*8*) prepared here had 92 % deuterium incorporation (D_0:D_1:D_2 = 3:10:87, calculated from the mass spectrum as in a preceding paper [5]) when using 160 times excess of CH_3OD and 87 % incorporation (D_0:D_1:D_2 = 5:16:79) when using 40 times excess of CH_3OD relatively to exchangeable hydrogen in the phosphonium bromide. A previously synthesized 7,7′-deuterated lycopene (*8*) obtained *via* a different route involving lithium aluminium deuteride reduction had 80 % incorporation (D_0:D_1:D_2 = 5:30:65).[4]

Relatively few IR data are available for deuterated carotenoids.[4-6] IR data for products *7*, *8*, and *9* are discussed in connection with data for the products of the model reaction, Scheme 2.

The olefinic region in the PMR spectra of hydrocarbons *14* and *15* were very complex due to a mixture of *cis* and *trans* isomers. GLC analysis revealed three components for *14*

Table 1. Infrared absorptions of some deuterated and undeuterated carotenoids and hydrocarbon model compounds.

Compound	a		b	c	d
Lycopene	961	—	—	—	
7,7′-Deuterated lycopene (*8*) [4]	960	925	727	—	—
7,7′-Deuterated lycopene (*8*)	961	927	730	—	—
11,11′-Deuterated ε-carotene [5]	965	922	732	—	—
7-Deuterated apo-8′-lycopenal (*7*)	966	920	729	—	—
Rhodopin	960	—	—	—	—
7,7′-Deuterated rhodopin (*9*)	960	925	728	—	—
14 (stereoisomeric mixture)	960s	—	—	772m	—
14A (1-*cis*-*14*)	(965w)	—	—	775s	—
14C (1-*trans*-*14*)	965s	—	—	—	—
15 (stereoisomeric mixture)	(960w)	918m	727s	(770w)	671w
15A (1-*cis*-*15*)	(960w)	(920m)	(730w)	(770w)	671m
15C (1-*trans*-*15*)	—	918s	727s	—	—
cis EtCH=CHEt [18]	—	—	—	709	—
cis EtCH=CDEt [18]	—	—	—	—	641

a = C−H out-of-plane deformation of *trans*-CH=CH.
b = C−H(D) out-of-plane deformation of *trans*-CD=CH.
c = C−H out-of-plane deformation of *cis*-CH=CH.
d = C−H(D) out-of-plane deformation of *cis*-CD=CH.
s = strong, m = medium, w = weak.
Parentheses indicate that these absorptions might be explained by incomplete separation on GLC or incomplete deuteration.

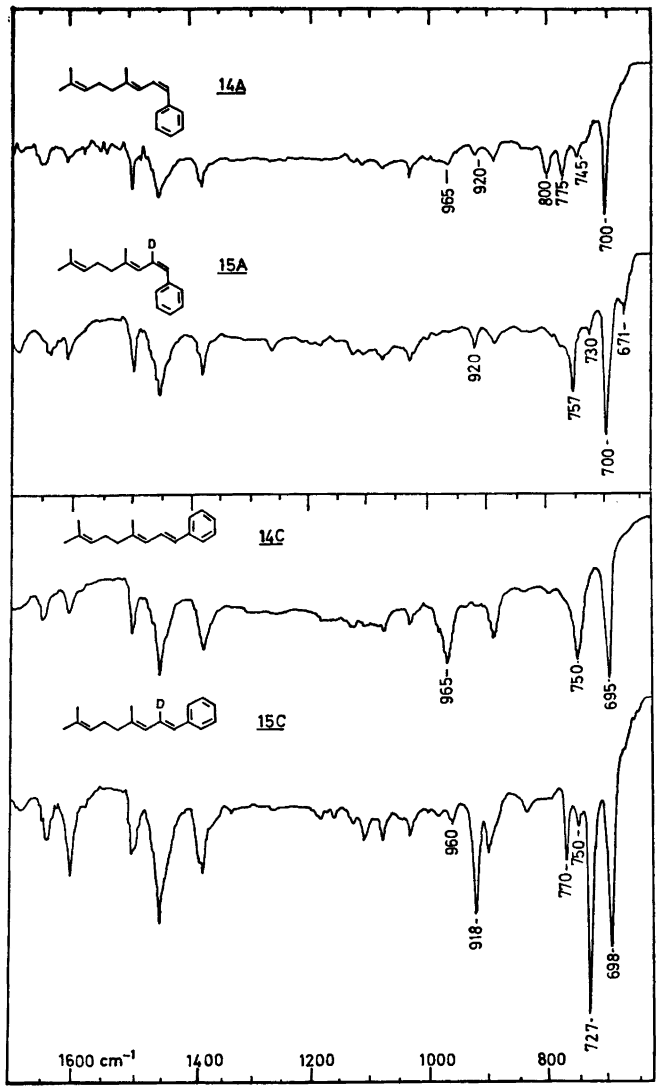


Fig. 1. Infrared spectra (liq.) of 1-*cis*-4,8-dimethyl-1-phenylnona-1,3,7-triene (*14A*), 1-*trans*-4,8-dimethyl-1-phenylnona-1,3,7-triene (*14C*), 1-*cis* 2-deuterated 4,8-dimethyl-1-phenylnona-1,3,7-triene (*15A*), and 1-*trans* 2-deuterated 4,8-dimethyl-1-phenylnona-1,3,7-triene (*15C*).

(*14A – C*) and four for *15* (*15A – D*). By preparative GLC *14A* and *14C*, and *15A* and *15C* were separated and isolated. IR data and PMR spectra revealed that *14A* and *15A* had *cis* configuration and *14C* and *15C* had *trans* configuration around the disubstituted double bond. The interpretation was supported by a computer-simulated (Program NMR 01 [16]) ABC spectrum with assumed chemical shifts and coupling constants. Available data do not permit definition of the stereochemistry of the adjacent double bond in these isomers (arbitrarily presented as *trans* in Scheme 2).

Characteristic IR data for the hydrocarbons *14* (stereoisomeric mixture), *14A*, *14C*, *15* (stereoisomeric mixture), *15A* and *15C* are given in Table 1, together with relevant data for the deuterated all-*trans* carotenoids here or previously prepared (*7*, *8*, and *9*) and undeuterated analogues. Assignments of the

~920 and ~730 cm^{-1} absorptions in deuterated carotenoids to *trans*-CD=CH is supported by data for the *trans* model compounds *14C* (undeuterated) and *15C* (deuterated). Absorption at 920–927 cm^{-1} has previously been considered characteristic of a monodeuterated *trans* double bond whereas absorption at 727–730 cm^{-1}, first considered due to an impurity,[4] later has been ascribed to this same group.[17] Data for the model compounds further suggest that absorption at 775 cm^{-1} (*14A*) is connected with C–H out-of-plane deformation of *cis*-CH=CH, whereas the corresponding vibration for *cis*-CD=CH occurs at 671 cm^{-1} (*14C*). Absorption at 641 cm^{-1} in *cis* EtCD=CHEt has previously been ascribed to such deformation at *cis*-CD=CH.[18]

The PMR spectra of 7,7'-deuterated lycopene (*8*), 7,7'-deuterated rhodopin (*9*), and 7-deuterated apo-8'-lycopenal (*7*) were as expected.

Discussion of the mass spectrometric data will be published separately.[19]

EXPERIMENTAL

Materials and methods. Methylene chloride (Merck *p.a.*) used in the exchange reactions had passed through a column of Al_2O_3 (Merck basic, activity grade 1 [20]). Ether had passed through a column of Al_2O_3 (Spence type H, activity grade 1 [20]). Technical grade acetone was distilled. Technical grade petroleum ether was distilled (b.p. 40–65°C) and then passed through a column of Al_2O_3 (Merck neutral, activity grade 1 [20]). Deuterated methanol (CH_3OD) was from Koch-Light (>99 % deuterated). Other solvents used were of analytical grade.

For column chromatography was used Merck, Spence or Woelm Al_2O_3, activity grade 1–3,[20] and Merck kieselgel (0.05–0.2 mm). TLC was performed on Merck Al_2O_3 or kieselgel (type 60) with or without indicator as stated in each case. Paper chromatography was carried out on Schleicher and Schüll Nos. 287 (kieselguhr) and 288 (aluminium oxide).

GLC was carried out on a Perkin Elmer F 11 flame ionization gas chromatograph. Other instruments used were as specified previously.[21] Complete spectra are available elsewhere.[11]

Deuterated carotenoids

Geranyltriphenylphosphonium bromide (2) and (7-hydroxy-3,7-dimethyloct-2-enyl)triphenylphosphonium bromide (3). Linalool (*1*, 145 g) and triphenylphosphonium bromide [286 g; m.p. 187–193°C; δ ($CDCl_3$) 7.50–8.15 (15 aromatic H) and 12.15 s (1 H); prepared by the procedure of Schwieter *et al.*[22]] in chloroform (0.7 l) were stirred at room temperature for 20 h. This gave a mixture of *2* (76 %) and *3* (24 %), yield 358 g (77 % based on *1*), on crystallization from methylene chloride-ethyl acetate; m.p. 164–165°C; λ_{max} (MeOH) 214, 261, 267, and 274 nm [*E*(1 %, 1 cm) 575, 31, 37 and 30]; ν_{max} (KBr) 3040–2770 (CH), 1660 (C=C), 1585, 1480 and 1445 (phenyl, CH_2, CH_3), 1435 (P-phenyl), 995 (*trans*-CH=CH), 750, 725 and 690 cm^{-1} (monosubst. phenyl and bromine); δ ($CDCl_3$) 1.17 s (H-8, CH_3-7 in *3*), 1.35 s and 1.42 s (3 H, CH_3-3 in *2* and *3*), 1.52 s and 1.59 s (H-8, CH_3-7 in *2*), 1.17, 1.52, and 1.59 counting for 6 H, 1.77 (H-6 in *2*?), 1.9–2.0 (4 H, H-3,4), 2.52 s (*ca.* 1/4 H, OH in *3*), 4.54 dd (2 H, J_{P-1}=14 Hz, J_{1-2}=8 Hz, H-1), 5.00 m (H-6 in *2*?), 5.08 t (J_{1-2}=8 Hz, H-2?) and 7.7–8.05 (15 aromatic H). An aliquot, after recrystallization from methylene chloride-ethyl acetate, melted at 177–178°C (reported 189°C for *2* [13] and 194°C for *3* [13]).

7-Deuterated apo-8'-lycopenal (7), 7,7'-deuterated lycopene (8) and 7,7'-deuterated rhodopin (9). Conditions for formation of the deuterated phosphoranes were investigated in model experiments described below.

(i). A solution of the phosphonium bromides *2* and *3* (35 mg) in CH_3OD (1.1 ml) and 0.28 N CH_3OLi (see below) was stirred at room temperature for 5 min. Crocetindial (*6*, 10 mg) in methylene chloride (5 ml) was added slowly, and the reaction mixture stirred at 30°C for 3 h. The reaction mixture was taken to dryness, the residue dissolved in chloroform and chromatographed by TLC on kieselgel G (chloroform), yield 0.34 mg (2 % based on *6*) of *8*; λ_{max} (acetone) 446, *472*, and 504 nm (most intense maximum is italicized); *m/e* (200°C) 538 (M); D_0:D_1:D_2=3:10:87; 92 % deuterium incorporation and 7.8 mg (52 % based on *6*) of *7*; λ_{max} (acetone) *446* nm; *m/e* (200°C) 417 (M); D_0:D_1=6:94; 94 % deuterium incorporation.

(ii). To a mixture of *2* and *3* (1.44 g) in CH_3OD (10.8 ml) and 0.28 N CH_3OLi, stirred for 5 min at room temperature, was added methylene chloride (3 ml). Crocetindial (*6*, 20 mg) in methylene chloride (4 ml) was added slowly, and the mixture stirred at 30°C for 3 h. The mixture was taken to dryness, the residue dissolved in 90 % methanol, extracted with petroleum ether and washed with aqueous 5 % NaCl-solution and water. The solution was taken to dryness, dissolved in ether, and the pigments purified by TLC on kieselgel G (15 % acetone in petroleum ether=15 % APE). This gave 7,7'-deuterated lycopene (*8*); yield 12.1 mg (31 % based on *6*), 7,7'-deuterated rhodopin (*9*); yield 5.6 mg (14 % based on *6*) and 7-deuterated apo-8'-lycopenal (*7*); yield 2.5 mg (8 % based on *6*).

7-Deuterated apo-8'-lycopenal (7) was rechromatographed by TLC on kieselgel G (15 % APE). Chromatography on paper (SS 287, 5 % APE) gave two zones: neo [15] a; R_F=0.90; λ_{max}

(acetone) 348 and *357* nm and all-*trans*; R_F = 0.76; λ_{max} (acetone) *466* nm. *7* was crystallized from acetone; m.p. 122–124°C (reported 141°C for the undeuterated analogue[23]); λ_{max} (acetone) 350 and *469* nm (reported λ_{max} (acetone) *470* nm[24]); ν_{max} (KBr) 3030–2850 (CH), 1670 (conj. CH=O), 1610 (CH_3), 1185, 1005, 966 (*trans*-CH=CH), 920 and 729 cm^{-1} (*trans*-CD=CH); δ ($CDCl_3$) 0.88 and 1.28 (imp.), 1.64 s and 1.70 s (isopropylidene CH_3), 1.85 s (end-of-chain CH_3), 1.90 (end-of-chain CH_3 at CHO), 2.02–2.40 (allylic CH_2) and 9.42 (aldehyde H); *m/e* (190°C), 417 (M), 348 (M–69), 325 (M–92), 322 (M–95) and 311 (M–106); D_0:D_1 = 12:88; 88 % deuterium incorporation.

7,7'-Deuterated lycopene (*8*) was rechromatographed by TLC on kieselgel G (5 % APE). Paper chromatography (SS 287, 2 % APE) gave three zones: neo b, R_F = 0.90, λ_{max} (ether) 361, (440), *464*, and 493 nm; neo a, R_F = 0.80, λ_{max} (ether) 360, 442, *467*, and 497.5 nm, and all-*trans* R_F = 0.73, λ_{max} (ether) 360, 445, *471*, and 503.5 nm. The all-*trans* isomer was inseparable from authentic lycopene. The same isomers were obtained on iodine catalyzed stereomutation of each isomer.[25] *8* was crystallized from acetone-petroleum ether, yield 6.0 mg; m.p. 157–158°C (reported 140–160°C for the deuterated[4] and 171°C for the undeuterated analogue[13]), undepressed on admixture with authentic lycopene; λ_{max} (acetone) 446, *473*, and 505 nm; % D_B/D_{II}[26] = 6, % III/II[26] = 78 (reported λ_{max} (acetone) 363, 446.5, *475*, and 506 nm[25] for undeuterated lycopene); ν_{max} (KBr) 3040–2855 (CH), 1625 (C=C), 1450 (CH_2), 1375 (CH_3), 961 (*trans*-CH=CH), 927 (*trans*-CD=CH), 825 (>C=CH–) and 730 (*trans*-CD=CH); δ ($CDCl_3$) 1.62 and 1.70 (4 isopropylidene CH_3), 1.83 (2 end-of-chain CH_3), 1.97 (4 in-chain CH_3), 2.10 and 2.15 (4 allylic CH_2), 5.13 (2 H, H-2,2'), 5.96 (2 H, H-6,6') and 6.78–6.25 (12 olefinic H); *m/e* (190°C) 538 (M), 469 (M–69), 446 (M–92), and 432 (M–106); D_0:D_1:D_2 = 5:16:79; 87 % deuterium incorporation.

7,7'-Deuterated rhodopin (*9*) was rechromatographed by TLC on kieselgel G (15 % APE). Paper chromatography (SS 287, 2 % APE) gave three zones: neo b, R_F = 0.69, neo a, R_F = 0.66, and *trans* R_F = 0.46. The *trans* isomer could not be separated from synthetic undeuterated rhodopin.[23] *9* had λ_{max} (ether), 360, 441, *467*, and 498; λ_{max} (acetone) 446, *471*, and 502 nm (reported λ_{max} (acetone) 365, 447.5, *477*, and 508 nm for the *trans* isomer[25]); ν_{max} (KBr) 3420 (OH), 3030–2850 (CH), 1630, 1450 (CH_2), 1375 (CH_3), 960 (*trans*-CH=CH), 925 and 728 cm^{-1} (*trans*-CD=CH); δ ($CDCl_3$) 0.88 and 1.27 (imp.), 1.60 and 1.67 s (isopropylidene CH_3), 1.82 s (end-of-chain CH_3), 1.96 s (in-chain CH_3), 2.10–2.15 (allylic CH_2), 5.13 m (isopropylidene), 5.96 (H-6,6'), and 6.0–6.9 (olefinic H); *m/e* (200°C) 556 (M), 538 (M–18), 464 (M–92), and 450 (M–106); D_0:D_1:D_2 = 7:13:80; 87 % deuterium incorporation.

Model compounds

Conditions for formation of undeuterated and deuterated phosphoranes. A 0.28 N solution of CH_3OLi in CH_3OH was prepared by addition of 20 % BuLi in hexane (9.6 ml) to CH_3OH (100 ml).

A 0.28 N solution of CH_3OLi in CH_3OD was prepared by addition of 20 % BuLi in hexane (1.2 ml) to CH_3OD (10 g).

Equivalent amounts of base and phosphonium bromide were used for phosphorane formation, unless otherwise stated. The reactions were monitored by TLC.

4,8-Dimethyl-1-phenylnona-1,3,7-triene (*14*). To a mixture of the phosphonium salts *2* and *3* (0.72 g) in CH_3OH (5.5 ml) and 0.28 N CH_3OLi, stirred for 5 min at 20°C, was added freshly distilled benzaldehyde (*13*, 0.16 g). The mixture was stirred at 20°C for 2 h and taken to dryness. TLC twice on kieselgel PF 254+366 (petroleum ether) gave *14*; yield 0.162 g (63 % based on *2*); λ_{max} (hexane) 287 nm; ν_{max} (liq.) 3080–2710 (CH), 1675, 1637 (conj. phenyl), 1598 (conj. C=C), 1491, 1448 (CH_2), 1374 (CH_3), 1180–1030 (phenyl), 960 (*trans*-CH=CH), 885, 827 (>C=CH–), 800, 772 (*cis*-CH=CH), 748 and 698 cm^{-1} (monosubst. benzene); δ ($CDCl_3$) 1.61 s, 1.70 s (6 H, H-9, CH_3-8), 1.84 s (3 H, CH_3-4), 2.11–2.24 (4 H, H-5,6), 5.13 m (1 H, H-7), 5.90–7.15 (3 H, H-1,2,3, complex coupling) and 7.15–7.42 (5 aromatic H); *m/e* (110°C) 226 (M), 157 (100 %) and 69 (98 %). *14*, analyzed on GLC (column OV-17) gave three peaks (*14A*, *14B* and *14C*) with retention time (210°C) 15, 17, and 22 min and relative integral 45:12:43, respectively. *14A* and *14C* were separated preparatively in the same GLC system.

1-cis-*4,8-Dimethyl-1-phenylnona-1,3,7-triene* (*14A*) had ν_{max} (liq.) 3080–2730 (CH), 1640 (conj. phenyl), 1600 (conj. C=C), 1570, 1490, 1450 (CH_2), 1375 (CH_3), 1180–1030 (phenyl), 965 (imp. from *6b*, *c*?), 885, 825 (>C=CH–), 800, 775 (*cis*-CH=CH), 745 and 700 cm^{-1} (monosubst. benzene); δ ($CDCl_3$) 1.61 s and 1.70 s (6 H, H-8, CH_3-8), 1.83 s (3 H, CH_3-4), 2.11–2.24 (4 H, H-5,6), 5.12 m (1 H, H-7), 6.39 (3 H, H-1,2,3) and 7.20–7.40 (5 aromatic H).

1-trans-*4,8-Dimethyl-1-phenylnona-1,3,7-triene* (*14C*) exhibited ν_{max} (liq.) 3080–2720 (CH), 1640 (conj. phenyl), 1598 (conj. C=C), 1575, 1495, 1449 (CH_2), 1375 (CH_3), 1180–1030 (phenyl), 965 (*trans*-CH=CH), 890, 845, 805, 750 and 695 cm^{-1} (monosubst. benzene); δ ($CDCl_3$) 1.63 s and 1.70 s (6 H, H-9, CH_3-8), 1.87 s (3 H, CH_3-4), 2.13–2.25 (4 H, H-5,6), 5.17 m (1 H, H-7), 5.92, 6.09, 6.28, 6.53, 6.82, 6.99, 7.12 (3 H, H-1,2,3), and 7.2–7.4 (5 aromatic H); the spectrum showed good agreement with a computer-simulated ABC spectrum assuming the given coupling constants and chemical shifts of the H-1, H-2, and H-3, protons, confirming the *trans* 1-double bond.

2-Deuterated 4,8-dimethyl-1-phenylnona-1,3,7-

triene (*15*). (i). To a mixture of the phosphonium bromides *2* and *3* (0.72 g) in CH_3OD (5.5 ml) and 0.28 N CH_3OLi stirred for 1 h at 20°C was added freshly distilled benzaldehyde (*13*, 0.16 g). The reaction mixture was stirred for 2 h at 30°C, and taken to dryness under vacuum. TLC as above gave 0.029 g (11 % based on *2*) of *15*; D_0:D_1 = 20:80; 80 % deuterium incorporation.

(ii). To a mixture of *2* and *3* (0.72 g) in CH_3OD (5.5 ml) and 0.28 N CH_3OLi stirred for 5 min at 20°C was added freshly distilled benzaldehyde (*13*, 0.16 g) and then treated as above. This gave *7*, yield 0.202 g (78 % based on *2*); λ_{max} (hexane) 287 nm; ν_{max} (liq.) 3080–2710 (CH), 2250 (CD), 1715, 1680, 1633 (conj. phenyl), 1595 (conj. C=C), 1491, 1447 (CH_2), 1376 (CH_3), 1180–1030 (phenyl), 960 (imp. from *14*?), 918 (*trans*-CD=CH), 899, 837 (>C=CH–), 770 (imp. from *14*?), 756, 727 (*trans*-CD=CH), 702–692 (monosubst. benzene), and 671 cm^{-1} (*cis*-CD=CH); δ ($CDCl_3$) 1.63 s, 1.69 s (6 H, H-9, CH_3-8), 1.82 (3 H, H-4), 2.10–2.23 (4 H, H-5,6), 5.13 m (1 H, H-7), 6.00 and 6.39 (2 H, H-1,3) and 7.05–7.35 (5 aromatic H); *m/e* (110°C) 227 (M), 158 (96 %) and 69 (100 %); D_0:D_1 = 18:82; 82 % deuterium incorporation. *15* was analyzed by GLC (column OV-17) and gave four peaks (*15A*, *15B*, *15C* and *15D*) with retention time (210°C) 15, 18, 24, and 27 min and relative integrals 28:19:43:10, respectively. *15A* and *15C* were separated preparatively in the same GLC system.

1-cis *2-Deuterated 4,8-dimethyl-1-phenylnona-1,3,7-triene* (*15A*) showed ν_{max} (liq.) 3080–2720 (CH), 2240 (CD), 1680, 1630 (conj. phenyl), 1600 (conj. C=C), 1492, 1447 (CH_3), 1376 (CH_2), 1180–1030 (phenyl), 920 (imp. from *15B*, *C*?, 885, 840 (>C=CH–), 757, 730 (imp. from *15B*, *C*?), 700 (monosubst. benzene), and 671 cm^{-1} (*cis*-CD=CH); δ ($CDCl_3$) 1.60 s and 1.68 s (6 H, H-9, CH_3-8), 1.83 (3 H, H-4, 2.06–2.25 (4 H, H-5,6), 5.16 (1 H?, H-7), 6.32–6.39 (2 H?, H-1,3) and 7.25–7.50 (5 aromatic H).

1-trans *2-Deuterated 4,8-dimethyl-1-phenylnona-1,3,7-triene* (*15C*) had ν_{max} (liq.) 3080–2720 (CH), 2250 (CD), 1680, 1630 (conj. phenyl), 1595 (conj. C=C), 1496, 1447 (CH_2), 1376 (CH_3), 1180–1030 (phenyl), 918 (*trans*-CD=CH), 895, 835 (>C=CH–), 770, 750, 727 (*trans*-CD=CH), and 693 cm^{-1} (monosubst. benzene); δ ($CDCl_3$) 1.65 s and 1.71 s (6 H, H-9, CH_3-8), 1.87 s (3 H, CH_3-4), 2.10–2.25 (4 H, H-5,6), 5.13 m (1 H, H-7), 5.87 s (1 H, H-3), 6.45 s (1 H, H-1), and 7.2–7.5 (5 aromatic H).

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Organic Hydroxylamine Derivatives. IX.* Structural Analogues of GABA of the Isoxazole Enol-betaine Type. Improved Synthesis and the Crystal Structure of 3-Hydroxy-5-(2-aminoethyl)isoxazole Zwitterion (Homomuscimol)

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An improved synthesis and the crystal structure determination of homomuscimol, 3-hydroxy-5-(2-aminoethyl)isoxazole zwitterion (IV), which is a structural analogue of δ-aminovaleric acid, are described. The synthesis is based on a reaction sequence in which a carboxylic acid is converted into the corresponding methoxycarbonylamino derivative without isolation of intermediate products. The pK_A values of homomuscimol have been determined to 5.12 ± 0.03 and 9.46 ± 0.02.

Crystals of homomuscimol, $C_5H_8N_2O_2$, are monoclinic, space group $P2_1/n$, $a = 11.530(1)$, $b = 6.203(1)$, $c = 8.782(2)$ Å, $\beta = 104.20(1)°$, $Z = 4$. The structure has been solved by a direct phasing technique using X-ray diffraction data and has been refined by full-matrix least-squares methods. The final R value is 0.042. The crystal structure is made up of homomuscimol zwitterions and is stabilized by a network of hydrogen bonds.

As part of our investigations of GABA analogues of the isoxazole enol-betaine type X-ray analyses of two crystal forms of muscimol have been performed.[1,2] This paper presents an improved synthesis and the crystal structure determination of homomuscimol, 3-hydroxy-5-(2-aminoethyl)isoxazole zwitterion (IV). Homomuscimol (IV) is a structural analogue of δ-aminovaleric acid, which exhibits a physiological activity very similar to that of GABA.[3,4] To our knowledge homomuscimol (IV) has not yet been examined for GABA activity. However, investigations of the biological properties of this compound will be initiated in the near future.

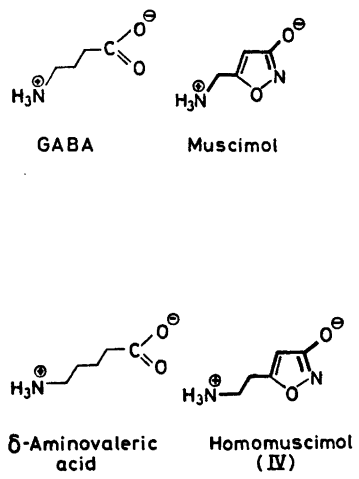
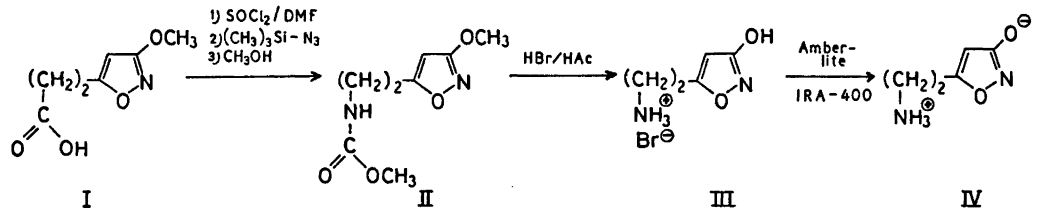


Fig. 1. Some common structural features of GABA/muscimol and δ-aminovaleric acid/homomuscimol.

RESULTS AND DISCUSSION

Homomuscimol (IV) was synthesized by Bowden *et al.*[5] The preparation described by these authors is based on 3-(3-methoxyisoxazolyl-5)-propionic acid (I) and proceeds through several steps, including a Curtius reaction. The reported yield of homomuscimol (IV) is rather low and, since several attempts made at the start of this investigation using the same reaction sequence confirmed this, we were prompted to develop and improve the synthesis. The present synthesis of homomuscimol is also based on 3-(3-

* Part VIII. *Acta Chem. Scand.* 27 (1973) 3251.



methoxyisoxazolyl-5)propionic acid (I). It is converted into 3-methoxy-5-(*N*-methoxycarbonyl-2-aminoethyl)isoxazole (II) without isolation of intermediate compounds. The reaction is an extension of the general sequence for the preparation of isocyanates,[6] in which acid chlorides are reacted with trimethylsilyl azide to isocyanates without isolation of the acyl azide intermediates. As the acid chloride of 3-(3-methoxyisoxazolyl-5)propionic acid (I) seemed to be very unstable it is allowed to react with trimethylsilyl azide without purification. The intermediate acyl azide is transformed into the corresponding isocyanate, and the reaction sequence is completed by addition of methanol to the reaction mixture to give 3-methoxy-5-(*N*-methoxycarbonyl-2-aminoethyl)isoxazole (II) in good yields. This is then cleaved by hydrogen bromide in glacial acetic acid to give 3-hydroxy-5-(2-aminoethyl)isoxazole hydrobromide (III). Homomuscimol (IV) is isolated from (III) in good yields and in a pure state using a strongly basic ion exchange resin. The stoichiometric pK_A values of homomuscimol are 5.12 ± 0.03 and 9.46 ± 0.02.

The X-ray diffraction analysis of homomuscimol confirms the zwitterionic structure. The molecular dimensions and the conformation of the molecule are shown in Figs. 2a and 2b.

The isoxazole ring is planar within the limits of experimental error; Table 1 lists the displacements of some atoms from the least-squares plane through the ring. The exocyclic oxygen atom O(2) is in this plane, but the exocyclic carbon atom C(6) is 0.055Å out of it. The plane of the aminoethyl side chain makes an angle of 83.1° with the isoxazole ring plane. The torsion angle τ_1, O(1)−C(5)−C(6)−C(7), is ±51.7° and τ_2, C(5)−C(6)−C(7)−N(1), is ±70.3°. The hydrogen atoms are tetrahedrally

Table 1. Distances of atoms from least squares plane (Å). The equation of the plane is in direct (unit-cell) space.

Isoxazole ring
Equation: $-3.5282x + 2.9683y + 7.6662z - 3.5116 = 0$

Atom	Deviation	Atom	Deviation
O(1)	0.002	C(6)[a]	−0.055
N(2)	−0.004	C(7)[a]	0.993
C(3)	0.005	N(1)[a]	2.361
C(4)	−0.003	H(41)[a]	−0.042
C(5)	0.001	H(61)[a]	−0.015
O(2)[a]	0.001	H(62)[a]	−0.947

[a] These atoms were not included in the calculation of the least squares plane.

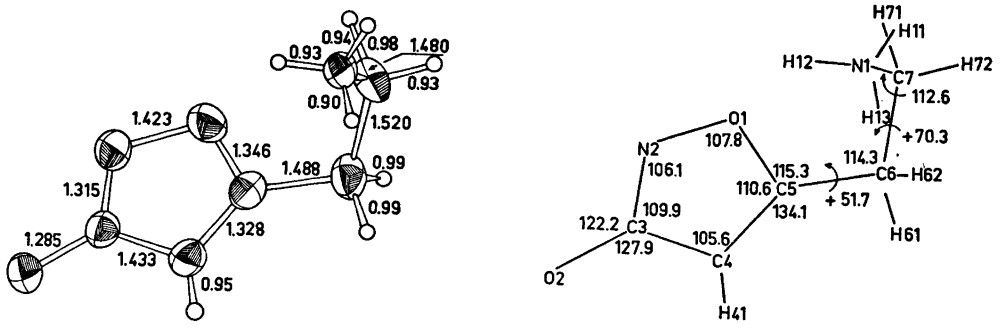


Fig. 2. (a) Bond lengths (Å), and thermal ellipsoids for the non-hydrogen atoms drawn to enclose 50 % probability. (b) The numbering of the atoms, and some bond angles (°) and torsion angles (°).

Table 2. Intra-molecular distances (Å). The estimated standard deviations ($\times 10^2$ for bonds to hydrogen, $\times 10^3$ for others) of the distances are given in parentheses.

O(1)–N(2)	1.423(2)	C(6)–H(61)	0.99(2)
N(2)–C(3)	1.315(2)	C(6)–H(62)	0.99(2)
C(3)–C(4)	1.433(2)	C(7)–H(71)	0.98(2)
C(4)–C(5)	1.328(3)	C(7)–H(72)	0.93(2)
C(5)–O(1)	1.346(2)	N(1)–H(11)	0.94(2)
C(5)–C(6)	1.488(2)	N(1)–H(12)	0.93(2)
C(6)–C(7)	1.520(3)	N(1)–H(13)	0.90(2)
C(7)–N(1)	1.480(3)	N(1)···N(2)	4.704(2)
C(3)–O(2)	1.285(2)	N(1)···O(1)	3.617(2)
C(4)–H(41)	0.95(2)	N(1)···O(2)	5.979(2)

arranged about the nitrogen atom. Their positions correspond to a 10–20° rotation about the C–N bond relative to that for a strictly staggered conformation.

Tables 2 and 3 and Figs. 2a and 2b show the *intra*-molecular distances and angles found. The bond lengths and angles agree well with their equivalents in other isoxazole compounds.[2,7-11] Thus, it is evident that there is significant resonance within the isoxazole ring, all the bonds except the N–O bond having a significant partial double bond character. The geometry of the aminoethyl side chain does not exhibit unusual

Table 3. Valency angles(°). The estimated standard deviations ($\times 10$ for angles not involving hydrogen) of the angles are given in parentheses.

C(5)–O(1)–N(2)	107.8(1)
O(1)–N(2)–C(3)	106.1(1)
N(2)–C(3)–C(4)	109.9(1)
N(2)–C(3)–O(2)	122.2(1)
C(4)–C(3)–O(2)	127.9(1)
C(3)–C(4)–C(5)	105.6(1)
C(4)–C(5)–O(1)	110.6(1)
C(6)–C(5)–O(1)	115.3(1)
C(6)–C(5)–C(4)	134.1(1)
C(5)–C(6)–C(7)	114.3(2)
C(6)–C(7)–N(1)	112.6(2)
C(3)–C(4)–H(41)	122(1)
C(5)–C(4)–H(41)	133(1)
C(5)–C(6)–H(61)	108(1)
C(5)–C(6)–H(62)	107(1)
C(7)–C(6)–H(61)	112(1)
C(7)–C(6)–H(62)	108(1)
H(61)–C(6)–H(62)	106(2)
C(6)–C(7)–H(71)	114(1)
C(6)–C(7)–H(72)	111(1)
N(1)–C(7)–H(71)	105(1)
N(1)–C(7)–H(72)	108(1)
H(71)–C(7)–H(72)	105(2)
C(7)–N(1)–H(11)	105(1)
C(7)–N(1)–H(12)	110(1)
C(7)–N(1)–H(13)	111(1)
H(11)–N(1)–H(12)	112(2)
H(11)–N(1)–H(13)	104(2)
H(12)–N(1)–H(13)	114(2)

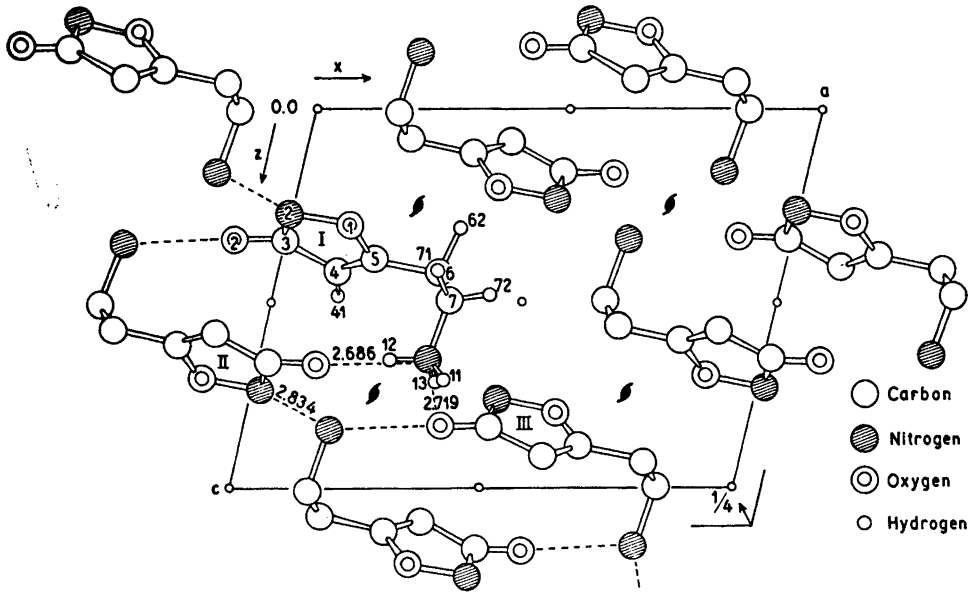


Fig. 3. Projection of the structure down the *b* axis. Some of the shorter *inter*-molecular contacts are indicated.

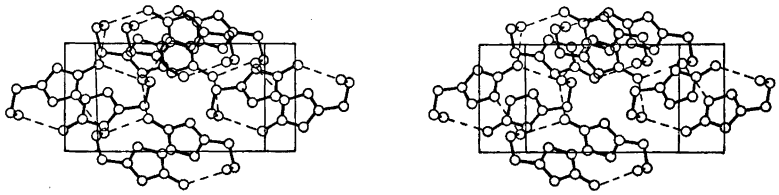


Fig. 4. Stereo diagram illustrating the molecular packing. The view is down c^*, with b vertical, and a horizontal. Hydrogen bonds are drawn with broken lines.

Table 4. Close *inter*-molecular contacts involving hydrogen and non-hydrogen atoms. Distances (Å) and angles (°).

A−H···B	B equipoint	A−H	A···B	H···B	∠AHB	∠HAB	∠HBA
$N(1)-H(11)\cdots N(2)_{IV}$	$(\frac{1}{2}+x,1\frac{1}{2}-y,\frac{1}{2}+z)$	0.94(2)	2.834(2)	1.90(2)	173(2)	5(1)	2.3(6)
$N(1)-H(12)\cdots O(2)_{II}$	$(\bar{x},1-y,1-z)$	0.93(2)	2.686(2)	1.77(2)	168(2)	8(1)	4.0(6)
$N(1)-H(13)\cdots O(2)_{III}$	$(\frac{1}{2}+x,\frac{1}{2}-y,\frac{1}{2}+z)$	0.90(2)	2.719(2)	1.83(2)	169(2)	7(1)	3.6(6)
$C(6)-H(62)\cdots O(1)_{V}$	$(\frac{1}{2}-x,-\frac{1}{2}+y,\frac{1}{2}-z)$	0.99(2)	3.366(3)	2.68(2)	127(1)	39(1)	13.6(5)
$C(7)-H(72)\cdots N(2)_{IV}$	$(\frac{1}{2}+x,1\frac{1}{2}-y,\frac{1}{2}+z)$	0.93(2)	3.420(2)	3.12(2)	101(1)	64(1)	15.4(4)
$C(4)-H(41)\cdots O(2)_{VI}$	$(\bar{x},\bar{y},1-z)$	0.95(2)	3.433(2)	2.53(2)	158(2)	16(1)	5.9(5)

features. The *intra*-molecular distance $N(1)^+\cdots O(2)^-$ is 5.979 Å.

The anisotropic thermal parameters of the non-hydrogen atoms are given in Table 5 and are drawn schematically in Fig. 2a.

Figs. 3 and 4 illustrate the packing of the homomuscimol molecules in the crystals. The crystal structure is made up of homomuscimol zwitterions and is stabilized by a network of hydrogen bonds, one for each hydrogen atom covalently bonded to nitrogen. Hydrogen bonded pairs result from the formation of bonds $[N(1)-H(12)\cdots O(2)_{II}$; $O(2)\cdots H(12)_{II}-N(1)_{II}]$ between pairs of molecules related by a centre of symmetry. The n-glideplane-related molecules are bound head to tail by the hydrogen bond $N(1)-H(13)\cdots O(2)_{III}$, thereby forming infinite chains in the [101] direction. These chains are connected in the b direction by the hydrogen bond $N(1)-H(11)\cdots N(2)_{IV}$. Thus, each molecule is involved in six hydrogen bonds to five different neighbouring molecules. A summary of the geometry of the hydrogen bonding between molecules together with a few other close contacts is shown in Table 4.

EXPERIMENTAL

The determination of melting points, the recording of IR, UV, and ^1H NMR spectra, and the performance of microanalyses were accomplished as described in a previous paper.[12] The pH values were measured on a Radiometer pH meter 26.

The computations were performed on a GIER computer and an IBM 360/75 computer using *INDIFF*,[13] a local version of *The N.R.C. 2A Picker Data Reduction Program*,[14] *The X-Ray System*,[15] and *ORTEP*.[16] The X-ray atomic scattering factors used were those of Cromer and Mann [17] for oxygen, nitrogen, and carbon, and of Stewart, Davidson, and Simpson [18] for hydrogen.

Synthesis

3-Methoxy-5-(N-methoxycarbonyl-2-aminoethyl)isoxazole (II). To a mixture of 1.71 g (10 mmol) of 3-(3-methoxyisoxazolyl-5)propionic acid (I) [19] and thionyl chloride (8 ml) 0.5 ml of *N,N*-dimethylformamide was added dropwise and with stirring. After stirring for further 2 min the solution was evaporated *in vacuo* to give an oil, which was extracted with three 6 ml portions of ether. The combined ether phases were evaporated *in vacuo* to give a brownish oil, which was dissolved in tetrachloromethane (6 ml), and upon addition of 1.27 g (11 mmol) of trimethylsilyl azide the solution was refluxed for 90 min. After cooling to room temperature 1.60 g (50 mmol) of methanol were added, and the solution was refluxed for further 90 min. After cooling to room temperature the solution was concentrated *in vacuo* to give an oil, which was dissolved in ether (15 ml). To the ethereal solution was added with stirring petroleum ether (5 ml) and diatomaceous earth

(0.5 g). After filtration the solution was evaporated *in vacuo* to give 1.7 g of a crystalline product. Recrystallization (ether-petroleum ether) afforded 1.21 g (61 %) of compound (II) as colourless crystals, m.p. 56.5 – 57.5°C. (Found: C 48.05; H 6.09; N 14.04. Calc. for $C_8H_{12}N_2O_4$: C 47.99; H 6.04; N 13.99). λ_{max} 212 nm ($\varepsilon = 6.50 \times 10^3$). IR data (KBr) cm^{-1}: 3265 (s, NH); 1708 and 1680 (both s, monosubstituted urethane C=O); 1618 and 1560 (both s, isoxazole-ring). ^1H NMR data (CCl_4) δ: 5.8 – 5.3 (broadened signal, 1 H, N*H*); 5.59 (s, 1 H, C=C*H*–C); 3.83 (s, 3 H, O–CH_3); 3.55 (s, 3 H, CO–OCH_3); 3.5 – 3.2 (t, 2 H, NH–CH_2–CH$_2$); 3.0 – 2.6 (t, 2 H, CH$_2$–CH_2–C=).

3-Hydroxy-5-(2-aminoethyl)isoxazole hydrobromide (III). A solution of 1.32 g (6.6 mmol) of (II) in 13 ml of glacial acetic acid containing 43 % of hydrogen bromide was heated in an oil bath (bath temperature: 90°C) for 45 min. After cooling to room temperature the reaction mixture was evaporated *in vacuo* to give an oil, which was dissolved in methanol (10 ml), filtered and evaporated *in vacuo* to give an oil, which crystallized after having been dried *in vacuo* over potassium hydroxide for 1 h. Recrystallization (methanol-ether) gave 1.13 g (82 %) of compound (III) as colourless crystals, m.p. 171 – 173°C (decomp.). (Found: C 28.60; H 4.29; N 13.33; Br 38.20. Calc. for $C_5H_9N_2O_2Br$: C 28.72; H 4.34; N 13.40; Br 38.22). λ_{max} 209 nm ($\varepsilon = 7.13 \times 10^3$). IR data (KBr) cm^{-1}: 3600 – 2100 and 1980 (s and w, respectively, NH_3^+); 1623 and 1535 (both s, isoxazole-ring). ^1H NMR data (DMSO-d_6) δ: 11.7 – 9.7 (broad signal, 1 H, O*H*); 8.5 – 7.4 (broad signal, 3 H, NH_3^+); 5.95 (s, 1 H, C=C*H*–C); 3.4 – 2.6 (m, 4 H, NH_3^+–CH_2–CH$_2$ and CH$_2$–CH_2–C=).

3-Hydroxy-5-(2-aminoethyl)isoxazole zwitterion (IV). 1.48 g (7.1 mmol) of (III) were dissolved in water (10 ml) and passed through a column containing an ion exchange resin (Amberlite IRA 400, (OH), 130 ml) using acetic acid (1 N) as an eluent. The fractions containing compound (IV) were collected and evaporated to dryness *in vacuo*. The residue was dissolved in water (10 ml) and the solution was treated with a mixture of activated charcoal and diatomaceous earth, filtered, and evaporated *in vacuo* to give an oil. Crystallization from water-ethanol gave 564 mg (62 %) of homomuscimol (IV) as colourless crystals, m.p. 167°C (decomp.) [Ref. 5, m.p. 158 – 160°C (decomp.)]. λ_{max} 210 nm ($\varepsilon = 6.05 \times 10^3$). The IR spectrum is identical with that of a sample prepared according to the method described by Bowden *et al.*[5]

Determination of the stoichiometric pK_A values

The stoichiometric pK_A values of homomuscimol (IV) have been determined according to the method described by Albert and Serjeant.[20]

Solutions of homomuscimol hydrobromide (III) 0.005 M and of homomuscimol (IV) 0.005 M were titrated with 0.1 N sodium hydroxide. pH Values were recorded after nine different additions of titrant between 10 and 80 % of neutralization, and the pK_A values were calculated using the formula:

$$pK_A = pH - \log \frac{[Base] + [H^+] - [OH^-]}{[Acid] - [H^+] + [OH^-]};$$

[Base] = the stoichiometric concentration of base, [Acid] = the stoichiometric concentration of acid. The final average stoichiometric pK_A values for homomuscimol (IV) are 5.12 ± 0.03 and 9.46 ± 0.02.

X-Ray analysis

The homomuscimol used for the X-ray examination was recrystallized by diffusion at room temperature of absolute ethanol into an aqueous solution of the compound. Colourless prisms somewhat elongated in the *c* direction were formed.

Crystal data. 3-Hydroxy-5-(2-aminoethyl)isoxazole (homomuscimol), $C_5H_8N_2O_2$, M = 128.14. Monoclinic, $a = 11.530(1)$, $b = 6.203(1)$, $c = 8.782(2)$ Å, $\beta = 104.20(1)°$, $U = 608.9$ Å^3, $D_m = 1.39$ g cm^{-3}, $Z = 4$, $D_c = 1.398$ g cm^{-3}. Linear absorption coefficient for X-rays [λ(Mo$K\alpha$) = 0.7107 Å], $\mu = 1.18$ cm^{-1}. Number of electrons per unit cell, $F(000) = 272$. Systematically absent reflections: $h0l$ when $h+l$ odd, $0k0$ when k odd; space group $P2_1/n$.

The unit-cell parameters were refined by least-squares techniques from the diffractometer-measured θ angles observed for 35 reflections well distributed in reciprocal space. The crystal density was measured by flotation in a chlorobenzene/bromobenzene mixture.

Data collection. Three-dimensional diffraction data were measured at room temperature on a Nonius three-circle automatic diffractometer using graphite monochromated Mo$K\alpha$ radiation. The ω scan technique with a scan speed of 1.2° min^{-1} was employed. Background counts were taken for half the scanning time at each of the scan range limits. One standard reflection was measured after every 25 reflections.

All the data were measured from a single crystal with approximate dimensions $0.25 \times 0.29 \times 0.39$ mm. The crystal was mounted with [001] along the ϕ axis of the goniostat.

Out of the 1323 independent reflections measured in the range $2.5° \leq \theta \leq 27°$, 996 had $I_{net} \geq 3.0\sigma(I)$, where $\sigma(I)$ is the standard deviation from counting statistics. These were regarded as observed reflections, whereas the remaining reflections were regarded as unobserved and excluded from the refinement procedure. Lorentz and polarization corrections were applied, but no absorption corrections were made owing to the low absorption coefficient.

Table 5. Final positional and thermal (Å²) parameters for non-hydrogen atoms. The estimated standard deviations of positional ($\times 10^4$) and thermal ($\times 10^2$) parameters are given in parentheses. The temperature expression is of the form:

$$\exp -\tfrac{1}{4}[(h^2a^{*2}B_{11}+k^2b^{*2}B_{22}+l^2c^{*2}B_{33}+2hka^*b^*B_{12}+2hla^*c^*B_{13}+2klb^*c^*B_{23})]$$

	x/a	y/b	z/c	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
O(1)	0.1106(1)	0.5446(2)	0.2984(2)	2.87(6)	3.11(6)	4.87(7)	−0.21(4)	0.29(5)	1.10(5)
N(2)	−0.0115(1)	0.4809(3)	0.2660(2)	2.47(6)	3.24(7)	4.76(8)	0.18(5)	−0.03(5)	0.78(6)
C(3)	−0.0134(1)	0.2956(3)	0.3381(2)	2.38(6)	2.47(7)	2.68(6)	0.07(5)	0.44(5)	−0.46(5)
C(4)	0.1061(1)	0.2330(3)	0.4162(2)	2.72(7)	2.54(7)	2.74(6)	0.29(5)	0.38(5)	0.19(5)
C(5)	0.1761(1)	0.3904(3)	0.3880(2)	2.59(6)	2.76(6)	2.25(6)	0.29(5)	0.30(5)	−0.35(5)
C(6)	0.3078(2)	0.4255(3)	0.4278(2)	2.52(7)	3.85(8)	3.43(7)	−0.22(6)	0.70(6)	−0.99(7)
C(7)	0.3471(2)	0.6457(3)	0.4973(2)	2.60(7)	3.66(8)	3.43(8)	−0.72(6)	0.77(6)	−0.22(6)
N(1)	0.3303(1)	0.6713(3)	0.6581(2)	2.20(5)	2.45(5)	3.16(6)	−0.10(5)	0.27(4)	−0.26(5)
O(2)	−0.1110(1)	0.1927(2)	0.3325(2)	2.49(5)	3.13(6)	4.77(6)	−0.27(4)	0.78(4)	−0.64(5)

Structure determination. The observed structure amplitudes were brought onto an absolute scale by Wilson statistics and normalized structure amplitudes, $|E(hkl)|$, were calculated.

The phase problem was solved in a straightforward way by direct methods using the programs of the X-Ray System, 1972 edition.[16]

In an E map based on 100 $E(hkl)$'s with $|E(hkl)| \geq 1.5$ the nine highest peaks formed a recognizable molecule and in fact all the peaks corresponded to the positions of the nine nitrogen, oxygen, and carbon atoms. Individual atomic parameters of this model were refined, first with isotropic and then anisotropic thermal parameters using the full-matrix least-squares method. On convergence the R value was 0.084. The quantity minimized was $\sum w(|F_o|-|F_c|)^2$, where weights were initially taken as unity.

A three-dimensional difference Fourier synthesis then showed maxima with peak heights of 0.5 − 0.7 e Å^{-3} in positions expected for all the hydrogen atoms, and there was no extraneous peaks greater than the lowest hydrogen atom. Introduction of the hydrogen atoms in the refinement, with the isotropic temperature factors of the connected non-hydrogen atoms as constant parameters, reduced the R value to 0.047.

The least-squares refinement was completed with the introduction of a weighting scheme of the form: $w = 1/(1+[(|F_o|-b)/a]^2)$, where $a = 3.5$e and $b = 6.0$e. On the last cycle of least-squares refinement shifts of all the parameters were less than one tenth of their estimated standard deviations and the final R value is 0.042. Tables 5 and 6 list the final positional and thermal parameters for the non-hydrogen atoms and hydrogen atoms, respectively. From these parameters the terminal set of structure factors, listed with the observed data in Table 7, was computed. Comparison of the 100 signs determined by direct methods with the corresponding phases computed from the refined structure shows that all had been correctly assigned.

Table 6. Final positional and thermal (Å²) parameters for hydrogen atoms. The estimated standard deviations ($\times 10^3$) of the coordinates are given in parentheses.

	x/a	y/b	z/c	B_{iso}
H(41)	0.122(2)	0.099(3)	0.471(2)	2.6
H(61)	0.347(2)	0.306(3)	0.497(2)	3.3
H(62)	0.334(2)	0.411(3)	0.329(2)	3.3
H(71)	0.303(2)	0.766(3)	0.437(2)	3.2
H(72)	0.427(2)	0.671(3)	0.501(2)	3.2
H(11)	0.381(2)	0.785(3)	0.702(2)	2.9
H(12)	0.251(2)	0.702(3)	0.653(2)	2.9
H(13)	0.359(2)	0.557(3)	0.719(2)	2.9

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Table 7. Observed structure amplitudes $|F_o|$ and calculated structure factors F_c (in electrons × 10). Within each groups of reflections with constant k and l, the columns list, from left to right, h, $10|F_o|$, and $10F_c$. Reflections considered unobserved are marked with an asterisk.

	H,0,0		13	86	85	-6	86	84	-6	73	70	-9	53	-56	-5	59	57
				H,1,1		-7	40	-39	7	44	-44	10	38	-40		H,0,4	
2	56	47				8	24	-22	-7	79	81	-10	32	-34			
4	488	-513				-8	57	61	8	159	-157	11	51	48			
6	344	326	0	468	542	-9	36	34	-8	118	-121	-11	107	-110	0	103	-102
8	159	155	1	344	-370	10	36	-35	9	54	59	12	28	-23	2	93	90
10	179	179	-1	215	-199				-9	80	80	-13	41	-43	-2	175	183
12	66	64	-2	156	161		H,6,1		10	45	-44	-14	24	-17	4	208	204
14	94	-83	-2	500	-559				-10	32	-32				-4	177	-179
			3	211	-223	0	42	-38	11	45	46		H,2,3		6	46	-46
	H,1,0		-3	104	-117	1	109	110	-11	26	27				-6	55	55
			4	95	-92	-1	51	-52	12	41	32	0	178	166	8	63	62
1	228	-225	-4	13	-19	2	89	94				1	16	-9	-8	100	102
2	211	-228	5	116	115	3	26	-17		H,4,2		-1	101	-97	10	36	30
3	494	-419	-5	40	36	-3	76	-75				2	59	-56	-10	146	-148
4	279	279	6	94	-24	4	107	103	0	163	-160	-2	228	-227	-12	43	-47
7	87	-79	-6	403	406	-4	144	146	1	92	93	3	69	68	-14	35	38
8	103	-97	7	67	-65	5	44	-46	-1	120	-123	-3	252	-240			
9	129	-123	-7	28	-23	-5	19	16	2	61	-61	4	131	-128		H,1,4	
10	134	132	8	132	-131	6	66	63	-2	43	-45	-4	62	63			
11	41	-38	9	193	-191	-6	63	64	3	197	201	5	28	-29	0	135	-137
12	58	55	-9	61	-60	7	43	46	4	63	60	-5	60	61	1	117	-112
14	27	-30	10	25	-21	-8	21	-23	-4	83	-81		68	71	-1	70	-72
			-10	31	34				5	23	24	-6	58	59	2	20	-30
	H,2,0		-11	50	-49		H,7,1		-5	102	99	7	162	-163	-2	58	-55
			12	33	32				6	124	-123	-7	150	147	3	49	47
0	158	-165	-12	25	20	1	60	-59	7	55	-55	8	82	-80	-3	318	-312
1	28	-33	-13	26	27	-1	67	65	-7	63	64	-8	55	-61	4	94	-92
2	39	39				3	96	-100	8	105	-108	9	41	-46	-4	59	59
3	136	-133		H,2,1		-3	47	-46	-8	22	24	-9	33	-31	6	46	-45
4	174	162				4	59	-58	9	21	21	10	37	-33	-6	128	-125
5	36	31	0	151	163	-4	31	-30	-9	44	46	-10	79	82	7	157	-158
6	43	45	1	181	-196	5	21	20	10	24	21	12	34	34	-7	198	199
8	25	-22	-1	387	403	-5	44	-45	-10	39	-37	-12	29	-32	8	33	-34
9	106	-104	2	274	-278	-6	27	-26	11	31	29				-8	46	-47
10	18	18	-2	63	62				-11	55	-54		H,3,3		9	54	-53
11	34	38	3	94	-96		H,0,2								-9	113	-113
12	18	-17	-3	191	202					H,5,2					10	34	-33
13	23	-17	4	280	282	0	292	-288				0	93	-96	-10	72	74
			-4	98	93	2	148	-142	0	37	-35	-1	106	102	11	54	-56
	H,3,0		5	64	60	-2	616	-677	1	116	118	2	105	-104	12	44	-47
			-5	195	-185	4	199	-196	-1	26	-28	-2	28	27	-13	32	32
1	347	-358	6	291	294	-4	59	57	-2	55	52	3	31	32			
2	93	93	-6	207	203	6	90	-88	3	43	47	-3	28	29			
3	220	219	7	48	49	-6	104	111	-3	125	123	-4	199	201		H,2,4	
4	42	35	-7	99	-97	8	132	-131	-4	93	93	5	110	114			
5	138	130	8	181	-184	-8	231	-225	5	28	20	-6	41	48	0	240	237
6	16	-13	-8	17	-22	10	181	-176	-5	56	56	6	110	114	1	191	-188
7	96	-99	9	100	101	12	20	-19	-6	38	-43	-6	87	85	-1	59	60
8	103	-102	-9	68	69				7	124	127	7	56	-58	2	56	-57
9	46	-44	10	19	18		H,1,2		-7	73	-73	-7	31	28	-2	377	374
11	19	-25	-10	83	-83				8	33	33	-8	54	-54	3	71	69
12	45	44	-11	55	57	0	695	814	-8	23	-24	-9	98	-104	-3	206	202
			12	96	91	1	149	147	-9	43	39	-10	77	80	4	61	-61
	H,4,0		-12	35	32	-1	621	680	-10	47	47	-12	52	-52	-4	113	112
			13	53	-49	2	267	-260	-11	24	25		46	46	5	153	149
0	63	61	-14	36	38	-2	297	289							-5	30	28
1	62	60				3	33	-34		H,6,2			H,4,3		6	106	110
2	203	197		H,3,1		-3	98	95				0	75	74	-6	87	-87
3	171	-170				4	258	-250	0	64	-66	1	72	68	7	35	-37
4	53	56	0	110	-106	-4	72	-68	1	63	64	2	181	-183	8	157	160
5	23	-24	1	259	260	5	124	118	-1	21	14	-2	87	87	-10	138	142
6	88	-86	-1	38	83	-5	44	-46	2	44	-40	-3	112	109	-12	87	92
7	206	206	2	39	-33	6	212	202	3	79	-83	4	63	-63	-14	35	-32
8	130	131	-2	189	183	7	121	114	-3	122	-123	-4	63	-66			
9	35	-32	3	185	-182	-7	63	59	4	67	-65	5	36	34		H,3,4	
10	87	88	-3	53	44	8	42	43	-4	40	42	-5	39	41	0	175	172
11	63	-61	4	70	69	-8	21	22	5	53	-56	6	56	60	1	57	-60
12	31	-28	-4	123	-121	9	139	139	-5	51	-49	-6	129	-128	-1	143	-140
			5	134	-131	-9	22	23	-6	58	-58	7	52	52	2	18	-16
	H,5,0		-5	133	-131	-10	148	148	-7	34	-33	-7	35	-33	-2	29	-30
			6	180	-179	11	74	77	8	21	21	8	24	19	3	76	-78
1	14	-12	-6	134	-131	12	19	15	-8	41	-44	-8	91	-88	-3	29	-23
2	121	121	7	116	123	-12	66	65	-9	28	-24	-9	68	71	-4	16	-20
3	172	-164	-7	121	-116	-14	45	-41				10	33	33	5	99	-104
4	84	-85	-8	99	102					H,7,2		-10	47	42	-5	57	-59
5	106	-105	9	54	53		H,2,2					-11	43	45	6	69	70
6	20	18	-9	185	184				1	27	23				-7	146	-148
7	42	-40	10	100	100	0	208	-209	-2	24	25		H,5,3		-8	27	-26
8	67	68	-10	34	33	1	165	160	-4	35	38				-9	22	-20
9	46	-42	12	68	-69	-1	108	103				-1	42	-43	10	27	31
10	21	-22	-12	38	-37	2	117	-110		H,0,3		2	68	-66	-10	48	53
			-13	55	-52	-2	183	-178				3	59	60	-11	25	26
	H,6,0					3	244	-246	1	208	209	-3	20	-19	-12	44	-42
				H,4,1		-3	153	-153	-1	754	815	4	85	-86	-13	53	-55
0	41	39				4	121	119	2	25	-9	-4	87	-92			
1	33	-31	0	16	-15	-4	91	-90	-3	37	-29	5	76	76		H,4,4	
2	34	32	1	110	-105	5	80	-77	5	64	-62	-5	107	108			
3	29	-30	-1	92	-90	-5	179	-173	-5	185	-180	6	34	-33	0	40	41
5	21	-17	2	119	126	6	60	-60	7	17	-8	-6	107	-109	1	75	-75
6	54	51	-2	141	-138	-6	66	58	-7	244	248	7	21	-23	-1	38	37
7	24	-23	3	70	69	7	33	34	9	164	167	-7	28	28	2	36	39
8	28	24	-4	79	81	-7	82	-80	-9	126	126	8	21	-19	-2	37	-33
			4	54	49	8	88	-84	11	21	-17	-9	54	-58	3	75	-77
	H,7,0		5	67	66	-8	61	-63	-11	75	77				-3	104	-103
			6	52	-56	-9	78	80	-13	23	19		H,6,3		4	41	40
2	65	67	7	32	32	10	23	27							-4	29	23
3	16	11	-7	112	-117	-10	98	-97		H,1,3		0	21	-21	5	38	40
4	43	40	-9	51	-54	11	41	41				1	69	72	-5	33	31
6	32	-35	10	46	44	-11	34	-29	0	209	-222	-1	47	-46	6	38	40
			-12	36	-36	-12	27	-26	1	64	62	3	42	47	-6	36	40
	H,0,1					-13	64	-65	-1	344	-356	-3	27	28	7	66	-67
				H,5,1					2	135	-140	4	26	-27	-7	36	34
1	254	-257					H,3,2		-2	72	76	-4	19	2	8	19	-15
-1	184	-181	0	61	-60				3	217	223	5	54	-54	-8	25	-26
3	204	-222	1	76	83	0	99	-99	-3	78	-79	6	47	-47	9	40	-38
-3	313	-337	-1	82	-81	1	72	68	-4	27	-19	-9	67	66	-9	51	-51
5	321	315	2	96	97	-1	180	179	5	70	72				-11	27	25
-5	214	211	3	20	-20	2	196	-190	-5	151	149		H,7,3				
7	84	82	-3	63	-66	-2	163	-153	-6	134	-138					H,5,4	
-7	129	-121	4	49	48	3	56	57	7	50	-51	1	78	82			
9	62	-58	-4	94	94	4	65	60	-7	55	56	-1	30	-27	0	109	114
-9	307	-311	5	116	-118	-4	114	112	8	63	64	2	55	60	-1	112	-113
11	19	-23	-5	22	-21	-5	16	-16	-8	121	-120	3	29	25	2	21	19
-11	48	-48	6	33	-39	6	138	138	9	22	-23	-3	37	-39			

Table 7. Continued.

	H.5.4		-9	63	-63	3	141	143	-8	39	-42		H.1.9		5	15*	17							
			-10	85	88	-3	212	214	-12	40	-40													
-2	51	-52	-12	22	-24	4	35	33				0	44	40		H.0.1								
3	88	94	-13	23	-21	-4	69	68		H.4.7		1	38	41										
-3	41	41				5	35	39				-1	27	-25	-13	13*	1							
4	31	-35		H.4.5		-5	28	25	0	65	-66	-3	41	-38										
-4	118	-120				7	27	27	-1	40	39	4	45	-43		H.1.1								
5	25	-24	0	87	89	-7	42	-37	-2	27	-32	5	30	-32										
-5	37	37	1	63	-63	-9	52	54	4	23	-26	-5	31	-25	-8	3*	5							
6	50	-46	-1	52	-52	-10	30	33	-4	23	-22	-7	25	-20	11	3*	7							
-6	31	29	2	103	106	-12	39	37	-5	48	-47	-8	20	15	13	17*	11							
7	58	-61	-2	98	-98				6	65	-63	-11	22	-17	-14	10*	6							
-7	38	37	3	18	21		H.4.6		-9	38	36													
-9	41	41	-3	78	79								H.2.9			H.2.1								
-10	19	8	4	74	72	0	63	-63		H.5.7														
-11	47	-47	-4	54	52	-1	63	62				1	56	-56	11	7*	-12							
			5	50	-49	2	33	31	0	23	22	-1	58	-56	-13	18*	-15							
	H.6.4		-5	55	-55	-2	61	-60	1	42	41	-2	42	40										
			-6	180	176	3	82	-87	2	34	38	3	28	-31		H.3.1								
0	43	37	-7	87	-86	-3	32	-32	-2	63	-63	-4	51	-51										
1	29	30	8	25	-25	-4	85	88	3	33	34	-6	22	22	8	3*	0							
-1	26	-23	-9	33	35	7	64	64	-4	44	43	-7	40	-39	11	12*	12							
-2	24	22	-12	26	20	-7	85	-87	-6	32	30	-8	50	51	-11	3*	2							
3	28	29				-8	33	-37				-9	42	-36										
-3	44	42		H.5.5		-9	55	-52		H.6.7						H.4.1								
5	32	32				-10	29	26					H.3.9											
-5	88	91	0	73	73	-11	64	65	-3	37	-33					3*	-2							
6	36	33	1	28	-28				-5	61	59	0	21	17	3	13*	9							
-7	27	-26	2	19	-19		H.5.6					1	42	-44	-5	16*	-14							
-9	21	-20	-2	30	26					H.0.8		-1	32	-31	8	8*	-4							
			3	73	-76	1	19	-25				-2	54	51	-8	3*	2							
	H.7.4		-4	31	33	-1	37	37	0	23	-21	3	24	20	9	5*	-7							
			-5	58	58	2	35	37	2	70	-69	-3	54	56	10	3*	-2							
0	49	-53	6	76	73	-2	67	-70	-2	70	65	-4	54	54	11	15*	12							
1	29	-25	7	44	47	3	41	-43	4	23	-24	-5	25	26	-11	6*	-5							
-2	20	13	-7	59	-63	-3	38	-34	-4	90	91		H.4.9			H.5.1								
3	74	-73	-8	34	-30	-4	20	16	6	98	94													
-5	44	-45	-9	70	-64	-5	44	-44	-8	60	-60													
			-10	29	22	-6	70	70	-12	80	81	-2	48	47	-2	6*	-5							
	H.0.5					-8	37	-35				-3	43	-45	7	3*	-13							
				H.6.5						H.1.8		-4	31	-32	9	14*	17							
1	249	-250					H.6.6					-5	32	29	-10	17*	-10							
-1	178	182	1	45	-45				0	46	44	-6	24	-19	-11	3*	-3							
3	195	-198	3	37	-32	0	73	73	1	36	36													
-3	80	-79	-4	41	-43	1	28	32	-1	57	-60		H.0.10			H.6.1								
5	83	84	5	36	35	2	25	-23	2	22	-25													
-5	49	-51	-5	23	-19	-2	41	-38	-2	81	82	-2	41	-42	-2	17*	-16							
7	19	22				3	40	37	-3	21	18	-4	28	-29	-7	3*	1							
9	77	-77		H.7.5		-4	29	-26	4	53	56	-6	39	-43	8	16*	12							
-9	178	-178				-6	23	20	5	77	-79				-9	15*	-12							
11	33	-27	-2	28	-30	-7	28	28	-5	43	-40		H.1.10											
-11	41	40	-3	29	29				6	32	30					H.7.1								
-13	88	92					H.0.7		-6	43	41	0	55	-54										
				H.0.6					7	34	29	2	37	-41	0	16*	18							
	H.1.5						158	155	-7	17	-12	-3	37	37	2	8*	-4							
0	108	110	-2	106	112	-1	36	-31	-8	47	51	-4	27	21	-2	3*	-4							
1	151	-149	4	28	25	3	91	-92	-11	33	-33	-8	23	-16	6	16*	15							
-1	287	286	-4	220	-225	5	102	-100					H.2.10											
-2	238	235	6	27	-30	-5	31	-27		H.2.8						H.0.2								
3	38	-42	-6	153	151	7	66	66				0	29	22	-10	5*	2							
-3	241	236	-8	127	134	-7	32	31	0	25	23	1	23	-14	-12	14*	7							
-4	147	-141	-10	122	-128	-11	20	-23	2	54	54	-1	23	-24	-14	3*	1							
5	88	86	-12	42	43	-13	20	-15	3	48	-49	2	24	23										
-5	112	-112							-3	66	-66	-2	29	-23		H.1.2								
6	37	41		H.1.6			H.1.7		4	37	41	-3	76	76										
-6	18	16							-4	24	-26	-4	21	27	-6	3*	-1							
8	61	62	0	69	-67	0	49	-47	-8	39	41	-6	27	21	10	12*	20							
-8	129	128	1	43	41	1	101	-101	-9	23	-26	-7	31	-30	11	3*	-7							
9	50	53	-1	97	95	-1	93	95	-10	22	-23	-8	20	17	13	9*	-1							
-9	76	77	2	64	65	2	43	-43							-13	3*	5							
-10	31	-27	-2	42	-42	3	110	-109		H.3.8			H.3.10											
-11	36	38	3	58	-55	-3	27	-29								H.2.2								
-12	46	48	-3	16	17	4	23	26	0	28	-27	0	31	34										
-13	30	26	4	121	116	-4	51	-48	2	51	-56	-1	24	-21	9	7*	-1							
			-4	123	-122	5	47	47	-2	71	72	-4	49	-54	12	3*	-1							
	H.2.5		5	18	23	-5	29	-27	-3	58	-54	-5	25	21	-14	8*	-4							
			-6	72	-74	-5	62	57	-4	87	-89													
0	30	-31	-6	28	31	7	25	-24	5	24	-24		H.0.11			H.3.2								
1	16	-15	8	99	101	-8	29	-27	-5	34	-37													
-1	212	-218	8	46	-48	-9	52	-51	-6	60	-56	-1	34	27	-3	7*	-9							
-2	49	45	-8	102	102	-10	95	-95	-7	56	52				5	6*	4							
3	127	128	-9	39	-39	-11	32	31	-9	30	28		H.1.11		-12	9*	-8							
-3	30	-27	-10	84	-86	-13	27	35	-10	27	-24				-13	14*	-11							
-4	42	-40	-11	23	23							-2	31	-35										
5	29	29	-12	97	-99		H.2.7			H.4.8		-3	44	44		H.4.2								
-5	65	64	-13	23	24							-5	38	-33										
6	43	-48				0	45	-47	0	36	-38				-3	7*	2							
-6	109	-104		H.2.6		-1	85	83	-1	20	-15		H.1.0		-6	12*	-16							
-8	36	34				2	92	-96	-2	41	42				-12	3*	-9							
-9	82	-83	0	148	-152	-2	141	142	3	76	75	5	4*	-9										
10	25	25	1	66	-66	3	19	17	-3	39	41	6	10*	0		H.5.2								
-10	41	43	-1	159	160	-3	174	173	-4	72	71	13	12*	-4										
-11	63	-65	-2	87	-88	4	91	94	-5	46	44				2	16*	-14							
-12	79	-80	3	18	-21	-4	26	-19	-6	37	-35		H.2.0		4	13*	-9							
-13	37	-39	-3	102	-106	7	47	47	-7	39	40				6	10*	0							
			4	52	-52	-9	77	81				7	5*	-1	9	15*	12							
	H.3.5		-4	81	-72	-10	45	-45		H.5.8					10	13*	-7							
			5	74	75	-11	34	40					H.3.0											
0	24	20	-5	63	-62	-12	22	23	0	30	-29					H.6.2								
1	107	-104	6	134	-140				-5	35	34	10	14*	13										
-1	44	40	-6	62	-61		H.3.7					13	3*	3	-2	9*	6							
2	138	141	7	90	-92					H.0.9					6	15*	19							
-2	90	-91	-7	121	121	0	128	-130					H.5.0		7	13*	-1							
3	60	-61	8	29	-29	1	61	62	1	59	63													
-3	144	-141	-8	19	16	-1	47	46	-1	52	-52	11	15*	11		H.7.2								
4	63	63	-11	29	-26	2	20	-21	3	80	81													
-4	38	39	-12	28	-27	3	43	40	-3	20	23		H.6.0		0	3*	2							
5	44	46				-3	81	-82	5	50	-50				-1	16*	-17							
-5	44	-39		H.3.6		-4	21	19	-5	42	-44	4	7*	11	2	9*	-5							
6	81	-84				5	31	35	-7	40	41	9	8*	-3	3	12*	-4							
-6	34	31	0	41	-39	-5	30	27	-9	67	69				-3	9*	9							
7	93	-95	1	28	-29	6	28	-27	-11	73	-76		H.7.0		4	4*	-5							
-7	19	15	-2	58	-61	-6	121	-117							5	19*	-18							
-8	59	58	-2	90	89	-7	78	75				1	8*	-2	-5	18*	18							

Table 7. Continued.

	H,7,2		-5	8*	10	8	14*	12	-11	17*	11		H,5,7		-2	14*	6
			11	5*	0	9	3*	-1							3	8*	-6
-6	4*	10	-14	17*	-1					H,4,6		-1	3*	-4	-4	11*	6
							H,3,5					-3	12*	8	-6	15*	-14
	H,1,3			H,2,4					1	17*	17	4	17*	-19	-9	3*	5
						8	8*	9	4	5*	3	-5	3*	1	-10	12*	18
4	14*	9	-7	16*	-24	9	7*	-15	-5	7*	-5	-7	3*	-1			
6	13*	11	-8	3*	8	-11	3*	-3	-5	17*	-19	-8	18*	-14		H,2,9	
-12	16*	-15	9	14*	9				6	3*	-1						
			-9	13*	12		H,4,5		-6	3*	-4		H,6,7		0	16*	17
	H,2,3		10	3*	2										2	11*	-9
			11	8*	2	6	18*	20		H,5,6		0	4*	6	-3	14*	-7
11	12*	21	-11	3*	-8	7	4*	-10				-1	15*	10	4	15*	21
-11	10*	-7	-13	14*	8	-8	3*	2	0	4*	-3	-2	20*	19	-5	10*	-14
-13	3*	-1				-10	12*	6	4	3*	4	-4	18*	22	-10	16*	-13
-14	15*	-12		H,3,4		-11	3*	5	5	19*	15						
									6	16*	-16		H,0,8			H,3,9	
	H,3,3		4	14*	-17		H,5,5		-7	3*	-3						
			-6	6*	-1				-9	5*	-5	-6	3*	5	2	4*	9
1	3*	1	7	3*	-1	-1	3*	5				-10	18*	-19	-6	13*	8
-5	6*	6	8	10*	17	-3	13*	3		H,6,6					-7	5*	-2
9	10*	17	9	3*	-6	4	3*	8					H,1,8		-8	3*	5
10	16*	12				5	15*	15	-1	18*	-18				-9	3*	-6
11	19*	21		H,4,4		-6	11*	8	-3	15*	-17	3	3*	0			
-11	11*	11							-5	16*	-4	-4	10*	-14		H,4,9	
-13	4*	1	-10	5*	11		H,6,5					-10	3*	-8			
			-12	3*	-6					H,0,7		-12	20*	22	0	19*	-14
	H,4,3					0	3*	1							1	18*	15
				H,5,4		-1	15*	5	-9	3*	6		H,2,8		-1	14*	-10
-1	11*	16				2	18*	-19							-7	3*	4
3	15*	13	1	12*	-19	-2	4*	-6		H,1,7		1	3*	0			
9	7*	18	-8	5*	-1	-3	15*	-13				-1	10*	21		H,0,10	
-12	15*	10	-8	17*	19	4	11*	2	2	12*	-4	-2	13*	7			
						-6	3*	6	6	3*	-1	5	12*	11	0	3*	12
	H,5,3			H,6,4		-7	17*	1	-7	4*	-1	-5	9*	-7	2	4*	-11
						-8	18*	-17	-8	15*	8	6	3*	8	-8	9*	-8
0	15*	-19	2	11*	0				-12	19*	19	-6	13*	0			
1	3*	3	4	12*	15		H,7,5					-7	3*	10		H,1,10	
-2	3*	0	-4	3*	-4					H,2,7		-11	3*	9			
8	4*	7	-6	7*	0	0	14*	13							1	17*	-8
9	17*	-8	-8	17*	21	-1	3*	0	1	11*	-20		H,3,6		-1	3*	-4
-10	11*	-6							5	9*	6				-2	14*	14
-11	15*	-4		H,7,4			H,0,6		-5	15*	-10	1	18*	-18	-5	12*	-4
									6	18*	15	-1	7*	-6	-6	9*	-6
	H,6,3		-1	6*	11	2	8*	1	-6	15*	-9	3	3*	4	-7	17*	17
			2	12*	-12	8	3*	-14	-7	10*	-1	4	3*	11	-9	3*	11
2	4*	-8	-3	8*	-7	10	18*	-2	8	4*	-9	-8	3*	13			
-2	14*	-18	-4	3*	-1				-8	14*	12	-11	19*	-18		H,2,10	
-5	3*	2					H,1,6										
-6	16*	6		H,0,5						H,3,7			H,4,8		-5	14*	-14
7	15*	9				-5	4*	1									
-7	12*	18	-7	10*	8	-7	14*	10	-2	17*	12	1	3*	15		H,3,10	
-8	16*	10				9	3*	-11	4	3*	11	2	10*	-7			
				H,1,5					-7	4*	-13	4	16*	19	-2	12*	19
	H,7,3						H,2,6		-9	11*	-14	-8	15*	-22	-3	11*	-14
			2	12*	-12				-10	3*	-7	-9	3*	10	-6	14*	13
0	17*	19	4	12*	10	2	15*	17	-11	9*	1						
-2	4*	-19	7	14*	18	9	4*	0					H,5,8			H,0,11	
4	14*	9	-7	9*	-7	-9	10*	-4		H,4,7							
-4	12*	15	10	16*	15	-11	16*	-16				1	6*	-9	-3	17*	15
-6	15*	13	11	19*	-15	-13	4*	2	1	7*	8	-1	14*	11	-5	4*	7
			-14	14*	18				2	3*	-2	-2	14*	2			
	H,0,4						H,3,6		-3	18*	21	-3	3*	-10		H,1,11	
				H,2,5					-3	18*	-18	-4	9*	14			
12	9*	9				-1	3*	-7	5	16*	-8	-6	4*	1	-4	4*	6
			2	3*	-2	6	15*	10	-6	3*	-14						
	H,1,4		4	3*	4	-6	3*	3	-7	18*	26		H,1,9				
			7	9*	2	8	15*	9	-8	16*	-10						
5	4*	-9	-7	3*	-14	-8	16*	-18	-10	5*	3	2	3*	4			

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Synthesis and Resolution of Vinylglycine, a β,γ-Unsaturated α-Amino Acid

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DL-Vinylglycine (2-amino-3-butenoic acid) has been synthesized for the first time from ethyl 2-bromo-3-butenoate by amination and saponification, and by a Strecker synthesis from acrolein. D-Vinylglycine has been produced by treatment of the racemate with L-amino acid oxidase or baker's yeast. The spectroscopic properties and chemical stability of vinylglycine have been determined. Vinylglycine, the simplest β-γ-unsaturated α-amino acid, has previously been postulated as an intermediate in various enzymatic processes. The natural occurrence of other β-γ-unsaturated α-amino acids is briefly discussed.

A number of aliphatic α-amino acids possessing β,γ-unsaturation have been found in natural sources in recent years as indicated in Table 1. Several biological actions have been ascribed to this type of amino acid, and a potential role as amino acid antagonists can be envisioned. Thus, (*E*)-2(*S*)-amino-4-methoxy-3-butenoic acid (Ie, Table 1) is an antimetabolite, inhibiting growth of a *Bacillus* species [4,5] and of *Escherichia coli*.[5] Rhizobitoxine (If, Table 1) in low concentrations strongly inhibits ethylene production by *Sorghum* seedlings and induces chlorosis in new leaf tissues in many plants,[7] whereas the saturated analogue, dihydrorhizobitoxine,[8] does not seem to induce any chlorosis.[9]

Vinylglycine is the simplest β,γ-unsaturated α-amino acid. It has been postulated as an intermediate in the enzymatic conversion of homoserine to threonine [10] and α-ketobutyrate [11] but has not so far been encountered as a compound of natural derivation, and no report of its synthesis has appeared. It is the purpose of the present paper to describe a synthesis of DL- and D-vinylglycine and the chemical and spectroscopic properties of these compounds.

RESULTS

Syntheses. Synthesis of vinylglycine in reasonable yields was impeded by the instability of the compound upon treatment with heat, acid, or base (see below). Even if substantial amounts of vinylglycine could be produced, the isolation of pure material was always accompanied by great losses. The most satisfactory yields were obtained by stirring ethyl 2-bromo-3-butenoate with concentrated aqueous ammonia, followed by cold saponification and isolation of vinylglycine by use of a cation exchange resin. The synthesis of ethyl 2-bromo-3-butenoate has been reported previously.[12] Later an improved version of this synthesis has been described and it has been shown that on treatment of this substance with diethyl sodiomalonate it undergoes displacement of bromine without rearrangement.[13] Vinylglycine was also produced in a Strecker reaction performed essentially as described in an analogous synthesis of β-methylene-DL-norvaline (Ia, Table 1).[1] The dark brown reaction mixture contained six components reacting with ninhydrin besides vinylglycine. However, after a cumbersome isolation procedure involving ion exchange chromatography, pure vinylglycine was obtained in a yield of 1.1 %.

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Table 1. Naturally occurring β,γ-unsaturated α-amino acids with the general formula $R_3(R_2)C=C(R_1)CH(NH_3^+)COO^-$ (I).

	R_1	R_2	R_3	Configuration at C_2	Configuration at double bond	Occurrence	Ref.
Ia	CH_3CH_2	H	H	*S* (L)	–	*Lactarius helvus*	1
Ib	$CH_3CH_2CH_2$	H	H	*S* (L)	–	*Amanita vaginata* var. *fulva*	2
Ic	CH_2OH	CH_3	H	*S* (L)	*Z*	*Bankera fuligineoalba*	3
Id	CHO	CH_3	H	*S* (L)	?	*Bankera fuligineoalba*	3
Ie	H	CH_3O	H	*S* (L)	*E*	*Pseudomonas aeruginosa*	4,5
If	H	$CH_2(OH)CH(NH_2)CH_2O$	H	?	*E*	*Rhizobium japonicum*	6

Double bonds can be produced by elimination reactions from sulphonium compounds [14] and sulphoxides.[15] Therefore production of vinylglycine was attempted by thermal decomposition of *S*-methylmethionine, *S*-adenosylmethionine and methionine sulphoxide. However, homoserine, probably produced *via* the lactone, was the only reaction product which could be identified in a number of experiments under varying conditions of temperature and solvent. This negative result is in agreement with previous experiments on the degradation of these compounds. Thus *S*-methylmethionine on heating gives homoserine and dimethyl sulfide. [16,17] Vinylglycine has been postulated as an intermediate in the acid degradation of methionine sulphoxide but has not been identified in the reaction mixture.[18]

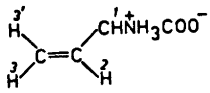
Resolution. Because of the instability of vinylglycine (see below) difficulties could be expected in attempts at chemical resolution of the racemate. Therefore the racemate was subjected to the action of L-amino acid oxidase,[19] which resulted in production of the levorotatory D-amino acid. However the optical purity obtained in this preparation was less than 25 % as indicated by the subsequent experiments.

Better results were obtained by subjecting racemic vinylglycine to the asymmetric action of baker's yeast during fermentation of sucrose. In this way a sample of D-vinylglycine was obtained possessing an $[\alpha]_D$ of $-94°$ in water. This product was hydrogenated to give a sample of 2-aminobutyric acid, $[\alpha]_D - 16.6°$ in hydrochloric acid. If it is assumed that the reduction proceeded without racemization, the D-vinylglycine must have contained about 9 % of the L-isomer, since a rotation of $+20.1°$ in hydrochloric acid is reported for L-2-aminobutyric acid (*cf.* Ref.[20], p. 116).

The D-configuration of the levorotatory stereoisomer is established by its production in the action of L-amino acid oxidase on the racemate and its reduction to D-2-aminobutyric acid. Furthermore the amino acid obeys the Clough-Lutz-Jirgensons rule (*cf.* Ref.[20], p. 85), showing a negative shift in $[\alpha]_D$ on acidification. Circular dichroism measurements (see below) also agree with the assignment of configuration.

Properties of vinylglycine. The UV-spectrum of vinylglycine in water shows a shoulder at about 210 nm, which can be attributed to the carboxylate group, and increasing absorption towards 190 nm due to the C=C bond. In hydrochloric acid the position of the shoulder is shifted to about 220 nm, in agreement with the change of the carboxylate group into a free carboxyl group. Measurements of CD-curves for the D-isomer (with optical purity more than 90 %, see above) demonstrated a negative Cotton effect at 208 nm in water and

212 nm in hydrochloric acid. No indication of additional Cotton effects could be observed down to 192 nm in water and 197 nm in hydrochloric acid. The strong UV-absorption prevented measurements at lower wavelengths. The Cotton effects observed must be attributed to the carboxylate and carboxyl groups. The numerically rather high $\Delta\varepsilon$-values found (−6.7 in water, −4.9 in hydrochloric acid) indicate, however, an influence from the C=C bond (*cf.* Ref. 21).



The ^{1}H NMR-spectrum of vinylglycine shows as expected the α-proton as a doublet and the three vinylic protons as a complex pattern. The spectrum was simulated using the LAOCN3 program.[22] With the following values for chemical shifts and coupling constants, agreement was obtained with the measured spectrum both with regard to position and intensity of the peaks (for designation of atoms see formula): δ_1 4.29 ppm; δ_2 5.97; δ_3 5.48; $\delta_{3'}$ 5.48; $J_{1,2}$ 7.1 cps; $J_{2,3}$ 10.8 cps; $J_{2,3'}$ 16.7 cps; $J_{3,3'}$ −1.2 cps.

On chromatograms vinylglycine produces with ninhydrin a yellow colour which changes through a brownish shade into the normal purple. The colour change is that expected for an α-amino acid with an electrophilic substituent in the β-position.[23]

When heated in water vinylglycine is slowly decomposed with the concomitant production of 2-aminobutyric acid and ammonia. However traces of vinylglycine can still be observed after 168 h at 100° or 68 h at 120°. In 1 N hydrochloric acid vinylglycine is decomposed without the production of other ninhydrin-reactive compounds. After 50 h at 100° no vinylglycine remained. When heated in aqueous ammonia vinylglycine is decomposed with the production of unidentified ninhydrin-reactive compounds. After 22 h at 100° in 2 N ammonia all vinylglycine had disappeared, whereas no decomposition was observed during 168 h in 2 N ammonia at room temperature.

When vinylglycine was heated in aqueous solution with 2-keto-4-methylpentanoic acid, leucine could be identified in the reaction mixture by paper chromatography. This result indicates that the production of 2-aminobutyric acid from vinylglycine in water takes place by production of 2-ketobutyric acid followed by transamination.

EXPERIMENTAL

^{1}H NMR spectra were measured on a JEOL-C-60 HL instrument. Chemical shifts are given in ppm downfield from sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propionate. Infra-red spectra (in KBr) were recorded on a Perkin-Elmer Model 337 instrument. UV-spectra were measured in 1 mm cells on a Perkin-Elmer Model 402 instrument. Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter in 1 dm tubes. CD-curves were recorded on a Roussel-Jouan Dichrograph CD 185 using 2 mm cells and concentrations of approximately 0.3 mg/ml. Microanalyses were performed by Mr. G. Cornali and his staff.

Paper chromatography was performed by the descending technique on Whatman No. 1 paper in A, BuOH−HOAc−H_2O [12 : 3 : 5 (v/v/v)], and B, PhOH−H_2O−conc. NH_3 [120 : 30 : 1 (w/v/v)]. The following R_F-values were found: Vinylglycine: A, 0.35; B, 0.66. 2-Aminobutyric acid: A, 0.47; B, 0.71.

DL-*Vinylglycine by Strecker synthesis.* The synthesis was performed as a modification of a standard procedure.[1,24] A solution of acrolein (28 g), NH_4Cl (64 g), and KCN (25 g) in water (270 ml) was kept at 5° for 30 min and at room temperature for 2 1/2 h. Conc. HCl (200 ml) was added and the solution concentrated by heating under a stream of nitrogen. After cooling, filtration, further concentration to 150 ml and filtration (to remove inorganic salts) the solution was applied to a strongly acidic cation exchange resin (Amberlite IR 120, 1 × 37 cm, H^+-form). After washing with water (300 ml) the column was eluted with ammonia (250 ml, 2 N). The eluate was concentrated to dryness (2.0 g), dissolved in water and passed through a column of deactivated carbon (1 × 5 cm). The effluent from the carbon was evaporated to dryness (1.75 g), the residue dissolved in water and the solution applied to a strongly acidic cation exchange resin (Dowex 50W × 8, 1.2 × 75 cm, particle size 45−70 μ, 3-chloropyridinium form). The column was washed with water (150 ml) and eluted with aqueous 3-chloropyridine (0.25 M). Fractions of 12 ml each were collected. Fractions 91−109 were concentrated to dryness to give chromatographically pure vinylglycine (480 mg, 1.1 %). Final purification was accomplished by passage of an aqueous solution through a small carbon column, concentration to dryness, redissolution in water (0.5 ml) and precipitation with ethanol (2 ml) to give colourless DL-vinylglycine (58 mg). (Found: C 47.51; H 6.99; N 13.70. Calc. for $C_4H_7NO_2$: C 47.52; H 6.98; N 13.85). IR:

ν_{max}(KBr) 2080 cm^{-1} (medium), 1660 (very strong), 1585 (vs), 1520 (vs), 1420 (vs), 1395 (vs), 1340 (vs), 1280 (strong), 1160 (m), 1120 (s), 1080 (m), 1040 (m), 990 (s), 935 (vs), 915 (vs), 880 (m), 790 (vs), 660 (s), 610 (m), 515 (m), 470 (s).

DL-*Vinylglycine from ethyl 2-bromo-3-butenoate.* Ethyl 2-bromo-3-butenoate [12,13] (74 g) was stirred with concentrated aqueous ammonia (450 ml) for 20 h. After addition of NaOH (30 %, 45 ml) the solution was kept at 5° for 14 days and at −15° for another 14 days. The solution was then applied to a cation exchange resin (Amberlite IR 120, 5 × 40 cm, H^+-form). The resin was washed with water (15 l). and eluted with aqueous pyridine (1 M, 2 l) All fractions showing a positive reaction with ninhydrin were pooled and evaporated to dryness, leaving a brown residue (13.6 g) which was dissolved in water (100 ml) and passed through a column of Sephadex G-10 (2.5 × 90 cm). The column was eluted with water, 6 ml fractions being collected. Fractions 25 – 51 contained vinylglycine as the major component. The residue (8.5 g) from fractions 36 – 51 was dissolved in a small amount of water and absolute ethanol was added. On storage in the icebox overnight a slightly yellow product precipitated (1.3 g). A second crop (1.2 g, total yield 6.6 %) was obtained on concentration of the mother liquor to near dryness followed by addition of absolute ethanol. The product was identical with the product from the Strecker synthesis as judged by paper chromatography and IR- and ^1H NMR-spectroscopy.

Production of partially racemic D-*vinylglycine by the action of* L-*amino acid oxidase on the racemate.* DL-Vinylglycine (200 mg) was dissolved in 500 ml of 0.2 M Tris (pH 7.5), and L-amino acid oxidase [50 mg, from *Crotalus adamanteus* venom (Sigma No. A-9378)] was added. The flask was stoppered and placed in a water bath at 37°. After shaking for 17 h, the stopper was removed and the shaking continued for 3 h. The solution was concentrated and applied to a cation exchange resin (Amberlite IR 120, 2.5 × 30 cm, H^+-form). The resin was washed with water (1100 ml) and eluted with aqueous pyridine (0.5 M). The fractions containing the amino acid were pooled and concentrated to a brown, sticky residue. Further purification was accomplished by ion exchange on a small column of Dowex 50W × 8 resin, preparative paper chromatography (solvent A) and passage through a small carbon column to give a colourless sample (136 mg), $[\alpha]_D^{23} - 23°$ (*c* 0.93, H_2O).

Production of D-*vinylglycine by the action of baker's yeast on the racemate.* DL-Vinylglycine (200 mg) and sucrose (7 g) were dissolved in water (50 ml), and baker's yeast (5 g) was suspended in the mixture by stirring. The suspension was set aside at room temperature for two days, and the liquid was decanted through a 1 cm layer of Filtercel. The filtrate was concentrated and applied to a cation exchange resin (Amberlite IR 120, 1.3 × 17 cm, H^+-form). The column was rinsed with water, and vinylglycine was eluted with aqueous pyridine (0.5 M), 5 ml fractions being collected. Fractions 8 – 12, which contained vinylglycine, were combined and evaporated to dryness. The residue (104 mg) was dissolved in a few ml of water and passed through a filter having a small layer of carbon. The filtrate was evaporated to dryness, leaving a colourless sample of D-vinylglycine (78 mg), m.p. 216 – 218° (decomp.), $[\alpha]_D^{22} - 93.8°$ (*c* 1.5, H_2O). The paper-chromatographic behaviour and IR- and ^1H NMR-spectra were identical with those observed for the racemate. A sample of D-vinylglycine with $[\alpha]_D^{22} - 83.1°$, produced in a preliminary experiment, revealed, dissolved in hydrochloric acid, an $[\alpha]_D^{22} - 95°$ (*c* 0.3, 2 M HCl).

D-*2-Aminobutyric acid by reduction of* D-*vinylglycine.* An aqueous solution of D-vinylglycine (29 mg) $[\alpha]_D - 93.8°$ was hydrogenated with platinum on carbon (30 mg, 10 % Pt) as a catalyst. Hydrogen uptake was completed in less than one hour, as demonstrated by paper chromatography. The catalyst was removed by filtration, and the filtrate was evaporated to dryness to give colourless and chromatographically pure D-2-aminobutyric acid (33 mg), $[\alpha]_D^{21} - 16.6°$ (*c* 0.9, 2 N HCl).

DL-*2-Aminobutyric acid and ammonia by heating an aqueous solution of* DL-*vinylglycine.* DL-Vinylglycine (100 mg) in water (10 ml) was heated in a sealed tube for 4 days at 120°. Ammonia could be identified in the reaction mixture by the use of Nessler's reagent. The solution was taken to dryness, dissolved in water and applied to a small column of cation exchange resin (Dowex 50W × 8, H^+-form). After flushing with water, neutral amino acids were eluted with aqueous pyridine (1 M). 2-Aminobutyric acid, which was the main component, was purified by passage through a small carbon column, preparative paper chromatography and a second carbon treatment. A colourless, crystalline sample (18 mg) was obtained. The paper-chromatographic behaviour and ^1H NMR-spectrum were identical with those observed for an authentic sample of L-2-aminobutyric acid. Small deviations in the IR-spectra must have been due to the difference between the racemate and the enantiomer.

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The Mechanism of the Nitrodeiodination of 2-Iodo-1,3,5-trineopentylbenzene

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When 2-iodo-1,3,5-trineopentylbenzene is nitrated in nitromethane with 90 % nitric acid, it undergoes nitrodeprotonation, as well as nitrodeiodination. The latter reaction was found to be a direct substitution of the iodine atom by a nitro group in analogy with the mechanism of the former reaction.

Results concerning the reaction between various alkyl-substituted iodobenzenes and fuming nitric acid in nitromethane were presented in a previous publication.[1] Two different reactions occurred with substrates having a hydrogen, as well as an iodine, atom attached to the benzene nucleus. These reactions were the usual nitrodeprotonation and a reaction in which the iodine atom is exchanged for a nitro group. The latter reaction was thought to be a direct nitrodeiodination.

Butler and Sanderson [2] showed recently that for the reaction between nitric acid and 4-iodoanisole in acetic acid, the observed nitrodeiodination takes place *via* an initial nitrosodeiodination and a subsequent oxidation. In Ref. 1, that mechanism was rejected for alkyl-substituted iodobenzenes since the rate of nitrodeiodination was not reduced when urea had been added to the reaction solution. As pointed out by Butler and Sanderson, such an experiment may not be completely conclusive and the dependence of the rate of nitrodeiodination on addition of nitrate may give more accurate information.

The present paper deals with the reaction between fuming nitric acid and 2-iodo-1,3,5-trineopentylbenzene (ITNB) in nitromethane solution, and evidence is presented for the view that the observed nitrodeiodination is a direct one.

ITNB was treated in nitromethane with 90 % nitric acid which was prepared with particular attention paid to minimizing the content of lower nitrogen oxides. The two products obtained were 2-iodo-1,3,5-trineopentyl-4-nitrobenzene (INO_2TNB) and 1,3,5-trineopentyl-2-nitrobenzene (NO_2TNB), resulting from nitrodeprotonation and nitrodeiodination, respectively.

From the composition of the product mixture, a quantity a was calculated; a is defined as twice the amount of NO_2TNB divided by the amount of INO_2TNB. In Ref. 1, it was reported that the value of a did not change during the course of reaction, and this result has been confirmed in the present work. It can thus be concluded that the value of a represents the ratio between the rates per available position of nitrodeiodination and nitrodeprotonation. The reaction was repeated several times with different amounts of sodium nitrite added to the reaction solution, and the value of a was determined in each case (see Table 1).

The a values increase with increasing molar ratio between added nitrite and ITNB, and a fairly good linear correlation can be found. The slope of the line is small, however, and the increase in a over the entire interval studied is less than half the value without addition of nitrite.

The observed nitrodeiodination could not be the result of a nitrosodeiodination followed by an oxidation because of the relatively high value of a in the case in which no nitrite was added and the small value of the slope of the correlation

Table 1. Nitration of 2-iodo-1,3,5-trineopentylbenzene (ITNB) with an excess of nitric acid containing varying amounts of sodium nitrite. For conditions, see Experimental section. The quantity a is defined as twice the ratio between the amounts of 1,3,5-trineopentyl-2-nitrobenzene and 2-iodo-1,3,5-trineopentyl-4-nitrobenzene.

mol $NaNO_2$ / mol ITNB	mol $NaNO_2$ / mol HNO_3	a
0	0	0.56
1.2	0.001	0.57
3.4	0.004	0.60
6.6	0.007	0.62
10.5	0.012	0.66
12.0	0.013	0.66
18.7	0.021	0.71
26.5	0.030	0.82
30.9	0.034	0.82

line. If this were the case, the nitric acid itself would have contained 7 mol % of nitrous acid, as can be found by extrapolation. The nitric acid prepared was not found to contain any detectable amounts of nitrous acid, within an experimental accuracy of 1 mol %.

The small increase in the a value observed when nitrite was added may be due to nitrodeiodination *via* a nitrosodeiodination, or to relative rate differences caused by medium effects. Neither of these possibilities can be ruled out.

The possibility that a catalytic effect of nitrite on the rate of nitrodeiodination may be hidden by a similar effect on the rate of nitrodeprotonation can be ruled out by means of the results from an estimate of the rate constants. In fact, the overall reaction rate decreased when sodium nitrite was added; see Experimental section.

A possible reaction sequence consisting of a protodeiodination followed by a nitrodeprotonation, leading to an overall nitrodeiodination has been ruled out.[1,3]

The reason for a difference between the mechanism found in the present case and that in the case of 4-iodoanisole may to some extent be rationalized in the following way. Butler and Sanderson [2] found a very low *ortho*:*para* ratio for the nitrosodeiodination reaction. Since the iodine in ITNB is placed between two *ortho* substituents the nitrosodeiodination may be hampered in this case, and the direct nitrodeiodination becomes the dominating path for the replacement of iodine by the nitro group.

EXPERIMENTAL

The NMR analyses were performed on a Varian A 60 NMR spectrometer. Gas chromatographic (GLC) analyses were made on a Perkin-Elmer 900 instrument fitted with SE-30 columns.

2-Iodo-1,3,5-trineopentylbenzene (ITNB) was prepared according to a method previously described.[3] The fuming nitric acid used was obtained according to Bennett *et al.*[4] The concentration was adjusted to 90 % by mixing with a proper amount of concentrated nitric acid. The acid was finally tested for nitrous acid according to a method based on reaction with *N*-chloro-4-toluenesulphonamide, described by Bennett *et al.*[4] No nitrous acid could be detected within the experimental accuracy of 1 mol %.

Nitration of ITNB with nitric acid. ITNB (100 mg, 0.24 mmol) was dissolved in 65 ml of nitromethane and the solution was cooled to 0°C. Fuming nitric acid (90 %, 10 ml, 215 mmol) at 0°C was added, and the reaction solution was kept at 0°C for 2 days. A small amount (ca. 10 ml) of water was added, and the new solution was extracted three times with cyclohexane. The combined cyclohexane phases were washed with water and dried with magnesium sulphate. The drying agent was removed and the solvent was evaporated. The residue was dissolved in carbon tetrachloride and analysed by means of NMR spectroscopy. The relative amounts of the two products, 1,3,5-trineopentyl-2-nitrobenzene and 2-iodo-1,3,5-trineopentyl-4-nitrobenzene, were determined by comparing the intensities of the peaks due to the different aromatic protons.

The experiment was repeated with varying amounts of sodium nitrite added to the nitric acid before the acid was added to the nitromethane solution. The results are summarized in Table 1.

When more than approximately 300 mg of sodium nitrite was added to the acid, a precipitate appeared when the acid was poured into the nitromethane solution. This precipitate was analysed by conventional chemical tests and was found to consist of sodium nitrate.

Estimate of the rate of reaction of ITNB with nitric acid with and without sodium nitrite. ITNB (20.0 mg, 0.048 mmol) was dissolved in 10.0 ml of nitromethane and the solution was cooled to 0°C. Fuming nitric acid (90 %, 2.0 ml, 43 mmol) at 0°C was added. The reaction mixture was kept at 0°C, and aliquots were withdrawn at proper time intervals. The aliquots were added to some water and the resulting solution was extracted with cyclohexane. The cyclohexane solution was analysed by means of GLC, and the relative amounts of the starting material and the two products were obtained as the

ratios of the peak heights. The value of the pseudo first-order rate constant for the consumption of ITNB was 2.1×10^{-4} s^{-1}.

The experiment was repeated with 64.6 mg (0.94 mmol) of sodium nitrite dissolved in the nitric acid before the addition to the nitromethane solution. In this case the value of the rate constant was 9.0×10^{-5} s^{-1}. The accuracy of these rate constants can be estimated at ± 10 %. Both experiments were repeated, and the values of the rate constants were found to be within the given error limits.

The relative amounts of the two products, NO_2TNB and INO_2TNB, determined by means of GLC as just described, were found to be constant throughout the reactions.

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Studies on Intermediates Involved in the Syntheses of Pentaerythritol and Related Alcohols. V.* On the Kinetics of the Base-catalyzed Aldol Condensation Reactions of Intermediate Aldehydes with Formaldehyde

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Rate constants for the base-catalyzed aldol condensation reactions of 3-hydroxypropanal, 2-propenal, 2-hydroxymethyl-2-propenal, 2-methyl-2-propenal, and 2-ethyl-2-propenal, respectively, with formaldehyde have been obtained from kinetic experiments.

In alkaline, dilute aqueous solutions the unsaturated aldehydes mentioned undergo hydration to give equilibrium mixtures with their saturated, α-hydroxymethyl-substituted counterparts. In these mixtures the unsaturated aldehyde dominates except in the pair 2-propenal ⇌ 3-hydroxypropanal. The hydration ⇌ dehydration reactions are rather rapid. By studying UV-spectrophotometrically the rates of disappearance of the latter three of the unsaturated aldehydes enumerated above, it was therefore thought possible indirectly to determine the rates whereby the corresponding three saturated aldehydes 3-hydroxy-2 hydroxymethylpropanal, 3-hydroxy-2-methyl propanal, and 2-ethyl-3-hydroxypropanal are converted into aldol condensation products with formaldehyde. Experimentally, however, the observed rates of condensation at higher formaldehyde concentrations were found to be faster than the known rates of hydration and this in spite of the activation energies for the condensations being higher. The explanation for this latter observation seems to be that the activation energy for dehydration of methylene glycol with formation of free formaldehyde, the reacting species, is included in the activation energies for the aldol condensations. The experimental findings thus indicate that the rate of condensation of each of the unsaturated aldehydes may differ from the corresponding rate for its saturated, hydrated counterpart and that equilibrated mixtures of these two aldehydes probably only are formed when the condensation reactions are carried out at relatively low concentrations of formaldehyde.

The condensation reactions with formaldehyde of 3-hydroxypropanal and of 2-propenal were followed by means of a gas chromatographic determination method. The sum of the concentrations of 2-propenal and of 3-hydroxypropanal was determined as an apparent concentration of 2-propenal after quantitative dehydration of 3-hydroxypropanal in the injection block. Both aldehydes were found, within experimental errors, to react with formaldehyde at the same rate. Furthermore, at higher formaldehyde concentrations, the reactions of these two aldehydes with formaldehyde, like those of the other aldehydes examined, are faster than their hydration and dehydration reactions.

This work is a sequel to a study [1] dealing with the base-catalyzed aldol condensations of the lowest molecular weight members of the alkanal series with formaldehyde. The hydroxyaldehydes 3-hydroxypropanal (I, monomethylolacetaldehyde, hydracrolein) and 3-hydroxy-2-hydroxymethylpropanal (II, dimethylolacetaldehyde) constitute the first and the second intermediate in the reaction sequence forming pentaerythritol, ultimately, from acetaldehyde and formaldehyde. Likewise, 3-hydroxy-2-methylpropanal (III, monomethylolpropionaldehyde) and 2-ethyl-3-hydroxypropanal (IV, monomethylolbutyraldehyde) are the first intermediates in

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the reaction series leading to trimethylolethane and to trimethylolpropane as products of the corresponding reactions of propionaldehyde and of butyraldehyde with formaldehyde:

$$CH_3CHO \xrightarrow[OH^-]{CH_2O} \underset{I}{HOCH_2CH_2CHO} \xrightarrow[OH^-]{CH_2O}$$

$$\underset{II}{(HOCH_2)_2CHCHO} \xrightarrow[OH^-]{CH_2O} (HOCH_2)_3CCHO$$

$$\xrightarrow{CH_2O,\ OH^-} C(CH_2OH)_4 + HCOO^-$$

$$CH_3CH_2CHO \xrightarrow[OH^-]{CH_2O} \underset{III}{HOCH_2CHCHO} \xrightarrow[OH^-]{CH_2O}$$

$$\underset{CH_3}{|}$$

$$(HOCH_2)_2CCHO \xrightarrow{CH_2O,\ OH^-}$$

$$\underset{CH_3}{|}$$

$$CH_3C(CH_2OH)_3 + HCOO^-$$

$$CH_3CH_2CH_2CHO \xrightarrow[OH^-]{CH_2O} \underset{IV}{HOCH_2CHCHO} \xrightarrow[OH^-]{CH_2O}$$

$$\underset{CH_2CH_3}{|}$$

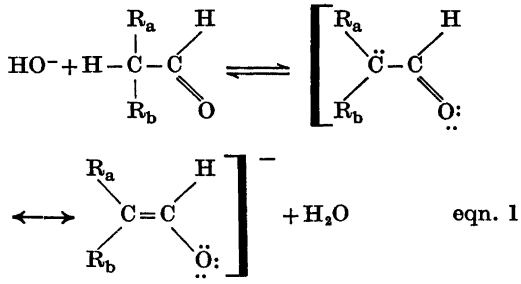
$$(HOCH_2)_2CCHO \xrightarrow{CH_2O,\ OH^-}$$

$$\underset{CH_2CH_3}{|}$$

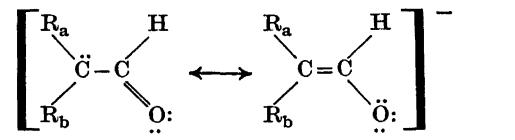
$$CH_3CH_2C(CH_2OH)_3 + HCOO^-$$

One of these reactions, namely the one between I and formaldehyde, has been the subject of a study by Ogata, Kawasaki, and Yokoi,[2] who in the same paper also present data concerning the aldol condensation between acetaldehyde and formaldehyde. Since some aspects of their method seem questionable, independent methods to study these reactions, preferably in a more direct way, have been sought. In the reaction with acetaldehyde its rate of disappearance could be followed by direct gas chromatographic analysis under reaction conditions where its rate of self-condensation was negligible.[1] 3-Hydroxypropanal, on the other hand, is not sufficiently thermally stable to allow direct determination by this method. It has now been found, however, that if a thin glass tube, the walls of which are covered with a mixture of acid sodium and potassium phosphates, is inserted into the heated injection block of the gas chromatograph, 3-hydroxypropanal, present in a neutralized reaction mixture, on injection is instantaneously converted into 2-propenal (V, acrolein) and can be quantitatively determined as such. It had to be ascertained, by separate experiments with no formaldehyde present, that the apparent rate of disappearance of V was negligibly small in aqueous solutions of the same nominal alkalinity as used in the experiments with formaldehyde present. Since it is known that in such solutions an equilibrium between I and V, in which I dominates, is rather rapidly established,[3] it follows that the mixture of I with V was quantitatively determined as V. Likewise, in an alkaline solution of the aldehyde I, its concentration, as measured by this method in the form of an apparent concentration of V, was found to be reasonably constant for a few hours in the temperature range investigated (20 – 40°C). In experimental kinetic runs performed in the presence of formaldehyde by this method, approximately straight pseudo first-order plots of the disappearance of aldehyde I (or V) were always obtained, the plot from a typical run being shown in Fig 1.

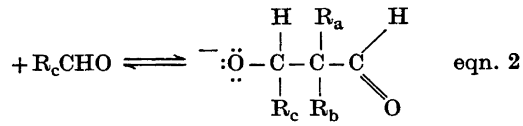
The problem to determine the rates of condensation with formaldehyde of the aldehydes II, III, and IV was more complicated, since so far attempts to work out similar gas chromato-



eqn. 1



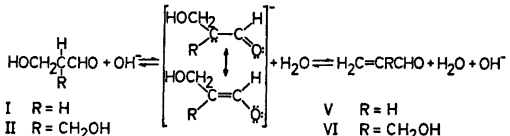
eqn. 2



eqn. 3

graphic determination methods have been unsuccessful. It could be expected, however, that these reactions follow the commonly accepted scheme for aldol condensations.[4]

Previous work,[3] made possible by the development of methods to obtain the aldehydes I, II, III, and IV,[5] had shown that II, III, and IV, in alkaline aqueous media, are rather rapidly converted into their dehydrated, unsaturated counterparts, 2-hydroxymethyl-2-propenal (VI), 2-methyl-2-propenal (VII, methacrolein), and 2-ethyl-2-propenal (VIII), respectively, until dynamic equilibria are established. In dilute aqueous solutions, at temperatures above 20°, the unsaturated aldehyde constitutes more than 90 mol % of each of these equilibrium mixtures.



eqn. 4

The equilibria presented in eqn. 4 therefore must be taken into consideration when aldol condensations with formaldehyde are investigated kinetically with compounds II, III, and IV.

At lower formaldehyde concentrations the second reaction step, presented in eqn. 2, can be assumed to be rate determining for the aldol condensations. Under these conditions the initial rate of formation of the aldol condensation product is not necessarily the same for the two possible reactants, the saturated and the unsaturated aldehyde, since the steady state concentrations of enolate ions they give rise to may well differ. After a period of time, however, an equilibrium is expected to be attained between the two aldehydes and from then on the same rate of formation of product must be observed, irrespective of which of the two aldehydes was chosen as starting material. A change in observed rate "constant" during the initial stage of the reaction is therefore not unexpected.

At higher formaldehyde concentrations the reaction given in eqn. 1, *i.e.* the formation of enolate ions, is expected to be rate determining. Again the saturated and the unsaturated aldehyde may show different rates of disappearance depending on their rates of formation of enolate ions. Besides, since the formaldehyde hydrates, methylene glycol and oligomers, buffer alkaline solutions, the maximum rates of condensation for a certain amount of sodium hydroxide added, would be expected to be lower than the rates of enolate ion formation in mixtures of the same nominal alkalinity, but with no formaldehyde present.

Unfortunately, as mentioned, it has so far not been possible to carry out kinetic investigations on the condensation reactions with formaldehyde of compounds II, III, and IV due to lack of analytical methods to follow the reactions. It then seemed of interest at least to determine the corresponding rates of reaction of the unsaturated aldehydes VI, VII, and VIII. In this way it would be possible to get rate constants for the formation of product from the equilibrium mixtures present at low formaldehyde concentrations. Analytically, it should be possible easily to follow the rates of disappearance of the unsaturated aldehydes by means of UV-spectrophotometry in the same way as the dehydration⇌hydration reactions were followed.[3]

In this earlier investigation it was observed that the addition of a small amount of hydroxide ion to a 10^{-4} M solution of any of the aldehydes VI, VII, or VIII caused a rapid decrease by less than 10 % of the UV-absorption, further changes occurring only comparatively slowly. By contrast, in similar experiments now performed with formaldehyde present, a rapid decrease in concentration of the unsaturated aldehyde, starting at once and continuing until this aldehyde had completely disappeared, was observed. In each run the rate "constant" was higher in the beginning of the reaction than later on but reached a fairly stable value after part of the unsaturated aldehyde had disappeared. In Fig. 2 this is illustrated with 2-hydroxymethyl-2-propenal (VI) and formaldehyde.

The assumption that the initial rate deviations could be ascribed to a change in temperature during the first few minutes of reaction can be ruled out, since the rate "constant" was always found to decrease, also at temperatures above ambient. The rate change observed for the disappearance of the unsaturated starting material is probably influenced by the rate change for formation of product expected, when

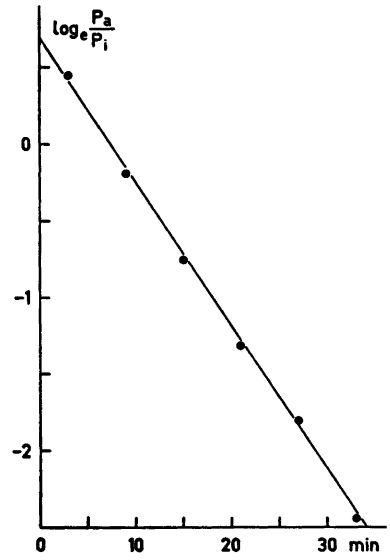


Fig. 1. Pseudo first-order disappearance of acrolein (V) in 0.25 M CH_2O. P_a/P_i: Peak area of signal from acrolein relative to that of 1-propanol. Nominal concentration of NaOH 0.0030 M. Temperature 20°C.

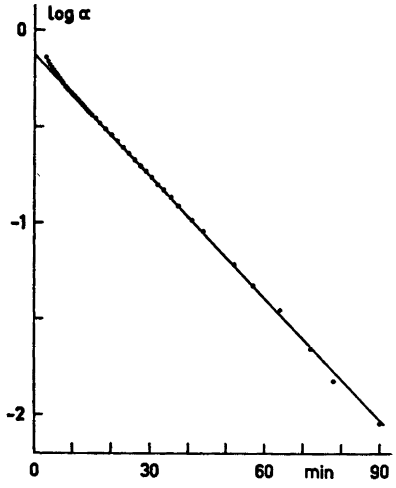


Fig. 2. Condensation of 2-hydroxymethyl-2-propenal (VI) with formaldehyde followed by UV-spectrophotometric measurement of the decreasing absorbance at 213.5 nm. Temperature 20.0° and nominal NaOH-concentration 0.0100 M.

part of this starting material is converted into the saturated aldehyde in their equilibrated mixture, as was discussed earlier. This hydration reaction in itself of course also contributes to the initial faster rate of disappearance of the unsaturated aldehyde but gives no direct contribution to the rate of formation of the aldol condensation product. Moreover, not only water can be reversibly added to the unsaturated aldehyde. Alcohol addition⇌elimination reactions also take place under alkaline conditions.[6] The rates of addition of some alcohols to the unsaturated aldehydes in question have been investigated in a separate study, which will be the subject of a forthcoming paper. These rates have been found to be considerably faster than the rates of addition of water, even in rather dilute aqueous solutions. Under the conditions used in the present work not only methanol, present in a low concentration in the formalin used, but also, and more important, methylene glycol and oligomeric formaldehyde hydrates may add in reversible reactions:

$$ROH + OH^- \rightleftharpoons RO^- + H_2O \qquad \text{eqn. 5}$$

$$RO^- + H_2C{=}CR'CHO \rightleftharpoons [ROCH_2\bar{C}R'CHO \leftrightarrow ROCH_2CR'{=}C\bar{H}O] \qquad \text{eqn. 6}$$

$$[ROCH_2\bar{C}R'CHO \leftrightarrow ROCH_2CR'{=}C\bar{H}O] + H_2O \rightleftharpoons ROCH_2CHR'CHO + OH^- \qquad \text{eqn. 7}$$

Since the reactions with methanol are known to be fast and the corresponding reactions with methylene glycol presumably are so too, such equilibrium reactions may well be part of the explanation for the initial faster rates observed. The curvatures of the pseudo first-order plots were also found to be more pronounced for experiments carried out at higher formaldehyde concentrations, where the reversible addition of methylene glycol can be expected to be of increasing importance. This state of things made it more difficult to graphically evaluate the rate constants "after stabilization" in these runs. Whereas the values of the rate constants obtained at lower formaldehyde concentrations were within ± 5 % of the mean value, reproducibility was only ± 10 % at higher formaldehyde concentrations.

If thus the observed rates of disappearance of the unsaturated aldehydes during the initial

period of each run do not represent the rate of any single reaction, once the various equilibria are attained, further changes must be due to the condensation reaction with formaldehyde. It is therefore assumed that the rate constants "after stabilization" represent the true rate of the condensation reaction with formaldehyde of the mixture of unsaturated and saturated aldehyde present at this stage of the reaction. These rate constants, like the ones obtained for the reactions of I and of V, were found to be directly proportional to the amount of NaOH added to the reaction mixtures. Second-order rate constants, k', may therefore be calculated referring to the rate expression:

$$-\mathrm{d[A]}/\mathrm{d}t = k'[\mathrm{A}][\mathrm{NaOH}]_{\mathrm{added}}$$

Here [A] denotes the concentration of the aldehyde under study, and k' times $[\mathrm{NaOH}]_{\mathrm{added}}$ is equal to k_{obs} for the pseudo first-order disappearance of A. The constants, k', were found to depend on the formaldehyde concentration in a manner somewhat similar to what was found for acetaldehyde and its homologs in the study referred to previously [1] (Fig. 3 and Table 1).

With the normal aldol condensation reaction mechanism operating one expects reaction step 1 (eqn. 1), that is the rate of formation of enolate ions, to become rate determining at higher formaldehyde concentrations. This simple picture is, however, partly obscured due to the behaviour of formaldehyde in alkaline, aqueous solutions. Firstly, the concentration of the reacting species, free, monomeric formaldehyde, is not linearly increasing with the total concentration of formaldehyde due to extensive formation of mono- and oligomeric hydrates. Secondly, because these hydrates are weakly acidic they buffer alkaline solutions increasingly with increasing concentration of formaldehyde. Of course it is still possible with the help of a pH-meter to adjust the hydroxide ion activity to the same value at the different formaldehyde concentrations. This would still leave open the question, however, to what extent the different amounts of anions of methylene glycol and oligomers present catalyze the condensation reaction. Furthermore, from an industrial viewpoint the interesting question is which rate is to be expected with a certain amount of alkali added, an amount determinable by titration. Anyhow, both the factors mentioned contribute to cause a less than proportional increase in rate constant for a certain increase in formaldehyde concentration. The levelling off of the values of the rate constants at concentrations of formaldehyde higher than about 1 M observed for the aldehydes VI, VII, and VIII does therefore not necessarily imply that the rates of enolate ion formation are becoming rate determining at this particular concentration.

In order to examine this, experiments have also been performed using the corresponding saturated aldehydes as starting materials for the condensations with formaldehyde. In these runs

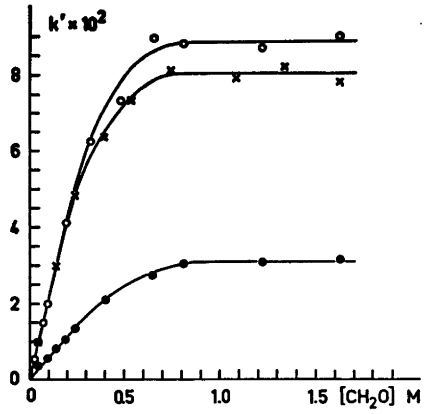


Fig. 3. Pseudo first-order rate constants for the condensation reactions of 2-hydroxymethyl-2-propenal (VI, ×), 2-methyl-2-propenal (VII, ○), and 2-ethyl-2-propenal (VIII, ●), respectively, with formaldehyde.

Table 1. Pseudo second-order rate constants, k' $\mathrm{M^{-1}\,sec^{-1}}$, for the disappearance of 3-hydroxypropanal (I) at different temperatures and concentrations of formaldehyde.

T°C	$[\mathrm{CH_2O}]_{\mathrm{tot}}$, M	k'
20	0.10	0.43
	0.25	0.52
	0.50	0.46
	0.75	0.39
	1.00	0.33
	1.50	0.27
	2.00	0.23
	3.00	0.17
	4.00	0.14
30	0.50	1.27
40	0.50	2.90

too the concentration of the unsaturated aldehyde in the equilibrium couple was followed by means of UV-spectrophotometry. The UV-absorptions of the unsaturated aldehydes were found at first to increase to a maximum and then to decrease to zero. This was the case both at lower and, more remarkably, also at higher formaldehyde concentrations in the region where the rates of the condensation reactions of the unsaturated aldehydes had been found to be unaffected by further increase of the formaldehyde concentration. The maximum concentration reached by the unsaturated aldehyde was highest with II as the starting material at equal formaldehyde concentrations. It was also higher when this concentration was low. This seems reasonable since the difference in rate between the dehydration of the saturated aldehyde and the condensation reaction of the unsaturated is greatest for the couple II and VI and also must be greater at lower formaldehyde concentrations. The very fact that unsaturated aldehyde is formed also at the higher of the formaldehyde concentrations used shows, anyhow, that not all enolate ions formed condense with formaldehyde even at these concentration levels. For this reason the rates of enolate ion formation cannot be completely rate determining even under these conditions.

The aldehydes I and V have a very pronounced rate maximum in about 0.25 M formaldehyde solutions, the rate constant having a four times smaller value for the reaction in 4.0 M solutions. The dehydration⇌hydration reactions cannot, as discussed earlier, give contributions to the experimentally observed rates of disappearance in these cases, since the analytical method used did not distinguish between I and V. The same is probably true for the alcohol addition ⇌ elimination reactions as well, β-alkoxyaldehydes being split when introduced into the injection block. Experimentally I and V were found to react with the same rate under the different concentration and temperature conditions used and this also helps to explain why changes in rate constant during a single run were not observed with these aldehydes. Their equality of rates is probably incidental since there seems to be no reason why this should be so for the unsaturated and saturated aldehyde in any given equilibrium couple, except under conditions where equilibrium already is attained.

The Arrhenius activation energies calculated from the experimental data are 17.3, 13.1, 13.1, and 12.6 kcal mol^{-1} for I (and V), VI, VII, and VIII, respectively. The experimental errors are estimated to be in the order of ± 0.4 kcal mol^{-1} for I (and V) and ± 0.8 kcal mol^{-1} for the others.

Since both the aldol condensation reactions and the hydration ⇌ dehydration reactions are believed to proceed *via* enolate ions it is of obvious interest to compare both rate constants and activation energies obtained.[3] It is then seen, that the condensation reactions have both the highest rates and, remarkably, the highest activation energies. At first sight this seems hard to reconcile with the assumption that all these reactions involve enolate ions. One must bear in mind, however, that only a very small part of the formaldehyde is present in reactive form, monomeric and unhydrated. The activation energies calculated for the condensation reactions thus reflect not only the activation energy for formation of the transition state from a formaldehyde molecule and the enolate ion. At least at lower formaldehyde concentrations, where the second reaction step (eqn. 2) is rate determining, they also contain the enthalpy and entropy changes for dehydration of methylene glycol, 5.7 kcal mol^{-1} and 5.4 e.u. according to Gruen and McTigue.[7] Thus, for all the aldehydes V to VIII the activation energies for the actual condensation reaction with formaldehyde are lower or about equal to the activation energies for the corresponding hydration reactions. For aldehyde II it is lower even without considering the influence due to dehydration of methylene glycol.

Ogata *et al.* in their paper report an activation energy of 22.9 kcal mol^{-1} for the aldol condensation of I with formaldehyde and the value of 22.1 kcal mol^{-1} for the corresponding reaction of acetaldehyde. As is evident from the spread in their presented primary rate data there is a considerable uncertainty inherent in their method. Besides, the temperature range they have investigated is rather narrow. Their values thus seem somewhat doubtful and they also seem high for normal aldol condensations. The rate constants one can calculate from their reported data for runs at 20° are very scattered. For acetaldehyde they are higher by factors between 1.5 to 3.5 but for I they are seven to ten times

smaller than the values reported in this series. It is believed, however, that the methods used in this work allow more accurate determinations with less sources of errors.

When comparing the rate constants and activation energies obtained for the aldehydes examined in this and the preceding study,[1] some notable differences can be observed. 3-Hydroxypropanal and 2-propenal are by far the most reactive ones of all the aldehydes investigated and react at 20° more than ten times faster than acetaldehyde, their precursor in the reaction series leading to pentaerythritol. 2-Methyl-2-propenal reacts in 1 M formaldehyde solutions at 20° with about the same rate as its precursor, propionaldehyde, but at formaldehyde concentrations lower than about 0.5 M this latter aldehyde reacts appreciably faster. 2-Hydroxymethyl-2-propenal and 2-ethyl-2-propenal, finally, react noticeably slower than their respective precursors, 3-hydroxypropanal and butyraldehyde, at all formaldehyde concentrations. The lowest molecular weight compounds among the alkanals and 3-hydroxypropanals, that is acetaldehyde and 3-hydroxypropanal, have distinctly higher activation energies than the other aldehydes. There does not seem to be any single factor causing this order of reactivities. The explanation must rather be sought in a combination of sterical and inductive effects of the various substituents. It could be that a 3-hydroxy group also has a neighbouring group effect in hydrogen bonding the formaldehyde molecule undergoing condensation.

EXPERIMENTAL

Materials. The formaldehyde used was of the LM-42 grade of Perstorp AB. This grade is an aqueous solution containing 42.0 ± 0.5 % of formaldehyde, 0.3 to 0.5 % of methanol, and 0.015 to 0.020 % of acid (calculated as formic acid; all percentages by weight). Acrolein and methacrolein were commercial practical grade chemicals. 2-Ethyl-2-propenal was prepared by the method of Marvel *et al.*[2] These three aldehydes were redistilled each day before use. 2-Hydroxymethyl-2-propenal and 3-hydroxypropanal were prepared in the form of their acetals, and 10^{-3} M stock solutions of the free aldehydes were prepared by the method described in an earlier paper [3] and used with the same precautions. 1-Propanol, used as internal standard in the gas chromatographic determinations, was of Fischer reagent grade. Sodium hydroxide stock solutions were freshly prepared each week from Merck Titrisol ampoules. These and all other aqueous solutions were made up with boiled, CO_2-free, distilled water.

The following stock solutions were prepared: of formaldehyde 1.00 and 10.0 M for use in the runs with I and V, 0.5 and 8.2 M for use in the runs with VII and VIII, and 0.5 and 10.9 M for use in the runs with VI; of I and V about 10^{-2} M and of VI, VII, and VIII about 10^{-3} M stock solutions were used; of NaOH 0.100 M and of 1-propanol about 0.08 % by weight solutions. The formaldehyde stock solutions were neutralized to pH 7.0.

Apparatus. The reaction mixtures were prepared in volumetric flasks and kept in a thermostatted bath controllable to within ± 0.05°C. Vapour phase chromatography was performed using a Varian 1740 gas chromatograph equipped with FI-detector and connected with an electronic integrator of type Varian 480. The UV-measurements were made using a Beckman DU instrument with cell compartment thermostatted by circulating water from the water bath.

General procedure. In the gas chromatographic runs this was identical with the one reported in the study on acetaldehyde and its homologs [1] and in the UV-spectrophotometric runs with the one used in the study on the hydration $\rightleftharpoons$ dehydration reactions of some 2-propenals and their saturated counterparts,[3] with the exception that formaldehyde was added to the mixtures used in the present study.

Gas chromatographic runs. At the start of the experimental runs, the reaction mixtures were about 0.001 M in I (or V) and about 0.004 % in 1-propanol, which was used as internal standard. The NaOH-concentration ranged from 0.001 to 0.005 M and the formaldehyde concentration from 0.10 to 4.0 M. The injection block was glass lined by insertion of a thin glass tube of commercial type to be used for this purpose. The inner wall of this tube was covered with a thin layer of a mixture of acid sodium-potassium phosphates. A syrupy aqueous solution of phosphoric acid and mono- and disodium and potassium phosphates had been prepared and was repeatedly deposited on the inner wall, the tube being dried in an oven after each treatment, until the wall was completely covered. The column was of stainless steel, 2 m × 0.22 cm. Stationary phase Chromosorb 101 80/100 mesh. Injection temperature 180°, column temperature 140°, detector temperature 150°. Carrier gas 30 ml per minute of N_2, attenuation 2×10^{-11} on the chromatograph and 1 on the integrator. Injected volume 1.0 µl.

UV-spectrophotometric runs. At the start of the experimental runs the reaction mixtures were about 10^{-4} M in VI, VII, or VIII, respectively. The concentration of formaldehyde was varied from 0.05 to 1.64 M. The NaOH-con-

centration was varied from 0.005 to 0.040 M in the runs with VI, from 0.005 to 0.030 M in the runs with VII, and from 0.010 to 0.080 M in the runs with VIII. A 1.000 cm quartz cell was used.

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Synthesis of Lignin Model Compounds for the Glyceraldehyde-2-aryl Ether Type of Structure

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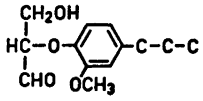
The preparation of lignin model compounds for the glyceraldehyde-2-aryl ether type of structure (I) is described. The occurrence of structural elements in lignin of type I is discussed on the basis of results from studies of lignin degradation products.

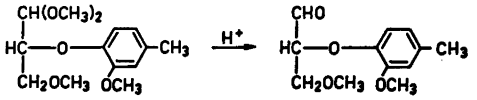
The occurrence of glyceraldehyde-2-aryl ether structures (I) in lignin has been suggested on the basis of the formation of pyruvaldehyde on "acidolysis" of lignin and model compounds.[1] Later studies [2-4] have supported the presence of such structures in lignin and some additional evidence is given in the present paper. Moreover, recent results from studies on the enzymatic oxidation of lignin model compounds [5] have provided indirect support for the occurrence of lignin structures of type I. Structure I contains a "detached side chain". The



I

role of oxidative side chain detachment in lignin chemistry has been discussed extensively the past few years (see Refs. 6 and 7). In the present paper, the synthesis of model compounds for the glyceraldehyde-2-aryl ether type of structure is described. Some aspects concerning the occurrence of "detached side chains" in lignin are also discussed.

Model compound II (liquid) was obtained by heating the sodium salt of 2-methoxy-4-methylphenol with 2-bromo-1,1,3-trimethoxypropane



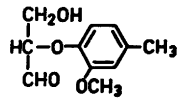
II III

Fig. 1. Preparation of aldehyde III by hydrolysis of acetal II.

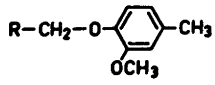
in ethanol solution. Although the yield was low, the product could be conveniently separated from the reaction mixture. Mild acidolysis of compound II gave aldehyde III (Fig. 1). This compound was obtained in a crystalline state (m.p. 44–46°). Aldehyde III is soluble in sulphite liquor and has been used as a model for structures of type I by Christofferson [4] in studies concerning the origin of pyruvaldehyde present in spent sulphite liquor (the formula given for the model compound in Ref. 4 is erroneous).

As reported previously,[1] attempts to prepare model compound IV by condensation of aldehyde VI with formaldehyde have been made. The product obtained gave 2-methoxy-4-methylphenol and pyruvaldehyde on "acidolysis", showed no infrared carbonyl absorption, and had a molecular weight which corresponded to a dimer of IV. On the basis of these properties, the product was suggested [1] to be a cyclic dimer of IV (in which the carbonyl groups take part in hemiacetal or acetal groups). The present paper describes experiments to prepare and characterize this product.

Starting material VI was prepared by hydrolysis of acetal V (obtained from the sodium



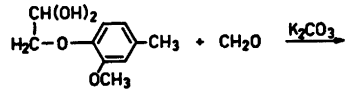
IV



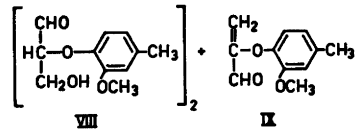
V R = $CH(OC_2H_5)_2$
VI R = CHO

salt of 2-methoxy-4-methylphenol and 2-bromo-1,1-diethoxyethane; *cf.* Ref. 8). The compound was obtained as a crystalline monohydrate (VII; m.p. 74 – 75°) and this was used in the condensation experiments with formaldehyde (Fig. 2). Distillation *in vacuo* of hydrate VII gave aldehyde VI (m.p. 50 – 52°).

The above discussed product, suggested to be a dimer of IV (VIII) (Fig. 2), was separated from reaction mixtures obtained in condensation experiments and examined. The NMR spectrum revealed the presence of a 2-methoxy-4-methylphenoxy group, but otherwise showed no clearly discernible signals. Gel filtration properties were in accord with a dimer of IV. Additional structural evidence for compound VIII was provided by the fact that borohydride reduction gave glycerol ether X (TLC) (Fig. 3) (*cf.* Ref. 9). The conversion of VIII into compound X was demonstrated by acetylation of the reaction product and identification of the acetate derivative with the independently prepared diacetate XII. For the preparation of X and XII, see Experimental.

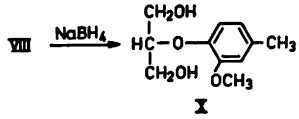


VII



VIII IX

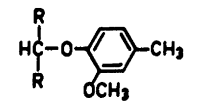
Fig. 2. Preparation of dimer VIII and aldehyde IX by condensation of compound VII with formaldehyde.



X

Fig. 3. Formation of glycerol ether X on reduction of dimer VIII with sodium borohydride.

In the preparation of dimer VIII an additional product was obtained, namely aldehyde IX. This compound is a likely intermediate in the acidolytic degradation [1] of compounds II – IV. In accord with expectations, it was found that compound IX gave pyruvaldehyde and 2-



XI R = $COOC_2H_5$
XII R = CH_2OCOCH_3

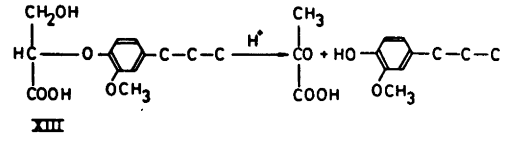
methoxy-4-methylphenol in high yields on brief "acidolysis". The fact that compound VI was stable during "acidolysis" [1] is also in accord with the assumption that an enol ether of type IX is an intermediate in the acidolytic cleavage of glyceraldehyde-2-aryl ethers.

In connection with studies of the formation of pyruvaldehyde on acidolysis of lignin, it was found that negligible amounts were formed from borohydride-reduced lignin (Ref. 3 and unpublished data). This is in accord with the proposal that the pyruvaldehyde originates from glyceraldehyde-2-aryl ethers, since such structures should be converted into glycerol-2-aryl ether structures on reduction with borohydride (*cf.* above and Ref. 9). The latter type of structure can be expected to give glycerol on "soda cooking".[10] In fact, borohydride treated lignin (but not nontreated lignin) gave glycerol on "soda cooking" (2 M NaOH, 170°, 2 h). The amount was somewhat smaller than expected from the studies of the pyruvaldehyde formation from nontreated lignin. Rather unexpectedly, it was found that glycerol was destroyed to a great extent during "soda cooking"; this makes quantitative evaluations of the results uncertain.

Comparison of the yields of pyruvaldehyde on "acidolysis" of lignin and model compounds suggests that about 2 % of the units in lignin are linked to glyceraldehyde with a 2-aryl ether bond. Calculations based on results from sulphite cooking studies [4] suggest a somewhat higher value.

During sulphite cooking structures of type I may be formed from arylglycerol-β-aryl ether structures in electrophilic displacement reactions.[10b] To what extent such or originally present structures of type I give rise to the pyruvaldehyde formed on sulphite cooking might be elucidated by sulphite cooking experiments with model compounds of the arylglycerol-β-aryl ether type and borohydride reduced lignin. In any case, it seems very likely that pyruvaldehyde (and pyruvic acid) present in sulphite liquor [4] arises from cleavage of glyceraldehyde-2-aryl ether groups.

Recent studies on lignin degradation products have demonstrated the occurrence of several types of structural elements containing units lacking propyl side chains (Refs. 7 and 11, see also Ref. 12). Based on yields of degradation products,[13,14] it seems that one of these types, namely the 1,2-diaryl-1,3-propanediol structure, is rather frequent in lignin. The figure obtained in the above-mentioned estimation of the frequency of "detached side chains" of the glyceraldehyde-2-aryl ether type therefore seems to be too low to fit with the number of units lacking side chains.



XIII

Fig. 4. Acidolytic cleavage of the proposed lignin structure XIII.

One explanation could be the occurrence of "detached side chains" of the glyceric acid-2-aryl ether type (XIII) (possibly with an esterified carboxylic group).[15] In connection with attempts to detect such structures in lignin the reaction shown in Fig. 4 was investigated. Model studies showed that ethers of type XIII were stable during "acidolysis". As expected, no pyruvic acid was formed on "acidolysis" of lignin. Additional model experiments showed that the reaction in Fig. 4 occurred to some extent on "acidolysis" at elevated temperature (140°) as indicated by the formation of pyruvic acid.

EXPERIMENTAL

IR spectra were recorded using KBr pellets (solids) or NaCl discs (liquids), with a Beckman IR 9 instrument. *NMR spectra* were recorded on a Varian A-60 instrument with TMS as internal standard. *Mass spectra* were taken on an AEI model MS 902 instrument. This equipment was also used for precise mass measurements of molecular ions. Elemental compositions were determined from these measurements using the tables of Beynon-Williams.[16]

Thin layer chromatography (TLC) was performed on silica gel plates. Eluents were benzene-ethyl acetate (1:1) (R_F values: X, 0.12; VIII, 0.17; 2-methoxy-4-methylphenol, 0.47; IX, 0.48; XII, 0.51) and benzene-ethyl acetate (4:1) (R_F values: III, 0.28; II, 0.36; 2-methoxy-4-methylphenol, 0.44). As developing agents iodine vapour (brown spots) and formalin-H_2SO_4 (1:9) were used (black spots after heating). Compounds VIII and IX were also made visible as purple spots by spraying with 2-methylindole in hydrochloric acid/ethanol and subsequent heating.

1,1,3-Trimethoxy-2-(2-methoxy-4-methylphenoxy)propane (II). 2-Methoxy-4-methylphenol (18.5 g) and 2-bromo-1,1,3-trimethoxypropane [17] (26.7 g) were added to a solution of 2.6 g Na in 50 ml ethanol and the mixture was heated in a steel autoclave at 150° for 5 h. After the NaBr which had formed was filtered off, the reaction mixture was diluted with dichloromethane, washed with 1 M NaOH and water, and dried over Na_2SO_4. Removal of the solvent by film evaporation gave an oil weighing 24.5 g. The product was chromatographed on a silica gel column (5 × 40 cm, 300 g SiO_2) with benzene-ethyl acetate (3:1) as eluent. The eluate fraction 700 – 950 ml contained compound II (TLC). The eluted material dissolved in ether was washed with 1 M NaOH and an essentially pure product (TLC) weighing 2.0 g was obtained. Final purification was made by distillation *in vacuo* (0.05 torr = 7 Pa) at 90°.

Precise mass measurements of the molecular ion gave *m/e* 270.14738. Calc. for $C_{14}H_{22}O_5^+$: *m/e* 270.146713. The molecular ion was 34 % of the base peak, *m/e* 75, which is attributed to the fragment ion $(CH_3O)_2CH^+$.

NMR spectrum (δ units; solvent, CCl_4): 2.24 (3 H, singlet; Ar-CH_3), 3.30 (3 H, singlet) and 3.33 (3 H, singlet) [$-CH(OCH_3)_2$], 3.41 (3 H, singlet; $-CH_2-O-CH_3$), 3.71 (1 H, doublet, $J = 4.8$ Hz) and 3.73 (1 H, doublet, $J = 3.4$ Hz) ($-CH_2-$), 3.78 (3 H, singlet; $Ar-O-CH_3$), 4.12 (1 H, doublet of triplets, $J = 3.4$ Hz and about 5 Hz; $Ar-O-CH<$), 4.42 (1 H, doublet, $J = 5.1$; $-CH(OCH_3)_2$), 6.49 – 6.91 (3 H, multiplet; aromatic protons).

3-Methoxy-2-(2-methoxy-4-methylphenoxy)propanal (III). Acetal II (0.33 g) was refluxed with 10 ml 0.2 M HCl in dioxan-water (9:1) for 10 min. The reaction mixture was neutralized with 10 ml 0.2 M $NaHCO_3$ and extracted with chloroform (20 + 2 × 10 ml). The extract was dried over Na_2SO_4. The amount of solvent was reduced to 10 ml by film evaporation. TLC showed one predominating spot. The solution was chromatographed on a silica gel column

(32 × 2 cm; 50 g SiO_2) with benzene-ethyl acetate (4:1) as eluent. From the eluate fraction 140 – 195 ml 0.21 g of an oil was obtained. Distillation (60 – 70°, 0.05 torr = 7 Pa) gave 0.12 g of an oil which crystallized on cooling (m.p. 44 – 46°). The IR spectrum showed a strong band at 1725 cm^{-1} (C = O). The molecular ion (*m/e* 224) was the base peak in the mass spectrum. Precise mass measurements of the molecular ion gave *m/e* 224.1038. Calc. for $C_{13}H_{16}O_4^+$: *m/e* 224.104851.

NMR spectrum (δ units; solvent, chloroform-*d*): 2.30 (3 H, singlet; $Ar-CH_3$), 3.39 (3 H, singlet; $-CH_2-O-CH_3$), 3.82 (3 H, singlet; $Ar-O-CH_3$), 3.82 (2 H, doublet, $J = 4.3$ Hz; $-CH_2-$), 4.45 (1 H, doublet of triplets, $J = 4.3$ and 1.5 Hz; $Ar-O-CH<$), 6.54 – 6.98 (3 H, multiplet; aromatic protons), 9.88 (1 H, doublet, $J = 1.5$ Hz; $-CHO$).

1,1-Diethoxy-2-(2-methoxy-4-methylphenoxy)-ethane (V) was prepared from 2-bromo-1,1-diethoxyethane and 2-methoxy-4-methylphenol according to a procedure (method B) described by Julia and Tchernoff [8] for the synthesis of similar compounds. The product was purified by distillation (4 torr = 500 Pa, bath temperature 147°). Yield: 59 %. (Found: C 66.51; H 8.89. Calc. for $C_{14}H_{22}O_4$: C 66.12; H 8.72.) NMR spectrum (δ units; solvent, chloroform-*d*): 1.21 (6 H, triplet, $J = 7.0$; $-CH_2-CH_3$), 2.26 (3 H, singlet; $Ar-CH_3$), 3.66 (2 H, quartet, $J = 7.0$) and 3.70 (2 H, quartet, $J = 7.1$) ($-CH_2-CH_3$), 3.80 (3 H, singlet; $-O-CH_3$), 4.01 (2 H, doublet, $J = 5.2$; $Ar-O-CH_2-$), 4.84 (1 H, triplet, $J = 5.2$ Hz; $>CH-$), 6.53 – 6.91 (3 H, multiplet; aromatic protons).

1,1-Dihydroxy-2-(2-methoxy-4-methylphenoxy)-ethane (VII). Acetal V (8.0 g) was refluxed with 100 ml 0.2 M HCl in dioxan-water (4:1) for 10 min. The reaction mixture was neutralized with 0.4 M $NaHCO_3$ to pH 6 and extracted with a total of 200 ml chloroform. The extract was dried over Na_2SO_4 and the solvent removed by film evaporation. The residual oil weighed 6.35 g. From ether saturated with water, 4.9 g crystals (m.p. 74 – 75°) was obtained. (Found: C 60.70; H 7.07; OCH_3 15.79. Calc. for $C_{10}H_{14}O_4$: C 60.59; H 7.12; OCH_3 15.66.)

NMR spectrum (δ units, 20°; solvent, DMSO-d_6): 2.24 (3 H, singlet; $Ar-CH_3$), 3.75 (3 H, singlet; $-O-CH_3$), 3.88 (2 H, doublet, $J = 5.2$ Hz; $-CH_2-$), 5.13 (1 H, approximately quintet, $J = 6.3$ and 5.2 Hz), 5.97 (2 H, doublet, $J = 6.3$ Hz; $-OH$), 6.43 – 6.91 (3 H, multiplet, aromatic protons). When the spectrum was recorded at higher temperatures, the presence of aldehyde VI was indicated.

2-(2-Methoxy-4-methylphenoxy)acetaldehyde (VI) was obtained upon distillation (0.04 torr = 5 Pa, 50 – 60°) of compound VII. The product melted at 51 – 52°. (Found: C 66.28; H 6.59; OCH_3 17.33. Calc. for $C_{10}H_{12}O_3$: C 66.65; H 6.71; OCH_3 17.22.) The IR spectrum showed a strong band at 1735 cm^{-1} (C = O). NMR spectrum (δ units; solvent chloroform-*d*): 2.29 (3 H, singlet; $Ar-CH_3$), 3.83 (3 H, singlet; $-O-CH_3$), 4.50 (2 H, doublet, $J = 1.3$ Hz; $-CH_2-$), 6.66 – 6.73 (3 H, multiplet; aromatic protons), 9.84 (1 H, triplet, $J = 1.3$ Hz; $-CHO$).

The compound gave a semicarbazone melting at 174 – 176° after recrystallization from ethanol-water. (Found: C 55.89; H 6.29; O 20.74; N 17.85; OCH_3 13.15. Calc. for $C_{10}H_{12}O_2N_3(OCH_3)$: C 55.69; H 6.37; O 20.23; N 17.71; OCH_3 13.08.)

Dimer (VIII) of 3-hydroxy-2-(2-methoxy-4-methylphenoxy)propanal (IV). Compound VII (5.0 g) was dissolved in 20 ml DMSO and 100 mg K_2CO_3 and 10 ml 37 % formaldehyde solution were added. The mixture was stirred for 20 min at room temperature. After the addition of 170 ml 0.1 M KH_2PO_4, the reaction mixture was extracted with chloroform (100 + 3 × 75 ml). The extract was dried over Na_2SO_4 and solvent removed by film evaporation. The residue, according to TLC, contained two components which gave purple spots with 2-methylindole in hydrochloric acid/ethanol, *i.e.* liberated pyruvaldehyde on acid treatment, *cf.* Ref. 1. The components were separated by chromatography on a silica gel column (2.5 × 40 cm; 170 g SiO_2) with benzene-ethyl acetate (1:1) as eluent. It may be noted that DMSO present in the residue was adsorbed at the top of the column. Eluate fraction 120 – 150 ml gave 0.78 g product (*fraction A*). Eluate fraction 190 – 260 ml gave 0.95 g product (*fraction B*).

Fraction A consisted essentially of 2-(2-methoxy-4-methylphenoxy)acrolein (IX). This was separated by chromatography on silica gel with dichloromethane as eluent. A product weighing 0.57 g was obtained. Purification by distillation (0.01 torr = 1 Pa, 55 – 63°) gave 0.40 g of an oil.

Mass measurements of the molecular ion gave *m/e* 192.077100. Calc. for $C_{11}H_{12}O_3^+$: *m/e* 192.078638.

The IR spectrum showed a strong band at 1705 cm^{-1} (C = O).

NMR spectrum (δ units; solvent, chloroform-*d*): 2.39 (3 H, singlet, $Ar-CH_3$), 3.93 (3 H, singlet; $-O-CH_3$), 5.03 (1 H, doublet, $J = 2.6$ Hz) and 5.21 (1 H, doublet, $J = 2.6$ Hz) ($=CH_2$), 6.61 – 6.98 (3 H, multiplet, aromatic protons), 9.43 (1 H, singlet, $-CHO$).

Fraction B was subjected to column chromatography on silica gel using gradient elution according to the previously described standard procedure [14a] to isolate the major component, which was subsequently purified by distillation (80 – 110°, 0.05 torr = 7 Pa). It has previously been suggested [1] that the product obtained in this way is a dimer of IV, in this paper denoted VIII. This was derived from the facts that no carbonyl band appeared in the IR, the molecular weight determined by osmometry fitted very well with a dimer of IV, and pyruvaldehyde and 2-methoxy-4-methylphenol were formed on "acidolysis".[1] We have made further studies which support the assumption that the product

is a dimer of IV. Thus gel filtration on Sephadex G-25 with dioxan-water (1:1) as eluent[18] supported a dimeric structure ($K_d' = 0.4$; K_d' values for compounds III and X were about 0.7). Furthermore, reduction with $NaBH_4$ gave glycerol ether X (see below). The NMR spectrum showed the presence of a 2-methoxy-4-methylphenoxy group, but was otherwise too complex to permit interpretation; possibly the product is a mixture of isomeric dimers. The mass spectrum corresponded to monomer IV. Mass measurements of the molecular ion gave *m/e* 210.091. Calc. for $C_{11}H_{14}O_4^+$: *m/e* 210.089 202. The molecular ion was 84 % of the base peak, *m/e* 138, which is proposed to be due to fragment ion (2-methoxy-4-methylphenol)$^+$.

Diethyl 2-(2-methoxy-4-methylphenoxy)-malonate (XI). Diethyl chloromalonate (35 g) was refluxed with the sodium salt of 2-methoxy-4-methylphenol (29 g) in 100 ml ethanol for 6 h (*cf.* Ref. 10a). The reaction mixture was poured into water and extracted with ether. The ether layer was dried over Na_2SO_4 and the ether removed by film evaporation. The residual oil was distilled (b.p. 116–120°/1 torr = 100 Pa). Yield: 24.5 g (46 %).

NMR spectrum (δ units, chloroform-*d*): 1.26 (6 H, triplet, $J = 7.1$; $-CH_2CH_3$), 2.26 (3 H, singlet; $Ar-CH_3$), 3.78 (3 H, singlet; $Ar-O-CH_3$), 4.26 (4 H, quartet, $J = 7.1$; $-CH_2-$), 5.16 (1 H, singlet, $-CH<$), 6.52–7.01 (3 H, multiplet; aromatic protons).

2-(2-Methoxy-4-methylphenoxy)-1,3-propanediol (X) was prepared by reduction of ester XI with $LiAlH_4$ (*cf.* Ref. 10a). The crude product was purified by chromatography on a silica gel column with ethyl acetate as eluent followed by distillation (110°, 0.02 torr = 3 Pa). The product was obtained as a colorless oil.

Precise mass measurements of the molecular ion gave *m/e* 212.101946. Calc. for $C_{11}H_{16}O_4^+$: *m/e* 212.104851.

NMR spectrum (δ units; solvent, chloroform-*d*): 2.27 (3 H, singlet; $Ar-CH_3$), 3.54 (2 H, singlet; $-OH$), 3.78 (3 H, singlet; $-O-CH_3$), 3.76 (4 H, doublet, $J = 4$ Hz; $-CH_2-$), 3.99 (1 H, multiplet; $-CH<$), 6.47–7.01 (3 H, multiplet; aromatic protons).

Diacetate (XII) of compound X. Compound X was acetylated with acetic anhydride-pyridine. Excess reagent was removed by distillation and the residual product purified by distillation *in vacuo*. (Found: C 61.10; H 6.63. Calc. for $C_{15}H_{20}O_6$: C 60.80; H 6.80.) The IR spectrum showed a strong band at 1745 cm^{-1} (C=O). NMR spectrum (δ units; solvent, chloroform-*d*): 2.03 (6 H, singlet; $-CO-CH_3$), 2.28 (3 H, singlet; $Ar-CH_3$), 3.79 (3 H, singlet; $-O-CH_3$), 4.3 (5 H, multiplet; $-CH_2-CH(OAr)-CH_2-$), 6.55–6.95 (3 H, multiplet; aromatic protons).

Reduction of compound VIII (dimer of IV). Compound VIII (100 mg) was dissolved in 5 ml dioxan and 2–3 ml $NaBH_4$ solution was added (2 g $NaBH_4$ in 100 ml 0.25 M NaOH). After 1 h the solution was extracted with chloroform. The extract was dried over Na_2SO_4 and the solvent evaporated. Examination by TLC indicated that the product was compound X (small amounts of contaminants were present). The product was acetylated with acetic anhydride-pyridine and the resulting acetate was purified by chromatography on silica gel with benzene-ethyl acetate (4:1) as eluent. A pure product (TLC) weighing 105 mg was obtained. This was shown to be identical with authentic diacetate XII by IR and NMR analyses.

Acidolysis of aldehyde IX. Aldehyde IX (6.0 mg) was acidolysed for 5 min in 10 ml 0.2 M HCl in dioxan-water (9:1). Five ml 0.4 M $NaHCO_3$ was added and the mixture extracted with chloroform ($10 + 2 \times 5$ ml). It was demonstrated that the major part of the pyruvaldehyde remains in the aqueous layer during this work up procedure. To the aqueous layer 50 ml 2,4-dinitrophenylhydrazine solution was added (3 g 2,4-dinitrophenylhydrazine was dissolved in 100 ml 72 % $HClO_4$ and 100 ml H_2O added). After 1 h the precipitate was filtered off and washed with 12 % $HClO_4$, water, and ethanol. The precipitate (11.6 mg) was identified as the bis[(2,4-dinitrophenyl)hydrazone] of pyruvaldehyde. The formation of 2-methoxy-4-methylphenol was demonstrated by examination of the organic layer with TLC.

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On the Reaction of Methylthio- and Methoxythiophenes with Tetracyanoethylene Oxide

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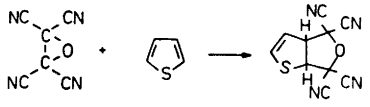
The reaction between methoxythiophenes or methylthiothiophenes and tetracyanoethylene oxides gives methoxy- or methylthiosubstituted thenoyl cyanides in low yields. A probable reaction mechanism for the formation of the thenoyl cyanides is given.

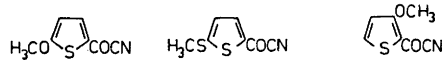
The mass spectra of the thenoyl cyanides are briefly discussed.

In connection with our work [1] on the effect of substituents on the cycloaddition of tetracyanoethylene oxide (I) to thiophene (II) [2] we used methoxy- and methylthiothiophenes as substrates. However, in these cases the normal substituted 1,1,3,3-tetracyano-1,3,3a,6a-tetrahydrothieno[2,3-*c*]furans (III) [2] were not obtained. Reacting for instance 2-methoxythiophene with I in benzene at 150° gave a crystalline compound, m.p. 68°C, which elemental analysis and mass spectrum showed to have the composition $C_7H_5NO_2S$. The IR spectrum indicated the presence of C≡N (2230 cm^{-1}) and C=O stretchings (1675, 1640 cm^{-1}) and the NMR spectrum showed aromatic methoxyl at δ 4.05 and two doublets at δ 6.44 and δ 7.87 with couplings of 4.5 Hz characteristic of a disubstituted thiophene. All these facts clearly show that the compound obtained is 5-methoxy-2-thenoyl cyanide (IV). 2-Thenoyl cyanide has recently been studied by Roques and Robba.[3] Using their substituent shifts in the proton NMR spectra and those earlier obtained by Gronowitz and Hoffman [4] for 2-methoxythiophene and assuming additivity, shifts of δ 7.67 and δ 6.34 for the 3- and 4-hydrogen were calculated for 5-methoxy-2-thenoyl cyanide, which are in good accordance with the observed values. Roques and Robba [3] also observed two carbonyl stretching frequencies at 1661 and 1683 cm^{-1} which they assigned to the presence of *s-cis* and *s-trans* conformers. This may also be the case in the 5-methoxy-substituted derivative, the somewhat lower frequencies being due to higher single bond character due to through-conjugation between the methoxy and the carbonyl cyanide group.[5]

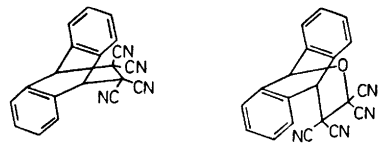
2-Methylthiothiophene reacted in the same way, although the yield of 5-methylthio-2-thenoyl cyanide (V) was only 6 %. Also in this case the structure followed from spectroscopic data and good accordance between the experimental chemical shifts (δ_3 = 8.03 ppm, δ_4 = 7.20 ppm) and those calculated by assuming additivity of



I II III



IV V VI



VII VIII

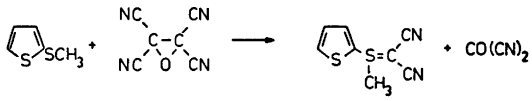
* Taken in part from the Ph.D. thesis of B. Uppström, University of Lund 1973.

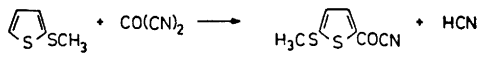
substituent shifts ($\delta_3 = 7.92$ ppm, $\delta_4 = 7.25$ ppm) was observed.

In the reaction of I with 3-methoxythiophene, 12 % of 3-methoxy-2-thenoyl cyanide (VI) was obtained. In this case larger deviations of the shifts from additivity were obtained. The observed shifts were 7.22 and 8.20 for the 4- and 5-hydrogen resonance, while the calculated values were δ 6.90 and δ 7.77, respectively. The deviation is probably due to steric interaction between the substituents.

It is known [3,6] that electron-rich olefins do not give normal adducts with I. Thus 2,3-dimethylbutene and I gave 2,3-dimethyl-2-butene epoxide. Anthracene and I gave a complex mixture of anthrone, anthraquinone, and bianthrone in addition to the anomalous adducts VII and VIII.[6] The side-reaction observed by us is most probably of another type. It has been found that I reacts with dimethyl sulphide to give dimethylsulphonium dicyanomethylide and carbonyl cyanide.[7] The corresponding reaction with dibutyl sulphide is indeed one of the most convenient methods for the preparation of carbonyl cyanide.[8] This reaction also occurs with aromatic sulphides. Thus *p*-anisyl methyl sulphide gave *p*-anisylmethylsulphonium dicyanomethylide in 67 % yield.[9]

It therefore seems likely that 2-methylthiothiophene first reacts with I to give the sulphonium dicyanomethylide and carbonyl cyanide, and the latter then reacts with excess of 2-methylthiothiophene to give V (*cf.* Formula Scheme).





V

An analogous route for the formation of IV and VI could be possible. However, the corresponding carbonyl ylide must be much less stable and more reactive than the sulphur analogue due to the absence of d-orbital stabilization. The formation of large amount of tars

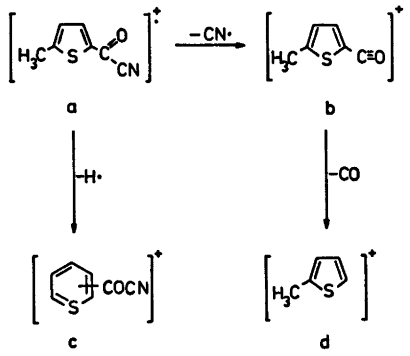


Fig. 1. Mass spectral fragmentation pattern for 5-methyl-2-thenoyl cyanide.

in this reaction could be due to the decomposition of the ylides.

The hypothesis that methylthio- and methoxythiophenes react with carbonyl cyanide at 130° to give thenoyl cyanides was confirmed with 2-methylthiothiophene and 3-methoxythiophene, which gave 33 % and 55 % yield of V and VI, respectively. Also less activated thiophenes such as 2-methylthiophene reacted with carbonyl cyanide to give the thenoyl cyanide, albeit in lower yield.

This way of introducing the COCN group into thiophenes appears to be more convenient than the $AlCl_3$-catalyzed reaction utilized earlier.[10]

We have also studied the mass spectral fragmentation of the thenoyl cyanides to some extent. The structures and mechanisms which are suggested should be considered as tentative. The simplest pattern is obtained for 5-methyl-2-thenoyl cyanide, Fig. 1. The molecular ion (a) loses a cyanogen radical to give the stable acylium ion (b) which further loses CO to give the ion (d). As with other methylthiophenes the molecular ion also loses hydrogen to give the thiopyrylium ion (c).[11]

Also in the mass spectra of the two methoxy-substituted thenoyl cyanides, the molecular ion is the most intense (Figs. 2 – 3). Fragmentation then occurs for IV by loss of either a cyanogen or a methyl radical to give the ion (f) (48 %) and (g) (60 %) of about equal intensity. For VI the loss of methyl is of much less importance, as the ion (o) has only an intensity of 5 %. The suggestion that the ions (g) (Fig. 2) and (o) (Fig. 3) lose the ring-carbonyl group as C=O

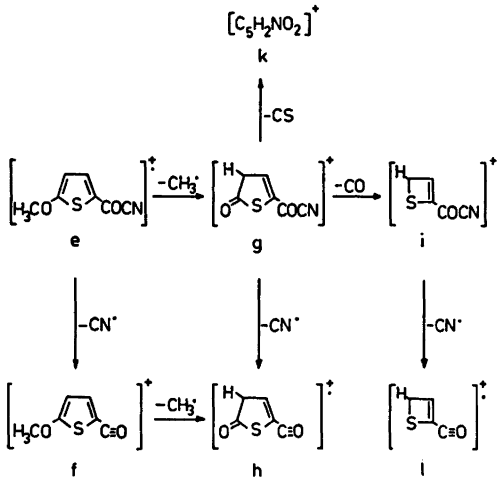


Fig. 2. Mass spectral fragmentation pattern for 5-methoxy-2-thenoyl cyanide.

is supported by the fact that the ion (x) does not lose CO but CS to give (y) (Fig. 4) and that ion (a) (Fig. 1) does not lose CO. The fragmentation with loss of ring-carbonyl groups is also observed in methoxythiophenes, which show step-wise loss of CH_3 and CO from the molecular

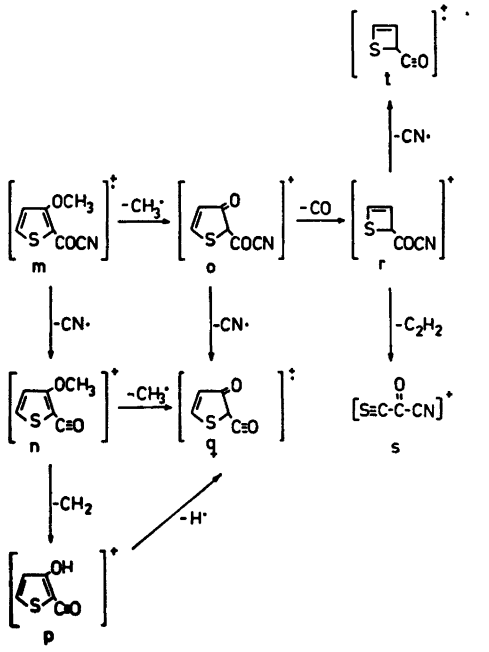


Fig. 3. Mass spectral fragmentation pattern for 3-methoxy-2-thenoyl cyanide.

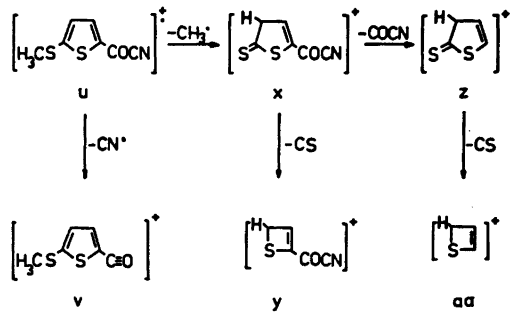


Fig. 4. Mass spectral fragmentation pattern for 5-methylthio-2-thenoyl cyanide.

ions.[13] The ion (i) (*m/e* 124) loses a fragment with mass 26 which could indicate loss of either a cyanogen radical or acetylene or both. High-resolution mass spectroscopy showed that the ion (l) had the composition C_4H_2OS. Thus the ion (i) loses only a cyanogen radical. On the other hand, the ion (r) (Fig. 3) loses both cyanogen and acetylene as with high-resolution mass spectroscopy both ion (s) and (t) could be observed. They had approximately the same intensity. Also the composition of ions (e), (g), (h), (i), (m), (n), (p), (q), and (r) was determined by high-resolution mass spectrometry. Further fragmentation is indicated in Figs. 2 – 3.

The mass spectrum of anisole shows the elimination of formaldehyde from the molecular ion.[12] This fragmentation path was not observed for IV and VI.

The fragmentation pattern of 5-methylthio-2-thenoyl cyanide is shown in Fig. 4. The loss of the cyanogen radical from the molecular ion is more important than the loss of methyl. No elimination of SH or CH_2S, which is characteristic for 2-fluoro-5-methylthiothiophene,[13] was observed. Neither was any intermediate ion observed on the path from the ion (x) to the ion (z) which might indicate the direct elimination of COCN.

EXPERIMENTAL

Reaction of methoxythiophenes with tetracyanoethylene oxide. 11.4 g (0.10 mol) of 2-methoxythiophene,[14] and 5.0 g (0.035 mol) of tetracyanoethylene oxide [15] in 100 ml of anhydrous benzene were heated in a glass ampoule at 150°C for 5 h. The mixture was filtered hot and evaporated to a volume of 20 ml. This

solution was chromatographed on silica gel, using benzene as eluent, and yielding 1.4 g (24 %) of 5-methoxy-2-thenoyl cyanide after recrystallization from 1,2-dichloroethane, m.p. 67 – 69°C. NMR ($CDCl_3$) $\delta_3 = 6.44$ ppm, $\delta_4 = 7.87$ ppm, $\delta_{CH_3} = 4.05$ ppm, $J_{3,4} = 4.5$ Hz. IR (KBr) C≡N: 2230 cm^{-1}, C=O: 1675, 1640 cm^{-1}. Mass spectrum (*m/e*, %): 169, 5; 168, 10; 167, 100; 153, 20; 152, 60; 141, 48; 126, 5; 124, 10; 108, 65; 98, 70; 96, 12; 70, 48; 69, 35; 64, 8; 57, 6; 54, 10; 53, 8; 45, 14; 38, 11; 37, 9. [Found: C 51.30; H 3.15; N 8.21; O 18.58; S 18.74. Calc. for $C_7H_5NO_2S$ (167.2): C 50.29; H 3.01; N 8.38; O 19.14; S 19.18].

From 11.4 g (0.10 mol) of 3-methoxythiophene,[16] 5.0 g (0.035 mol) of tetracyanoethylene oxide and 100 ml of benzene, 0.70 g (12 %) of the title compound, m.p. 134 – 135°C after recrystallization from 1,2-dichloroethane/cyclohexane, was obtained in the same way as described above. NMR ((CD_3COCD_3): $\delta_5 = 8.20$ ppm, $\delta_4 = 7.22$ ppm, $\delta_{CH_3} = 4.13$ ppm, $J_{45} = 5.5$ Hz. IR (KBr) C≡N: 2210 cm^{-1}, C=O: 1610 cm^{-1}. Mass spectrum (*m/e*, %): 169, 5; 168, 10; 167, 100; 152, 5; 150, 12; 142, 5; 141, 60; 140, 25; 139, 6; 138, 12; 137, 5; 128, 5; 127, 60; 126, 45; 124, 12; 113, 8; 112, 25; 111, 60; 110, 10; 109, 25; 98, 30; 96, 12; 85, 6; 84, 5; 83, 12; 82, 10; 81, 5; 73, 13; 69, 12; 64, 5; 58, 7; 56, 5; 54, 28; 53, 9; 45, 35; 44, 10; 41, 10; 39, 12; 38, 7; 37, 2. [Found: C 51.34; H 3.13; N 8.19; O 18.72; S 18.67. Calc. for $C_7H_5NO_2S$ (167.2): C 50.29; H 3.01; N 8.38; O 19.14; S 19.18].

2-Methylthiothiophene. This compound was prepared in a manner analogous to that described for 3-methylthiothiophene.[17] To 42.0 g (0.50 mol) of thiophene in 450 ml of anhydrous ether, 0.55 mol of 1.64 N butyllithium in hexane was added dropwise. The solution was cooled to −70°C and 47.1 g (0.50 mol) of dimethyl disulphide was added dropwise. The mixture was left over night at room temperature and then poured into ice-water. The ether phase was separated, the aqueous layer extracted with ether and the combined organic phases washed with water, sodium hydroxide solution and water. The organic layer was dried over magnesium sulphate, the ether and hexane evaporated and the residue distilled in vacuo to yield 50.0 g (77 %) of 2-methylthiothiophene, b.p. 65 – 70°/15 mmHg. Literature value[18] b.p. 80 – 82°/22 mmHg.

Reaction of 2-methylthiothiophene with tetracyanoethylene oxide. From 13.0 g (0.10 mol) of 2-methylthiothiophene, 5.0 g (0.035 mol) of tetracyanoethylene oxide and 100 ml of anhydrous benzene, 0.40 g (6 %) of the title compound, m.p. 69 – 70°C after recrystallization from 1,2-dichloroethane/cyclohexane mixture was obtained in the same way as described above. NMR ($(CD_3)_2CO$): $\delta_3 = 8.03$ ppm, $\delta_4 = 7.20$ ppm, $\delta_{CH_3} = 2.75$ ppm, $J_{34} = 4.4$ Hz. IR (KBr) CN: 2220 cm^{-1}, C=O: 1640 cm^{-1}. Mass spectrum (*m/e*, %): 185, 7; 184, 8; 183, 100; 168, 15; 159, 6; 158, 5; 157, 90; 124, 5; 114, 62; 108, 5; 96, 5; 88, 5; 85, 28; 82, 9; 81, 7; 70, 30; 69, 55; 57, 10; 54, 8; 53, 5; 45, 52; 41, 5; 38, 10; 37, 5. [Found: C 45.97; H 3.12; N 7.54; O 8.38; S 35.10. Calc. for $C_7H_5NOS_2$ (183.2): C 45.88; H 2.75; N 7.64; O 8.73; S 34.99].

3-Methoxy-2-thenoyl cyanide. To a solution of 2.6 g (0.033 mol) of carbonyl cyanide,[8] in 50 ml of anhydrous benzene, 9.5 g (0.083 mol) of 3-methoxythiophene was added, and the solution heated to 130°C for 2 h in a glass ampoule. The reaction mixture was worked up as described in method A yielding 1.8 g (33 %) of the title compound, m.p. 133 – 134°C and with the same spectroscopic data as the sample described above.

5-Methylthio-2-thenoyl cyanide. To a solution of 2.3 g (0.029 mol) of carbonyl cyanide [8] in 50 ml of anhydrous benzene 12.0 g (0.092 mol) of 2-methylthiothiophene was added and the solution heated in a glass ampoule to 130°C for 2 h. The reaction mixture was worked up as described above yielding 3.0 g (55 %) of the title compound, m.p. 68 – 69°C, having the same spectroscopic properties as the sample described above.

5-Methyl-2-thenoyl cyanide. To a solution of 1.8 g (0.023 mol) of carbonyl cyanide [8] in 50 ml of anhydrous benzene, 13.0 g (0.13 mol) of 2-methylthiophene [19] was added and the solution heated in a glass ampoule to 130°C for 3 h. The reaction mixture was filtered, evaporated almost to dryness and the residue chromatographed on silica gel using benzene as eluent. The product was recrystallized from carbon tetrachloride, yielding 0.6 g (17 %) of the title compound, m.p. 73 – 74°. NMR ($(CD_3)_2CO$): $\delta_3 = 8.03$ ppm, $\delta_4 = 7.12$ ppm, $\delta_{CH_3} = 2.68$ ppm, $J_{34} = 4.0$ Hz, $J_{CH_3-4} = 1.0$ Hz, $J_{CH_3-3} = 0.6$ Hz. IR (KBr) CN: 2220 cm^{-1}, C=O: 1640 cm^{-1} (broad). Mass spectrum (*m/e*, %): 153, 5; 152, 10; 151, 100; 150, 15; 127, 5; 126, 8; 125, 95; 122, 8; 98, 5; 97, 58; 96, 8; 95, 8; 93, 5; 82, 5; 71, 8; 70, 10; 68, 22; 63, 5; 59, 7; 58, 7; 57, 12; 54, 30; 53, 40; 52, 5; 51, 12; 50, 10; 45, 32; 39, 18; 38, 13; 37, 10. [Found: C 54.81; H 3.36; N 9.10; S 20.94. Calc. for C_7H_5NOS (151.2): C 55.61; H 3.33; N 9.26; S 21.21].

IR spectra were recorded on a Perkin-Elmer 257 Grating Infrared Spectrophotometer, NMR spectra on a Varian A-60 spectrometer and mass spectra on an LKB 9000 mass spectrometer. The compounds were injected through a direct inlet at approximately 25°C. The energy of the ion beam was 70 eV and the electron current was set to 60 μA. High-resolution mass spectra were obtained with an MS 902 AEI spectrometer.

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The Synthesis and Stereochemistry of 3-Alkyl-1,2-indandiol Diacetates

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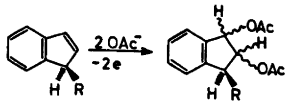
A series of alkylindenes, with the alkyl group methyl, ethyl, propyl, isopropyl, butyl, and *t*-butyl has been subjected to (a) permanganate oxidation and (b) oxidation by iodine/silver acetate under "wet" and "dry" conditions, These methods, in combination with acetylation by acetic anhydride/pyridine, give access to the four possible 3-alkyl-1,2-indandiol diacetates from each 1-alkylindene (except for the *t*-butyl compound, where the all-*trans*-isomer is not available by these methods). Structures have been assigned to all compounds by synthetic, mechanistic, and NMR spectral considerations.

Studies on the stereochemistry of the anodic side-chain acetoxylation of 2-*t*-butylindan [1] and 1-*t*-butylacenaphthene [2] have indicated that the anodic surface might exert a certain degree of steric control on the reaction. In order to extend these studies to other reaction types we have chosen to investigate the anodic addition of two acetoxy groups to 1-alkylindenes (eqn. 1). Since the steric relationships among the



(1)

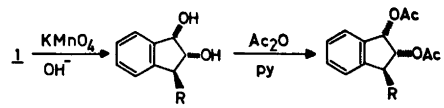
1 2

R = CH_3, C_2H_5, C_3H_7, i-C_3H_7, C_4H_9, and *t*-C_4H_9

four possible isomeric 3-alkyl-1,2-indandiol diacetates (*2*) are not known, it was first necessary to establish these by independent methods. This paper is a report of the synthesis and elucidation of the structures of all possible diacetates of the type *2* with R defined as in eqn. 1.

RESULTS AND DISCUSSION

It is known that potassium permanganate will oxidize alkenes to *cis* glycols in alkaline solution,[3] as exemplified for a 1-alkylindene in eqn. 2. It has also been shown that potassium permanganate will attack a properly substituted alkene preferentially from the sterically least hindered side and thus should give the less hindered *cis* glycol (*3*) from a 1-alkylindene [4–7] (eqn. 2). From *3*, the corresponding diacetate (*4*) should be easily available by reaction with acetic anhydride/pyridine.

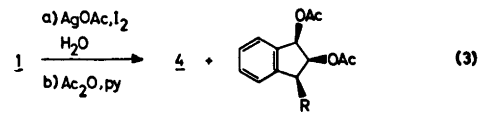


(2)

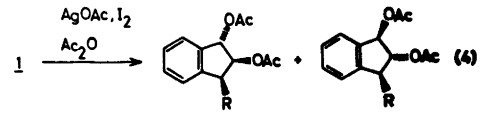
3 4

Another method of preparing *cis* glycols is the reaction between an alkene and iodine/silver acetate in "wet" acetic acid.[8] Again exemplifying with a 1-alkylindene, this reaction should give a mixture of *cis* glycol monoacetates from which the two possible *cis* diacetates *4* and *5* should be easily available (eqn. 3). The same reaction in "dry" acetic acid, [5b] known to give *trans* glycol monoacetates, should in the case of a 1-alkylindene directly give access to the two possible *trans* glycol diacetates *6* and *7* (eqn. 4).

The more often used method of preparing glycols from alkenes by peracid oxidation cannot be used here, since one usually obtains a mixture of *cis* and *trans* glycols [9,10] unless the reaction conditions are controlled very carefully.[11]



(3)



(4)

Table 1. Yields and physical data of *4*, synthesized *via* permanganate oxidation of 3-alkylindenes (*1*) and subsequent acetylation of the product (eqn. 2).

R	Yield of *4*, %	B.p. °C at mmHg	Retention time, min[a]
CH_3	35	117–120/0.8	15.0
C_2H_5	75	130–132/1.5	20.1
C_3H_7	73	137–139/1.0	15.0
i-C_3H_7	68	127–128/0.7	13.6
C_4H_9	47	135–137/0.8	19.0
t-C_4H_9	60	125–120/1.0	17.0

[a] 2 m × 0.3 mm 5 % neopentylglycol succinate on Chromosorb W column at 180°C; carrier gas N_2, flow rate 20 ml/min.

In the oxidation of 1-alkylindenes (*1*) with potassium permanganate/water only one of the possible *cis* glycols is formed in each case. In line with the known stereochemistry of the reaction (see eqn. 2) structure *3* is assigned to these glycols. Table 1 gives overall yields of the reaction sequence (eqn. 2) and some physical data for identification of the diacetates *4*, R = CH_3, C_2H_5, C_3H_7, i-C_3H_7, C_4H_9, and *t*-C_4H_9.

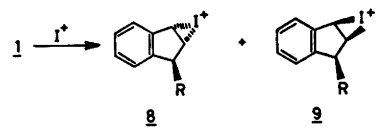
The reaction of 1-alkylindenes (*1*) with silver acetate/iodine in "wet" acetic acid, followed by acetylation of the monoacetates formed, gives a mixture of two *cis* diacetates, one of them being identical to *4* and the other one accordingly assigned structure *5*. By using GLC and/or NMR analysis it was possible to determine the relative amounts of *4* and *5* in the product mixtures (Table 2).

Table 2. Yields and retention times of products from the reaction between 1-alkylindenes (*1*) and iodine/silver acetate in "wet" acetic acid and subsequent acetylation (eqn. 3).

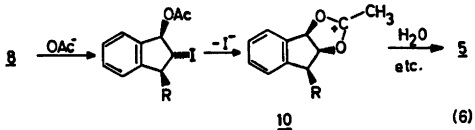
R	Yield of *4*+*5*, %	Relative yields, %		Retention time, min[a]	
		4	*5*	*4*	*5*
CH_3	52	69	31	15.0	17.2
C_2H_5	55	65	35	20.1	22.2
C_3H_7	72	65	35	15.0	15.5
i-C_3H_7	57	29	71	13.6	15.2
C_4H_9	49	72	28	19.0	20.1
t-C_4H_9	59	< 0.1	100	17.0	20.1

[a] 2 m × 0.3 mm 5 % neopentylglycol succinate on Chromosorb W column at 180°C; carrier gas N_2, flow rate 20 ml/min.

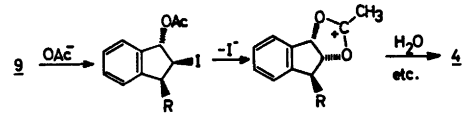
The reaction between an alkene and iodine/silver acetate in "wet" acetic acid is supposed [12] to follow the mechanism given in eqns. 5 – 7 for a 1-alkylindene:



(5)



(6)



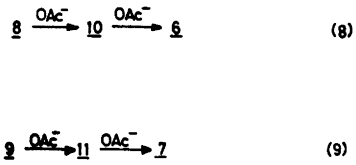
(7)

The initial step is the formation of iodonium ions *8* and *9* which then undergo S_N2 displacement by acetate ion followed by internal nucleophilic displacement of iodide ion to give acetoxonium ions *10* and *11*. These hydrolyze to give a mixture of *cis* hydroxy acetates, from which a mixture of diacetates *4* and *5* are prepared by treatment with acetic anhydride/pyridine. In the first step which controls the overall stereochemistry of the reaction sequence,

an increase in the steric demands of R should increase the percentage of iodonium ion *8* and hence the proportion of *5* in the final product. The results given for $R=C_2H_5$, i-C_3H_7 and *t*-C_4H_9 in Table 2 show this prediction to be correct.

The same reaction, run in "dry" acetic acid, gave a mixture of diacetates which by GLC and/or NMR spectral analysis were not identical with either *4* or *5*. Accordingly, the product mixture must consist of *trans* diacetates *6* and *7*. The assignment of structures to *6* and *7* is based on predictions from mechanistic considerations in the following way: Under "dry" conditions the mechanism is identical to that given in eqns. 5–7, except for the hydrolysis step which is exchanged for an S_N2 attack at the benzylic carbon of acetoxonium ions *10* and *11* by acetate ion [5b, 12] (eqns. 8 and 9). Since the first part of the reaction is performed in the same way under both "wet" and "dry" condition, *8* and *9* and hence *10* and *11* should be formed in approximately the same ratios in both cases. From this it follows that the *4*/*5* ratio should be approximately equal to the *7*/*6* ratio, and this prediction has been the basis of the structural assignments of Table 3.

The NMR spectra of compounds *4*–*7* are in good agreement with the general structure *2* but cannot be reliably used for detailed stereostructural assignments due to the irregularities of vicinal coupling constants in the indan system.[1,13,14] Table 4 illustrates these difficulties



(8)

(9)

for two representative sets of compounds *4*–*7*, $R=CH_3$ and C_4H_9. In these cases there is no possibility of assigning *cis* or *trans* stereochemistry of H_a, H_b and H_c from the relative magnitudes of the coupling constants J_{ab} and J_{bc}.

The mass spectra of diacetates *4*–*7* are closely similar. (For an example, see Table 5). No molecular ion is detectable, the fragment with highest mass being formed by elimination of acetic acid from M^+.

Further evidence for the correctness of the structural assignments above was obtained by studying the pseudocontact shifts in the NMR

Table 3. Yields and retention times of products from the reaction between 1-alkylindenes and iodine/silver acetate in "dry" acetic acid (eqn. 4).

R	Yield of *6*+*7*, %	Relative yields, %		Retention time, min[a]	
		7	*6*	*7*	*6*
CH_3	53	68	32	13.1	15.1
C_2H_5	64	68	32	16.2	18.4
C_3H_7	61	67	33	12.0	13.8
i-C_3H_7	53	22	78	10.7	11.9
C_4H_9	51	72	28	15.6	17.7
t-C_4H_9	57	<0.1	100	14.0[b]	16.1

[a] 2 m×0.3 mm 5 % neopentylglycol succinate on Chromosorb W column at 180°; carrier gas N_2, flow rate 20 ml/min. [b] This compound was prepared by anodic oxidation of 1-*t*-butylindene.[15]

Table 4. NMR parameters of the five-ring hydrogens of 3-alkyl-1,2-indandiol diacetates (H_A on the 3-carbon; *t* denotes *trans* coupling, *c* *cis*).

R	Compound	H_a,δ	H_b,δ	H_c,δ	J_{ab},Hz	J_{bc},Hz
CH_3	*4*	3.5	5.0	6.2	7.4 (*t*)	5.2 (*c*)
	5	3.3	5.6	6.2	5.6 (*c*)	5.6 (*c*)
	6	3.2	5.5	6.2	6.6 (*c*)	4.2 (*t*)
	7	3.2	5.2	6.2	6.0 (*t*)	4.8 (*t*)
C_4H_9	*4*	3.3	5.6	6.1	5.6 (*t*)	5.6 (*c*)
	5	3.5	5.2	6.2	5.2 (*c*)	5.2 (*c*)
	6	3.1	5.5	6.2	5.0 (*c*)	6.8 (*t*)
	7	3.1	5.3	6.1	4.6 (*t*)	3.6 (*t*)

Table 5. Mass spectral data for diacetates *4*–*7*, $R=C_2H_5$.

Fragment *m/e*	Relative abundance, % *4*	*5*	*6*	*7*
202	6	9	8	8
161	12	16	12	12
160	100	100	100	100
145	36	43	40	48
132	10	12	11	11
131	33	52	33	39
115	13	15	18	20
91	8	9	7	7
43	50	74	48	57

spectrum of *4* – *7*, R = *t*-Bu, caused by the addition of tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionato)europium(III), Eu(fod)$_3$. Since Eu(fod$_3$) is sterically very demanding, one would expect it to complex at the 3-acetoxy group of the sterically most crowded diacetate, the all-*cis* isomer *5*. Hence the 3-acetoxy methyl should be more shifted than that of the 2-acetoxy group, and H_a and the methyl groups should be affected to a relatively low extent (see Table 6). The second isomer with *cis* acetoxy groups, *4*, should have nearly equivalent acetoxy groups from the steric point of view, resulting in nearly equal shifts of the acetoxy methyl signals. On the other hand, both the H_a and the R methyl signal should be relatively more shifted than in *5*.

Isomer *6*, with the 2-acetoxy group *cis* to the *t*-butyl group, again should have the 3-acetoxy group sterically more accessible for complexing with Eu(fod)$_3$. On the other hand, the all *trans* isomer *7* should have nearly equivalent acetoxy groups and hence show similar shifting of the acetoxy methyl signals.

A study of the anodic addition of two acetoxy groups across the double bond of 1-alkylindenes will be reported later.[15]

Table 6. Pseudocontact shifts in the NMR spectra of *4* – *7*, R = *t*-C_4H_9 by addition of Eu(fod)$_3$; solvent $CDCl_3$.[a]

Compound	Shift, Hz CH_3 in *t*-C_4H_9	CH_3 in 3-acetoxy	CH_3 in 2-acetoxy	H_a	[Eu(fod)$_3$]/[substrate]
4	43	166	160	143	0.39
5	38	210	143	62	0.39
6	52	134	110	100	0.42
7	68	148	137	154	0.36

[a] Recorded on a Varian T-60 NMR spectrometer.

EXPERIMENTAL

1-Alkylindenes. These compounds were prepared as described earlier.[16]

trans,cis-*3-Alkyl-1,2-indandiol diacetates* (*4*). To a cooled solution of 1-alkylindene (10.0 mmol) in acetone-water (80 + 20 ml) a solution of potassium permanganate (1.5 g) and potassium hydroxide (0.3 g) in water (30 ml) was added at a rate allowing for the purple colour caused by one drop to disappear before the next drop was added. The temperature was not allowed to exceed 0°C during the addition. After 2 h the manganase dioxide was filtered off and the solvent removed from the filtrate in a rotating-film evaporator. The residue was treated with a mixture of acetic anhydride (25 ml) and pyridine (1 ml) and refluxed for 5 min. After cooling and addition of water (100 ml) the organic material was extracted into pentane. The pentane solution was then worked up by distillation, giving the pure isomers *4* (see Table 1).

Mixtures of trans,cis- *and all*-cis-*3-alkyl-1,2-indandiol diacetates* (*4 and* 5). Iodine (6.5 g) was added all at once at room temperature to a mixture of acetic acid (125 ml), silver acetate (8.6 g, 96 %) and 1-alkylindene (25 mmol). The solution was stirred for 1 h at 20°C and water (2 ml) was added. The solution was then stirred at 80 – 90° for 2 h. From the cooled solution silver iodide was removed by filtration and acetic acid by evaporation. The residue was refluxed for 5 min with acetic anhydride (25 ml) and pyridine (1 ml). The same work-up procedure as above yielded a mixture of *4* and *5* (see Table 2; for R = *t*-Bu, only *5* is formed).

Mixtures of cis-trans- *and all*-trans-*3-alkyl-1,2-indandiol diacetates* (*6 and* 7). This reaction was carried out exactly as described in the preceding case, except that the water was replaced with acetic anhydride (5 ml) and the acetylation procedure was omitted.

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Synthesis of 19,19′-D_6-β-Carotene

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The total synthesis of 19,19′-D_6-β-carotene (*10*) with two fully deuterated in-chain methyl groups is described. The deuterium was introduced by base-catalyzed deuterium exchange of enolizable hydrogen. Two alternative routes were investigated for optimum deuterium incorporation in the final product.
Spectroscopic and other physical properties of intermediates and products are reported.

Previous reports on the preparation of deuterated carotenoids by reduction methods have just been summarized.[1] In the preceding paper we reported the first synthesis of deuterated carotenoids using the exchange technique on a phosphonium salt to give a deuterated phosphorane.[2]

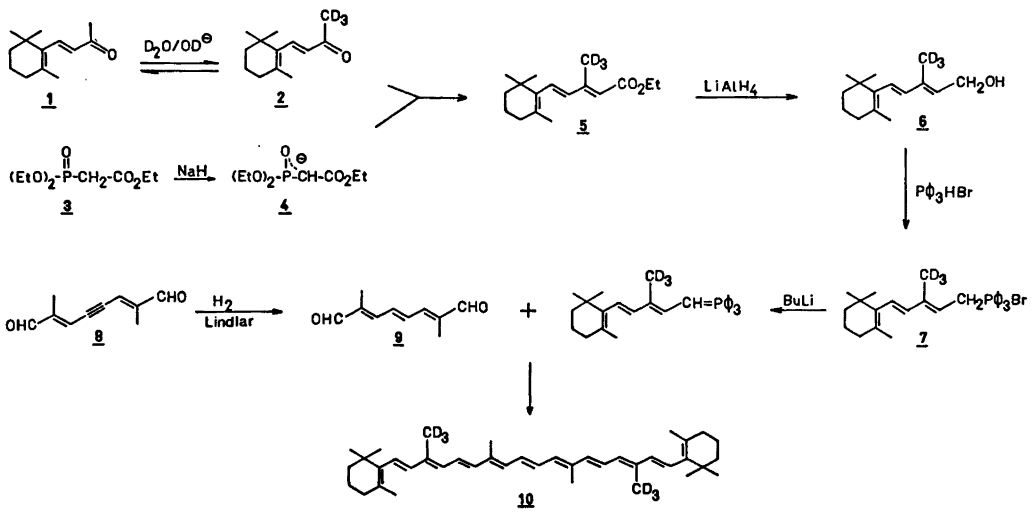
We now report the synthesis of β-carotene with two fully deuterated in-chain methyl groups, involving base-catalyzed deuterium exchange of enolizable hydrogen.

The present work forms part of a larger project comprising synthesis of specifically deuterated carotenoids for mass-spectrometric studies.[3]

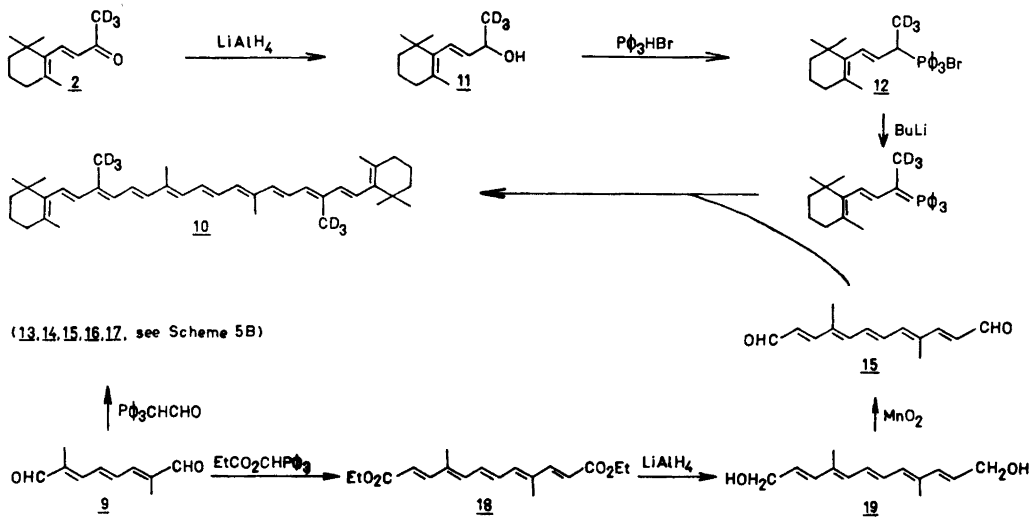
RESULTS AND DISCUSSION

Two alternative approaches were considered for the synthesis of 19,19′-D_6-β-carotene (*10*), shown in Schemes 1 and 2. Both routes involved labelling of β-ionone (*1*) in a base-catalyzed exchange reaction as briefly described by Thomas *et al.*[4]

The shortest, but also least safe route (Scheme 1, route 1), was first pursued. Horner reaction between α,α,α-D_3-β-ionone (*2*, Scheme



Scheme 1.

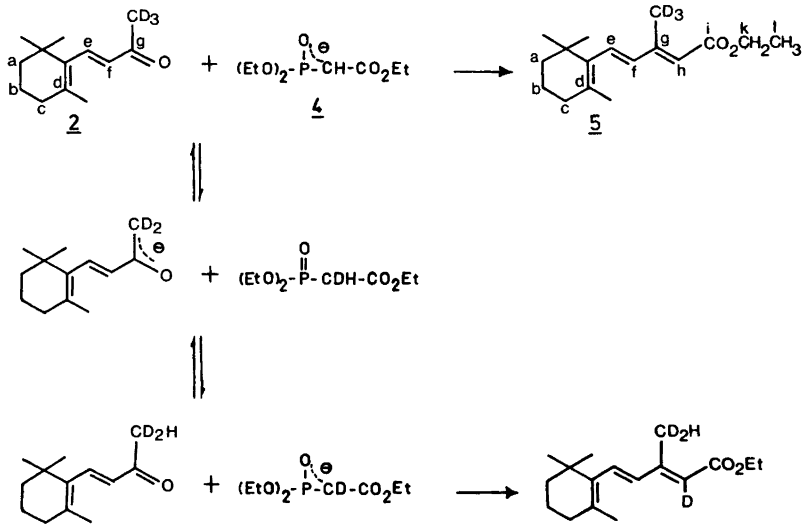


Scheme 2.

2) and triethylphosphonoacetate (*3*) resulted in scrambling of the deuterium. To prevent scrambling *via* enolization of α,α,α-D_3-β-ionone (*2*), route 2 (Scheme 2) involved reduction of the deuterated ketone *2*.

Route 1. Labelled β-ionone (*2*) was obtained by using 0,1 % NaOD in D_2O/pyridine; 1 % NaOD in D_2O/dioxane was employed by Thomas *et al.*[4,5] Also 0.28 N CH_3OLi in CH_3OD effected the same. Maximal incorporation was obtained within 18 min in the NaOD case, while few seconds were sufficient in the latter system. Three deuterium atoms only were incorporated in both cases.[4]

The attempted synthesis of D_3-β-ionylidene acetate (*5*) was performed from α,α,α-D_3-β-ionone (*2*) and triethylphosphonoacetate (*3*).[6] The anion of *3* (*4*) was generated *in situ* with equivalent amount of base (NaH) to prevent enolization of *2*. The resulting ester *5a* was reduced with $LiAlH_4$, and the corresponding alcohol *6a* converted to the phosphonium salt *7a*.



Scheme 3.

Examination of the PMR spectra of the ester *5a* and the alcohol *6a* revealed that some deuterium was lost from the methyl group and present at position *h* (Scheme 3). Thus the phosphonate anion *4* was a sufficiently strong base to effect enolization of α,α,α-D_3-β-ionone (*2*), and served as a deuterium acceptor as shown in Scheme 3. Compounds *5a*, *6a*, and *7a* thus represent mixtures with deuterium in both positions *g* and *h* (Scheme 3; *5*, *6*, *7* and *7*-phosphorane also considered to comprise *cis*-isomers as to the trisubstituted double bond).

The trienedial *9*, obtained by selective, catalytic hydrogenation [7,8] of the corresponding acetylene *8*, was condensed with the phosphorane of the phosphonium bromide *7a* to give deuterated β-carotene (*10a*) with uneven and unsatisfactory deuterium incorporation (D_0:D_1:D_2:D_3:D_4:D_5:D_6:D_7:D_8 = 2:5:12:20:24:21:13:3:0).

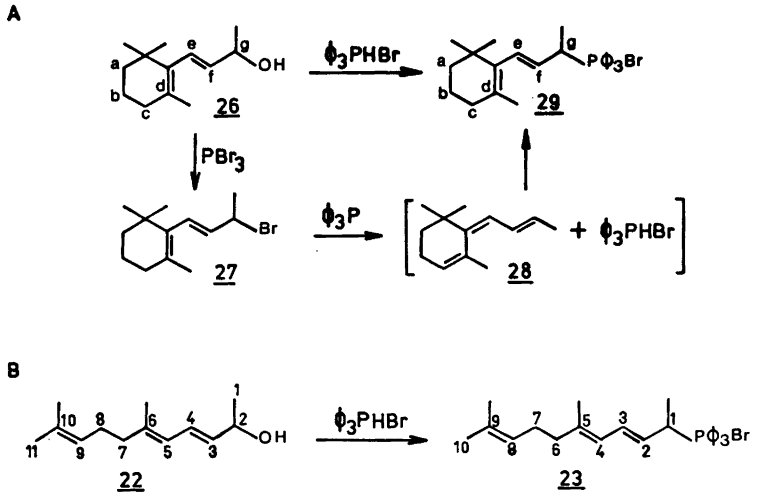
Route 2. On formation of phosphonium salts by S_N2 mechanism primary substrates are favoured.[9] Synthesis of the secondary phosphonium bromide *29* (Scheme 4A) either from the corresponding alcohol *26* [10] or bromide *27* [11] are described in patents only. In the latter case the reaction is presumed to occur *via* the triene *28* intermediate.[12]

As a model was checked the reaction of ψ-ionol (*22*, Scheme 4B) with triphenylphosphonium bromide, which gave the corresponding phosphonium bromide *23* without complications.

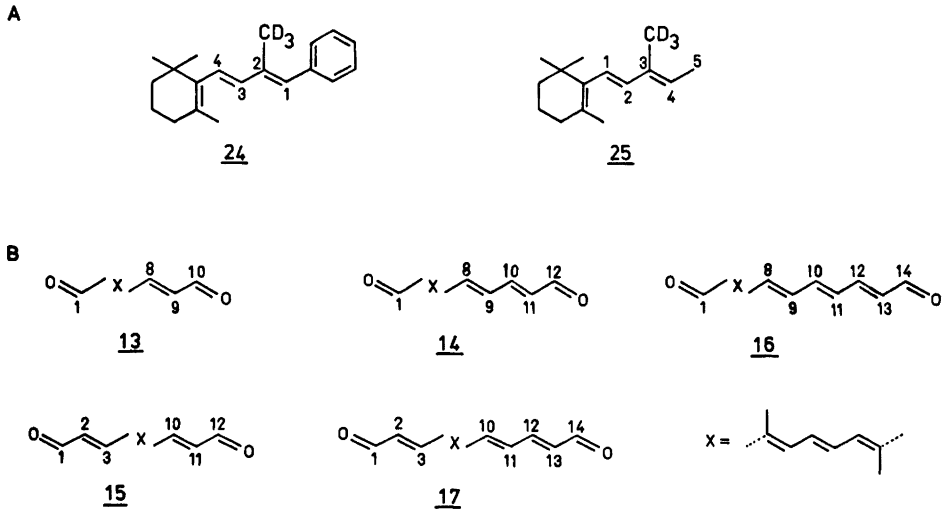
α,α,α-D_3-β-Ionone (*2*) from Route 1 was reduced with $LiAlH_4$, and the corresponding alcohol *11* converted to the phosphonium salt *12* with triphenylphosphonium bromide in like manner and in good yield.

The PMR spectrum of the phosphonium salt *12* showed unexpectedly two singlets at δ 0.70 and 0.88 (6 H), attributed to the *gem.* dimethyl group. Normally the *gem.* dimethyl in an unsubstituted β-end group give rise to a singlet around δ 1.1.[13] The compounds *24* and *25* (Scheme 5A), also prepared, showed singlets at δ 1.02 and 1.05 and δ 1.00 and 1.03, respectively. The abnormal chemical shift of the *gem.* dimethyl group in the phosphonium salt *12* is ascribed to the anisotropic effect of the triphenylphosphonium group, whereas the magnetic non-equivalence may be due to *cis* and *trans* isomerism.

The C_{14}-dialdehyde *15*, used as the central component, was synthesized from the C_{10}-dial *9*. Condensation of the C_{10}-dial *9* with formylmethylene triphenylphosphorane [14] gave a mixture of different products (Schemes 2 and 5B), not readily separable. However, condensation of the C_{10}-dial *9* with carbethoxymethyl triphenylphosphonium bromide to the diethyl ester *18*, followed by reduction with $LiAlH_4$ to the corresponding diol *19*, and allylic oxidation with activated MnO_2 gave the desired C_{14}-dialdehyde *15*.



Scheme 4.



Scheme 5.

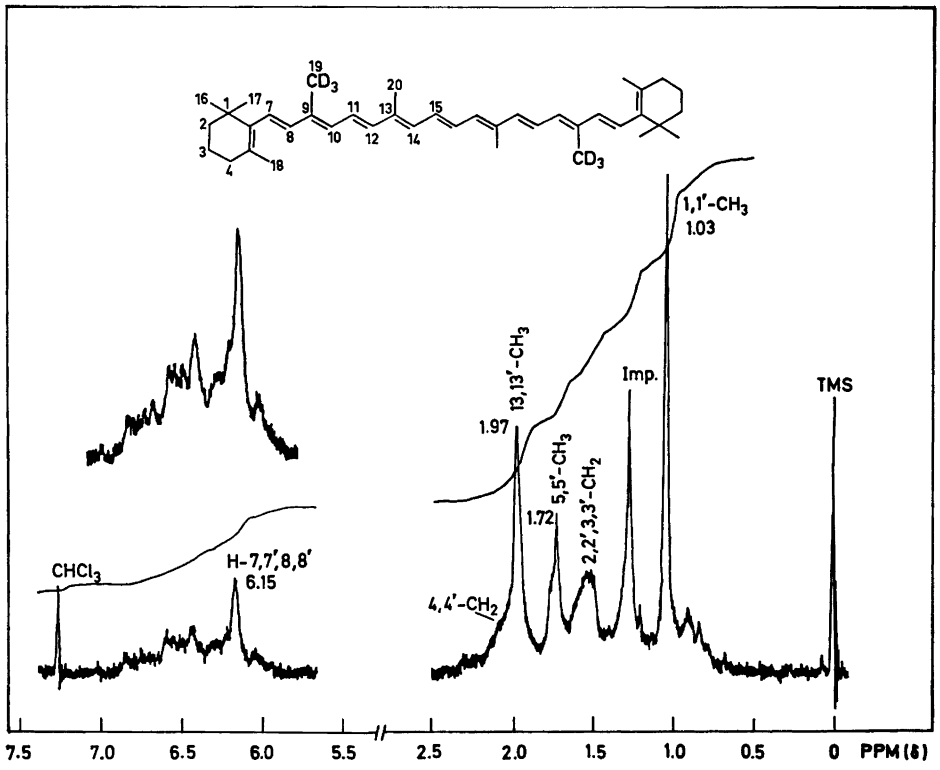


Fig. 1. ^{1}H NMR spectrum ($CDCl_3$) of 19,19'-D_6-β-carotene.

19,19'-D_6-β-Carotene (*10*) was obtained by condensation of the dialdehyde *15* and the phosphorane of the phosphonium bromide *12*. Deuterated β-carotene (*10*) thus prepared had 85 % deuterium incorporation (D_0:D_1:D_2:D_3:D_4:D_5:D_6 = 0:1:2:4:10:42:41). The PMR spectrum of *10* is given in Fig. 1. Specific deuterium incorporation in 19,19' is consistent with the integral for the in-chain methyl signal at δ 1.97 (6 rather than 12 hydrogens). IR absorption at 2110 and 2160 cm^{-1} was ascribed to C–D stretching vibrations.

For the deuterated β-carotene (*10a*), prepared by Route 1, labelled at C-10,10', absorption at 761 cm^{-1} was also observed, compatible with C–D out-of-plane vibration of a trisubstituted double bond (>C=CD–).

The mass spectrum of 19,19'-D_6-β-carotene (*10*) is discussed elsewhere.[3]

EXPERIMENTAL PART

Materials and methods were as described elsewhere.[2,15]

Heavy water was from Norsk Hydro (>99.8 % deuterated). Pyridine *p.a.* (Merck) was distilled and kept over barium oxide. NaH (50 %), delivered in mineral oil, was washed with petroleum ether prior to use. NaOD was from Merck (40 % in D_2O). β-Ionone (Firmenich, synthetic) contained 13 % α-ionone, judged by the ^1H NMR integral.

Complete spectra of the compounds prepared are available elsewhere.[16]

Deuterium incorporation is calculated from the mass spectra, taking naturally occurring isotopes into account, *cf.* Ref. 1.

Route 1

α,α,α-D$_3$-β-Ionone (*2*). β-Ionone (*1*, 9.0 g), D_2O (28.1 g) and pyridine (60 ml) were mixed, 10 drops NaOD added and the mixture stirred at room temperature for 1 h. The product was extracted with ether, washed thoroughly with H_2O (7 times), dried (Na_2SO_4) and evaporated. This gave *2*; yield 8.95 g (98 %); n_D^{20} = 1.5173; λ_{max} (hexane) 281 nm [*E*(1 %, 1 cm) = 400]; ν_{max}(liq.) 3015–2730 (CH), 2227 and 2212 (CD), 1690, 1665 (conj. C=C), 1460–1365 (CH_2,CH_3), 1265, 980 (*trans*-CH=CH), 920 and 735 cm^{-1} (the two last absorptions were absent or very weak in the spectrum of undeuterated β-ionone (*1*)); δ ($CDCl_3$, Scheme 3) 0,87 s and 0.94 s (*gem.* dimethyl in α,α,α-D_3-α-ionone), 1.08 s (6 H, *gem.* dimethyl), 1.77 s (3 H, CH_3-*d*), 1.98–2.13 (2 H, H-*c*), 6.12 d (1 H, J_{e-f} = 16.5 Hz, H-*e*) and 7.31 (1 H, J_{e-f} = 16.5 Hz, H-*f*); *m/e* (130°C) 195 (M), 180 (M–15, 100 %) and 139 (M–56); D_0:D_1:D_2:D_3 = 0:3:21:76; 90 % deuterium incorporation.

Deuterated ethyl β-ionylidene acetate (*5a*). To triethylphosphonoacetate (*3*, 7.5 g) was slowly added NaH (50 %, 1.6 g) in dry ether (20 ml) and the mixture stirred at room temp. for 1 h. *2* (5 g) in dry ether was added during 30 min, and the mixture stirred for 24 h; 80 % conversion judged by TLC. The mixture was transferred to water (1 l), extracted with ether, washed with water, dried (Na_2SO_4) and evaporated; yield 6.12 g *2* and *5a*, plus *ca.* 13 % α-isomers. The reaction was repeated with the product mixture, and the same amount of reagent *4* generated *in situ* as above. The mixture, worked up as above after 2 h, gave *5a*, yield 6.48 g (95 %); n_D^{20} = 1.5361; λ_{max} (hexane) 262 [*E*(1 %, 1 cm) = 550] and (290) nm; ν_{max} (liq.) 3015–2725 (CH), 2210, 2165, 2110, and 2060 (CD), 1715 (C=O), 1605 (conj. C=C), 1455=1360 (CH_2,CH_3), 1235 (C–O), 1215 [not in the undeuterated analogue *20* prepared below, 1150 (C–O), 1080 (not in *20*) and 970 (*trans*-CH=CH); δ (CCl_4), Scheme 3] 0.82 s and 0.92 s (*gem.* dimethyl in the α-isomer), 1.02 s (6 H, *gem.* dimethyl), 1.25 t (3 H, J_{k-l} = 7 Hz, H-*l*), 1.68 s (3 H, CH_3-*d*), 2.29 (*ca.* 1 H, CH_3-*g*), 4.11 k (2 H, J_{k-l} = 7 Hz, H-*k*), 5.66 s (0.7 H, H-h), 6.02 d (1 H, J_{e-f} = 16 Hz, H-*f*) and 6.53 d (1 H, J_{e-f} = 16 Hz, H-*e*); *m/e* (130°C) 266 (M+1), 265 (M), 264 (M–1), 263 (M–2), 262 (M–3), 250 (M–15), 236 (M–29), 220 (M–45), and 59 (100 %); D_0:D_1:D_2:D_3:D_4 = 13:21:31:28:7; 63 % deuterium incorporation (assuming 3 exchangeable H).

Deuterated β-ionylidene ethanol (*6a*). To a mixture of *5a* (5.5 g) in dry ether (10 ml) was added $LiAlH_4$ (0.85 g) in dry ether (50 ml) at 0°C during 30 min. Excess $LiAlH_4$ was destroyed with moist ether and water after 1 h stirring. The mixture was poured into 1 N H_2SO_4 (0°C) and the product extracted with ether. The extract was washed with water, aqueous $NaHCO_3$ solution and water, dried (Na_2SO_4) and evaporated. This gave *6a* plus *ca.* 13 % α-isomer; yield 4.2 mg (91 %); $n_D^{20.5}$ = 1.5363; λ_{max} (hexane) 239 [*E*(1 %, 1 cm) = 560] and (265) nm (reported λ_{max} 237 and 265 in methanol[17]); ν_{max} (liq.) 3320 (OH), 3020–2725 (CH), 2240, 2210, 2155, 2110, and 2065 (CD), 1455–1360 (CH_2, CH_3), 970 (*trans*-CH=CH), 915 and 735 cm^{-1}; δ ($CDCl_3$, reference letters as for *5a* in Scheme 3) 0.82 s and 0.90 s (*gem.* dimethyl in the α-isomer), 1.02 s (6 H, *gem.* dimethyl), 1.68 s (3 H, CH_3-*d*, 2.08 (–OH), 4.27 s and 4.27 d (2 H, J_{h-i} = 7 Hz, H-*i*), 5.62 s and 5.62 t (*ca.* 0.8 H, J_{h-i} = 7 Hz, H-*h*) and 6.07 (2 H, H-*e*, *f*); *m/e*(130°C) 224 (M+1), 223 (M), 222 (M–1), 221 (M–2), 220 (M–3), 179 (M–44), 149 (M–74), 136 (M–87), and 41 (100 %), D_0:D_1:D_2:D_3:D_4 = 14:23:28:27:8; 61 % deuterium incorporation (assuming 3 exchangeable H).

Deuterated β-ionylidene triphenylphosphonium bromide (7a). To triphenylphosphonium bromide (6.15 g) in methanol (65 ml) was added *6a* (4.0 g), and the mixture was stirred for 48 h. The solvent was removed, and the residue washed with ether and ethyl acetate gave *6a* plus *ca.* 13 % α-isomer; yield 5.1 g (51 %). Recrystallization three times from CH_2Cl_2-ethyl acetate gave white, cubic crystals; m.p. 157–158°C (reported 123°C [17]); λ_{max} (methanol) 204, 225, 261, 267, and 274 nm with E(1 %, 1 cm) = 1060, 730, 212, 221, and 208, respectively; ν_{max} (KBr) 3020–2775 (CH), 1440, 1115, 995, 975 (*trans*-CH=CH) and 760–685 (phenyl and bromine); δ ($CDCl_3$, reference letters as for *5a* in Scheme 3) 0.96 (6 H, *gem.* dimethyl), 1.64 (3 H, CH_3-*d*), *ca.* 1.9 (2 H, H-*c*), 4.78 dd (J_{H-P} = 16 Hz, J_{h-i} = 7 Hz, =CH–CH_2P), 4.78 d (J_{H-P} = 16Hz, =CD–CH_2P), 4.78 (total 2 H), 5.29 t (*ca.* 0.6 H, J_{h-i} = 7 Hz, H-*h*), 5.99 (2 H, H-*e,f*) and 7.6-8.1 (15 aromatic H).

2,7-Dimethylocta-2,4,6-triene-1,8-dial (9). 2,7-Dimethylocta-2,6-diene-4-yne-1,8-dial (*8*, 5 g) in ethyl acetate (150 ml) was hydrogenated in the presence of Lindlar catalyst [7] (3.5 g) until one equivalent of hydrogen was absorbed as described by Mildner and Weedon.[8] The catalyst was removed by filtration, the solvent evaporated, and the product crystallized from benzene; *9* had m.p. 133–135°C (reported 135–140°C[8]); λ_{max} (MeOH) 236, 3235 [E(1 %, 1 cm) = 2800] and 334 nm; ν_{max} (KBr) 2810, 2710 (CH), 1655 (C=O), 1600 (conj. C=C), 1435–1330 (CH_3), 1225, 1010, 900, 830, 800, 730, and 670 cm^{-1}; δ($CDCl_3$) 1.95 s (6 H, 2 CH_3), 6.75–7.52 (4 olefinic H, complex coupling) and 9.63 s (2 aldehyde H); *m/e* (75°C) 164 (M).

10,10',19,19'-Deuterated β-carotene (10a). To a suspension of deuterated β-ionylidenethyltriphenyl-phosphonium bromide (*7a*, 0.262 g) in dry ether (20 ml) was added dropwise a solution of 0.06 N BuLi in dry ether (20 ml). Excess BuLi was destroyed by addition of CH_2Cl_2 (3 ml). After 10 min C_{10}-dial (*9*, 33 mg) in CH_2Cl_2 (3 ml) was dropwise added over a period of 20 min, and the mixture stirred at room temp. for 5 h. The solvent was removed and the residue dissolved in 90 % aqueous methanol. The pigments were extracted with petroleum ether, washed with water, dried (Na_2SO_4) and the solvent removed. Chromatography on alumina (Woelm neutral, grade 2 [18]) and elution with benzene gave *10a* yield 15.0 mg (7 %); δ ($CDCl_3$) 0.84 s, 0.92 s (imp. or *gem.* dimethyl in α-carotene), 1.03 s (12 H, CH_3-1,1'), 1.27 s (imp.), 1.49–1.54 (8 H, H-2,2',3,3'), 1.72 s (6 H, CH_3-5,5'), 1.97 s (6 H, CH_3-13,13'), 2.09 (4 H, H-4,4'), 6.14 (H-7,7',8,8'), and 6.0–6.8 (olefinic H). Crystallization from $CHCl_3$-petroleum ether yielded 8.8 mg; m.p. 150–151°C (reported 178–179°C for undeuterated β-carotene [19]), undepressed on admixture with authentic β-carotene; λ_{max} (petroleum ether) 340, 424, *447*, and 474 nm; % D_B/D_{II}[20] = 9.5, % III/II[20] = 42 (reported λ_{max} (petroleum ether, b.p. 80–105°C) *453* and 481 nm[21]); ν_{max} (KBr) 3020–2820 (CH), 2220 (?), 1550 (C=C), 1450 (CH_2), 1395–1360 (CH_3), 1212, 1174, 966 (trans-CH=CH), 761 (>C=CD–?), and 710 cm^{-1}; *m/e* (190°C) 543–536 (M); D_0:D_1:D_2:D_3:D_4:D_5:D_6:D_7:D_8 = 2:5:12:20:24:21:13:3:0; 64 % deuterium incorporation (assuming 6 exchangeable H).

Route 2

α,α,α-D$_3$-β-Ionol (11). α,α,α-D_3-β-ionone (*2*, 3.5 g) in dry ether was reduced with $LiAlH_4$ (0.60 g) in dry ether (50 ml) by the procedure used above for the synthesis of *6a* yield 3.1 g (88 %); n_D^{17} = 1.4923; λ_{max} (hexane) and 233 [E (1 %, 1 cm) = 245]; ν_{max} (liq.) 3300 (OH), 3020–2720 (CH), 2215, 2120 and 2060 (CD), 1445 (CH_2), 1380, 1370 and 1360 (CH_3), 1115, 1045, 1030 (C–O) and 970 cm^{-1} (*trans*-CH=CH); δ (CCl_4, Scheme 4A) 0.80 s and 0.88 s (*gem.* dimethyl in the α-isomer), 0.99 s (6 H, *gem.* dimethyl), 1.64 s (3 H, CH_3-*d*), 2.78 s (1 H, –O*H*), 4.25 d (1 H, J_{f-g} = 6 Hz, H-*g*), 5.40 dd (1 H, J_{f-g} = 6 Hz, J_{e-f} = 16 Hz, H-*f*) and 5.99 d (1 H, J_{e-f} = 16 Hz, H-*e*); *m/e* (110°C) 197 (M), 179 (M–18), 164 (M–33), 141 (M–56), 136 (M–61), 123 (M–74) and 121 (M–76, 100 %); D_0:D_1:D_2:D_3 = 2:5:24:69; 87 % deuterium incorporation.

α,α,α-D$_3$-β-Ionyl-triphenylphosphonium bromide (12). A mixture of α,α,α-D_3-β-ionol (*11*, 2.95 g) and triphenylphosphonium bromide (5.14 g) in methanol (65 ml) was stirred at room temperature for 48 h. The solvent was removed and the residue washed with ether. The product (*12*) yield 6.4 mg (80 %) had λ_{max} (methanol) 207.5, (225), 261, 267, and 274 nm with E(1 %, 1 cm) = 577, 426, 130, 134, and 107, respectively; ν_{max} (KBr) 3050–2830 (CH), 2225 (CD), 1435 (CH_2), 1110, 995, 975 (*trans*-CH=CH), 755, 725 and 695 cm^{-1} (bromide and monosubst. benzene; δ($CDCl_3$, Scheme 4A) 0.70 s and 0.88 s (6 H, *gem.* dimethyl), 1.43 s and *ca.* 1.90 (9 H, H-*a*, *b*, *c*, CH_3-*d*), 4.87–5.37 (1 H, H-*g*, complex coupling), 5.97–7.01 (2 H, H-*e,f*, complex coupling, *cis-trans*?) and 7.6–8.3 (15 aromatic H).

2,7-Dimethyldeca-2,4,6,8-tetraene-1,10-dial (13), 2,7-dimethyldodeca-2,4,6,8,10-pentaene-1,12-dial (14), 4,9-dimethyldodeca-2,4,6,8,10-pentaene-1,12-dial (15), 2,7-dimethyltetradeca-2,4,6,8,10,12-hexaene-1,14-dial (16) and 4,9-dimethyltetradeca-2,4,6,8,10,12-hexaene-1,14-dial (17). A mixture of C_{10}-dial (*9*, 1.52 g) and formylmethylenetriphenylphosphorane (5.69 g) in benzene (300 ml) was refluxed for 22 h as described by Trippett and Walker.[14] An aliquot of the reaction mixture was submitted to TLC on alumina G 254 (20 % acetone in petroleum ether = 25 % APE) giving *8:13:(14+15):(16+17)* = 57:37:5:1, calculated from the electronic spectra using extinction coefficients based on those reported for *9*, *14* and crocetindial.[22]

13 had $R_F = 0.32$; λ_{max} (ether) *ca.* 335, 349 and 369 nm; δ ($CDCl_3$) 1.92 s and 1.94 s (3 H, CH_3-2), 2.05 (3 H, CH_3-7), 6.0–7.5 (6 olefinic H) and 9.0–9.7 (2 H, aldehyde). *14* + *15* had $R_F = 0.28$; λ_{max} (ether) 360, 378, and 401 nm; δ ($CDCl_3$) 1.93 s (CH_3-2 in *14*), 2.05 (CH_3-7 in *14* and CH_3-4,9 in *15*), 6.0–7.5 (8 olefinic H) and 9.0–9.7 (2 H, aldehyde); *14*:*15* = 1:1, calculated from the ^{1}H NMR spectrum. *16* + *17* exhibited $R_F = 0.24$; λ_{max} (ether) 381, 403, and 427 nm; δ ($CDCl_3$) 1.26 (imp.), 1.90 s (CH_3-2 in *16*), 2.02 s (6 H, CH_3-7 in *16* and CH_3-4 in *17*), 6.0–7.5 (10 olefinic H) and 9.5–9.7 (2 H, aldehyde); *16*:*17* = 1:4, calculated from the ^{1}H NMR spectrum.

Diethyl 4,9-dimethyldodeca-2,4,6,8,10-pentaene-1,12-dioate (18). A mixture of C_{10}-dial (*9*, 0.55 g) and carbethoxymethylenetriphenylphosphorane (8.5 g) in $CHCl_3$ (60 ml) was stirred at room temp. for 12 h. Chromatography twice on kieselgel (eluent 25 % benzene in $CHCl_3$) gave *18*; yield 1.02 g (100 %); λ_{max} (ether) 272, 352, *370*, and 392 nm; ν_{max}(KBr) 2980–2850 (CH), 1705 (conj. ester), 1605 (conj. C=C), 1365 (CH_3), 1300, 1175, 1035, 980–970 (*trans*-CH=CH), 850 and 800 cm^{-1}; δ ($CDCl_3$) 1.32 t (6 H, $J = 7$ Hz, 2 CH_3 in ethyl), 1.95 s (6 H, CH_3-4,9), 4.23 q (4 H, $J = 7$ Hz, 2 CH_2 in ethyl) and 5.75–7.60 (8 olefinic H, complex coupling); *m/e* (140°C) 304 (M), 259 (M–45) and 157 (100 %).

4,9-Dimethyldodeca-2,4,6,8,10-pentaene-1,12-diol (19). The C_{14}-diester *18* (1.0 g) in dry ether (50 ml) was reduced with $LiAlH_4$ (0.30 g) in dry ether (50 ml) by the procedure described above for preparation of *6a*. This gave crude *19*; yield 0.47 g (65 %). An analytical amount, purified by TLC on kieselgel (30 % APE), exhibited λ_{max}(ether) 248, 324, 340, and 359 nm; δ ($CDCl_3$) 1.25 (imp.), 1.90 s (6 H, CH_3-4,9) 2.62 s (2 H, -O*H*), 4.23 d (4 H, $J_{1-2(11-12)} = 6$ Hz, H-1,12) and 5.7–6.5 (8 olefinic H, complex coupling); *m/e* (130°C) 220 (M) and 95 (100 %).

4,9-Dimethyldodeca-2,4,6,8,10-pentaene-1,12-dial (15). A mixture of crude *19* (140 mg) and activated MnO_2 (1.40 g) in acetone (75 ml) was kept at room temp. for 12 h. TLC on kieselgel G (20 % APE) gave *15*; yield 41 mg (29 %); λ_{max}(ether) 280, 362, *379*, and 401 nm (previously found λ_{max} (petroleum ether) 358, *378*, and 400 [22]), ν_{max} (KBr) 3040–2710 (CH), 1665 (conj. C=O), 1585 (conj. C=C), 1385 and 1370 (CH_3), 1170, 1125, and 975 cm^{-1} (*trans*-CH=CH); δ ($CDCl_3$) 2.02 s (6 H, $CH_3-4,9$), 6.05–7.35 (8 olefinic H, complex coupling) and 9.64 d (2 H, $J_{1-2(11-12)} = 7.5$ Hz, H-1,12); *m/e* (140°C) 216(M).

19,19′-D_6-β-Carotene (10). To a suspension of α,α,α-D_3-β-ionyl-triphenylphospohonium bromide (*12*, 389 mg) in a dry ether (20 ml) was added dropwise a solution of 0.06 N BuLi in dry ether (30 ml). Excess BuLi was destroyed by addition of CH_2Cl_2 (3 ml). After 10 min was C_{14}-dial (*15*, 40 mg) in CH_2Cl_2 (10 ml) added slowly during 20 min, and the mixture stirred at room temperature for 3 h. The solvent was removed and the residue dissolved in 90 % aqueous methanol. The pigments were extracted with petroleum ether, washed with water, dried (Na_2SO_4) and the solvent removed. Chromatography on alumina (Merck neutral, grade 2 [18]) and elution with 10 % ether in petroleum ether gave *10*; yield 41.2 mg (41 %). Paper chromatography of *10* on Schleicher & Schüll No. 288 paper (1 % ether in petroleum ether) gave two zones: neo a $R_F = 0.61$; λ_{max} (petroleum ether) *442* and 475 nm and all-*trans* $R_F = 0.54$; λ_{max} (petroleum ether) *447* and 471 nm. The *trans* isomer was inseparable from authentic β-carotene, whereas both zones were separated from authentic α- and ε-carotene, also theoretically present. *10* exhibited δ ($CDCl_3$, Fig. 1) 0.85 s (imp.), 0.91 (imp.), 1.03 s (12 H, *gem.* dimethyl), 1.27 s (imp.), 1.5–1.6 (8 H, H-2,2′,3,3′), 1.72 s (6 H, end-of-chain CH_3), 1.97 (6 H, in-chain 13,13′-CH_3), 6.15 (H-7,7′,8,8′) and 6.05–6.85 (14 olefinic H). Crystallization from acetone gave 2.3 mg, *m.p.* 163–164°C (reported 178–179°C for undeuterated β-carotene [19]), undepressed on admixture with authentic β-carotene; λ_{max} (petroleum ether) 339, *447.5* and 474 nm, % D_B/D_{II}[20] = 13, % III/II[20] = 28 (reported λ_{max} in petroleum ether *453* and 481 nm[21]); ν_{max} (KBr) 3020–2820 (CH), 2110 and 2060 (CD), 1705, 1623, 1556 (C=C), 1455 (CH_2), 1395–1360 (CH_3), 967 (*trans*-CH=CH) and 830 (>C=CH–); *m/e* (200°C) 542 (M), 463 (M–79), 450 (M–92), 433 (M–109), 406 (M–136) and 481 (M–161), D_0:D_1:D_2:D_3:D_4:D_5:D_6 = 0:1:2:4:10:42:41; 85 % deuterium incorporation.

Model compounds

Ethyl β-ionylidene acetate (20) was prepared from triethylphosphonoacetate (*3*, 1.5 g), NaH (50 %, 0.32 g) and β-ionone (*1*, 1.0 g) by the same procedure as for *5a* above; conversion 70 %, judged by TLC. Preparative TLC on kieselgel HF 254 + 366 (10 % ether in petroleum ether) gave *5a*, yield 0.65 g (48 %); $n_D^{20.5}$ = 1.5361; λ_{max} (hexane) and ν_{max} (liq.) see *5a* above; δ (CCl_4, Scheme 5A) 2.29 s (3 H, CH_3-*g*) and 5.66 s (1 H, H-*h*), otherwise as for 5a above.

ψ-Ionol (22). A solution of ψ-ionyl acetate [23] (*21*, 8.5 g) in 10 % KOH in methanol (0.5 l) and ether (0.5 l) was stirred for 12 h. Water was added and the product transferred to ether. The ether extract was washed, dried (Na_2SO_4) and the solvent evaporated. This gave *22* yield 6.5 g (93 %); $n_D^{17} = 1.5059$; λ_{max} (hexane) 241 [*E*(1 %, 1 cm) = 1210]; ν_{max} (liq.) 3330, 3020–2725, 1665, 1448, 1375, 1138, 1057, and 965; δ (CCl_4, Scheme 4B) 1.20 d (3 H, $J_{1-2} = 6.5$ Hz, H-1), 1.58 s, 1.65 s and 1.73 s (3 × 3 H, H-11, CH_3-6,10), 2.23 s (1 H, -O*H*), 4.21 dq (1 H, $J_{1-2} = 6.5$ Hz, $J_{2-3} = 6$ Hz, H-2), 5.04 (1 H, H-9), 5.47 dd (1 H, $J_{2-3} = 6$ Hz, $J_{3-4} = 15$ Hz, H-3), 5.71 d (1 H, $J_{4-5} = 10.5$

Hz, H-5) and 6.27 dd (1 H, $J_{3-4}=15$ Hz, $J_{4-5}=10.5$ Hz, H-4); *m/e* (110°C) 194 (M), 176 (M − 18), 151 (M − 43), and 133 (M − 43 − 18).

ψ-Ionyl-triphenylphosphonium bromide (23). A mixture of *ψ*-ionol (*22*, 3.0 g) and triphenylphosphonium bromide (5.3 g) in methanol (40 ml) was stirred at room temp. for 24 h. The solvent was removed and the product washed with ether and water. This gave *23* yield 4.4 g (53 %); λ_{max} (MeOH) 204.5, (225), 261, 267, and 274 [*E*(1 %, 1 cm) = 1050, 630, 192, 184, and 121]; ν_{max}(KBr) 3050 − 2850, 1435, 1375, 1110, 995, 755, 725 and 695 cm^{-1}; δ ($CDCl_3$), Scheme 4B) 1.58, 1.63 (H-10, CH_3-1,5 and 9), 4.70 − 6.50 (H-1,2,3,4, and 8, complex coupling) and 7.60 − 8.20 (aromatic H).

2-D_3-Methyl-1-phenyl-4-(2,6,6-trimethyl-cyclohex-1-enyl)-1,3-butadiene (24). To a mixture of α,α,α-D_3-*β*-ionyl-triphenylphosphonium bromide (*12*, 0.52 g) in dry ether (10 ml) was added 0.06 N BuLi in dry ether (20 ml). Excess BuLi was destroyed by addition of CH_2Cl_2 (3 ml) after 10 min. Freshly distilled benzaldehyde (0.11 mg) was added and the mixture stirred for 2 h at 35°C. TLC twice on kieselgel HF 254 + 366 (petroleum ether) gave *24* (0.11 g, 41 %); λ_{max} (hexane) 288 [*E*(1 %, 1 cm) = 730]; ν_{max} (liq.) 3020 − 2830, 2140, 1595, 1490, 1445 − 1360, 975, 965, 920, 740, and 700; δ ($CDCl_3$, Scheme 5A) 1.02 s and 1.05 s (6 H, *gem.* dimethyl), 1.45 − 1.63 (4 H, H-4′,5′), 1.70 (s?, 3 H, 2′-CH_3), 2.00 m (2 H, H-3′), 6.09 − 6.73 (3 H, H-1,3,4, complex coupling, *cis-trans* around double bonds 1,2 and 3,4?) and 7.22 − 7.37 (5 aromatic H); *m/e* (110°C) 269 (M, 100 %), D_0:D_1:D_2:D_3 = 2:6:21:71; 88 % deuterium incorporation.

3-D_3-Methyl-1-(2,6,6-trimethylcyclohex-1-yl)-1,3-pentadiene (25) was made by the above procedure, using large excess of acetaldehyde (2 ml) instead of benzaldehyde. This gave *25*; yield 0.10 g (48 %); λ_{max} (hexane) 363 [*E*(1 %, 1 cm) = 620]; ν_{max} (liq.) 3020 − 2720, 2230, 2200, 2110, 2060, 1445 − 1360, 970, and 735 cm^{-1}; δ ($CDCl_3$), Scheme 5A) 1.00 s and 1.03 s (6 H, *gem.* dimethyl), 1.72 (s?, CH_3-2′, 1.73 d (H-5, $J_{4-5}=6.5$ Hz), 2.00 m (2 H, H-3′), 5.43 p or dq (1 H, $J_{4-5}=6.5$ Hz, H-4, *cis-trans*?), 6.00 s (H-1,2, *trans*), 6.06 d and 6.50 d ($J_{1-2}=16.5$ Hz, H-1,2, *cis*), *m/e* (110°C) 207 (M), 180 (M − 27), and 46 (100 %), D_0:D_1:D_2:D_3 = 3:9:29:59; 82 % deuterium incorporation.

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Thermodynamics of Vinyl Ethers. VIII.* A Study of the Relative Ring Strain Energies and Relative Ring Entropies of Three- to Eight-membered Carbocyclic Rings with One or No sp^2-Hybridized Carbon Atom

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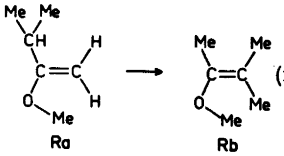
The relative stabilities of three- to eight-membered 1-methoxyvinylcycloalkanes and 1-methoxyethylidenecycloalkanes have been studied by means of chemical equilibration at several temperatures. The thermodynamic functions of isomerization, in comparison with the corresponding functions for the isomerization of 2-methoxy-3-methyl-1-butene to 2-methoxy-3-methyl-2-butene, allowed an estimation of the relative ring strain energies and relative ring entropies of carbocyclic rings with one or no sp^2-hybridized carbon atom.

Although the values of ring strain energy have been established for a great number of saturated and unsaturated cyclic hydrocarbons (cycloalkanes and cycloalkenes, respectively), relatively few reliable data exist of ring strain energies in carbocyclic hydrocarbons with an sp^2-hybridized carbon atom in the ring.[1–4] This is mainly due to a lack of appropriate thermochemical data for such compounds; in fact, in the case of methylenecycloalkanes the standard enthalpy of formation in the ideal gas state at 298.15 K is known only for methylenecyclobutane.[4,5] In addition, the values of ΔH_f° (l, 298.15 K) are known for methylenecyclopentane, ethylidenecyclopentane, and the corresponding cyclohexanes,[4] but no actual enthalpy of vaporization measurements have been reported for these compounds. This lack is not very severe since the standard enthalpy of vaporization can be estimated with good accuracy from the normal boiling point.[6] For other cycloalkanes with an *exo*-cyclic double bond, the enthalpy of formation must be estimated indirectly, which of necessity brings about additional uncertainty in the values of derived quantities, such as ring strain energy. Moreover, there are no gas-phase entropy data for alkylidenecycloalkanes. Gas-phase entropies of many organic compounds can be estimated by the group increment scheme described by Benson *et al.*[7] but, unfortunately, the values of "ring corrections" for carbocyclic rings with an sp^2-hybridized carbon atom are not known.

This paper reports the results of an approach to the evaluation of the relative ring strain energies and relative ring entropies of three- to eight-membered carbocyclic rings with one or no sp^2-hybridized carbon atom. The work is based on the assumption that comparison of the values of the standard enthalpy change, ΔH° (g, 298.15 K), and standard entropy change, ΔS° (g, 298.15 K), of the reactions (1) and (2) should reflect the changes in ring strain energy and ring entropy on going from the saturated ring to one containing an sp^2-hybridized carbon atom:



(reference reaction) (1)

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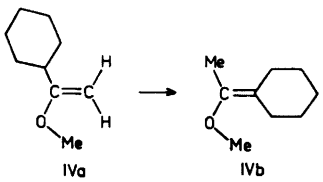
and, for example,



IVa IVb (2)

The degree of double bond substitution by alkyl groups undergoes similar changes in each reaction, and as the stabilizing effect of alkyl groups on olefinic double bonds is virtually independent of the alkyl group,[8] the difference $\Delta H°$ (reference reaction) − $\Delta H°$ [reaction (2)] should indeed reflect the change in ring strain energy for the six-membered ring on going from IVa to IVb, provided that the effects of steric factors across the double bond are equal in both reactions. This is a critical point, and certain corrections are necessary for small-ring compounds up to cyclopentane.

EXPERIMENTAL

NMR spectra. The NMR spectra of the vinyl ethers were recorded on a 60 MHz Perkin Elmer R10 spectrometer at 307 K. Carbon tetrachloride was used as solvent and tetramethylsilane as internal standard. In many cases the spectra were recorded from a mixture of isomers containing mainly one of the two isomers and hence the signals due to the protons of the minor component could not always be detected. The spectra are shown in Tables 1 and 2.

Materials. 1-Methoxyvinylcyclopropane (Ia) and its isomer (Ib). A mixture of cyclopropyl methyl ketone (0.225 mol, Fluka AG), trimethylorthoformate (0.236 mol), and methanol (0.63 mol) was allowed to stand five days at room temperature in the presence of a small amount of *p*-toluenesulfonic acid, after which the mixture was made alkaline by adding a small volume of sodium methoxide in methanol.[9] Fractionation of the mixture at reduced pressure gave the dimethyl acetal of cyclopropyl methyl ketone (b.p. 312.7 – 313.2 K at 3.3 kPa) in 40 % yield. The acetal was distilled from a small amount of *p*-toluenesulfonic acid at atmospheric pressure to give a mixture of methanol and the desired vinyl ethers, boiling temperature 336 – 371 K. The mixture was extracted with water to remove the methanol, after which the organic layer was dried over K_2CO_3. Distillation from $LiAlH_4$ gave pure Ia (b.p. 377.0 K at 99.3 kPa) in 44 % yield.

1-Methoxyvinylcyclobutane (IIa) and its isomer (IIb). The preparation was analogous to that of Ia except that the acetal of cyclobutyl methyl ketone (the ketone was from Ash Stevens Inc., Mich.) was not isolated but the acid reaction mixture was directly subjected to fractional distillation. After a forerun below *ca.* 380 K, a mixture of IIa and IIb (in the mol ratio of 21 to 1) could be collected at 397.8 – 398.2 K at 101.1 kPa. The yield was 85 %.

1-Methoxyvinylcyclopentane (IIIa) and its isomer (IIIb). 1-Cyclopentylethanol (Aldrich) was oxidized with sodium dichromate in aqueous sulfuric acid to cyclopentyl methyl ketone using the procedure described by Brown and Garg.[10] The yield of the product boiling at 431.7 – 432.7 K at 102.5 kPa was 71 %. The ketone was converted to the desired vinyl ethers as described above for IIa and IIb. An approximately equimolar mixture of IIIa and IIIb was collected at 426 – 431 K at 103.3 kPa. The yield was 82 %.

1-Methoxyvinylcyclohexane (IVa) and its isomer (IVb). The preparation was analogous to that of IIIa and IIIb. The ketone (b.p. 340 – 341 K at 1.7 kPa) was obtained in 79 % yield from 1-cyclohexylethanol (Aldrich). A

Table 1. NMR data for some 1-methoxyvinylcycloalkanes in CCl_4 solution. Peak positions are given in τ values (ppm) and the coupling constant in Hz.

Ring size	H *cis* to MeO	H *trans* to MeO	Me in MeO	Ring protons	$J(=CH_2)$
C_3	6.20	6.26	6.44	8.4 – 8.9, 9.3 – 9.6	2.2
C_4	6.21	6.21	6.51	6.8 – 7.4, 7.7 – 8.4	0.0
C_5	6.16	6.32	6.50	7.4 – 8.0, 8.2 – 8.6	2.0
C_6	6.23	6.33	6.54	7.8 – 9.0	2.3
C_7	6.20	6.37	6.53	? [a]	2.0

[a] The spectrum was recorded from a mixture of isomers containing only 30 % of the isomer in question; hence the signals of the major component eclipsed those of the isomer concerned.

Table 2. NMR data for some 1-methoxyethylidenecycloalkanes in CCl_4 solution. Peak positions are in τ values (ppm).

Ring size	Me	Me in MeO	Ring protons
C_5	8.25	6.56	7.6 – 8.1, 8.2 – 8.6
C_6	8.28	6.64	7.6 – 8.2, 8.3 – 8.6
C_7	8.26	6.59	7.6 – 8.1, 8.2 – 8.6
C_8	8.26	6.58	7.6 – 8.1, 8.2 – 8.6

93 % yield of IVa and IVb (in the mol ratio of 4 to 1) was collected at 449 – 451 K at 102.1 kPa.

1-Methoxyvinylcycloheptane (Va) and its isomer (Vb). Cycloheptylmagnesium chloride was prepared from cycloheptyl chloride (0.50 mol, Aldrich) and magnesium (0.55 mol) in 250 cm^3 of dry ether. The Grignard reagent was cooled to *ca.* 263 K, after which 0.50 mol of acetaldehyde in 70 cm^3 of ether was added to the Grignard reagent during about 0.3 h. The product was allowed to warm to room temperature, after which it was poured on ice. Dilute sulfuric acid was added to dissolve the precipitate. The organic layer was separated, and the aqueous layer was extracted with ether. The combined extracts and the original organic layer were washed with sodium bicarbonate solution and water. After drying over K_2CO_3, the ether was evaporated and the residue fractionated to give 1-cycloheptylethanol, b.p. 367 – 369 K at 1.3 kPa, in 37 % yield. The alcohol was oxidized to cycloheptyl methyl ketone as described above for cyclopentyl methyl ketone. The yield of the product boiling at 355 – 357 K at 1.5 kPa was 69 %. The ketone was converted into a mixture of Va and Vb in the usual manner. The yield was 46 %, and the mixture of isomers containing 75 mol % of Vb was collected at 351 – 353 K at 1.2 kPa.

1-Methoxyvinylcyclooctane (VIa) and its isomer (VIb). Cyclooctanol (0.34 mol, Fluka AG) was dissolved in chloroform (150 cm^3), after which thionyl chloride (0.39 mol) was added with stirring so that the chloroform refluxed gently. Refluxing was continued for an hour, or until the evolution of gas ceased. The mixture was poured into water, the layers were separated, and the organic layer was washed with saturated sodium bicarbonate solution and water. The product was dried over $MgSO_4$ and distilled to give a 55 % yield of cyclooctyl chloride, b.p. 345.2 – 346.2 K at 1.1 kPa. 1-Cyclooctylethanol was then prepared from cyclooctylmagnesium chloride and acetaldehyde as described above for 1-cycloheptylethanol. The product, obtained in 15 % yield, was collected at 376 – 378 K at 0.9 kPa. It was oxidized to cyclooctyl methyl ketone (b.p. 362 – 364 K at 0.8 kPa) in 63 % yield. The ketone was converted into a mixture of VIa and VIb (in the mol ratio of 1 to 20, respectively) in the usual manner. The product boiled at 358 – 359 K at 0.8 kPa, and the yield was 62 %.

Purification of the vinyl ethers. Prior to the equilibrations, the mixtures of the isomeric vinyl ethers were purified by preparative GLC using a Carbowax 20M column.

Determination of normal boiling points. The normal boiling points of the isomeric vinyl ethers were determined by the gas-chromatographic method described earlier.[6] The following compounds were used as the reference compounds (compound, normal boiling point): isobutyl vinyl ether (R1), 356.2 K; isobutylidene ethyl ether (R2), 366.7 K; 1-methoxycyclopentene (R3), 387.0 K; 1-ethoxycyclopentene (R4), 409.0 K; 1-methoxycyclohexene (R5), 417.1 K; 1-ethoxycyclohexene (R6), 435.0 K; 1-propoxycyclohexene (R7), 455.9 K; 1-cyclopentoxycyclopentene (R8), 479.7 K. The normal boiling points of IIa and IIb were determined by means of the following reference compounds (compound, relative retention time): R1, 0.255; R2, 0.286; R3, 0.417; R4, 0.574; R5, 0.708; R8, 1.000. The relative retention times of IIa and IIb were 0.501 and 0.656, respectively, corresponding to normal b.p.'s of 399.0 and 413.9 K. *IIIa and IIIb*, reference compounds and their relative retention times: R2, 0.149; R3, 0.226; R5, 0.411; R6, 0.604; R7, 1.000. The relative retention times of IIIa and IIIb (0.461 and 0.633, respectively) led to normal b.p.'s of 422.6 and 437.4 K. *IVa and IVb*, reference curve: R2, 0.180; R3, 0.262; R5, 0.440; R6, 0.619; R7, 1.000. The relative retention times of IVa and IVb (0.817 and 0.971, respectively) corresponded to normal b.p.'s of 447.5 and 454.6 K. *Va and Vb*, reference curve: R2, 0.153; R3, 0.202; R5, 0.303; R6, 0.393; R7, 0.573; R8, 1.000. The relative retention times of Va and Vb (0.844 and 0.985, respectively) led to normal b.p.'s of 473.0 and 479.2 K. *VIa and VIb*, reference curve: R2, 0.187; R3, 0.239; R5, 0.341; R6, 0.429; R7, 0.604; R8, 1.000. The relative retention times of VIa and VIb were 1.420 and 1.547 corresponding to normal boiling points of *ca.* 493.0 and 496.0 K, respectively.

Procedure. Details of the experimental performance of the equilibrations have been described previously.[6] Iodine was used as catalyst and cyclohexane as solvent. The purities of the vinyl ethers were checked by GLC (Carbowax 20 M and SE-30 columns) and NMR spectra.

Table 3. Values of the mean equilibrium constant K and its standard error for the iodine-catalyzed reaction 1-methoxyvinylcycloalkane (a) ⇌ 1-methoxyethylidenecycloalkane (b) in cyclohexane solution at various temperatures. The time of equilibration varied from a few days at the lowest temperatures to a few tens of minutes at the highest temperatures employed; n denotes the number of independent determinations.

Ring size	T/K	$c_{catalyst}$ mol dm^{-3}	$c_{substrate}$ mol dm^{-3}	Isomer composition at start/mol %	n	K(b/a)
C_4	273.2	0.008	1.0	96 (a), 4 (b)	3	0.0551 ± 0.0020
	283.2				3	0.0667 ± 0.0015
	299.2				2	0.0829 ± 0.0015
	323.2				3	0.1047 ± 0.0013
	342.2				3	0.1284 ± 0.0009
	373.2				2	0.1668 ± 0.0001
	394.2				3	0.1912 ± 0.0023
	398.2				3	0.1989 ± 0.0001
C_5	293.2	0.0008	0.3	100 (a)	2	1.288 ± 0.001
	313.2				2	1.332 ± 0.002
	323.2				2	1.363 ± 0.002
	343.2				2	1.388 ± 0.005
	354.7			100 (b)	2	1.413 ± 0.007
	366.2			100 (a)	2	1.433 ± 0.002
	384.2				2	1.463 ± 0.008
	390.2			100 (b)	2	1.467 ± 0.001
C_6	283.2	0.005	0.7	100 (a)	4	0.0629 ± 0.0026
	293.2				7	0.0781 ± 0.0008
	298.2				4	0.0818 ± 0.0009
	303.2				6	0.0861 ± 0.0009
	313.2				9	0.1016 ± 0.0007
	323.2				6	0.1177 ± 0.0010
	333.2				5	0.1291 ± 0.0011
	355.7				2	0.1670 ± 0.0004
	375.2				2	0.1997 ± 0.0007
	389.7				2	0.2326 ± 0.0004
C_7	283.2	0.008	0.6	30 (a), 70 (b)	3	2.69 ± 0.01
	313.2				3	2.74 ± 0.02
	343.2				2	2.85 ± 0.01
	373.2				3	2.88 ± 0.03
	403.2				2	2.94 ± 0.01
C_8	293.2	0.008	0.5	8 (a), 92 (b)	3	16.24 ± 0.27
	313.2				2	14.55 ± 0.07
	343.2				2	12.65 ± 0.04
	373.2				2	11.64 ± 0.08
	402.7				3	10.42 ± 0.04

Peak separation was good, and the compounds were eluted in the sequence a,b. Peak areas, which were considered to be proportional to the molar amounts of the vinyl ethers,[6] were integrated by means of a Hewlett-Packard 3370B integrator.

In cases when the synthetic product contained only a small amount of the less stable isomer, the isomers were not separated by preparative GLC, and then only one initial mixture of isomers was used in the equilibrations. To be sure that true equilibrium had been reached, equilibration times were extended well beyond the time necessary for the isomer ratio to become constant. Moreover, the studied compounds are fairly reactive under the experimental conditions employed so that the state of equilibrium is readily attained without noteworthy side-reactions except for the formation of small amounts of hydrolysis products [$R(MeO)C=CH_2 + H_2O \rightarrow RCOMe + MeOH$; $MeOH + R(MeO)C=CH_2 \rightarrow RMeC(OMe)_2$].

If the less stable isomer could not be separated for its spectral characterization, it was identi-

fied by its retention time in the gas-chromatographic analyses, and by its behavior in the equilibrations. In no cases did the identification provide any difficulty since in addition to the peaks of the two isomers, the only noteworthy peaks could be shown to be due to the hydrolysis products.

Equilibrium constants could not be determined for the reaction Ia⇌Ib, since the position of equilibrium lay so far to the left that the peak of Ib could not be detected in the gas chromatogram.

RESULTS

The values of the mean equilibrium constant K and its standard error at various temperatures are shown in Table 3. As mentioned earlier (see EXPERIMENTAL), equilibrium constants could not be determined for the reaction Ia⇌Ib because of the low thermodynamic stability of the b isomer. For each isomerization reaction the standard Gibbs free energy change $\Delta G°$, calculated from the mean equilibrium constant, proved to be a linear function of temperature (within experimental error, of course). The values of the standard enthalpy change $\Delta H°$ and standard entropy change $\Delta S°$ were obtained by a least-squares treatment of $\Delta G°$ against temperature T ($\Delta G° = \Delta H° - T\Delta S°$). The values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ in the liquid phase at 298.15 K are collected in Table 4. The normal boiling points of the isomeric compounds (see EXPERIMENTAL) were then used for the evaluation of the values of the standard enthalpy and standard entropy of vaporization at 298.15 K.[6] Table 5 gives the values of $\Delta H°$ and $\Delta S°$ in the gas phase at 298.15 K. The calculations have been described in more detail in Refs. 6 and 11.

DISCUSSION

Both molecular models and the available thermochemical data suggest that the compounds studied in this work can be divided into two groups according to the extent of steric *cis* interactions across the double bond in the b isomer: (i) compounds in which the steric interactions can be assumed to be equal to those in the reference compound Rb, and (ii) compounds in which this is not the case. The latter group includes the three-, four-, and five-membered compounds, whereas all the other ring compounds belong to the former group. This can be verified by means of thermochemical data as follows.

From available enthalpy of hydrogenation data,[12] the enthalpy of isomerization of methylenecyclopentane to 1-methylcyclopentene is *ca.* -16.2 kJ mol^{-1} in acetic acid at 298.15 K, but the corresponding value for the isomerization of ethylidenecyclopentane to 1-ethylcyclopentene is -5.4 kJ mol^{-1}. The difference in these values shows that the methyl group of ethylidenecyclopentane stabilizes the *exo*-cyclic double bond by 10.8 kJ mol^{-1}, which is practically equal to the hyperconjugative stabilization (11.3 kJ mol^{-1}) in propene,[8] an apparently "strain-free" molecule. The similarity of these two stabilization energies indicates that there is no measurable destabilization between the methyl group and ring hydrogens of ethylidenecyclopentane. This is supported by the fact that the enthalpy of isomerization of isopropylidenecyclopentane to 1-isopropylcyclopentene is 4.3 kJ mol^{-1} in acetic acid at 298.15 K,[13] which shows that the second *exo*-cyclic methyl group in isopropylidenecyclopentane causes an additional stabilization (relative to ethylidenecyclopentane) of $4.3 - (-5.4) = 9.7$ kJ mol^{-1}, *i.e.*, not significantly less than the first

Table 4. Thermodynamic data for the reaction 1-methoxyvinylcycloalkane → 1-methoxyethylidenecycloalkane in cyclohexane solution at 298.15 K. The errors are twice the standard errors.

Ring size	$\Delta G°$/kJ mol^{-1}	$\Delta H°$/kJ mol^{-1}	$\Delta S°$/J K^{-1} mol^{-1}
C_4	6.271 ± 0.046	9.02 ± 0.26	9.2 ± 0.8
C_5	-0.654 ± 0.012	1.27 ± 0.07	6.46 ± 0.21
C_6	6.211 ± 0.040	10.98 ± 0.30	16.0 ± 0.9
C_7	-2.483 ± 0.023	0.73 ± 0.13	10.76 ± 0.38
C_8	-6.831 ± 0.048	-3.89 ± 0.27	9.9 ± 0.8

Table 5. Thermodynamic data for the reaction 1-methoxyvinylcycloalkane → 1-methoxyethylidenecycloalkane in the gas phase at 298.15 K.

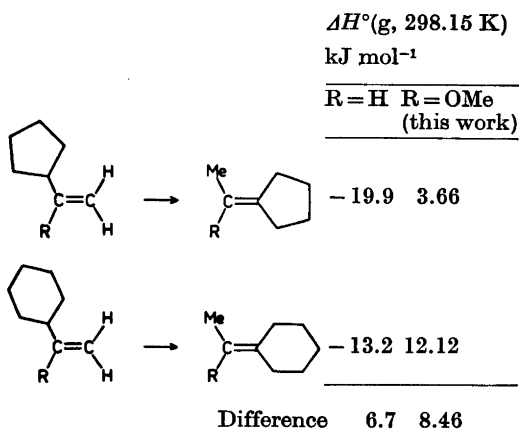
Ring size	$\Delta H°$/kJ mol^{-1}	$\Delta S°$/J K^{-1} mol^{-1}
C_4	11.42 ± 0.40	11.9 ± 0.9
C_5	3.66 ± 0.31	9.0 ± 0.5
C_6	12.12 ± 0.42	17.1 ± 1.0
C_7	1.73 ± 0.33	11.7 ± 0.6
C_8	−3.41 ± 0.40	10.3 ± 0.9

methyl group. Hence it appears that in alkylidenecycloalkanes with three-, four-, and five-membered rings the hydrogen atoms of the *exo*-cyclic methyl groups are not capable of interacting with the hydrogen atoms of the ring.

On the other hand, the enthalpy of isomerization of methylenecyclohexane to 1-methylcyclohexene (acetic acid, 298.15 K) is only 5.0 kJ mol^{-1} more negative than that of ethylidenecyclohexane to 1-ethylcyclohexene,[12] which shows that there are considerable destabilizing interactions between the *exo*-cyclic methyl group and ring hydrogens in ethylidenecyclohexane. As the methyl group of ethylidenecyclopentane brings about a stabilization of 10.8 kJ mol^{-1} relative to methylenecyclopentane, the strain between the methyl group and ring hydrogens of ethylidenecyclohexane is about 10.8 − 5.0 = 5.8 kJ mol^{-1}. This is somewhat higher than the strain in (*Z*)-2-butene, 4.2 − 4.5 kJ mol^{-1},[14] but Skinner [1] has pointed out that for compounds like 2-methyl-2-butene (and, by analogy, ethylidenecyclohexane) carrying two alkyl groups on one of the two sp^2-hybridized carbon atoms and one alkyl group on the other, steric crowding is enhanced so that the total strain between the three alkyl groups amounts to *ca.* 5.8 kJ mol^{-1}, or exactly the same that was found to exist between the methyl group and ring hydrogens of ethylidenecyclohexane. This strain is released when the *cis* interaction is absent, as in ethylidenecyclopentane and the other small-ring compounds.

Moreover, it can be deduced by means of molecular models that the strain between the methyl group and ring hydrogens of ethylidenecycloalkanes larger than ethylidenecyclohexane should be close to that in ethylidenecyclohexane.

Applied to the present compounds, in addition to the disappearance of the destabilizing interaction between the *exo*-cyclic methyl group and ring hydrogens on going from 1-methoxyethylidenecyclohexane (IVb) to IIIb, IIb, and Ib, there remains the question of the possible change in the interaction energy between the methoxy group and ring hydrogens in the same process. This problem can be solved as follows. From available data,[4] the values of $\Delta H°_f$(l, 298.15 K) of vinylcyclopentane and ethylidenecyclopentane are −34.8 and −56.7 kJ mol^{-1}, respectively. The normal boiling points of the above compounds are 373.5 and 385.8 K, respectively.[15] Thus the standard enthalpy of vaporization at 298.15 K of vinylcyclopentane is calculated [6] to be about 36.7 and that of ethylidenecyclopentane 38.7 kJ mol^{-1}. Hence the values of $\Delta H°_f$(g, 298.15 K) of the above compounds are calculated to be about 1.9 and −18.0 kJ mol^{-1}, respectively. Similarly, for vinylcyclohexane and ethylidenecyclohexane, the values of $\Delta H°_f$(l, 298.15 K) are −88.7 and −103.5 kJ mol^{-1}, respectively. The normal boiling points of vinylcyclohexane [15] (400.2 K) and ethylidenecyclohexane [15] (410.0 K) lead to the standard enthalpies of vaporization at 298.15 K of 41.0 and 42.6 kJ mol^{-1}, respectively. Hence the values of $\Delta H°_f$(g, 298.15 K) of vinylcyclohexane and ethylidenecyclohexane are *ca.* −47.7 and −60.9 kJ mol^{-1}, respectively. Now the following standard enthalpies of isomerization can be written:



The difference $\Delta H°$(6-membered ring) − $\Delta H°$(5-membered ring) is *ca.* 1.8 kJ mol^{-1} more positive in the case R = OMe. Hence the destabilizing

interaction energy between the methoxy group and ring hydrogens increases slightly on going from the 5-membered to the 6-membered ring compound.

To summarize, the following discussion of ring strain energies is based on three assumptions: (i) in the 6-, 7-, and 8-membered ring compounds IVb, Vb, and VIb, the strain between the *exo*-cyclic substituents (Me and MeO) and ring hydrogens is taken to be equal to that in the reference compound Rb, (ii) this strain (fixed at $5.8+1.8=7.6$ kJ mol^{-1}) is absent in the 3-, 4-, and 5-membered compounds Ib, IIb, and IIIb, and, (iii) the strain in the a isomers (excluding the strain of the saturated ring) is equal to that in the reference compound Ra.

As to the values of $\Delta H°$(g, 298.15 K) and $\Delta S°$(g, 298.15 K) for the reference reaction, the liquid phase data given in Ref. 11 lead to the following values for the thermodynamic functions in question, if the standard enthalpies and standard entropies of vaporization at 298.15 K are estimated from the appropriate equations given in Ref. 6: $\Delta H°$(g, 298.15 K) $= 9.46 \pm 0.40$ kJ mol^{-1}, $\Delta S°$(g, 298.15 K) $= 17.9 \pm 0.9$ J K^{-1} mol^{-1}.

Relative ring strain energies. Three-membered ring compounds. In this case only a qualitative result was achieved since the peak of Ib could not be detected by GLC, owing to the low thermodynamic stability of the three-membered ring with an sp^2-hybridized carbon atom. Hence the following discussion is based on literature data. From available data,[4] the value of $\Delta H°_f$(g, 298.15 K) of methylenecyclopropane is found to be *ca.* 175 kJ mol^{-1} higher than that of a strain-free methylenecyclopropane molecule calculated from the group increments given by Cox and Pilcher.[4] On the other hand, the experimental enthalpy of formation of gaseous cyclopropane [4] is 115 kJ mol^{-1} higher than the calculated value. Hence the introduction of an sp^2-hybridized carbon atom into the three-membered saturated ring leads to an increase of ring strain of *ca.* 60 kJ mol^{-1}, which causes Ib to be highly unfavored at equilibrium.

Four-membered ring compounds. The enthalpy change $\Delta H°$(g, 298.15 K) for the reaction 1-methoxyvinylcyclobutane → 1-methoxyethylidenecyclobutane is 1.96 kJ mol^{-1} more positive than that for Ra → Rb. If the correction term of 7.6 kJ mol^{-1} (due to the absence of steric interactions across the double bond in IIb) is taken into account, the introduction of an sp^2-hybridized carbon atom into the cyclobutane ring is seen to increase the ring strain by $1.96 + 7.6 \simeq 9.6$ kJ mol^{-1}. This may be compared with the value obtainable from literature data as follows. The experimental enthalpy of formation of gaseous cyclobutane [4] and the group increments of Cox and Pilcher lead to a strain energy of *ca.* 111 kJ mol^{-1} for the cyclobutane ring. The enthalpy of formation of methylenecyclobutane has not been determined experimentally but it can be estimated as follows. The enthalpy of hydrogenation data by Turner and Garner [12] reveal that the enthalpy of 1-methylcyclobutene is *ca.* 3.8 kJ mol^{-1} lower than that of methylenecyclobutane in acetic acid at 298.15 K. Methylenecyclobutane is reported to boil *ca.* 5 K higher than 1-methylcyclobutene,[16,17] and thus the standard enthalpy of vaporization (at 298.15 K) of the latter should be about 0.80 kJ mol^{-1} lower than that of the former.[6] Accordingly, the standard enthalpy change in the reaction methylenecyclobutane → 1-methylcyclobutene is about -4.6 kJ mol^{-1} in the gas phase at 298.15 K, if it is assumed that both isomers have equal enthalpies of solution in acetic acid. The standard enthalpy of formation in the gas phase at 298.15 K of 1-methylcyclobutene can be estimated by assuming that the difference in the values of $\Delta H°_f$(g, 298.15 K) between 1-methylcyclobutene and cyclobutene is equal to that between 1-methylcyclopentene and cyclopentene. The value of $\Delta H°_f$(l, 298.15 K) of 1-methylcyclopentene is -36.2 kJ mol^{-1},[4] and as the normal boiling point [19] (348.7 K) corresponds to a standard enthalpy of vaporization of 32.7 kJ mol^{-1} at 298.15 K,[6] the value of $\Delta H°_f$(g, 298.15 K) of 1-methylcyclopentene is obtained as -3.5 kJ mol^{-1}. As the corresponding value for cyclopentene is 32.4 kJ mol^{-1},[4] the difference in the values of $\Delta H°_f$(g, 298.15 K) between cyclopentene and 1-methylcyclopentene is about 35.9 kJ mol^{-1}. For cyclobutene, $\Delta H°_f$(g, 298.15 K) is *ca.* 156.7 kJ mol^{-1},[4] which leads to a $\Delta H°_f$(g, 298.15 K) of $156.7 - 35.9 = 120.8$ kJ mol^{-1} for 1-methylcyclobutene. Hence the standard enthalpy of formation of gaseous methylenecyclobutane is calculated to be $120.8 + 4.6 = 125.4$ kJ mol^{-1} at 298.15 K. The group increments suggest a standard enthalpy of formation of 5.1 kJ mol^{-1} for a strain-free

methylenecyclobutane, and thus the strain in methylenecyclobutane is obtained as 120 kJ mol^{-1}, which is 9 kJ mol^{-1} higher than the strain in cyclobutane. The result achieved is practically equal to that obtained in this study.

Five-membered ring compounds. If the correction term of 7.6 kJ mol^{-1} is added to the difference (−5.80 kJ mol^{-1}) between the gas phase standard enthalpies of isomerization of 1-methoxyvinylcyclopentane (IIIa) to 1-methoxyethylidenecyclopentane (IIIb) and Ra to Rb, the introduction of an sp^2-hybridized carbon atom into the cyclopentane ring is seen to cause a slight increase (1.8 kJ mol^{-1}) in ring strain energy. This result is in good agreement with the value obtainable from literature data. The standard enthalpy of formation of liquid methylenecyclopentane is −20.1 kJ mol^{-1} at 298.15 K,[4] and the standard enthalpy of vaporization at 298.15 K is estimated to be *ca.* 32.7 kJ mol^{-1} (normal boiling point 348.8 K [15]) leading to a $\Delta H°_f$(g, 298.15 K) of 12.6 kJ mol^{-1}, which is 28.1 kJ mol^{-1} more positive than the value −15.5 kJ mol^{-1}, calculated by means of the group increments. On the other hand, the strain in cyclopentane is obtained to be 25.8 kJ mol^{-1} from its experimental [4] and estimated enthalpy of formation. Thus the literature data lead to an increase of *ca.* 2 kJ mol^{-1} in ring strain energy on going from cyclopentane to methylenecyclopentane.

Six-membered ring compounds. The enthalpy of isomerization of 1-methoxyvinylcyclohexane (IVa) to 1-methoxyethylidenecyclohexane (IVb) is 2.66 kJ mol^{-1} more positive than that of Ra to Rb showing that the introduction of an sp^2-hybridized carbon atom into the saturated six-membered ring increases the strain by this amount. The data of Turner and Garner [12] show that the enthalpy of methylenecyclohexane is *ca.* 10.0 kJ mol^{-1} higher than that of 1-methylcyclohexene in acetic acid at 298.15 K. The value of $\Delta H°_f$(l, 298.15 K) of 1-methylcyclohexene is about −81.2 kJ mol^{-1},[4] and thus the standard enthalpy of formation of liquid methylenecyclohexane is about −71.2 kJ mol^{-1} at 298.15 K. The normal boiling point of methylenecyclohexane is 376.5 K [15] corresponding to a standard enthalpy of vaporization at 298.15 K of about 37.2 kJ mol^{-1}, and hence the value of $\Delta H°_f$(g, 298.15 K) of methylenecyclohexane is *ca.* −34.0 kJ mol^{-1}. The group increments [4] suggest a $\Delta H°_f$(g, 298.15 K) of −36.1 kJ mol^{-1} for a strain-free methylenecyclohexane. Thus the strain in this compound is about 2 kJ mol^{-1}. From the experimental enthalpy of formation of gaseous cyclohexane [4] the strain of the saturated six-membered ring is calculated to be about 0 kJ mol^{-1} which is 2 kJ mol^{-1} less than the strain in methylenecyclohexane. This result is in reasonable agreement with the value 2.7 kJ mol^{-1} achieved in this study.

Seven-membered ring compounds. The enthalpy of isomerization of 1-methoxyvinylcycloheptane (Va) to 1-methoxyethylidenecycloheptane (Vb) is 1.73 kJ mol^{-1} while that of Ra to Rb is 9.46 kJ mol^{-1}. Hence the seven-membered ring containing an sp^2-hybridized carbon atom is about 7.7 kJ mol^{-1} less strained than the saturated ring. From enthalpy of hydrogenation measurements,[12] the enthalpy of isomerization of methylenecycloheptane to 1-methylcycloheptene is *ca.* −9.6 kJ mol^{-1} in acetic acid at 298.15 K. As the normal boiling point of the former is about 1 K lower than that of the latter,[12] the enthalpy difference is about −9.4 kJ mol^{-1} in the gas phase. Moreover, if it is assumed that the difference in the values of $\Delta H°_f$(g, 298.15 K) between cycloheptene [$\Delta H°_f$(g, 298.15 K) = −9.2 kJ mol^{-1}, Ref. 4] and 1-methylcycloheptene is the same as the corresponding difference between cyclohexene and 1-methylcyclohexene, 38.7 kJ mol^{-1},[4] the value of $\Delta H°_f$(g, 298.15 K) for 1-methylcycloheptene is obtained as −9.2 −38.7 = −47.9 kJ mol^{-1}. Hence for methylenecycloheptane, $\Delta H°_f$(g, 298.15 K) = −47.9 + 9.6 = −38.3 kJ mol^{-1}, which is *ca.* 18 kJ mol^{-1} more positive than the value −56.7 kJ mol^{-1} calculated from the group increments. On the other hand, the experimental standard enthalpy of formation of gaseous cycloheptane [4] is 26 kJ mol^{-1} more positive than the calculated value. Thus the decrease in ring strain energy on going from cycloheptane to methylenecycloheptane is about 8 kJ mol^{-1}, in agreement with the result (7.7 kJ mol^{-1}) obtained in this study.

Eight-membered ring compounds. The results of the present work indicate that the introduction of an sp^2-hybridized carbon atom into cyclooctane decreases the ring strain energy by 9.46 − (−3.41) ≃ 12.9 kJ mol^{-1}. In the absence of suitable literature data, a comparison cannot be made.

Summary of the changes of ring strain energy. From the above discussion it appears that on going from a cycloalkane to the corresponding alkylidenecycloalkane, the values of ring strain energy undergo the following changes:

Ring size	Increase of ring strain energy/kJ mol^{-1}
C_3	60 from literature data
C_4	9.6
C_5	1.8
C_6	2.7
C_7	−7.7
C_8	−12.9

These results have been combined with some literature data in Table 6, which shows the values of ring strain energy in carbocyclic rings with no, one, or two sp^2-hybridized carbon atoms. The literature data are based on the difference between the experimental [4] and estimated standard enthalpies of formation of the gaseous compounds at 298.15 K.

The increase of ring strain energy in the three- and four-membered rings with increasing unsaturation of the ring carbons is undoubtedly due to increased bending of the carbon-carbon bond angles from their normal values. On the other hand, the decrease of ring strain in the large-ring compounds (C_7 and C_8) in the same sequence points to decreased destabilizing interactions between incompletely staggered adjacent bonds, or/and to decreased transannular interactions (see, for example, Ref. 18).

Relative ring entropies. For each isomerization reaction studied in this work, the value of $\Delta S°$(g, 298.15 K) is less positive than the entropy change in the reference reaction. As the differences in the values of $\Delta S°$ for the reference reaction and the a → b reactions must be due to differences in ring entropy between a saturated carbocyclic ring and the corresponding ring containing an sp^2-hybridized carbon atom the entropy data show that the saturated ring has a greater entropy value. This result is in agreement with the decreasing values of intrinsic entropy [7,19] on going from cycloalkanes to cycloalkenes or to more highly unsaturated cyclic olefins. O'Neal and Benson [19] have described a method for estimating gas phase entropies of carbocyclic compounds with one or more *endo*-cyclic double bonds from the entropies of the corresponding cycloalkanes by making certain corrections the most important of which is the "double bond tightness correction". This correction is negative for cycloalkenes up to cyclooctene except for cyclopropene for which it is zero. It is of interest to compare the value of the "*endo*-cyclic double bond tightness corrections" of O'Neal and Benson with the "*exo*-cyclic double bond tightness corrections" obtainable from this study by subtracting 17.9 J K^{-1} mol^{-1} (the value of $\Delta S°$ for the reference reaction) from the entropy changes in the a − b reactions involving four- to eight-membered carbocyclic rings:

Ring size	*Exo*-cyclic *vs.* *endo*-cyclic double bond tightness correction /J K^{-1} mol^{-1}
C_4	−6.0/−7.9
C_5	−8.9/−16.3
C_6	−0.8/0.0
C_7	−6.2/−10.4
C_8	−7.6/−13.4

The negative values of the double bond tightness corrections show that in general, increasing unsaturation of the ring carbons leads to increased rigidity of the ring. This effect is least marked for the six-membered ring; on the other hand, it attains a maximum value for the five-membered ring. Hence, on going from cyclohexane to methylenecyclohexane, the rigidity of the ring undergoes only a slight increase. This is understandable since methylene-

Table 6. Values of ring strain energy (in kJ mol^{-1}) in some saturated and unsaturated carbocyclic rings.

Ring size	Ring strain energy in cycloalkane	alkylidene-cycloalkane	cycloalkene
C_3	115	175	225
C_4	111	120	126
C_5	26	28	24
C_6	0	3	5
C_7	26	18	21
C_8	40	27	24

cyclohexane exists in a chair conformation,[20] and thus there is no change in conformation on going from cyclohexane to methylenecyclohexane. However, on going from cyclopentane to methylenecyclopentane, the flexible envelope conformation of cyclopentane is changed to a more rigid half-chair conformation in methylenecyclopentane.[18]

As an application of the *exo*-cyclic double bond tightness corrections given above, the entropy change $\Delta S°$(g, 298.15 K) for the reaction methylenecyclopentane → 1-methylcyclopentene is estimated by means of the group increment method proposed by Benson *et al.*[7] For cyclic compounds, a ring correction term is necessary. This is obtained as 105.3 J K^{-1} mol^{-1} for the five-membered ring in methylenecyclopentane by summing the ring correction term for a saturated five-membered carbocyclic ring (114.2 J K^{-1} mol^{-1}) given by Benson *et al.*, and the *exo*-cyclic double bond tightness correction term (−8.9 J K^{-1} mol^{-1}) for a five-membered ring. Now the entropies $S°$(g, 298.15 K) of methylenecyclopentane (symmetry number = 2) and 1-methylcyclopentene (external symmetry number = 1) are estimated to be 322.9 and 327.7 J K^{-1} mol^{-1}, respectively. Hence the entropy change for the reaction in question is calculated to be about 4.8 J K^{-1} mol^{-1}. From equilibrium [21] and enthalpy of hydrogenation [12] studies in acetic acid at 298.15 K, the values of $\Delta G°$ and $\Delta H°$ are −17.45 and −16.2 kJ mol^{-1}, respectively, and hence the standard entropy change $\Delta S°$(l, 298.15 K) is obtained as 4.2 J K^{-1} mol^{-1}. Since the normal boiling points of the compounds in question are equal within 1 K,[15,22] they are expected [6] to have practically equal standard entropies of vaporization at 298.15 K, and hence the value of $\Delta S°$(g, 298.15 K) is *ca.* 4.2 J K^{-1} mol^{-1}, in agreement with the estimated value.

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Short Communications

Formation of α-(5-Phenyl-1,3-dithiol-2-ylidene)propanethione from Thioacetic Acid and Phenylacetylene

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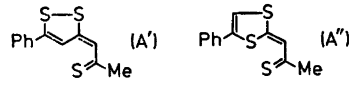
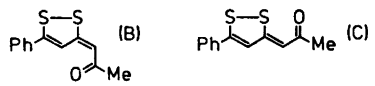
It has been reported that the reaction of thioacetic acid with phenylacetylene in the presence of anhydrous sodium acetate resulted in the formation of an intensely coloured compound, $C_{12}H_{10}S_3$, (A).[1] This compound has later been assigned the *trans* trithiapentalene structure (A').[2-4]

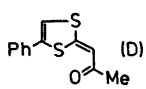


We want to present evidence which conclusively establishes the structure α-(5-phenyl-1,3-dithiol-2-ylidene)propanethione (A'') for this substance.

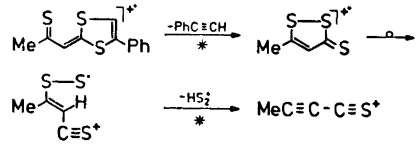
If compound (A) is dissolved in concentrated sulfuric acid a compound with the composition $C_{12}H_{10}OS_2$ can be isolated after dilution with water. The compound has been assigned the structure (B).[2-4]



This structure is unlikely since dithiolylidene ketones (C) have been shown to photoisomerize to *trans* compounds of the same type as (B).[5,6] These *trans* isomers, however, are not stable but revert to starting material *via* a thermal process. The lifetime of the *trans* isomers varies from milliseconds to some minutes. The compound obtained by partial desulfurization is actually α-(5-phenyl-1,3-dithiol-2-ylidene)propanone (D).[7]



The mass spectrum of (A), Fig. 1, is fully in accordance with structure (A''). The dominant fragmentation is loss of phenylacetylene from the molecular ion, a metastable peak corresponding to this fragmentation being present. The loss of acetylenes has been observed to be a general for 1,3-dithioles of the same type as (A'').[8] The $[M-PhC{\equiv}CH]^{+}$ ion further loses $HS_2^{\cdot}$ to give rise to the abundant ion *m/e* 83. These fragmentations can be rationalized for structure (A'') in the following way.



The loss of HS_2 is caracteristic for 5-substituted 1,2-dithiol-3-thiones.[9]

When compound (A) is refluxed for several hours in xylene very little isomerisation to the corresponding 2-methyl-5-phenyl-1,6,6a^{IV}*S*-trithiapentalene occurs; however, if thioacetamide is added to the refluxing solution rapid isomerisation takes place in accordance with previous observations.[10-12]

1,2-Dithiol-3-thiones react with activated acetylenes in a cycloaddition reaction forming α-(1,3-dithiol-2-ylidene)thioketones.[10,13,14] We have succeeded in preparing a compound identical with (A) by reacting 5-methyl-1,2-dithiol-3-thione with phenylacetylene in boiling xylene. By partial desulfurization in concentrated sulfuric acid a compound identical to the compound which had been assigned structure (D) was obtained.

We have observed, that 5-methyl-1,2-dithiol-3-thione is formed in a refluxing solution of anhydrous sodium acetate in thioacetic acid. It has further been observed that the amount of 5-methyl-1,2-dithiol-3-thione is augmented by addition of diacetyl disulfide. We therefore propose that diacetyl disulfide is a precursor for the thione which reacts in a cycloaddition forming (A). This is further substantiated by the observation that the yield of (A) increased in proportion to the amount of diacetyl disulfide added to the reaction mixture.

Experimental. *α-(5-Phenyl-1,3-dithiol-2-ylidene)propanethione(A'').* Phenylacetylene (10 g), thioacetic acid (40 g), and anhydrous sodium acetate (1 g) were refluxed for 3 h, and the

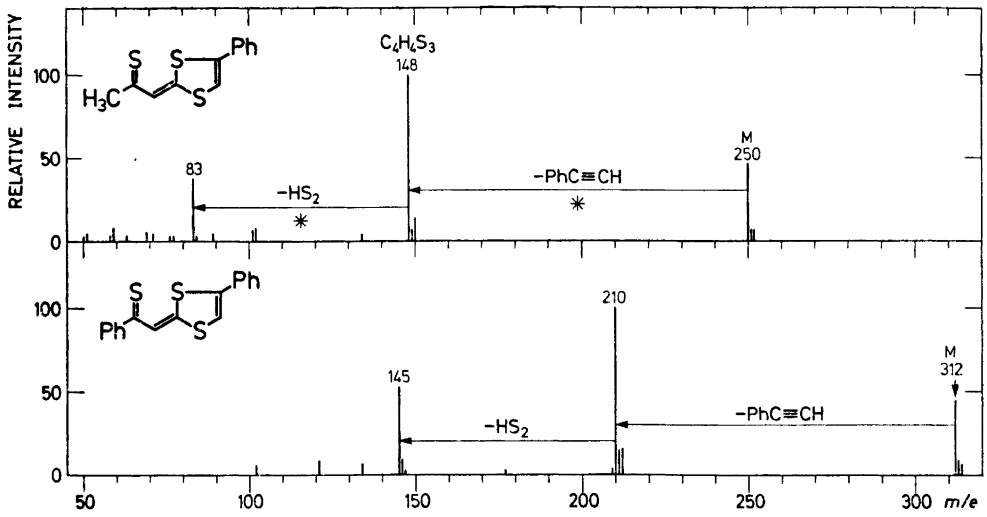


Fig. 1. Mass spectra of α-(5-phenyl-1,3-dithiol-2- ylidene) propanethione, and α-(5-phenyl-1,3-dithiol-2-ylidene)thioacetophenone.

reaction mixture was left over-night. The crystals that separated were washed with water. Yield 1.8 g, 7 % based on phenylacetylene. Recrystallized from glacial acetic acid; m.p. 185 – 187 °C (isomerization). M^+ 249.9945; calc. for $C_{12}H_{10}S_3$ 249.9904, M – 102 148.0060; calc. for $C_4H_4S_3$ 148.0071. (Found: C 57.55; H 4.13; S 38.43. Calc. for $C_{12}H_{10}S_3$: C 57.60; H 4.03; S 38.37).

α-(5-Phenyl-1,3-dithiol-2-ylidene)propanone (D) (A″) (500 mg) was dissolved in cold concentrated sulfuric acid (10 ml); after 10 min at room temperature the yellow solution was warmed for 5 min on the water bath. After cooling the solution was poured into ice. The product was extracted with chloroform. After evaporation the compound was recrystallized from ethanol. Yield 300 mg; m.p. 157 – 158 °C, (lit. 157 – 158 °C,[4] 152 – 154 °C [7]).

2-Methyl-5-phenyl-1,6,6a^{1V}*S-trithiapentalene.* Compound (A″) (300 mg) was refluxed in xylene (25 ml). After 4 h small amounts of trithiapentalene were detectable by means of TLC. Thioacetamide (300 mg) was added, and the solution was refluxed for a further 4 h, after which (A″) was 75 % converted to trithiapentalene. After evaporation and chromatography on alumina 100 mg of crystals were isolated, Recrystallized from cyclohexane, m.p. 168 – 169 °C, (lit. 169 °C [15]).

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On the Rearrangement of 1-Aralkylindenes

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In connection with studies [1,2] on the tautomerism of indene, an unusual effect of the triphenylmethyl group on the base-catalyzed prototropic rearrangement has been observed. 1-Benzyl-, 1-benzhydryl-, and 1-triphenylmethylindene were rearranged to the corresponding 3-substituted indenes in benzene with diazabicylo[2.2.2]octane (DABCO) as catalyst. The rate constants observed are given in Table 1, together with that found for the rearrangement of 1-methylindene. The trend of moderate increase in the rate constant observed on substituting the hydrogens in the methyl group by one and two phenyl groups is drastically changed for 1-triphenylmethylindene. This compound rearranges 30 times slower than 1-benzhydrylindene and about 5 times slower than 1-methylindene.

If one of the hydrogens in the methyl group of 1-methylindene is substituted, the transition state for proton abstraction (which is rate-determining in the rearrangement of 1-substituted indenes) can adopt a conformation in which steric hindrance between the catalyst is of minor importance. Thus, in this case, the retarding effect of a methyl substituent (1-ethylindene) as well as the accelerating effect of the methoxy-, acetoxy-, and thioacetoxy groups can be qualitatively understood on the basis of electronic effects as shown by Ahlberg.[3] Furthermore, in benzhydrylindene, rotation about the bond between the indenyl ring and benzylic carbon can minimize the steric interaction between the catalyst and the substrate, and electronic factors may thus account for the moderate rate acceleration found when going from 1-methylindene to benzhydrylindene (*cf.* Tafts' σ^*-substituent constants [4]).

The abrupt drop in the rearrangement rate which is observed when going to 1-triphenylmethylindene (Table 1) must to a large extent be ascribed to the lack of a conformationally favourable transition state. The series methyl-, benzyl-, benzhydryl-, and triphenylmethylindene is very suitable for the study of such effects, since the fully substituted compound deviates from the trend in the series. Successive substitution of the hydrogens of methylindene by methyl groups gives a monotonic decrease in the rate constant.

As regards the equilibrium constant between the 3- and the 1-substituted indene isomer, the triphenylmethyl group differs from the *t*-butyl group. The equilibrium constant, [3-*t*-butylindene]/[1-*t*-butylindene], is 33 $\pm$1,[5] a value smaller than that found for [3-methylindene]/[1-methylindene] (about 100).[5] This effect can, at least partly, be accounted for by the increased *peri*-interaction between the substituent and the aromatic hydrogen in the 3-isomer on going from methyl- to *t*-butylindene. In an analogous way, the equilibrium constant [3-triphenylmethylindene]/[1-triphenylmethylindene] would be expected to be smaller than 33, but it is in fact larger than 50. It is possible that steric interactions also in the 1-isomer in this case may be responsible for this observation. However, all peculiarities of the triphenylmethyl-group are still not understood.

Experimental. The aralkylindenes were prepared and purified according to procedures previously recorded.[2] Stock solutions of diazabicyclo[2.2.2]octane (Kebo), m.p. 158 – 159 °C, recrystallized twice from hexane, in benzene (Mallinckrodt), dried over molecular sieves and distilled, were prepared. The aralkylindenes were weighed into 10 ml flasks and base solution added, so that the substrate concentration was about 0.3 M. The flask was placed in a thermostat at a temperature of 35.0 $\pm$ 0.1 °C. Samples were withdrawn at intervals, quenched with 2 M hydrochloric acid and analyzed in the NMR-apparatus. The signals of the *x*-protons (*cf.* Ref. 1) of 1- and 3-aralkylindenes were examined and the corresponding integrals re-

Table 1. Comparison of the isomerization rates of 1-methylindene and some 1-aralkylindenes with diazabicylo[2.2.2]octane in benzene at 35 °C.

Substrate	Conc. of catalyst [B] (mol/l)	k_1 pseudo-first order $\times 10^5$ (sec^{-1})	$k_1 \times 10^3$/[B] (l mol^{-1} sec^{-1})
1-Methylindene	2.41×10^{-2}	11.3 $\pm$0.2	4.69 $\pm$ 0.09
1-Benzylindene	4.15×10^{-3}	2.56 $\pm$ 0.12	6.17 $\pm$ 0.35
1-Benzhydrylindene	4.40×10^{-3}	12.1 $\pm$0.6	27.5 $\pm$1.4
1-Triphenylmethylindene	2.64×10^{-2}	2.40 $\pm$ 0.15	0.91 $\pm$ 0.05

corded. The reaction was followed for at least 3 half-life times. No 1-substituted indene could be detected at equilibrium. From the equilibrium solutions, the 3-substituted indenes were isolated and recrystallized from isopropanol. The distinct melting points of the indenes isolated and the NMR-spectra showed that the products were uniform in character. The equilibrium constants were determined by the NMR-technique with the aid of a computer of average transients. All equilibrium constants were larger than 50. The experimental technique did not allow a more precise determination.

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Isoelectric Focusing in Deuterium Oxide Density Gradient

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The present paper is a preliminary report on the use of deuterium oxide as a substitute for sucrose when preparing density gradients for small isoelectric focusing columns. Full details of the technique used and the results obtained will be given elsewhere.

The isoelectric focusing column utilized in the present work is a modification of the 1.5 ml column described by Jonsson *et al.*[1] *Inter alia*, the bottom electrode of platinum wire has been exchanged for a sheet of palladium covering the bottom of the focusing chamber.

The D_2O density gradient has been created directly in the column by free interdiffusion of three D_2O solutions for 3 min.[2] The resulting D_2O concentration course, as obtained by measurement of the refractive index gradient,[2] has a very high degree of linearity. The initial D_2O density gradient (0.018 g cm^{-4}) is as strong as the initial sucrose density gradient used by Jonsson *et al.* As expected, the stability in time is less for the D_2O density gradient than for the sucrose density gradient. However, the D_2O density gradient remaining after the column has been standing in an upright position for two hours (0.016 g cm^{-4}), is still more than twice as strong as the sucrose density gradient obtainable in an LKB 110 ml column with 500 g of sucrose per litre as initial bottom concentration. Thus, the D_2O density gradient described should normally be quite sufficient for stabilization of protein zones. This conclusion is supported by results obtained in this laboratory at isoelectric focusing of β-lactoglobulin (*cf.* Fig. 1) and sperm whale myoglobin.

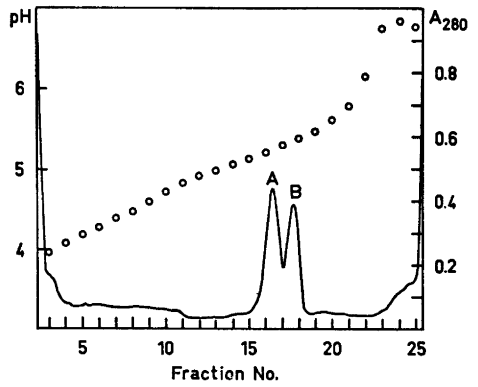

Fig. 1. Scan of 1.5 ml column [1] at 280 nm (solid curve) obtained after D_2O density gradient isoelectric focusing of 100 μg β-lactoglobulin in 1 % Ampholine pH 4 – 6 for 75 min. Average field strength 30 V cm^{-1}. Superimposed are pH values of 60 μl fractions of the column contents (circles).[3] (A represents β-lactoglobulin A, and B β-lactoglobulin B.)

Isoelectric points evaluated from isoelectric focusing runs in D_2O density gradients were found to be about 0.1 pH unit higher than those obtained in sucrose density gradients. This increase seems to be the net result of two effects: the intrinsic pK_a values of the protolytic groups in proteins are higher in D_2O than in H_2O but simultaneously the pH meter reading in D_2O solution is lower than in H_2O solution for solutions of comparable acidity.

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Isoelectric Focusing in Deuterium Oxide Density Gradient

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The present paper is a preliminary report on the use of deuterium oxide as a substitute for sucrose when preparing density gradients for small isoelectric focusing columns. Full details of the technique used and the results obtained will be given elsewhere.

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The D_2O density gradient has been created directly in the column by free interdiffusion of three D_2O solutions for 3 min.[2] The resulting D_2O concentration course, as obtained by measurement of the refractive index gradient,[2] has a very high degree of linearity. The initial D_2O density gradient (0.018 g cm^{-4}) is as strong as the initial sucrose density gradient used by Jonsson *et al.* As expected, the stability in time is less for the D_2O density gradient than for the sucrose density gradient. However, the D_2O density gradient remaining after the column has been standing in an upright position for two hours (0.016 g cm^{-4}), is still more than twice as strong as the sucrose density gradient obtainable in an LKB 110 ml column with 500 g of sucrose per litre as initial bottom concentration. Thus, the D_2O density gradient described should normally be quite sufficient for stabilization of protein zones. This conclusion is supported by results obtained in this laboratory at isoelectric focusing of β-lactoglobulin (*cf.* Fig. 1) and sperm whale myoglobin.

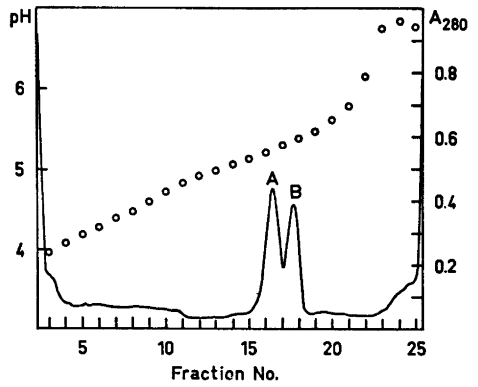

Fig. 1. Scan of 1.5 ml column [1] at 280 nm (solid curve) obtained after D_2O density gradient isoelectric focusing of 100 μg β-lactoglobulin in 1 % Ampholine pH 4 – 6 for 75 min. Average field strength 30 V cm^{-1}. Superimposed are pH values of 60 μl fractions of the column contents (circles).[3] (A represents β-lactoglobulin A, and B β-lactoglobulin B.)

Isoelectric points evaluated from isoelectric focusing runs in D_2O density gradients were found to be about 0.1 pH unit higher than those obtained in sucrose density gradients. This increase seems to be the net result of two effects: the intrinsic pK_a values of the protolytic groups in proteins are higher in D_2O than in H_2O but simultaneously the pH meter reading in D_2O solution is lower than in H_2O solution for solutions of comparable acidity.

1. Jonsson, M., Pettersson, S. and Rilbe, H. *Anal. Biochem. 51* (1973) 557.
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3. Fredriksson, S. *Anal. Biochem. 50* (1972) 575.

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High Resolution Mass Spectra of Aromatic γ-Dilactones

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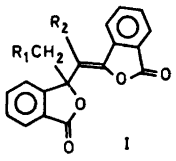
The mass fragmentation of some α,β-unsaturated γ-dilactones formed by condensation of certain γ-keto-acids [1] was recently reported on.[2] When aromatic γ-keto-acids were condensed similar γ-dilactones were formed where the aromatic ring constitutes the α,β-unsaturation (I). The availability of the proper aromatic γ-keto-acids [1] gave in hand γ-dilactones well suited for a study of the effect of other substituents (*i.e.* at the double bond connecting the two lactone rings and at the sp^3 ring-carbon-atom) on the fragmentation of such systems. The main fragments in the high mass region are given in Table 1.

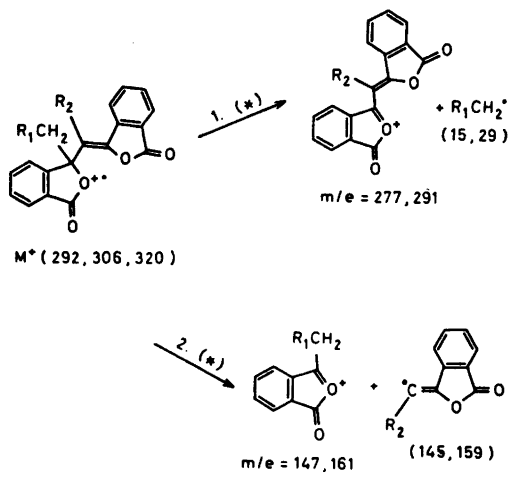


I

a : $R_1 = R_2 = H$
b : $R_1 = H$, $R_2 = Me$
c : $R_1 = R_2 = Me$

The molecular ions are involved in two simple bond cleavage processes, both being associated with a change in hybridization at the only sp^3 ring-carbon atom in the molecules (Scheme 1). The first involves splitting off the R_1CH_2-radical and the other route leads to the carbonium ion believed to be one of the key intermediates in the selfcondensation of the γ-keto-acids.[1]

Scheme 1 is interesting in many respects. The structural difference between Ib and Ic lies in the substituent R_1 and therefore the observed

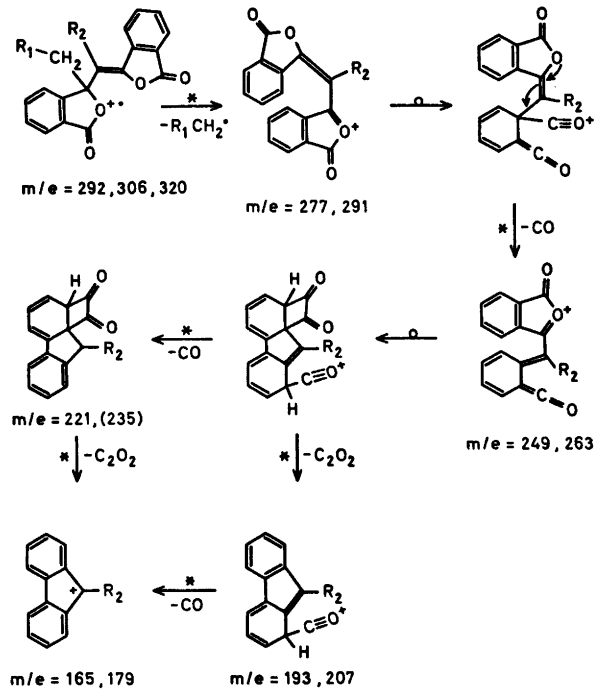


Scheme 1. Consecutive ejection of carbon monoxide (or C_2O_2) leading to fluorenyl cations. Ia. Preferred route: 1. Ib. Preferred route: 2. Ic. Preferred route: 1.

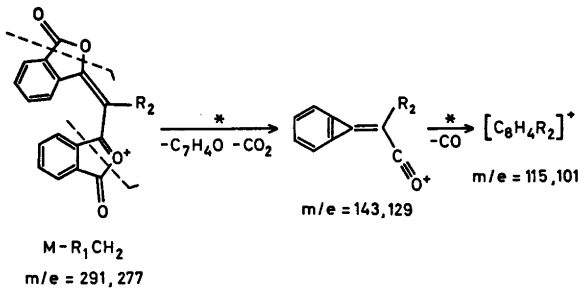
variation in fragmentation must be due to the difference in stabilities of the radicals R_1CH_2· and/or the relative stabilities of the carbonium ions formed by route 2. From measurements of ionization potentials an ethyl group is found to stabilize a carbonium ion better than a methyl group by about 1.9 kcal/mol,[3] and thus the carbonium ion formed by route 2 should be more stable from Ic than from Ib. However, the limit of error of the ionization potential measurements was estimated to 1.2 kcal/mol. On the other hand, most values for stabilities of radicals favor the ethyl radical in comparison to the methyl radical by 3 – 6 kcal/mol. Thus this greater difference in radical stabilities explains the preference for route 1 for Ic and

Table 1. Relative intensities of main high masses in the fragmentation of I.

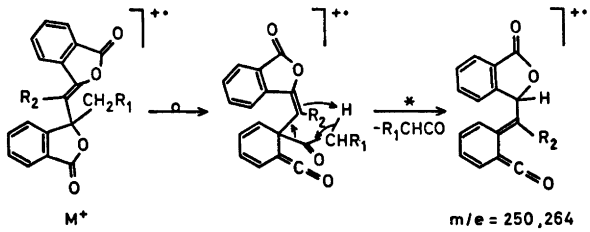
Ia		Ib		Ic		Fragment
Rel. int.	*m/e*	Rel. int.	*m/e*	Rel. int.	*m/e*	
8	292	6	306	3	320	M^+
100	277	20	291	100	291	$M-R_1CH_2$
22	250	3	264	1	264	$M-R_1CH=C=O$
26	249	4	263	3	263	$M-R_1CH_2CO$ (or $M-R_1CH_2,-CO$)
3	221	0	235	0	235	$M-R_1CH_2,-2CO$
15	193	5	207	5	207	$M-R_1CH_2,-3CO$
16	165	1	179	0.4	179	$M-R_1CH_2,-4CO$
19	147	100	147	8	161	$M-R_2C_9H_4O_2$
29	129	22	143	54	143	$M-R_1CH_2,-C_7H_4O,-CO_2$
9	101	10	115	20	115	$M-R_1CH_2,-C_7H_4O,-CO_2,-CO$



Scheme 2. Fragmentation of M-$R_1\dot{C}H_2$.



Scheme 3. Competitive fragmentation of molecular ion.



Scheme 4. Ketene ejection from molecular ion.

route 2 for Ib. The different fragmentation preference for Ia and Ib must lie in the relative stabilities of the entities carrying the substituent R_2. Again the radical stabilities seems to be more important, *i.e.* a methyl group (compared to a hydrogen atom at a double bond) must be more effective in stabilizing radicals than carbonium ions.

Similar to the aliphatic analogues, fragmentations involving consecutive losses of carbon monoxide are observed. The metastable defocusing technique once again revealed that two molecules of carbon monoxide (or C_2O_2) are ejected simultaneously. These fragmentations leading to fluorenyl cations are dealt with in Scheme 2. The rearrangement of $[M-R_1CH_2]^+$ and the intermediate formation of α-diketones before ejection of C_2O_2 are processes discussed earlier.[2]

As seen from Table 1 the path through $m/e=221$ (Ia) or $m/e=193$ (Ia) and 207 (Ib and Ic) is preferred only to some extent. In fact, the fragmentation route in Scheme 1 is very prominent only for Ia. When $R_2=Me$ (Ib and Ic) another route seems to be of more importance. Here fragmentation of $M-R_1CH_2$ directly to $m/e=143$ is preferred, demonstrated by the observation of the proper metastable peak (Scheme 3).

As for the aliphatic analogues,[2] metastable peaks indicating the elimination of ketenes from I are observed. In these aromatic dilactones such processes must involve rearrangements destroying the aromatic structure in one of the aromatic rings.

However, compared with other processes, only for Ia this represents a major fragmentation path.

Experimental. Mass spectra (70 eV, ion source temperature 230 °C) were obtained on an AEI MS 902 mass spectrometer connected to an AEI DS 30/64/H data system. Peak compositions were within 4 ppm of calculated values. Metastable peaks were observed in the spectra and confirmed by the metastable defocusing technique.

1. Kolsaker, P. and Berner, E. *Tetrahedron 29* (1973) 1095.
2. Kolsaker, P. *Org. Mass Spectrom. 7* (1973) 535.
3. Lossing, F. P. *Mass Spectrometry*, McGraw, New York 1963, p. 442.

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The Dimerization of Coniferyl Alcohol in Aqueous Sodium Hydroxide*

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p-Hydroxyarylglycerol β-aryl ether structures (*1*), present originally as such or formed during degradation, have been shown to be the major site of lignin fragmentation in kraft pulping.[1] Structures of type *1* fragment to give predominantly *trans*-coniferyl alcohol (*2*).[2] This undergoes further transformation, in part via its extended quinone methide *3*. By this process the side chains of units derived from *2* and *3* condense to the final product, kraft lignin. The present paper is concerned with the elucidation of some of the reactions by which *2* reacts with the kraft lignin being formed.

Heating of *2* in dilute aqueous sodium hydroxide for 2 h at 170 °C gives a polymer.[2a,3] Little monomeric or dimeric material remains. The most characteristic feature of this polymer (in a strict sense it should be termed a telomer) is the absence of the γ-hydroxymethyl group present in its precursor *2*. This was demonstrated by recording the NMR-spectrum of the acetylated polymer. A similar polymer can be made by alkaline treatment of γ-coniferyl benzoate, which is believed to give primarily the quinone methide *3*.[4]

Suitable choice of the conditions of heating coniferyl alcohol in dilute sodium hydroxide (120 °C 1 h) afforded considerable amounts of dimeric material. Gas chromatography of the trimethylsilylated reaction mixture [bis(trimethylsilyl)trifluoracetamide in pyridine] showed that the dimer fraction has two major components (*A* and *B*). Small amounts of these were collected and analyzed by high resolution mass spectroscopy. The fragmentation pattern and determinations of exact masses of the molecular ion (*B*) and of a prominent fragment (*A*) suggested structures *4* and *5* for *A* and B, respectively. Compound *4* is the 1,8-addition product of the carbanion of *2* to the quinone methide *3*. Proton abstraction from solvent by the anion of *4* gives a quinone methide (*6*) which undergoes cyclization with preferential reaction by the less hindered primary hydroxyl group, yielding *5*.

p-Coumaryl alcohol reacted in the same way as *2*, giving major amounts of the non-meth-

* Part IV of the series "Degradation of Lignin by Kraft Cooking", Part III, Ref. 2a.

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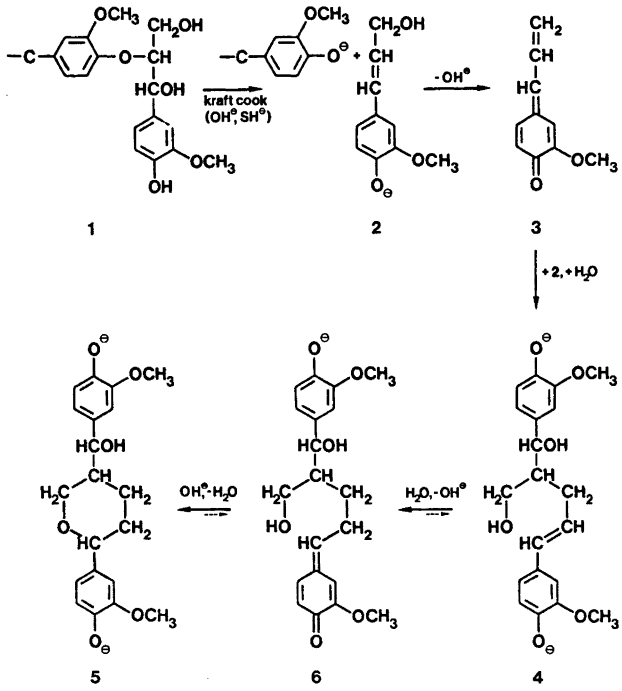

Compounds *2*, *4*, *5*, and *6* are shown as anions.

oxylated dimers corresponding to *4* and *5* (at a slightly higher reaction temperature, 135 °C).

The structural assignment for *4* was confirmed by the reaction of γ-coniferyl benzoate with an excess of *2* in aqueous NaOH at room temperature. Apart from some polymeric material the major product of this reaction was *4*. The absence of *5* (GLC) is due to the low rate at room temperature of the protonation of the carbanion of *4* leading to formation of *6*.

These experiments indicate that the dominant reactions in the base-catalyzed transformation of *2* to dimeric and polymeric products are Michael-type additions, in which *3* provides the unsaturated carbonyl substrate (1,8-addition being preferred to 1,6-addition), and *2* reacts as a carbanion with attack via C_β. The condensation products can react in much the same way as do *2* and *3*. It should be noted, however, that in contrast to the experiments described here, under the conditions of a kraft cook of lignin, *2* is present in a low, approximately steady-state concentration. Thus dimerization of *2* should be suppressed in favour of Michael-type addition to other enone structures, and of addition to *3* of other carbanions present.

Precise mass measurement. Tetrakis(trimethylsilyl)ether of *1,5-bis(4-hydroxy-3-methoxyphenyl)-2-hydroxymethyl-4-penten-1-ol* [*tetrakis(trimethylsilyl)ether* of *4*]. Peak at $m/e = 558$ [molecular ion at $m/e = 648 - (CH_3)_3SiOH$]. Found: 558.2659 ± 3 mmu. Calc. for $C_{29}H_{46}O_5Si_3$: 558.2653.

Tris(trimethylsilyl)ether of *2-(4-hydroxy-3-methoxyphenyl)-5-[(4-hydroxy-3-methoxyphenyl)-hydroxymethyl]-tetrahydropyran* [*tris(trimethylsilyl)ether* of *5*]. Molecular ion. Found: 576.2749 ± 3 mmu. Calc. for $C_{29}H_{48}O_6Si_3$: 576.2759.

Acknowledgement. The authors are indebted to Dr. B. Thomas, Lund, for valuable comments.

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2. a. Brunow, G. and Miksche, G. E. *Acta Chem. Scand. 26* (1972) 1123; b. Gierer, J., Pettersson, I., Smedman, L.-Å. and Wennberg, I. *Ibid. 27* (1973) 2083.
3. Brunow, G. and Miksche, G. E. *Acta Chem. Scand. 23* (1969) 1444.
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Strained Heterocyclic Compounds. 5. The Synthesis of [Bromochloro(*N*,*N*-pentamethylenecarbamoyl)methyl]-phenylmercury and Its Thermal Decomposition to a Chloro-β-lactam

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In the last few years considerable efforts have been devoted to the synthesis of compounds related to penicillin.[1] Our own efforts have been directed towards the synthesis of penicillin analogues in which the thiazolidine nucleus has been modified. We have recently been able to synthesize bromo-β-lactams like *3* and *4* by thermolysis of phenylmercury compounds of the type *2* in boiling bromobenzene.[2] While the compound *3* could be prepared in good yield,[2b] only a low yield of the compound *4* was obtained.[2c] One reason for this is the low thermal stability of the compound *4*. Since the analogous chloro compound *4a* is considerably more stable,[3] attempts were made to prepare a chloro-β-lactam by thermolysis of the dichloro compound *2b*. However, due to the sluggishness of this reaction, the product chloro-β-lactam was extensively decomposed despite its relative stability.[2b] To circumvent these difficulties we have now prepared the bromochloro compound *2a*, which on thermolysis gave the desired chloro-β-lactam *3a* in good yield.

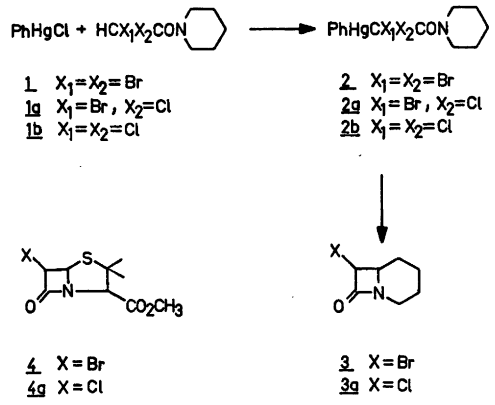

Fig. 1.

The phenylmercury compound *2a* was prepared essentially as the compounds *2* and *2b*. Bromochloroacetyl chloride, prepared by distillation of 1,2-dibromo-1,2-dichloro-1-ethoxyethane,[4] was reacted with piperidine to give the amide *1a*. This was condensed with phenylmercury chloride in THF using potassium *t*-butoxide as a base.[2] Interestingly, the condensation of the bromochloroamide *1a* and phenylmercury chloride gave lower yields than the corresponding reactions with either of the dichloro- and dibromoamides *1b* and *1*. Also, it is much more important that the reaction temperature is kept low with the bromochloroamide *1a*. A maximum yield of about 50 % of the compound *2a* was obtained when phenylmercury chloride and the bromochloroamide *1a* in THF solution were added rapidly from two dropping funnels to a cooled (−75°C) solution of the potassium *t*-butoxide in THF. If the butoxide and phenylmercury chloride are first mixed, the yield of condensation product is negligible. If the base and the bromochloroamide are first mixed, the yield of the mercury compound *2a* is lowered to about 20 %.

The thermal generation of the chloro-β-lactam *3a* from the mercury compound *2a* proceeded 10 times faster than the corresponding reaction from the dichloro analogue *2b*.[2b] In the reaction of the bromochloro compound *2a*, both phenylmercury chloride and bromide could be formed, although the elimination of phenylmercury bromide should be favoured (*cf.* Ref. 4b). Mass spectrometric analysis of the products gave no indication of the formation of phenylmercury chloride or bromo-β-lactam *3*.

The yield of the chloro-β-lactam *3a* from the compound *2a* is only slightly higher than the yield of the bromo-β-lactam *3* from the compound *2*. However, the exclusive generation of the *trans*-isomer of the chloro-β-lactam (*trans*:*cis* > 15:1) is an advantage since the correct configuration should be obtained on introduction of the proper side-chain (*cf.* Ref. 2). Furthermore, the facile generation of a relatively stable halogen derivative may be advantageous when labile systems of structures similar to *4a* are involved.

Experimental. All melting points were determined on a micro hot stage and are uncorrected. Elemental analyses were carried out by Centrala Analyslaboratoriet, Uppsala, Sweden, and by Alfred Bernhardt, Mikroanalytisches Laboratorium, 5251 Elbach über Engelskirchen, West Germany. IR spectra were recorded on a Perkin-Elmer No. 421 spectrophotometer, solids were measured in KBr discs, and oils as liquid films. The numbers are given in cm^{-1}. NMR spectra were recorded on a Varian A 60 instrument, the spectra refer to $CDCl_3$-solutions and the chemical shifts are given as δ-values relative to TMS as internal standard. Mass spectra were recorded on an LKB 9000 instrument. All THF used was freshly distilled from potassium metal/benzophenone under a N_2-atmosphere. Column chromatography was made on silica (Merck 0.05 – 0.2 mm) using increasing amounts of dry ether in distilled light petroleum as eluent.

N-(Bromochloroacetyl)piperidine (1a). Bromochloroacetyl chloride (38.4 g, 0.2 mol) in 200 ml of dry ether was added during 15 min from a dropping funnel to a magnetically stirred, ice-cooled solution of piperidine (34 g, 0.4 mol) in 800 ml dry ether. The reaction mixture was left for 3 h at room temperature and the precipitate of piperidine hydrochloride was filtered off. The ether solution was washed with two portions of 2 M HCl, two portions of saturated $NaHCO_3$-solution and one portion of water, then dried over Na_2SO_4 and evaporated. Recrystallization of the crude product from diethyl ether gave white crystals, m.p. 60–62°C. Yield 31.7 g (66 %). (Found: C 35.08; H 4.63; Br 33.07; Cl 14.61; N 5.77. Calc. for $C_7H_{11}BrClNO$: C 35.14; H 4.60; Br 33.05; Cl 14.64; N 5.85.) IR: CO 1635 cm^{-1}. NMR 1.68 (S, CH_2); 3.64 (S, $N-CH_2$); 6.34 (S, CHBrCl). MS: *m/e* 239, 241, 243 (M).

[Bromochloro(N,N-pentamethylenecarbamoyl)methyl]phenylmercury (2a). Phenylmercury chloride (3.1 g, 0.01 mol) in THF (80 ml) and *N*-(bromochloroacetyl)piperidine (2.4 g, 0.01 mol) in THF (50 ml) were added rapidly and simultaneously from two dropping funnels to a magnetically stirred solution of unsolvated *t*-BuOK (0.01 mol) in THF (100 ml), which was maintained at −75°C during the reaction. During the addition there was always a small excess of the amide over the phenylmercury chloride. When the addition was complete the reaction mixture was kept at −75 °C for an additional 45 min. The temperature was quickly raised to +10°C, and the solvent was evaporated on a rotatory evaporator at room temperature. The residue was dissolved in benzene (200 ml), washed with distilled water (40 ml) and dried over $MgSO_4$. Crystallization from dry diethyl ether gave [bromochloro(*N,N*-pentamethylenecarbamoyl)-methyl]phenylmercury (*2a*) 2.46 g (48 %), m.p. 111–113 °C (dec.). When the reaction was performed at −40 °C, the yield was lowered to 0.55 g (11 %). A longer reaction time also lowered the yield; thus, 75 min gave 1.26 g (27 %), 120 min gave 0.94 g (18 %). (Found: C 30.7; H 3.00; Br 16.0; Cl 7.14; N 2.69. Calc. for $C_{13}H_{15}BrClHgNO$: C 30.2; H 2.94; Br 15.5; Cl 6.85; N 2.71). IR: CO 1615 cm^{-1}. NMR: 1.75 (S, CH_2); 3.90 (S, $N-CH_2$); 7.60 (S, aromatic protons).

Thermolysis of [bromochloro(N,N-pentamethylenecarbamoyl)methyl]phenylmercury (2a). 0.8 g (1.55 mmol) of *2a* was refluxed in 240 ml freshly distilled bromobenzene. The decomposition of *2a* was followed by the use of IR spectrometry (β-lactam absorption at 1760 cm^{-1}) with samples removed after 1.3 h, 1.7 h, 2 h, 2.3 h, and 3.5 h. The best result was obtained at 2.3 h. The solvent was removed *in vacuo*, ether was added and the insoluble phenylmercury bromide was filtered off. Separation of the ether soluble products was accomplished by chromatography on a column of silica gel, cooled to −20 °C by cold circulating ethanol. Elution with increasing amounts of diethyl ether in light petroleum afforded 7-chloro-8-oxo-1-azabicyclo[4.2.0]octane (*3a*) (0.125 g, 54 %). Only the *trans*-isomer of *3a* could be detected by NMR: 1.2–1.3 (M, H-3, H-5); 2.6–4.1 (M, H-2, H-6); 4.45 (D, $J = 1.3$ Hz, H-7, *cf. cis*-isomer 4.95 (two D, $J = 1.4$ and 4.4 Hz, H-7).

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Synthesis of Bradykinin by Fragment Condensation on a Solid Support

SUNE M. KARLSSON and ULF RAGNARSSON

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Solid phase peptide synthesis (SPPS) was introduced by Merrifield.[1] In a series of papers he developed the technique further and demonstrated the exceptional scope of his method.[2]

To simplify purification, which is the crucial step of this method, Weygand and one of the present authors [3] attempted to couple a peptide fragment instead of an amino acid derivative, *i.e.*, to use fragment condensation instead of a step-wise approach in analogy with common strategy in conventional peptide synthesis. We found that a peptide could be coupled satisfactorily with respect to the yield and with a very low degree of racemization when *N,N'*-dicyclohexylcarbodiimide (DCC) plus *N*-hydroxysuccinimide (HOSu) together [4] were used in CH_2Cl_2, the solvent of choice in SPPS, although admittedly the evidence concerning the yield was weak. Both considerations are of equally fundamental importance in this context. Since our initial work in this area, a couple of papers

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To simplify purification, which is the crucial step of this method, Weygand and one of the present authors [3] attempted to couple a peptide fragment instead of an amino acid derivative, *i.e.*, to use fragment condensation instead of a step-wise approach in analogy with common strategy in conventional peptide synthesis. We found that a peptide could be coupled satisfactorily with respect to the yield and with a very low degree of racemization when *N,N'*-dicyclohexylcarbodiimide (DCC) plus *N*-hydroxysuccinimide (HOSu) together [4] were used in CH_2Cl_2, the solvent of choice in SPPS, although admittedly the evidence concerning the yield was weak. Both considerations are of equally fundamental importance in this context. Since our initial work in this area, a couple of papers

on the use of fragment condensation in SPPS have appeared,[5] but to date progress has been slow.

This communication describes the preparation of bradykinin on a solid support from three protected peptide fragments (I – III below). The coupling procedure used was DCC plus HOSu when there was a risk for racemization and DCC only when that risk was absent. However, only one equivalent of HOSu was used as compared with two earlier,[3] when much more dilute solutions were used. When possible, completeness of coupling was verified using the 2-hydroxy-1-naphthaldehyde procedure [6] before proceeding to the next step. The loss in time was more than compensated for by the information gained.

The three protected peptide fragments used, Z-Arg(NO_2)-Pro (I), Z(OCH_3)-Pro-Gly-Phe (II) and Z(OCH_3)-Ser(Bzl)-Pro-Phe (III),* were prepared in step-wise fashion by the mixed anhydride procedure without a carboxyl protecting group. They all gave only one spot on thin-layer chromatography and satisfactory elemental analyses as well as, after hydrolysis, amino acid analyses. II and III are amorphous solids. The reason for selecting two peptide fragments with C-terminal phenylalanine was that racemization, if any, could be simply established and consequently compared with that obtained earlier.[3]

We started our synthesis from 1.32 g of Boc-Arg(NO_2)-resin with a substitution degree of 0.151 mmol/g of Arg. All operations were performed manually in a sintered glass vessel, and apart from those explicitly mentioned below consisted of washings with proper solvents and neutralizations of amine trifluoroacetates. Initially, Boc was removed with 50 % trifluoroacetic acid (TFA) in CH_2Cl_2. Coupling was effected in 99.5 % yield, determined as described above, with 2.5 equiv. of III, DCC and HOSu each in CH_2Cl_2 for 4 h. Z(OCH_3) was removed using 10 % TFA in CH_2Cl_2.[7] The following coupling was effected also in 99.5 % yield as before with II substituted for III. After removal of Z(OCH_3), I was attached. In this case HOSu was omitted. This coupling step was repeated once, since a preliminary experiment indicated some residual heptapeptide. Finally, the peptide was split off from the resin using HF.[8]

Crude bradykinin was obtained in 81 % yield. After hydrolysis in 6 N HCl at 110 °C for 24 h it gave the following amino acid analysis: Arg 2.00 (2), Gly 1.03 (1), Phe 2.06 (2), Pro 2.93 (3) and, after correction for decomposition during hydrolysis, Ser 0.98 (1). Another sample was hydrolyzed as just mentioned and coupled with Leu-*N*-carboxyanhydride.[9] In a parallel experiment, analytically pure, biologically fully active bradykinin was carried through the same procedure as a control. On analysis the D-Phe/L-Phe ratio was found to be 3.76 % compared to 2.91 % for the control. These determinations were performed under such conditions that the L-Leu-D-Phe peaks could be properly integrated. The difference, 0.85 %, reflects the average racemization taking place during the coupling of the two fragments II and III to the resin. The original sample of phenylalanine used in this work contained less than 0.10 % D-Phe.

The crude bradykinin was purified by ion-exchange chromatography on carboxymethyl cellulose. The recovery of pure bradykinin in this step was 87 %. This is at least 25 % higher than the average we have obtained using step-wise procedure. Less than 5 % of the total material was obtained in two minor peaks, the rest we believe are inevitable losses. Amino acid analysis after hydrolysis now gave: Arg 2.00, Gly 1.02, Phe 2.03, Pro 2.98, and Ser 0.98. Purity was further established by high voltage paper electrophoresis at pH 6.46 and elemental analysis.

The conclusions reached in the earlier paper [3] have now been further verified. DCC together with HOSu in CH_2Cl_2 gives a very efficient coupling to the resin for the two tripeptides II and III. This might not always be the case.[5bf] As concerns racemization, the conditions used are certainly not perfect. However, a value as low as ours can in most cases, we believe, be tolerated. It may have been lower if we had used more than one equivalent of HOSu.

Many more experiments are needed to establish the scope of this approach to SPPS. Steric hindrance or an enhanced degree of racemization may in some cases exclude its general use. Even so, however, cases can be envisaged where it would be possible to incorporate a difficult amino acid into the interior of a fragment which could then be coupled to the resin by the proposed procedure. A mixed procedure, in which some amino acids are coupled step-wise and others as oligopeptides, may in other cases be a practical alternative.

Small protected peptides with a free C-terminal carboxyl group, as needed in the present scheme, are generally simple to prepare in satisfactory quality and quantity by conventional solution procedures. Somewhat larger ones, if needed, can perhaps be made as proposed recently.[5g] A definite disadvantage of any fragment condensation approach is that time is lost in the preparation of the partial sequences. For bradykinin,[10] which is small enough to be easily purified, our procedure is hardly an improvement. Nevertheless, our experiences with this synthesis as concerns the near absence of smaller peptides in the crude product make us believe that for larger peptides the purification will be much less time-consuming, *i.e.*, make a fragment condensation approach competitive even as regards the time needed.

* All amino acids used were of L-configuration except glycine.

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The Oligomerization of Ethylene Oxide to Macrocyclic Ethers, Including 1,4,7-Trioxacyclononane

JOHANNES DALE, GERD BORGEN and KARI DAASVATN

Kjemisk Institutt, Universitetet i Oslo, Oslo 3, Norway

Cyclic homologues of ethylene oxide, $-[-CH_2-CH_2-O-]-_n$, have attracted much attention due to their remarkable complexing power for alkali and other cations.[1-3] Laboratory methods for the preparation of the higher members ($n > 4$) have been described [2] which involve several steps and use di-, tri-, or tetraethylene glycol as starting materials; these methods fail to yield the medium rings ($n = 3$ and 4). Clearly, the simplest conceivable way to prepare this class of cyclic ethers is the direct oligomerization of ethylene oxide. In fact, it has been reported [4] that the cyclic tetramer (1,4,7,10-tetraoxacyclododecane) is obtained together with mainly dioxan and polymer from ethylene oxide in the presence of trialkylaluminium. On the other hand, although BF_3 is reported [5] to catalyse the conversion of propylene oxide to isomeric cyclic tetramers and pentamers, ethylene oxide under the same conditions gave only dioxan and polymers.

We can now report that a mixture of all the possible cyclic oligomers, including the hitherto unknown trimer 1,4,7-trioxacyclononane, m.p. 0 °C, and unaccompanied by open-chain oligomers and polymers, can be easily obtained from ethylene oxide at room temperature and atmospheric pressure in the presence of BF_3 or similar acidic fluorine compounds (PF_5, SbF_5). The important point is to exclude any substance capable of furnishing permanent end groups to polymeric chains. Thus, the common practice of using BF_3 as its etherate leads to a mixture of the rings and open-chain compounds terminated by ethoxy groups. Another common practice, to add water as a cocatalyst to speed up the reaction,[6] leads to hydroxyl-terminated open-chain compounds. Only dry HF gas proved acceptable as a cocatalyst, in accordance with the low nucleophilicity of fluoride ion. Likewise, any solvent must be inert (benzene, saturated hydrocarbons); even diethyl ether suppresses totally the formation of macrocyclic compounds, while dioxan, being one of the products, is a suitable solvent.

Other Lewis acids are either ineffective as catalysts ($AlCl_3$, $FeCl_3$) or give waxy polymers ($SnCl_4$, $SbCl_3$), while oxygen acids ($HClO_4$, *p*-toluene sulfonic acid) are rapidly transformed to half-esters of ethylene glycol.

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Surprisingly, the product composition is little dependent on the concentration of monomer, so that similar products are obtained from

undiluted liquid ethylene oxide as from 5 % solutions. The most convenient procedure is to add cooled liquid ethylene oxide gradually to a small volume of dioxan containing initially about 1 % BF_3/HF (1:1). The catalyst is destroyed with NH_3 gas.

A typical product distribution as determined by distillation and gas chromatography is:

n:	2	3	4	5	6	7	8	9	10	11	higher
%:	40	1	15	5	4	3	2	2	1	1	25

With PF_5 and SbF_5 the distribution of ring sizes is similar, except that relatively more trimer and pentamer are formed.

The composition of the mixture does not change with reaction time, although for entropy reasons the smallest unstrained ring, dioxan, corresponding to the largest number of molecules, must be the thermodynamically stable end product. Since furthermore the isolated products are stable in the presence of the catalyst, one might be led to the conclusion that the reaction is kinetically controlled. In the presence of ethylene oxide and catalyst, however, the larger rings are degraded to dioxan. The product composition seems therefore to be determined by the balancing rates of oligomerization and of degradation of the products to dioxan, both dependent on ethylene oxide concentration. This is in accord with observations [6] on the degradation of the polymer.

Ethylene oxide may in part be dehydrated by BF_3 in the gas phase, in non-basic solvents (hexane), and generally at higher temperatures. Dark-coloured tars are then formed and the resulting hydrated catalyst gives rise also to open-chain polyethers.

Only very little of cyclic acetals are observed. 2-Methyl-1,3-dioxolan is present in the dioxan fraction only when the reaction is carried out at higher temperatures. 2-Methyl-1,3,6-trioxacyclooctane occurs as impurity in the cyclic trimer fraction and must be removed by mild hydrolysis.

Experimental. Example of oligomerization procedure. A stock solution of catalyst is prepared by absorbing first gaseous BF_3 (7 g), thereafter gaseous HF (2 g), in dioxan (50 ml). This solution does not attack glass and is stable for months at room temperature. A portion of this solution (2 ml) was diluted with dioxan (40 ml) and cooled liquid ethylene oxide (250 g) added, with vigorous stirring, at a rate slow enough to prevent the temperature to rise above 30 °C. After 18 h, the solution was neutralized with gaseous NH_3 and fractionally distilled. Unreacted ethylene oxide (20 – 40 %) and dioxan distilled at atmospheric pressure, the trimer and most of the tetramer at 10 mmHg, and the remaining tetramer and the higher fractions including the octamer at 0.1 mmHg; in the last fractions at 200 °C rings up to the undecamer were identified.

Purification of 1,4,7-trioxacyclononane. The trimer fraction (100 mg), b.p. 110 °C/10 mmHg, was dissolved in ether (10 ml) containing water (0.1 ml) and a trace of *p*-toluenesulfonic acid, and the solution refluxed for one hour. After evaporation of the ether, the residue was taken up in pentane, stirred with basic alumina and filtered. The filtrate was concentrated and distilled to give pure 1,4,7-trioxacyclononane, b.p. 168 – 172 °C/760 mmHg, m.p. 0 °C. The NMR-spectrum in $CDCl_3$ showed a single line at δ 3.77 at room temperature, showed the molecular ion in MS, and had the following main infrared bands in the liquid: 2910, 2890, 2840, 1385, 1370, 1305, 1285, 1275, 1260, 1155, 1145, 1135, 1120, 1065, 1005, 910, 885, 835, 525 and 505 cm^{-1}.

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Synthesis and Configurational Assignment of the Two Stereoisomeric Methyl 3,4-*O*-Ethylidene-β-D-galactopyranosides

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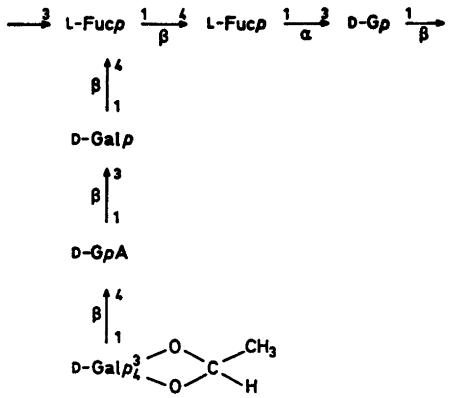
The two stereoisomers of methyl 3,4-*O*-ethylidene-β-D-galactopyranoside have been prepared. Their configurations have been assigned from an X-ray crystallographic structural determination of one of them and correlated with the observed chemical shift of the ethylidene methine proton for each of the stereoisomers.

Previous structural studies on the extracellular M-antigen which can be isolated from the mutant *Salmonella typhimurium* 395 MRO-M1 have demonstrated the following polysaccharide frame-work.[1,2]



The configuration at the asymmetric acetal carbon of the ethylidene group linked to the terminal D-galactose residues is so far not known.

In the present paper, we report the synthesis of the two stereoisomeric methyl 3,4-*O*-ethylidene-β-D-galactopyranosides. These were required as reference compounds for the determination of the configuration at the above 3,4-*O*-ethylidene groups. We have determined the configuration of the ethylidene group in one of these acetals by X-ray crystallography. This has made it possible for us to correlate the NMR chemical shifts of the acetal methine protons with the structures of the two possible stereoisomers.

We have recently reported a method of benzylidenation, whereby the appropriate diol is treated with a benzal halide in pyridine at reflux temperature. The method is applicable in the presence of acetyl or trityl groups on other positions of a pyranose ring.[3,4] It therefore gives easy access to methyl 3,4-*O*-benzylidene-β-D-galactopyranoside (I),[4] which was the starting material in the present synthetic work. Benzylation of I produced II, the stereoisomers of which (IIa and IIb) were obtained by chromatography. Removal of the benzylidene group from II under mild acidic conditions produced methyl 2,6-di-*O*-benzyl-β-D-galactopyranoside III. This, on treatment with excess 1,1-dimethoxyethane and a catalytic amount of sulphuric acid afforded the 3,4-*O*-ethylidene acetal IV, the stereoisomers of which, IVa and IVb, were obtained by chromatographic separation. Catalytic hydrogenation of IVa and IVb yielded the stereoisomeric methyl 3,4-*O*-ethylidene-β-D-galactopyranosides Va and Vb, respectively.

The assignments of configuration at the asymmetric benzylidene acetal carbons for compounds Ia, Ib, IIa, and IIb described in the experimental part, are based on the observations of Baggett and co-workers that the benzylic proton in a dioxolane ring fused to a

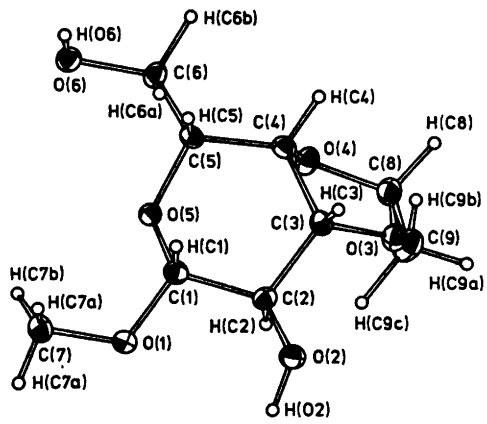


Fig. 1. Molecular structure of methyl 3,4-*O*-ethylidene-β-D-galactopyranoside, isomer Vb.

pyranoside (of Ia and Ib) has different chemical shifts depending on the configuration. An *exo*-benzylidene proton resonates at a higher field than does an *endo* one, and this was used in the structural assignment of benzylidene acetals previously described.[5,6] By similar reasoning the ethylidene acetals IVa and Va have an *endo* acetalic hydrogen and IVb and Vb correspondingly an *exo* one. In view of the fact that such correlations for pyranoside ethylidene acetals do not seem to have been made, we decided to determine the structure of one of the acetals, Vb, by X-ray crystallography. The molecular structure is shown in Fig. 1. Intramolecular distances and angles are listed in Table 1. Having thus determined the configuration at the ethylidene carbon in Vb, the corresponding configuration in Va, IVa, and IVb follows. The results (*cf.* Table 2) clearly indicate the possibility of applying the NMR correlations of Baggett and co-workers (for the configuration of benzylidene acetals) to ethylidene acetals. However, no such correlation for the chemical shift of the methyl protons of the ethylidene group with the configuration at the ethylidene acetal carbon was found.

EXPERIMENTAL

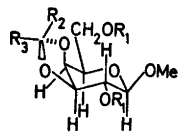
General methods were the same as those described in a previous paper.[4] Unless otherwise stated, chemical shifts are measured in ppm downfield from tetramethylsilane (internal).

Methyl 2,6-di-O-benzyl-3,4-O-benzylidene-β-D-galactopyranoside (IIa, IIb). Methyl 3,4-*O*-benzylidene-β-D-galactopyranoside (Ia, m.p. 139–141°, 530 mg) was converted into the 2,6-di-*O*-benzyl ether by treatment with benzyl bromide (3.25 g) and sodium hydride (from 0.91 g 50 % suspension in oil, thoroughly washed with light

Table 1. Intramolecular nonhydrogen bond distances (Å) and angles (°) for methyl 3,4-*O*-ethylidene-β-D-galactopyranoside, isomer Vb. Estimated standard deviations are given in parentheses.

C(1)–C(2)	1.515 (6)	C(1)–C(2)–C(3)	110.6 (4)
C(2)–C(3)	1.530 (6)	C(2)–C(3)–C(4)	113.4 (4)
C(3)–C(4)	1.530 (7)	C(3)–C(4)–C(5)	115.8 (4)
C(4)–C(5)	1.510 (7)	C(4)–C(5)–O(5)	110.7 (4)
C(5)–C(6)	1.505 (6)	C(5)–O(5)–C(1)	110.6 (3)
C(8)–C(9)	1.495 (9)	C(5)–C(1)–C(2)	109.3 (4)
C(1)–O(1)	1.393 (6)	C(4)–C(5)–C(6)	113.2 (4)
C(7)–O(1)	1.421 (7)	O(5)–C(5)–C(6)	108.1 (4)
C(2)–O(2)	1.429 (6)	C(5)–C(6)–O(6)	112.1 (4)
C(3)–O(3)	1.420 (5)	C(1)–O(1)–C(7)	113.5 (4)
C(4)–O(4)	1.441 (6)	C(1)–C(2)–O(2)	110.9 (4)
C(1)–O(5)	1.423 (5)	C(3)–C(2)–O(2)	108.3 (4)
C(5)–O(5)	1.442 (6)	C(2)–C(3)–O(3)	111.1 (4)
C(6)–O(6)	1.426 (6)	C(4)–C(3)–O(3)	102.9 (3)
C(8)–O(3)	1.439 (6)	C(2)–C(1)–O(1)	109.1 (4)
C(8)–O(4)	1.416 (6)	C(5)–C(1)–O(1)	107.9 (4)
		C(3)–C(4)–O(4)	101.4 (4)
		C(5)–C(4)–O(4)	111.3 (4)
		C(3)–C(8)–O(4)	106.3 (4)
		O(3)–C(8)–C(9)	111.5 (4)
		O(4)–C(8)–C(9)	110.4 (5)
		C(3)–O(3)–C(8)	108.6 (3)
		C(4)–O(4)–C(8)	104.4 (4)

Table 2. Chemical shifts for benzylidene or ethylidene methine protons in compound I – V.



	R_1	R_2	R_3	δ
I a	H	H	Ph	6.15[a]
I b	H	Ph	H	5.97[a]
II a	CH_2Ph	H	Ph	5.98[a]
II b	CH_2Ph	Ph	H	5.92[a]
IV a	CH_2Ph	H	CH_3	5.25[a]
IV b	CH_2Ph	CH_3	H	5.13[a]
V a	H	H	CH_3	5.45[b]
V b	H	CH_3	H	5.25[b]

[a] In ppm downfield from TMS. [b] In ppm downfield from sodium 3-(trimethylsilyl)propanesulphonate.

petroleum) in dry dimethylformamide (30 ml), essentially as described by Brimacombe and co-workers.[7] Excess benzyl bromide was converted into benzyl methyl ether by adding methanol (15 ml) and continuing the reaction at room temperature for 3 h. The product was partitioned between benzene and water and concentrated. The resulting syrup was purified by TLC to yield syrupy II (580 mg) $[\alpha]_D$ +11° (*c* 0.9, $CHCl_3$). (Found: C 72.5; H 6.37. $C_{28}H_{30}O_6$ requires: C 72.7; H 6.54).

An NMR spectrum showed that although the starting material contained one stereoisomer only (Ia), the product contained approximately equal quantities of the isomers IIa and IIb. A small-scale benzylation of Ia with benzyl bromide and silver oxide in dimethyl formamide essentially as described by Kuhn and co-workers,[8] however yielded pure IIa. Benzylation of a mixture of Ia and Ib (4.2 g) with benzyl bromide and sodium hydride in dimethyl formamide as described above afforded an 80 % yield of a stereoisomeric mixture of IIa and IIb, used in the subsequent synthesis. A small quantity of this mixture was separated by TLC ($CHCl_3$ – Et_2O 9:1) yielding pure, syrupy, IIa (faster-moving isomer), $[\alpha]_D$ +10° (*c*, 0.6, $CHCl_3$) and IIb, $[\alpha]_D$ +13° (*c* 0.3, $CHCl_3$). (Found: IIa: C 72.6; H 6.36. IIb: C 72.9; H 6.69. $C_{28}H_{30}O_6$ requires: C 72.7; H 6.54). NMR, IIa ($CDCl_3$): δ 3.60 (s and broad one-proton signal, 4 H), methoxyl and one ring proton; δ 4.62 (2 H) and 4.90 (2 H), benzylic protons; δ 5.98 (s, 1 H), benzylidene proton. NMR, IIb ($CDCl_3$): δ 3.57 (s and broad one-proton signal, 4 H), methoxyl and one ring proton; δ 4.67 (2 H) and 4.75 (2 H), benzylic protons; δ 5.92 (s, 1 H), benzylidene proton.

Methyl 2,6-O-benzyl-β-D-galactopyranoside (III). A mixture of the stereoisomers IIa and IIb (9.2 g) was treated with 85 % aqueous trifluoroacetic acid (100 ml) at room temperature for 15 min. The solution was concentrated and the product purified on a silica gel column, ($CHCl_3$, $CHCl_3$ – Et_2O 9:1 and 1:1, EtOAc in sequence), to yield III (4.7 g), which after recrystallization from cyclohexane had m.p. 78 – 80°, $[\alpha]_D$ +10° (*c* 0.4, $CHCl_3$). (Found: C 67.1; H 7.16. $C_{21}H_{26}O_6$ requires: C 67.4; H 7.00). NMR ($CDCl_3$): δ 3.54 (s, 3 H), methoxyl protons; δ 4.58 (2 H) and 4.73, 4.87 (2 H), benzylic protons.

Methyl 2,6-di-O-benzyl-3,4-O-ethylidene-β-D-galactopyranoside (IVa, IVb). The dibenzyl ether II (100 mg) in 1,1-dimethoxyethane (1.5 ml) containing sulphuric acid (1 drop) was allowed to stand at room temperature for 15 min. Excess chloroform was added and the resulting solution was shaken with aqueous sodium bicarbonate. The chloroform layer was dried (Na_2SO_4), filtered, and concentrated to a syrup (IVa and IVb, 78 mg) $[\alpha]_D$ +19° (*c* 0.5, $CHCl_3$). (Found: C 69.8, H 6.91, $C_{23}H_{28}O_6$ requires: C 70.0; H 7.05). The syrup was subsequently fractionated by TLC (light petrol (40 – 60°) – Et_2O – EtOAc 5:2:1) to yield IVa (minor component), $[\alpha]_D$ +13° (*c* 0.3, $CHCl_3$), and IVb (major component), $[\alpha]_D$ +12° (*c* 0.3, $CHCl_3$). These substances were not quite pure and tended to decompose on standing which may account for the rotations being lower than that recorded above for the mixture. NMR, IVa, ($CDCl_3$): δ 1.33 (d, J = 5 Hz, 3 H), ethylidene methyl protons; δ 3.58 (s, 3 H), methoxyl protons; δ 4.65 (2 H) and 4.83 (2 H), benzylic protons; δ 5.25 (q, J = 5 Hz, 1 H), ethylidene methine proton. NMR IVb, ($CDCl_3$): δ 1.30 (d, J = 5 Hz, 3 H), ethylidene methyl protons; δ 3.54 (s, 3 H), methoxyl protons; δ 4.62 (2 H) and 4.82 (2 H), benzylic protons; δ 5.13 (q, J = 5 Hz, 1 H), ethylidene methine proton.

Methyl 3,4-O-ethylidene-β-D-galactopyranoside (Va, Vb). The above dibenzylated ethylidene acetals, IVa and IVb, were hydrogenated in ethanol using 5 % palladium on charcoal as catalyst to give, in quantitative yields, respectively, Va, $[\alpha]_D$ +13° (*c*, 0.3, H_2O), m.p. 169 – 171° (recrystallized from ethanol), and Vb, $[\alpha]_D$ +8° (*c*, 0.3, H_2O), m.p. 193 – 196° (recrystallized from ethanol). (Found, Va: C 49.2; H 7.48. Vb: C 49.0; H 7.27. $C_9H_{16}O_6$ requires: C 49.1; H 7.32). NMR, Va (D_2O, δ values in ppm downfield from sodium 3-(trimethylsilyl)-propane sulphonate: δ 1.35 (d, J = 5 Hz, 3 H), ethylidene methyl protons; δ 3.57 (s, 3 H), methoxyl protons; δ 5.45 (q, J = 5 Hz, 1 H), ethylidene methine proton. NMR, Vb (D_2O, δ values in ppm downfield from sodium 3-(trimethylsilyl)propane sulphonate: δ 1.45 (d, J = 5 Hz, 3 H), ethylidene methyl protons; δ 3.58 (s, 3 H), methoxyl protons; δ 5.25 (q, J = 5 Hz, 1 H), ethylidene methine proton.

Crystallography. Vb crystallized in space groups $P2_12_12_1$, $a=16.678$ (1), $b=12.828$ (2), $c=4.9165(9)$, $Z=4$. The X-ray data were collected using a Philips PW 1100 computer-controlled single-crystal diffractometer with graphite monochromatized Cu$K\alpha$ radiation. The determination of the phases were carried out by a computerized application of direct methods using the weighted phase-sum formula described by Norrestam.[9]

Several cycles of full-matrix least-squares refinements (anisotropic non-hydrogen and fixed isotropic hydrogen temperature parameters) gave an R-value of 0.052. Full details of the X-ray diffraction investigation will be published elsewhere.[10]

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Mass Spectrometry of Carotenoids— In-chain Fragmentations of Deuterium Labelled Carotenoids

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In-chain fragmentations of specifically labelled 7,7′-D_2- (*1*—*3*), 11,11′-D_2- (*4*—*6*) and 19,19′-D_6- (*7*) carotenoids have been studied.

The data support the modified Edmunds-Johnstone mechanism for in-chain eliminations of toluene, xylene, and dimethylcyclodecapentaene, and the previously given ranges for the sites of these eliminations.

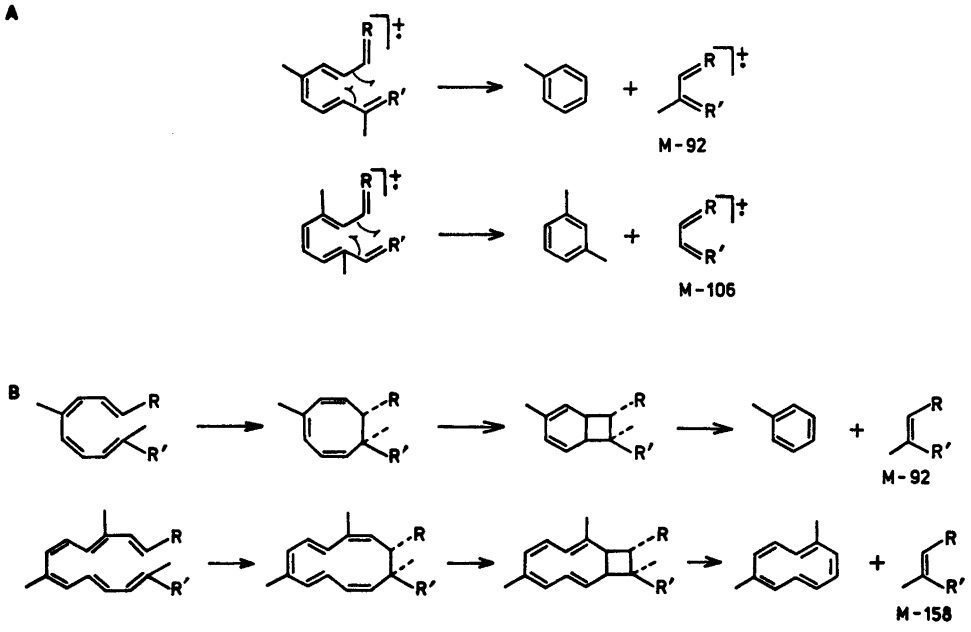
In bicyclic carotenoids the expulsion of dimethylcyclodecapentaene is restricted to the C(10)—C(10′) range.

Rationalization by others of a common M − 79 ion as elimination of a cyclopentadienyl radical from the polyene chain seems valid and appears to be restricted to the C(11)—C(11′) range for bicyclic carotenoids.

Formal loss of methylbenzyne instead of toluene may be general for bicyclic 15,15′-didehydro carotenoids.

Evidence for some in-chain cleavages with hydrogen transfer was also obtained.

Fragmentations leading to losses of 92 (toluene), 106 (xylene), and 158 (dimethylcyclodecapentaene) from the polyene chain due to electron impact induced and thermal processes are con-



Scheme 1.

sidered characteristic of carotenoid mass spectra.[1,2] A comprehensive review on carotenoid mass spectrometry was recently published.[3] Further systematic studies have since appeared.[4–9]

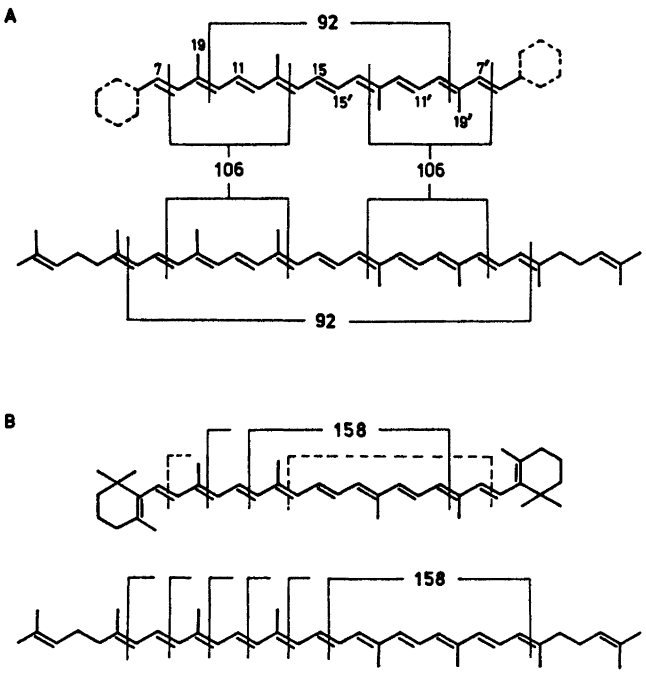
For the eliminations from the polyene chain the alternative mechanisms A[1] and B[4] have been considered. Mechanism A, exemplified in Scheme 1A for the loss of toluene and xylene involves rupture of single bonds, whereas mechanism B implies cleavage of double bonds in the original carotenoid. Mechanism B, as recently indepently modified by Vetter *et al.*[3] and Francis [6] according to the principle of conservation of orbital symmetry,[10] is illustrated for the expulsion of toluene in Scheme 1B. As dicussed by others [6,7] this modification involves an eight-electron conrotatory electrocyclic reaction followed by a disrotatory six-electron electrocyclic reaction and scission of the four-membered transition state. The formation of toluene and dimethylcyclodecapentaene may be envisaged by analogous reactions; transitions through twelve- and ten-electron electrocyclic reactions [3,6,7] being involved in the latter case as illustrated in Scheme 1B.

From consideration of the fragmentation pattern of 15,15′-D_2- and 7,7′-D_2-carotenes mechanism B has been favoured, and the origin of toluene, xylene, and dimethylcyclodecapentaene from acyclic and bicyclic carotenoids has been proposed.[11,12] Scheme 2A, B gives the presumed range for these eliminations. Several modes of elimination are considered involved [4,11,13] as illustrated for the M − 158 elimination in Scheme 2B.

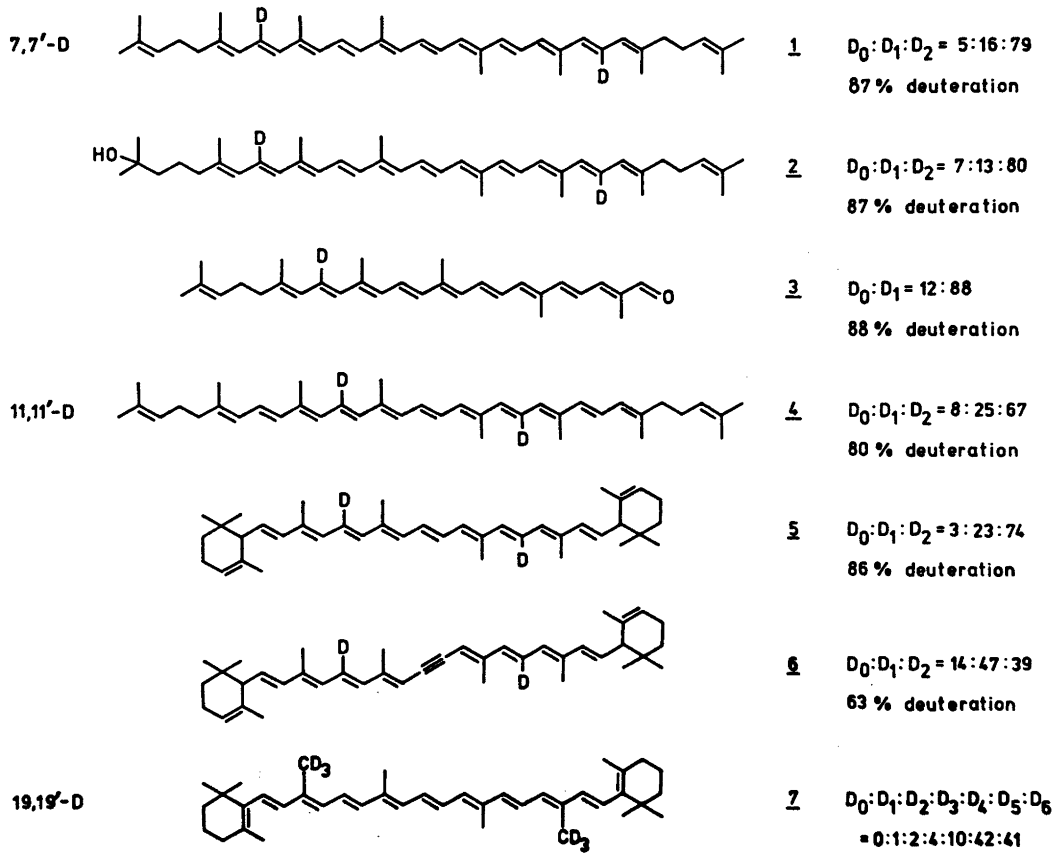
RESULTS AND DISCUSSION

In order to obtain further evidence for these in-chain eliminations 7,7′-D_2-[13], 11,11′-D_2-[14] and 19,19′-D_6-carotenoids [15a] have been synthesized. The carotenoids here studied and their degree of deuteration estimated by mass spectrometry are given in Scheme 3. The ratio of deuterated species has been calculated taking into account ^{13}C isotope contributions, *cf.* Ref. 15b. Percentage deuteration reflects average number of deuterium atoms incorporated relative to the theoretically possible incorporation.

In the following considerations are made as to



Scheme 2.



Scheme 3.

the preferred mechanism (A or B) and sites of in-chain eliminations. The ratio of eliminated, labelled, and unlabelled species, calculated theoretically on the basis of a valid mechanism, should within experimental error agree with values calculated from observed spectra. Conclusions provide that the rate of elimination be the same from all parts of the aliphatic polyene chain. Also hydrogen-deuterium scrambling through sigmatropic shifts in the mass spectrometer should not take place. This has been considered and rejected.[7,11]

Steric conflicts in the intermediate are considered responsible [1,6,12] for the reduction of the theoretically possible modes of elimination of toluene [11] and dimethylcyclodecapentaene [5] in bicyclic carotenoids. Such limits become obvious when figures obtained for a given compound do not fit a generally preferred mechanism.

Mechanism and range for elimination of toluene, xylene and dimethylcyclodecapentaene. Taking into consideration all possible modes of elimination of toluene, xylene, and dimethylcyclodecapentaene by mechanisms A and B the calculated and observed ratios of deuterium incorporation in the expelled fragments from *1*, *2*, *4*, *5*, and *7* are given in Table 1.

For the 7,7'-D_2-compounds *1* and *2* the results for toluene and xylene formation is consistent with those previously obtained for acyclic 7,7'-D_2-carotenoids [11] in favour of mechanism B. The deuterium incorporation in the expelled toluene and xylene from the 11,11'-D_2-carotenes *4* and *5* also shows a clear preference for

Table 1. Calculated and observed ratios for deuterated and non-deuterated toluene, xylene, and dimethylcyclodecapentaene, based on examination of ions derived from the molecular ions of carotenoids *1*, *2*, *4*, *5*, and *7* by loss of these fragments.

Carotenoid		Toluene $D_0:D_1:D_2:D_3$			Xylene $D_0:D_1:D_2:D_3$			Dimethylcyclodecapentaene $D_0:D_1:D_2:D_3$		
		Observed	Calc. mech. A	Calc. mech. B	Observed	Calc. mech. A	Calc. mech. B	Observed	Calc. mech. A	Calc. mech. B
Acyclic	7,7′-D_2-lycopene (*1*)	65:35:0:0	50:30:0:0	67:33:0:0	100:0:0:0	60:40:0:0	100:0:0:0	59:41:0:0	43:57:0:0	67:33:0:0
	7,7′-D_2-rhodopin (*2*)	70:30:0:0	50:50:0:0	67:33:0:0	100:0:0:0	60:40:0:0	100:0:0:0	—	—	—
	11,11′-D_2-lycopene (*4*)	38:62:0:0	0:100:0:0	33:67:0:0	0:100:0:0	60:40:0:0	0:100:0:0	0:100:0:0	0:86:14:0	0:100:0:0
Bicyclic	11,11′-D_2-ε-carotene (*5*)	37:63:0:0	0:100:0:0	50:50:0:0	0:100:0:0	33:67:0:0	0:100:0:0	0:100:0:0	0:80:20:0	0:100:0:0[a] 0:100:0:0[b]
	19,19′-D_2-β-carotene (*7*)	100:0:0:0	50:0:0:50	100:0:0:0	0:0:0:100	33:0:0:67	0:0:0:100	100:0:0:0	20:0:0:80	50:0:0:50[a] 100:0:0:0[b]

[a] Assuming C(8)—C(8′) range of elimination. [b] Assuming C(10)—C(10′) range of elimination.

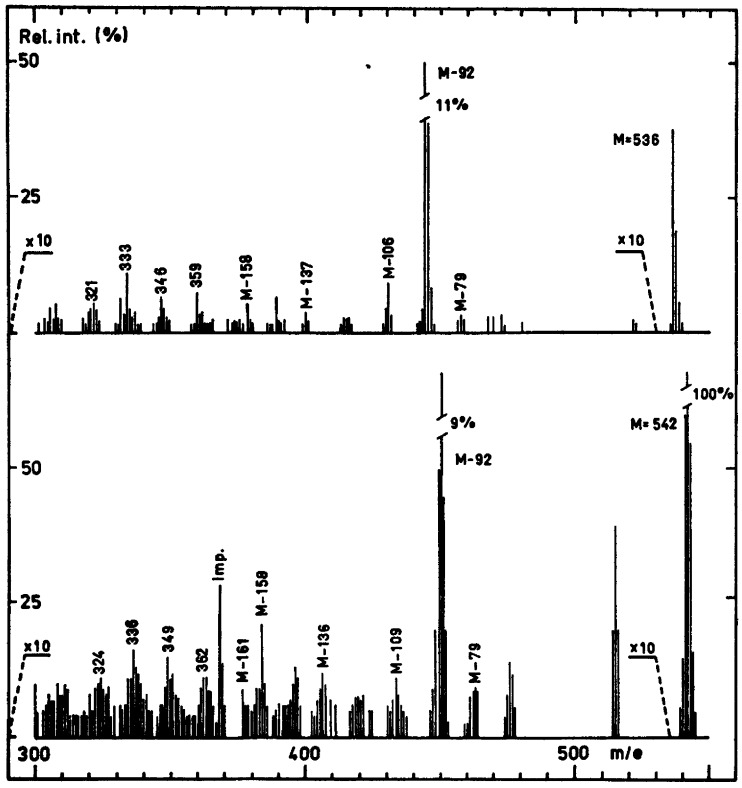


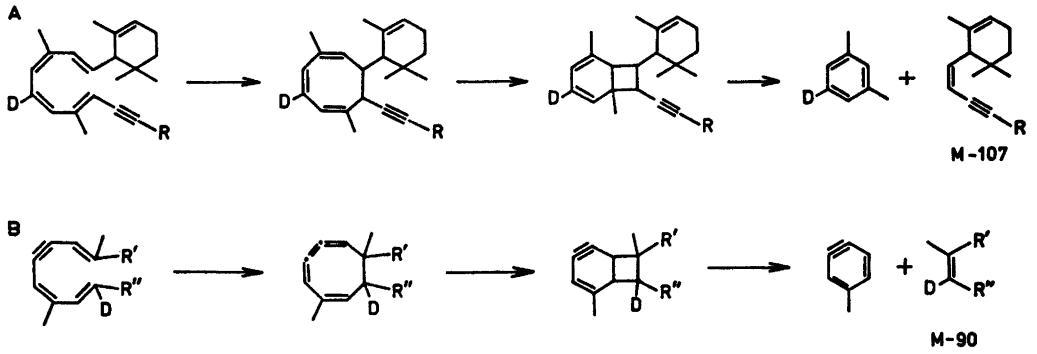
Fig. 1. Mass spectra of 19,19′-D_6-β-carotene (*7*, lower curve) and undeuterated β-carotene (upper curve).

mechanism B. The preference is still more obvious for the most heavily deuterated test compound, 19,19′-D_6-β-carotene (*7*), judged by the data for toluene and xylene in Table 1 and Fig. 1.

Considered jointly these results for toluene and xylene clearly support mechanism B, rather than A, and the previously assumed ranges for the origin of these species.

Regarding the origin of the less abundant M − 158 ion the possible modes of elimination by mechanism B are given in Scheme 2B. Satisfactory agreement is observed (Table 1) for mechanism B and the C(6) − C(6′), range for acyclic carotenoids. However, the range appears to be limited to C(10) − C(10′) in the bicyclic series, if the reasonable assumption is made that mechanism B also is valid here. Thus no loss of trideuterio-dimethylcyclodecapentaene (161 mass units) is observed from 19,19′-D_6-β-carotene (*7*). This situation was already suggested in previous work [11] and is later documented by Brzezinka [5] on the basis of results for 11,12,-11′,12′-deuterated carotenoids. The steric argument previously advanced [12] and further elaborated [3,6,7] is likely to be valid in this case, see Scheme 1B.

Eliminations from 15,15′-didehydrocarotenoids. The fragmentation pattern of the acetylenic analogue *6* of 11,11′-dideuterio-ε-carotene (*5*) was studied. It has previously been claimed that a triple bond in 15,15′-position totally inhibits the loss of toluene and xylene.[3] According to mechanism B and the ranges given in Scheme 2 for toluene and xylene eliminations, this should be true only for the toluene case (Scheme 4A). However, a small peak at M − 90, ascribed to the loss of methylbenzyne has previously been observed.[3,16,17] This loss may also be rationalized by mechanism B, although this implies a



Scheme 4.

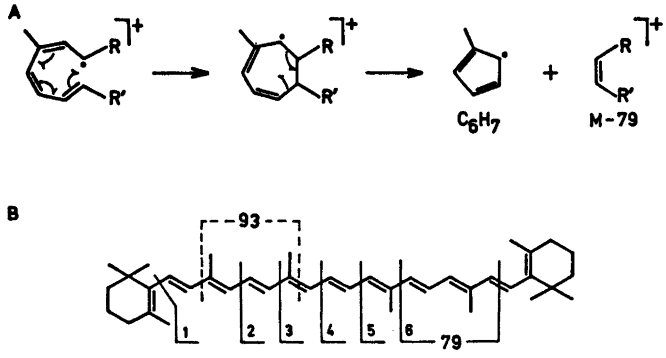
strongly deformed, energetically unfavourable transition state (Scheme 4B). The 11,11'-labelled acetylenic compound *6* should according to mechanism B and the ranges given in Scheme 2 give rise to monodeuterated xylene and undeuterated methylbenzyne. Indeed M − 107 and M − 90 ions were observed.

An M − 156 peak has previously been reported for undeuterated 15,15'-didehydro-β-carotene,[5] and the expected M − 158 peak for the 11,11'-D_2-15,15'-didehydro compound *6* was observed. It is also mentioned that the M − 15 peak, usually encoutered in the spectra of 15,15'-didehydro compounds,[5] was also observed for *6*.

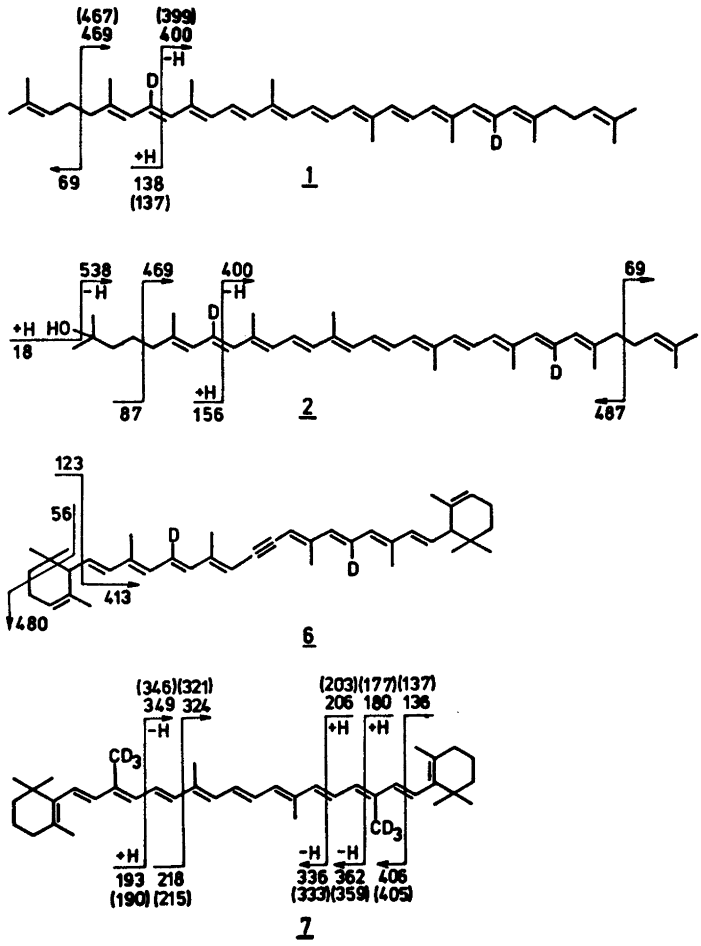
Apo-carotenoids. Apo-carotenoids, here represented by 7-D-8'-apolycopenal (*3*), Scheme 3, are known to give weak and inconsistent M − 92 and M − 106 ions.[16] Only very weak M − 92 and M − 106 ions were observed for *3*, not suitable for mechanistic considerations.

Mechanism for elimination of methylcyclopentadienyl radical. It was early recognized that in addition to M − 92, M − 106 and M − 158 ions a less abundant M − 79 ion is characteristic of carotenoid mass spectra. A methylcyclopentadienyl radical was considered the species involved.[2] Kjøsen [7] has recently rationalized the formation of this common M − 79 ion as loss of a methylcyclopentadienyl radical from the polyene chain, also explaining the formation of M − 78 and M − 80 ions from a common seven-membered transition state (*8*), here cited in Scheme 5A for the loss of 79 mass units.

Six alternative modes of eliminations by this mechanism for the bicyclic case are illustrated in Scheme 5B. Particularly modes 1 and 6 are considered less likely due to steric hindrance, leaving the C(11) − C(11') range as likely site of elimination. Only the D_6-model compound has sufficient number of labels to provide information in this case. It appears from the mass spectrum of 19,19'-D_6-β-carotene (*7*), as compared with that of undeuterated β-carotene, Fig. 1, that the fragment lost is undeuterated. This



Scheme 5.



Scheme 6.

may support the origin suggested by Kjøsen [7] of the M − 78, M − 79, and M − 80 ions, and the C(11) − C(11′) range of expulsion in the bicyclic case.

In acyclic undecaenes such as lycopene the entire C(6) − C(6′) range is expected to serve as sites for these eliminations.

In-chain cleavages. In-chain cleavages are not abundant amongst carotenoids of type *1*, *2*, *4* − *7*.[3] However, unlabelled *β*-carotene and 19,19′-D_6-*β*-carotene (*7*) showed fragment ions consistent with the in-chain cleavages given in Scheme 6, generally occurring with hydrogen transfer. Values in parenthesis refer to unlabelled compounds. Some of these cleavages have not been previously reported.[3,8] Cleavage of the 7-double bond with hydrogen transfer to the smaller, uncharged fragment was also confirmed for unlabelled and labelled lycopene (*1*) and indicated for labelled rhodopin (*2*).

EXPERIMENTAL

The syntheses of the compounds studied are described elsewhere.[13–15] 11,11′-D_2-15,15′-didehydro-*ε*-carotene (*6*) was here prepared in small scale by a procedure analogous to that used for *7*, using an acetylenic center-piece. *6* had *m/e* 536 (M), M − 15, M − 56, M − 90, M − 107 M − 123 (*cf.* Scheme 6), M − 158 and no M − 91, M − 92 or M − 93 ions.

Mass spectra were recorded on an AE1 MS 902 mass spectrometer with direct insertion probe. Spectra were recorded at 70 eV, 6 kV

and with the ion source at minimum temperature required to achieve vapourization (190 – 200°C).

The accuracy of the present intensity ratio calculations depends on absolute peak intensity and is estimated to *ca.* ± 5 % in most cases.

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A New One Step Indole Synthesis

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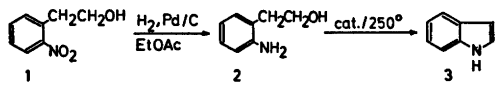
A simple conversion of 2-(*o*-nitrophenyl)ethanol to indole in the gas phase over a copper catalyst was presented. The probable way of formation of indole was discussed. The most probable intermediates were thought to be 2-(*o*-aminophenyl)-ethanol (*2*) and 2,3-dihydroindole (*8*). Part of the reaction might proceed *via* *o*-aminophenylacetaldehyde (*9*).

Most of the methods for the synthesis of indole (*3*) result primarily in substituted indoles.[1] Lately an interest in simple, direct methods for the synthesis of indole itself has been noted. Thus, a number of groups have been working on the high temperature conversion of *o*-aminoethylbenzene to indole,[2,3] and lately Suvorov, Avramenko, Shilkova and Zamyshlyaeva [4] were able to convert acetaldehyde phenylhydrazone to indole in 60 % yield by catalytic reaction in the vapour phase.

Some time ago, we reported the addition of mononitrotoluenes to aldehydes.[5] The products from the reaction between some *o*-nitrotoluenes and various aldehydes have been used to synthesize both substituted and unsubstituted indoles and 2,3-dihydroindoles.[6] 2-(*o*-Nitrophenyl)ethanol (*1*), readily available from *o*-nitrotoluene and formaldehyde, gave indole (*3*) or 2,3-dihydroindole (*8*) by that reaction sequence. Although the yields in each step were high, the number of steps necessary made it desirable to find a more direct synthesis of indole and 2,3-dihydroindole.

By running reactions in gas phase it is sometimes possible to carry out several consecutive synthetic steps without isolation and purification of the intermediate products. The possibility of a gas phase conversion of 2-(*o*-nitrophenyl)-ethanol to indole was therefore investigated.

Several years ago, Ufer and Breuers [7] described the formation of indole from 2-(*o*-aminophenyl)ethanol (*2*) by gas phase reaction over a copper catalyst. As 2-(*o*-aminophenyl)ethanol was readily available from the nitro analogue this method was tried:



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No yield was stated in the German patent, and our reproduction indicated the catalyst to have a rather short working life (less than 10 min). Ufer *et al.*[7] had prepared the catalyst by shaking bauxite with dry copper carbonate, followed by reduction. In that way, copper would be present only on the outer surface of the catalyst particles, and not in the internal pore structure.

A far superior catalyst was made by soaking silica gel in a solution of copper nitrate, drying and reducing with hydrogen. This is a well known method for the preparation of metal catalysts on carriers, and in this way even the inner pores of the carrier will contain metal catalyst. By using this type of preparation, catalysts with a working life of several hundred hours were obtained. The yield (95 %) and purity (96 %) of the indole produced was high. We had thus obtained a two step synthesis of indole from 2-(*o*-nitrophenyl)ethanol: reduction of the nitro group to an amino group in the liquid phase, followed by the vapour phase synthesis of indole.

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However, the vapour phase reduction of aromatic nitro compounds to the corresponding amines is a well known process.[8] If therefore 2-(*o*-nitrophenyl)ethanol could be reduced in the vapour phase without decomposition, the reaction would proceed directly to indole, and a one step synthesis of indole from 2-(*o*-nitrophenyl)-ethanol would be obtained.

When 2-(*o*-nitrophenyl)ethanol was passed over the copper catalysts used for the reaction of 2-(*o*-aminophenyl)ethanol, indole was obtained and the yield was almost as high (90 – 95 %) as in the case of 2-(*o*-aminophenyl)-ethanol. The catalysts and reaction conditions could be varied as shown in Table 2. The table indicates the catalysts containing copper to be of advantage as compared to those with other metals. Hydrogen was used for the reduction in most of the experiments, but other reducing gases, *e.g.* ammonia (run 12), could be used.

A typical run with copper chromite as catalyst gave indole in 90 % yield, and with the by-products given in Table 1.

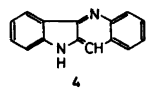
Table 1. Formation of indole and byproducts from 2-(*o*-nitrophenyl)ethanol over copper chromite at 250°, with hydrogen [12 mol/mol 2-(*o*-nitrophenyl)ethanol)] as reducing gas.

Products	Yields (%)[a]
Aniline	0.1
o-Toluidine	2.6
o-Aminoethylbenzene	2.1
2,3-Dihydroindole	4.4
Indole	90
Unidentified	0.8

[a] Determined by GLC.

The byproducts are readily accounted for by expected side reactions: retro-Knoevenagel condensation of 2-(*o*-nitrophenyl)ethanol would give *o*-nitrotoluene which would be reduced to *o*-toluidine. Dehydration of 2-(*o*-nitrophenyl)ethanol followed by hydrogenation of the *o*-nitrostyrene formed would give *o*-aminoethylbenzene, and incomplete dehydrogenation of 2,3-dihydroindole shows up in the presence of this compound. That this is the probable source of 2,3-dihydroindol and not hydrogenation of indole, was indicated by an increase in the content of 2,3-dihydroindole as the catalyst aged.

One byproduct which showed up in a very low yield (less than 10^{-2} %) was a crystalline, high melting compound, almost insoluble in common solvents. The IR spectrum indicated a strongly hydrogen bonded hydrogen to be present and the electronic spectrum that it contained a longer chromophore than indole itself. The mass spectrum indicated an elemental formula of $C_{18}H_{10}N_2$. From these indications both structures *4* and *5* (or the 5H-isomers) seemed possible for the compound. Compound *4* is quindoline, synthesized by Fichter and Boehringer,[9] and compound *5* quinindoline, synthesized by Gabriel and Eschenbach.[10]



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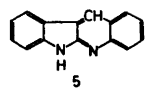

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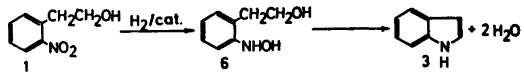
M.p. of the substance from the indole synthesis indicated it to be quinindoline (*5*), and comparison of the IR spectrum with that of an authentic sample [10] together with mixed m.p. showed the substance to be quinindoline.

The formation of indole from 2-(*o*-nitrophenyl)ethanol (*1*) probably proceeds *via* 2-(*o*-aminophenyl)ethanol (*2*) as indicated above. However, 2-(*o*-hydroxylaminophenyl)ethanol (*6*) can not be excluded as an intermediate. The hydrogenation conditions used would normally give the amino compound in high yield.[8] However, in the present case, the hydroxylamine (*6*) might be trapped in the cyclization reaction before further reduction could take place.



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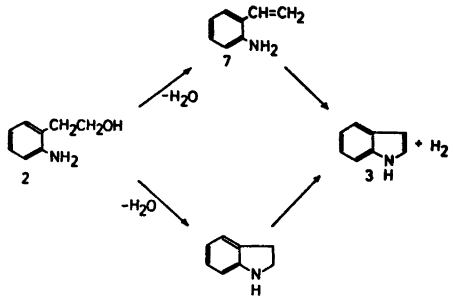
This posibility has not been investigated further. However, because of the ready reduction of nitro aromatics to the corresponding amines [8] it seems reasonable to assume that at least part of the indole was formed *via* 2-(*o*-aminophenyl)-ethanol.

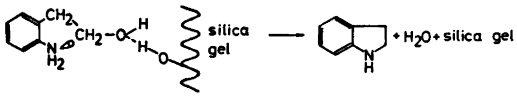
The formation of indole from 2-(*o*-aminophenyl)ethanol can be envisaged by several routes. One possibility is by *o*-aminostyrene (*7*) followed by cyclisation and dehydrogenation,

another by cyclisation to 2,3-dihydroindole (*8*) followed by dehydrogenation:



Experiments were undertaken to investigate these and other ways of formation. The route *via* o-aminostyrene was excluded by an experiment showing that this compound did not give indole at the comparatively low temperature (250°) used for the reaction of 2-(*o*-aminophenyl)ethanol.

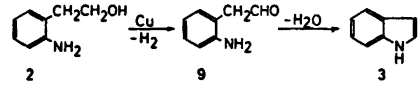
On the other hand, dihydroindole gave indole in high yield (98 %) under the reaction conditions. Further, if 2-(*o*-aminophenyl)ethanol vapour was passed over silica gel without copper metal, dihydroindole was obtained (98 % yield). This cyclisation of aminophenylethanol was shown to be catalyzed by silica gel, since no cyclisation took place over Vycor chips under the same reaction conditions. The catalytic effect of silica gel may be explained by the hydroxyl groups present.



From these results, it seemed plausible to assume that the indole had been formed by cyclisation of 2-(*o*-aminophenyl)ethanol followed by dehydrogenation of 2,3-dihydroindole.

However, another possible reaction path was revealed when metallic copper alone (from copper oxide) was used as catalyst. Again, indole in high yield was obtained from 2-(*o*-aminophenyl)ethanol. In the case of metallic copper, the catalytic cyclisation above is harder to envisage as the copper has no hydroxyl groups like silica gel.

A possible path for the formation of indole over metallic copper would be by dehydrogenation of the alcohol group of 2-(*o*-aminophenyl)-ethanol to an aldehyde group. The product *o*-aminophenylacetaldehyde (*9*) would probably cyclize to indole under the reaction conditions.



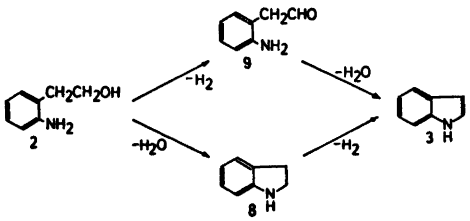
The catalytic dehydrogenation of alcohols to the corresponding aldehydes is a well known reaction: $RCH_2OH \xrightarrow{cat.} RCHO + H_2$.[11] The equilibrium is usually displaced towards the aldehyde by trapping the hydrogen formed. In the present reaction, the equilibrium would be displaced by the removal of *o*-aminophenylacetaldehyde (to indole).

To test the hypothetical reaction path *via* the aldehyde, 2-(*p*-aminophenyl)ethanol vapour was passed over silica gel and metallic copper catalysts. With 2-(*p*-aminophenyl)ethanol, no cyclization reaction would be possible, and *p*-aminostyrene was expected from the reaction over silica gel (by catalytical dehydration). From the reaction over copper it was hoped to identify traces (due to ready polymerization) of *p*-aminophenylacetaldehyde.

However, from both experiments, only unreacted 2-(*o*-aminophenyl)ethanol was isolated and in low yields, indicating polymerization of the products in the reactor. No conclusions as to presence of an aldehydic intermediate (*9*) in the indole synthesis could thus be drawn.

To test if an aldehyde nevertheless could be formed under the reaction conditions, 2-phenylethanol vapour was passed over copper at 250° in a nitrogen stream. The main product was indeed phenylacetaldehyde (50 % yield). Maihle [12] had earlier obtained phenylacetaldehyde from 2-phenylethanol over copper at 300°. Our result thus indicates that the copper catalyzed synthesis of indole from 2-(*o*-aminophenyl)ethanol might proceed *via* *o*-aminophenylacetaldehyde.

The reaction path *via* 2,3-dihydroindole was obviously not excluded by the results, and the only conclusion possible was that the reaction might proceed by both paths.



By the method presented here, it has thus been possible to synthezise indole in one step and in high yield from 2-(*o*-nitrophenyl)ethanol. This should be compared to the three steps necessary if a synthetic route in liquid phase is used.[1] On the other hand, the synthesis in liquid phase is probably more advantageous for the synthesis of substituted indoles.[6] The presented synthesis in vapour phase has not yet been investigated in the case of substituted indoles.

EXPERIMENTAL

The IR spectra were recorded on a Perkin-Elmer infrared spectrophotometer Model 257 and the electronic spectra on a Beckman DK-2 spectrophotometer. Gas chromatographic separations were made on a Perkin-Elmer F-11 gas chromatograph, equipped with a hydrogen flame ionisation detector and a 10 % SE-30 column. Mass spectra were recorded on an A. E. I. M. S. 902 mass spectrometer.

The gas phase reactor was made of a 60 cm long Vycor tube (o.d. 18 mm) equipped with a thermocouple well (o.d. 8 mm). The reactor was heated in an electrically heated fluidized bed with silicon carbide as heat transferring medium.[18] The reactor was half filled with catalyst (catalyst volume 30 ml) and the rest of reactor filled with Vycor chips (to heat reactants before reaching the catalyst). The liquid reactants were passed onto the top of the reactor by a syringe pump, together with the appropriate gas. The effluents from the reactor were analyzed by GLC. The stated percentages of the various components in the reaction mixtures were calculated from the gas chromatograms after corrections had been made for differences in the ionizing power of the various components.

Reactions of 2-(o-aminophenyl)ethanol in the vapour phase. Indole synthesis. When the experiment of Ufer *et al.*[7] was reproduced, indole was formed in 70 % yield during the first 10 min. The catalyst deteriorated rapidly, and later indole was found in low yield together with 2,3-dihydroindole and unreacted starting material. The reaction was then tried with a catalyst made by soaking silica gel in an aquous solution of copper nitrate, evaporating the water, drying the silica gel at 100°, heating it at 250° until the evolution of nitrous gasses ceased and finaly reducing it with hydrogen at 250°. The catalyst contained 7 % copper. With this catalyst (30 ml) in the reactor, 2-(*o*-aminophenyl)ethanol (10 g/h) was evaporated in a stream of hydrogen (200 ml/min) and passed over the catalyst. The yield of indole was 95 % and the main byproducts were 2,3-dihydroindole (2 %) and *o*-aminoethylbenzene (1.5 %). The indole from the reactor was 96 % pure and had m.p. 45–47°. Several commercial copper or copper chromite catalysts were tried and gave essentially the same result. The working lives of the catalysts were several hundred hours. A copper metal catalyst made by hydrogen reduction of copper oxide (Merk AG, Kupferoxid Draht Form) gave the same result.

2,3-Dihydroindole from 2-(o-aminophenyl)ethanol. 2-(*o*-Aminophenyl)ethanol (10 g/h) and hydrogen (200 ml/min) were passed through the reactor containing silica gel as catalyst at 250°. The yield of 2,3-dihydroindole was 98 %.

Gas phase reaction of 2-(o-aminophenyl)ethanol without catalyst. This reaction was carried out as the previous one, except that the reactor was filled with Vycor chips alone. The product consisted of unreacted starting material. No 2,3-dihydroindole was detected.

Gas phase reaction of o-aminostyrene. o-Aminostyrene (10 g/h, prepared by reduction of *o*-nitrostyrene with iron turnings) together with hydrogen (200 ml/min) was passed through the reactor containing silica gel (30 ml) as catalyst at 250°. The product consisted of unreacted *o*-aminostyrene. No 2,3-dihydroindole was detected. When 7 % copper on silica gel was used as catalyst, *o*-aminoethylbenzene was obtained in 97 % yield.

Gas phase reaction of 2,3-dihydroindole. When 2,3-dihydroindole was vapourized and passed with hydrogen over the copper/silica gel catalyst, as described for 2-(*o*-aminophenyl)ethanol, indole was obtained in 98 % yield.

Reactions of 2-(p-aminophenyl)ethanol in the vapour phase. 2-(*p*-Aminophenyl)ethanol (2.5 g) was dissolved in 1,2-dimethoxyethane (37.5 g) and the solution (19.6 g/h) pumped into the reactor at 250° together with N_2 (30 ml/min). When silica gel (30 ml) was used as catalyst, 21.10 g solution was reacted (containing 1.9 g *p*-aminophenylethanol).

The product (0.1 g concentrated, 5 % of starting material) gave 8 peaks on GLC, one of which was possibly 2-(*p*-aminophenyl)ethanol. By extracting the catalyst for 6 h with a 1:1 mixture of chloroform:methanol, 0.3 g of a product consisting mainly of starting material (as judged by TLC) was obtained.

When copper metal (from copper oxide) was used as catalyst and the other conditions retained as described above, 31.5 g of the *p*-aminophenylethanol solution (containing 2.06 g of *p*-aminophenylethanol) was reacted. A certain amount of product was isolated (*ca.* 450 mg),

consisting mainly of unreacted starting material, (GLC).

As the conditions for the reactions of *p*-aminophenylethanol (due to the crystallinity of the substance) were slightly different from those originally used for *o*-aminophenylethanol, control experiments were run: A solution of 2-(*o*-aminophenyl)ethanol (3.5 g) in 1,2-dimethoxyethane (50 g) was reacted at the same rate and under the same conditions as those for the *p*-isomer described above. When silica gel was used as catalyst, 2,3-dihydroindole was obtained. When copper was used as catalyst, indole was the product. Both substances were obtained in the same yields and purities as described for the reaction without solvent and with H_2 as carrier gas.

Formation of 2-phenylacetaldehyde. 2-Phenylethanol (1.85 g/h) was pumped into the reactor at 250° together with nitrogen (85 ml/min). The reactor contained copper (from copper oxide) (13 ml) and Vycor chips (10 ml). Totally 3.7 g of phenylethanol was reacted. The condensate from the reactor (3.05 g) contained phenylacetaldehyde (35 %), ethylbenzene (26 %), and phenylethanol (31 %) (analyzed by GLC, internal standard method). This corresponded to a yield of phenylacetaldehyde of 50 % (calculated on basis of reacted alcohol). Phenylacetaldehyde was identified after isolation by column chromatography, by comparison of IR and TLC with those of an authentic sample.

Indole from 2-(o-nitrophenyl)ethanol. 2-(*o*-nitrophenyl)ethanol (5.6 g/h) was pumped into the reactor containing 30 ml of catalyst and 30 ml of Vycor chips together with a reducing gas. The products were analyzed by GLC. Results and reaction conditions are given in Table 2. From the reaction mixture with copper chromite as catalyst, 0.01 % of a crystalline compound was isolated. The compound had mp. 341 – 343°, IR (KBr) 3140 (broad, from 3300 to 2300 cm^{-1}), 1640, 1610, 1580, 1490, 1480, 1460, 1410, 1330, 1280, 1260, 1230, 1130, 1020, 990, 950, 910, 880, 860, 820, 790, 760, 740, 700, 640 cm^{-1}. The electronic spectrum (in ethanol) had maxima (in nm) numbers in parantheses give ε_{max} (l mol^{-1} cm^{-1}): 368 (3600), 331 (17 000), 317 (12 000), 270 (52 000), 265 (shoulder) (45 000). The mass spectrum indicated an elemental formula of $C_{15}H_{10}N_2$ with the molecular ion giving the base peak.

The isolated compound was in every respect identical with an authentic one synthesized by the method of Gabriel *et al.*[10] (In the synthesis,[10] catalytic reduction was used instead of the sulfide reduction used by these authors).

Table 2. Synthesis of indole from 2-(*o*-nitrophenyl)ethanol (ONPE) in the vapour phase, (5.6 g ONPE/h reacted). Reducing gas H_2.

Run	Catalyst	Reaction temperature °C	Mol reducing gas/mol ONPE	Yield of indole %
1	7 % Cu/silica gel	250	12	99
2		330	12	66
3		400	12	26
4		250	3	70
5		250	18	97
6	7 % Cu/Al_2O_3	250	6	97
7	7 % Ni/silica gel	250	12	59
8	7 % Co/silica gel	250	12	78
9	0.5 % Pd/C	250	3	66
10	Cu (from CuO)	250	12	95
11	V_2O_5/K_2SO_4-silica gel	250	12	85
12	V_2O_5/K_2SO_4-silica gel	250	12[a]	84
13	Copper chromite	250	12	90

[a] Reducing gas NH_3.

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Effects of Alkyl-substitution on the Base-catalysed 1,3-Proton Transfer in the Indene System

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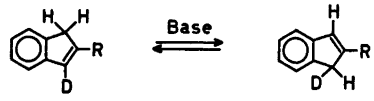
Syntheses of 2-alkyl substituted, deuterated indenes and their isomerization catalyzed by some tertiary aliphatic amines in pyridine solution, have been studied. Optically active 1,2-dimethylindene has been prepared and its rearrangement to 2,3-dimethylindene has been studied polarimetrically. Some steric and electronic consequences of substitution in the indene nucleus are discussed. The substitution pattern of the indene and the nature of the catalyst seem to influence the reaction rate in a complex way. Rate constants and activation parameters for the isomerization reactions are given.

The prototropic 1,3-shift in the indene system has been investigated in detail.[1–19] Base-catalyzed isomerization of 1-,[1–7] 1,2-,[9] and 1,3 [10]-alkyl substituted indenes have been studied with regard to stereospecificity,[2,19] intramolecularity and deuterium isotope effect.[1,20,21] Recently, a report has been published [22] concerning the influence of some β-substituents in alkylsubstituted methylindenes on the isomerization rate.

This communication reports the results of some experiments with 2-alkyl substituted indenes. The isomerization *1* ⇌ *2* (Scheme 1), where R is a methyl or an ethyl group, was studied by the NMR-technique. Further, optically active 1,2-dimethylindene *7* (Scheme 3)

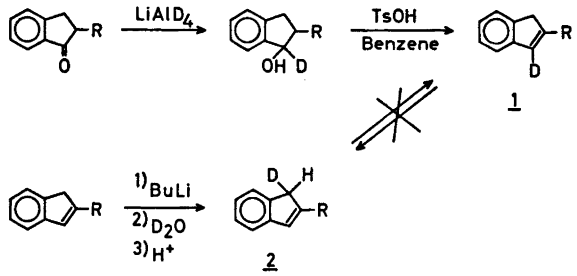


1 2

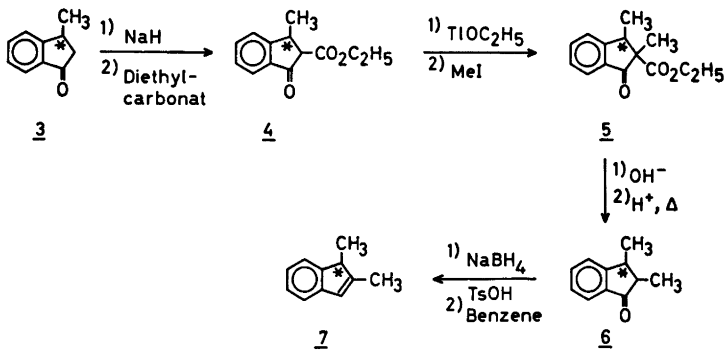
Scheme 1.

was synthesized and its mutarotation to the non-chiral 2,3-dimethylindene was studied polarimetrically. As in previous investigations, aliphatic amines were used as catalysts and the reactions were run in pyridine solutions. The catalytic effect of 1,4-diazabicyclo[2.2.2]-octane (DABCO) and triethylamine (TEA) were investigated. Earlier, it was found that the rearrangements under these conditions in the indene system were completely intramolecular and highly stereospecific.[1,21]

In order to kinetically follow the reaction by NMR it was necessary to label the 2-alkylindenes with deuterium in position 1 or 3. The 2-alkyl-3-deuterioindenes *1* were prepared by reducing the corresponding 2-alkylindanones



Scheme 2.



Scheme 3.

with lithium aluminium deuteride, followed by dehydration with *p*-toluenesulphonic acid of the indanol obtained (Scheme 2). In order to get access to the 2-alkyl-1-deuterioindenes *2*, 2-methylindene (or 2-ethylindene) was treated with butyllithium in dry ether at −30°C and the indenyllithium thus formed was decomposed with D_2O, followed by rapid acidification (Scheme 2). As judged from the NMR-spectrum, no rearranged product *1* was formed during this synthesis.

In the case of the 1,2-dialkylindene, the isomerization [9] can be followed more accurately by the polarimetric technique since the indene mutarotates with the same rate as it isomerizes. The conventional synthesis of an optically active 1,2-substituted indene starts with the resolution of an α-alkyl-β-phenyl substituted alkanoic acid. This may be a complicated procedure since the molecule contains two asymmetric carbon atoms. An alternative route to the optically active indene was therefore undertaken. Optically active 3-methylindanone *3* was acylated in the 2-position with diethylcarbonate and the thallium salt of 2-carbethoxy-3-methylindanone *4* was prepared by treatment of *4* with thallous ethoxide. Alkylation with methyl iodide gave almost exclusively C-alkylation of the carbethoxy-indanone in good yield. Alkaline hydrolysis and decarboxylation gave the 1,2-dimethylindene *7* after some conventional steps (Scheme 3).

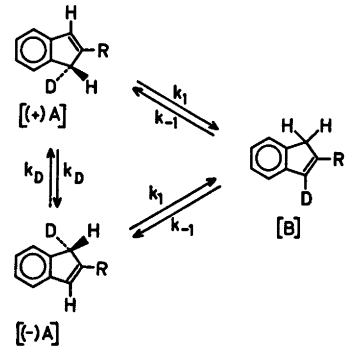
TREATMENT OF RATE DATA

The course of the reaction in Scheme 1 was measured by integration of the NMR-signals from the vinylic proton and/or the saturated ring protons. The signal from the 2-alkyl group was used as an internal standard. In discussing the rate and equilibrium properties of the system, it should be remembered that the equilibrium constant *2/1* is equal to 2, due to the asymmetry of *2* (*cf.* Scheme 1). Using the notations given in the phenomenological reaction Scheme 4, we get the following rate equations:

$$\frac{d[(+)A]}{dt} = -(k_1+k_D)[(+)A]+k_D[(-)A]+k_{-1}[B] \quad (1)$$

$$\frac{d[(-)A]}{dt} = k_D[(+)A]-(k_1+k_D)[(-)A]+k_{-1}[B] \quad (2)$$

$$\frac{d[(B)]}{dt} = k_1[(+)A]+k_1[(-)A]-2k_{-1}[B] \quad (3)$$



Scheme 4.

If $[A]=[(+)A]+[(-)A]$, these equations can be written as:

$$\frac{d[A]}{dt} = -k_1[A]+2k_{-1}[B] \quad (4)$$

$$\frac{d[B]}{dt} = k_1[A] - 2k_{-1}[B] \quad (5)$$

[The (+)- and (−)-signs have no configurational relevance.] Thus, deuterium migration, which brings (+)A to (−)A and *vice versa*, does not influence our kinetic measurements. Furthermore, if secondary isotope effects are neglected, *i.e.* if $k_{-1}=k_1$, the rate equations, in integrated form, will be:

$$[A] = ([A]_0 - [A]\infty) \exp(-3k_1t) + [A]\infty \quad (6)$$

$$[B] = ([B]_0 - [B]\infty) \exp(-3k_1t) + [B]\infty \quad (7)$$

If x and y denotes the area of the saturated and the vinylic protons, respectively, and λ is a proportionality constant we have

$$x = \lambda([A] + 2[B]) \quad (8)$$

$$y = \lambda[A] \quad (9)$$

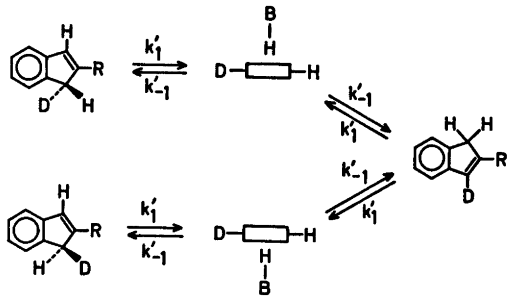
Combining eqns. (6 – 7) with eqns. (8 – 9) we get:

$$x = (x_0 - x\infty) \exp(-3k_1t) + x\infty \quad (10)$$

$$y = (y_0 - y\infty) \exp(-3k_1t) + y\infty \quad (11)$$

Thus, the observed rate constant is $3k_1$. This fact is of importance for the comparison of the rate constants obtained for alkylindenes with different substitution pattern. Thus, if we consider a phenomenological reaction scheme equivalent to Scheme 4 for a 1- or a 1,2-substituted indene, the observed rate constant will be k_1,[1,9] since the collapse ratio is large and for the symmetrically substituted 1,3-dimethylindene [10] the observed rate constant will be exactly $2k_1$.

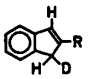
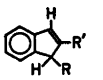
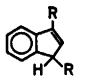
As pointed out before,[14] however, the rearrangements proceed most probably *via* an ion-pair intermediate as in mechanistic reaction, Scheme 5.



Scheme 5.

In this case, a proper comparison should involve the mechanistic rate constant k'_1, and it is easily shown that $k_{obs}=3k'_1/2$ for 2-substituted indenes and that $k_{obs}=k'_1$ in other cases of interest, *i.e.* the last expression is exactly valid for 1,3-dimethylindene and approximately valid for the 1-methylindene since the collapse ratio is large. The comparisons should thus be made according to the directions given in Table 1. These considerations should

Table 1.

Substrate:	Phenomenological Scheme 4 k_1	Mechanistic Scheme 5 k'_1
	$\frac{1}{3}k_{obs}$	$\frac{2}{3}k'_{obs}$
	k_{obs}	k_{obs}
	$\frac{1}{2}k_{obs}$	k_{obs}

be kept in mind when rate constants are to be compared. Enthalpies of activation can of course be calculated and compared directly, using k_{obs}. The entropies of activation will depend on which rate constant is chosen, but the experimental error will, in most cases, be larger than the difference between the entropy values calculated using different rate constants (*i.e.* k_{obs}, k_1 or k'_1). Finally, it should be noted that k_{obs} used in the present discussion is the second order rate constant, obtained by multiplying the experimentally observed pseudo-first order constant with 1/[Base].

RESULTS AND DISCUSSION

It was found earlier that the introduction of a methyl group in position 1 of the indene nucleus slows the TEA-catalyzed reaction at 30°C by a factor of 5.8.[6] Introduction of a substituent in the 2-position of 1-methylindene further shows the reaction by a factor of 25.[9] The retarding effect of a substituent in the 2-position is thus considerable. If 1-methylindene is substituted with a methyl group in the 3-

position, only a very slow rearrangement occurs with TEA.[10]

Turning to the DABCO-catalyzed reactions, it can be found from the present investigation and from earlier results in this field [6] that 1-methylindene rearranges about 25 % slower than indene itself, but a further substitution to 1,2-dimethylindene slows the reaction rate by a factor about 7.[9] Alkyl substitution to 1,3-dimethylindene shows that the mechanistic rate constant is about 40 times smaller than that for indene. The effect of alkyl-substitution on the reaction rate is thus markedly dependent on the situation of the substituent.

In the ground state, a 2-alkylindene should be more stable than a 1-alkylindene, due to hyperconjugation between the double bond and the methyl group, as well as between the double bond and the CH_2-group in position 1. In the transition state, the indene part is probably very much like the indenyl anion. In this anion, positions 1 and 3 have higher electron densities than position 2 [12] and a 2-substituted indenyl anion is therefore expected to be more stable than a 1-substituted anion.

Starting with indene, the introduction of an alkyl substituent in the 1-position would thus raise the energy level of the ground state but also increase the energy level of the transition state (Fig. 1). In accordance with this view, the situation in the 2-alkylindene case should be a lower ground state energy level, but with a transition state of about the same magnitude as in indene. The introduction of a methyl group in the 1-position of 2-methylindene should also raise the energy of the ground state to about the same extent as a 1-methyl group does on substitution in indene. In the transition state, a 1-methyl substituent would act in the same direction, *i.e.* raising the energy level.

The introduction of a methyl group in the 1-position does not always retard the proton abstraction rate (k'_1) when DABCO is used as a catalyst. This might seem puzzling since a retardation in reaction rate is quite obvious with TEA as catalyst. TEA should of course be sterically more hindering in the transition state than DABCO. However, a retardation in reaction rate would be expected also in the DABCO-case when a methyl group is introduced in the 1-position of 2-methylindene. Thus if $\Delta G^{\ddagger}_0$, $\Delta G^{\ddagger}_1$, $\Delta G^{\ddagger}_2$, and $\Delta G^{\ddagger}_{12}$ denote the free energies of activation (as calculated from the k'_1-values) for indene, 1-methylindene, 2-methylindene, and 1,2-dimethylindene, we get, using the values given in Table 2:

$(\Delta G^{\ddagger}_1 - \Delta G^{\ddagger}_0)_{\mathrm{DABCO}} = +0.1$ kcal mol^{-1};
$(\Delta G_1^{\ddagger} - \Delta G^{\ddagger}_0)_{\mathrm{TEA}} = +1.0$ kcal mol^{-1}

$(\Delta G^{\ddagger}_{12} - \Delta G^{\ddagger}_2)_{\mathrm{DABCO}} = -0.1$ kcal mol^{-1};
$(\Delta G^{\ddagger}_{12} - \Delta G_2^{\ddagger})_{\mathrm{TEA}} = +1.1$ kcal mol^{-1}

Thus, in the case of TEA the 1-methyl substituent increases the free energy of activation by 1.1 kcal mol^{-1} whether or not a methyl group is present in the 2-position. With DABCO the introduction of a methyl substituent in the 2-position has little or no influence on the reaction rate, the average value for the difference in free energy of activation being 0.0 kcal mol^{-1}.

The difference between these values, 1.1 kcal mol^{-1}, may be ascribed to the difference in steric hindrance in the transition state (steric hindrance in the ground state being equal and negligible) between the 1-methyl and TEA and DABCO, respectively. If y'_1 denotes the steric hindrance in kcal mol^{-1} between the 1-methyl group and the catalyst in the transition state, we get:

$(y'_1)_{\mathrm{TEA}} - (y'_1)_{\mathrm{DABCO}} = +1.1$ kcal mol^{-1}

In the same way, one may use the activation energy differences between 2-methylindene (and 1,2-dimethylindene) and indene to estimate the difference in steric effects in the transition state between a 2-methyl group and TEA and DABCO, respectively. If y'_2 denotes the steric hindrance between the 2-methyl group and the catalyst in the transition state, we get:

$(y'_2)_{\mathrm{TEA}} - (y'_2)_{\mathrm{DABCO}} = (\Delta G_2^{\ddagger} - \Delta G^{\ddagger}_0)_{\mathrm{TEA}} - (\Delta G_2^{\ddagger} - \Delta G^{\ddagger}_0)_{\mathrm{DABCO}}$

and

$(y'_2)_{\mathrm{TEA}} - (y'_2)_{\mathrm{DABCO}} = (\Delta G^{\ddagger}_{12} - \Delta G^{\ddagger}_0)_{\mathrm{TEA}} - (\Delta G^{\ddagger}_{12} - \Delta G^{\ddagger}_0)_{\mathrm{DABCO}} - \{(y'_1)_{\mathrm{TEA}} - (y'_1)_{\mathrm{DABCO}}\}$

The right hand sides of these equations are, using the previously deduced value for the y'_1-difference and the $\Delta G^{\ddagger}$-values of Table 2, +0.4 and +0.6 kcal mol^{-1}. As an average value +0.5 kcal mol^{-1} can be chosen and thus

$(y'_2)_{\mathrm{TEA}} - (y'_2)_{\mathrm{DABCO}} = +0.5$ kcal mol^{-1}

A 2-methyl group thus causes a less pronounced selectivity between different catalysts than a

Table 2. Rate constants and activation parameters for the base-catalyzed tautomerization of some indene derivatives. Indene concentration: 2 M. Temperature: 30.0°C. Solvent: Pyridine.

Indene derivative	Catalyst Nature	Conc. [B] mol l^{-1}	k_{obs} sec^{-1}	$k_{obs}/[B]$ 1 mol^{-1} sec^{-1}	k'_1 1 mol^{-1} sec^{-1}	$\Delta G^{\ddagger}$ kcal mol^{-1}	$\Delta H^{\ddagger}$ kcal mol^{-1}	$\Delta S^{\ddagger}$ cal mol^{-1} degree
Indene	DABCO	3.40×10^{-3}	1.53×10^{-4} [c]	4.51×10^{-2}	3.02×10^{-2}	19.87 ± 0.05		
1-Methyl-	»	3.41×10^{-3}	8.15×10^{-5} [b]	2.39×10^{-2}	2.39×10^{-2}	20.00 ± 0.03	10.9 ± 0.2 [d]	-30 ± 1
1,2-Dimethyl	»	2.21×10^{-2}	7.32×10^{-5} [a]	3.31×10^{-3}	3.31×10^{-3}	21.20 ± 0.01	12.7 ± 0.2	-28 ± 1
2-Methyl	»	1.72×10^{-2}	7.24×10^{-5} [c]	4.21×10^{-3}	2.82×10^{-3}	21.29 ± 0.05	11.4 ± 0.6	-33 ± 3
2-Ethyl	»	1.72×10^{-2}	5.21×10^{-5} [c]	3.03×10^{-3}	2.03×10^{-3}	21.49 ± 0.05	11.4 ± 0.6	-33 ± 3
1,3-Dimethyl	»	2.79×10^{-1}	2.13×10^{-4} [b]	7.65×10^{-4}	7.65×10^{-4}	22.06 ± 0.03	13.6 ± 0.2	-28 ± 1
Indene	TEA	5.40×10^{-3}	5.75×10^{-5} [c]	1.06×10^{-2}	7.10×10^{-3}	20.74 ± 0.05	— [e]	—
1-Methyl	»	2.57×10^{-1}	3.15×10^{-4} [b]	1.23×10^{-3}	1.23×10^{-3}	21.79 ± 0.03	9.6 ± 0.2 [f]	-40 ± 1
1,2-Dimethyl	»	1.32	6.61×10^{-5} [a]	5.01×10^{-5}	5.01×10^{-5}	23.72 ± 0.01	11.8 ± 0.2	-39 ± 1
2-Methyl	»	1.22×10^{-1}	6.26×10^{-5} [c]	5.13×10^{-4}	3.44×10^{-4}	22.56 ± 0.05	9.8 ± 0.6	-42 ± 3
2-Ethyl	»	1.20×10^{-1}	4.68×10^{-5} [c]	3.90×10^{-4}	2.61×10^{-4}	22.73 ± 0.05	9.2 ± 0.6	-45 ± 3

[a] $2\sigma\simeq2$ %. [b] $2\sigma\simeq5$ %, [c] $2\sigma\simeq8$ %, [d] Ref. 14 Indene conc. ~0.1 M. [e] Ref. 3. [f] Ref. 6.

1-methyl substituent, and TEA exhibits a larger steric hindrance than DABCO. These conclusions are quite reasonable from the chemical point of view.

The above analysis invites an attempt to describe the substituent effects, both steric and electronic, by a linear model. This cannot lead anywhere, however, without further data. Important information can be obtained from the previous study of the rearrangement of 1,3-dimethylindene with DABCO. We may assume that the transition state is unsymmetrical, so that only one of the methyl groups gives rise to steric repulsion. Both methyl groups have other effects, however, which are mainly electronic on the ground state as well as on the transition state. Another important fact is the equilibrium constant between 3-methylindene and 1-methylindene. It has been found that 1-methylindene is present to about 1 % in equilibrium. The free energy difference between the ground states of these two indenes must therefore be close to 2.8 kcal mol^{-1}. This difference is caused by substituent effects on the ground states, which for simplicity one may interpret as electronic (hyperconjugative) effects. Introducing the symbols given below, one gets the energy levels given in Fig. 1. The rate determining transition state for 3-methylindene is, of course, the same as for 1-methylindene. Referring to Fig. 1 we obtain the equations:

$$\Delta G^{\ddagger}_1 - \Delta G^{\ddagger}_0 = x'_1 + y'_1 + x^0_0 \quad (12)$$

$$\Delta G^{\ddagger}_{13} - \Delta G^{\ddagger}_0 = 2x'_1 + y'_1 + x^0_0 - x^0_1 \quad (13)$$

$$\Delta G_0 = x^0_0 + x^0_1 \quad (14)$$

$$\Delta G^{\ddagger}_2 - \Delta G^{\ddagger}_0 = x'_2 + y'_2 - x^0_1 \quad (15)$$

$$\Delta G^{\ddagger}_{12} - \Delta G^{\ddagger}_0 = x'_1 + y'_1 + x'_2 + y'_2 + x^0_0 - x^0_1 \quad (16)$$

The symbols have the following meaning:

x^0_0 = effect on the ground state of introducing a ring-CH_2-group

x^0_1 = effect on the ground state of introducing a CH_3-group in hyperconjugation with a double bond

x'_1 = effect on the transition state of introducing a CH_3-group in the 1- or 3-position

x'_2 = effect on the transition state of introducing a CH_3-group in the 2-position

y'_1 = steric effect on the transition state of a CH_3-group in the 1-position

y'_2 = steric effects on the transition state of a CH_3-group in the 2-position

$\Delta G_0 = -RT \ln K_{eq.}$, where $K_{eq.}$ is the equilibrium constant between 3-methylindene and 1-methylindene.

It should be noted, that x^0_0, x^0_1, x'_1 and x'_2 are independent of the nature of the catalyzing base, whereas y'_1 and y'_2 are, of course, different for DABCO and TEA, as explained above.

Eqns. (12), (13), and (14) can be solved for y'_1, and we get:

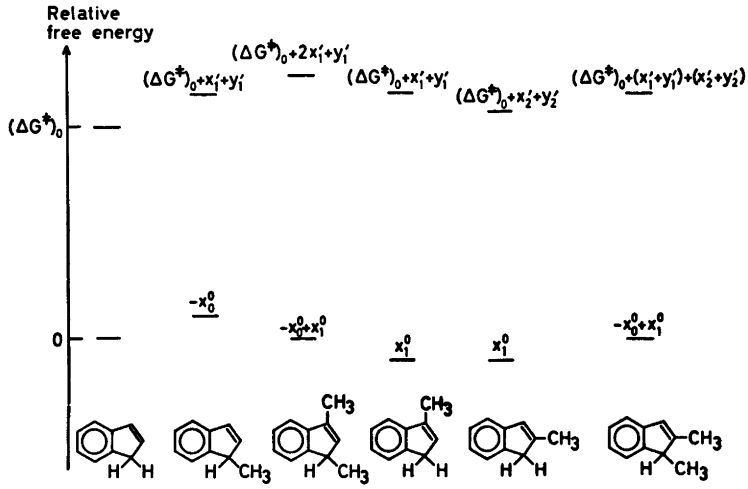


Fig. 1. Relative free energies between ground state and transition state for alkylindenes. The symbols are explained in the text.

$$y'_1 = 2\Delta G^{\ddagger}_1 - \Delta G^{\ddagger}_{13} - \Delta G^{\ddagger}_0 - \Delta G_0 \quad (17)$$

The rate constant (7.65×10^{-4} l mol^{-1} sec^{-1}) reported previously for the DABCO-catalyzed reaction for 1,3-dimethylindene, gives $\Delta G^{\ddagger}_{13} =$ 22.1 kcal mol^{-1}. Using this value and the $\Delta G^{\ddagger}$-values reported in Table 2 (together with $\Delta G_0 = -2.8$ kcal mol^{-1} as given above), we find that $(y'_1)_{DABCO} = +0.8$ kcal mol^{-1}. Since we already have concluded that $(y'_1)_{TEA} - (y'_1)_{DABCO}$ was +1.1 kcal mol^{-1}, we now get $(y'_1)_{TEA} = +1.9$ kcal mol^{-1}.

Combination of eqns (12), (15), and (16) gives:

$$\Delta G^{\ddagger}_1 - \Delta G^{\ddagger}_0 = \Delta G^{\ddagger}_{12} - \Delta G^{\ddagger}_2 = x'_1 + y'_1 + x^0_0 \quad (18)$$

Using the average value for the free energy differences used above for the DABCO-case and the value +1.1 kcal mol^{-1} for the TEA catalyzed reaction together with the y'_1-values just obtained, we get $x'_1 + x^0_0 = -0.8$ kcal mol^{-1}. With the parameter values thus obtained, eqns (15) and (16) are best satisfied using $x'_2 - x^0_1 + (y'_2)_{DABCO} = +1.4$ kcal mol^{-1}

Thus, the data now available gives us the following parameters:

$(y'_1)_{DABCO} = +0.8$ kcal mol^{-1}
$(y'_1)_{TEA} = +1.9$ kcal mol^{-1}
$x'_1 + x^0_0 = -0.8$ kcal mol^{-1}
$x^0_0 + x^0_1 = -2.8$ kcal mol^{-1}
$(x'_2 - x^0_1) + (y'_2)_{DABCO} = +1.4$ kcal mol^{-1}

Table 3 summarizes the activation free energy differences calculated using these parameter values and compares them with experimental results.

Thus, the eight parameters used in the model [eqns. (12)–(16)] have been fixed to six numerical values. An attempt can now be made, using chemical intuition, to create a set for all eight parameters. The steric parameters already given

Table 3.

	DABCO		TEA	
	Exp.	Calc.	Exp.	Calc.
$\Delta G^{\ddagger}_1 - \Delta G^{\ddagger}_0$	0.1	0	1.0	1.1
$\Delta G^{\ddagger}_{13} - \Delta G^{\ddagger}_0$	2.2	2.0	–	3.1
$\Delta G^{\ddagger}_2 - \Delta G^{\ddagger}_0$	1.4	1.4	1.8	1.9
$\Delta G^{\ddagger}_{12} - \Delta G^{\ddagger}_0$	1.3	1.4	2.9	3.0

makes it plausible to set $(y'_2)_{DABCO} = 0$, and the steric parameters would then be as follows:

$(y'_1)_{DABCO} = +0.8$ kcal mol^{-1};
$(y'_1)_{TEA} = +1.9$ kcal mol^{-1}

$(y'_2)_{DABCO} = 0.0$ kcal mol^{-1};
$(y'_2)_{TEA} = +0.5$ kcal mol^{-1}

Using this value for $(y'_2)_{DABCO}$, we see that $x'_1 - x'_2 = +0.6$ kcal mol^{-1}. As long as $(y'_2)_{DABCO}$ is greater than -0.6 kcal mol^{-1}, which it seems safe to assume, the difference between x'_1 and x'_2 will be greater than zero. Thus, a 1-methyl group will have a more destabilizing effect on the transition state than a methyl group in the 2-position. This agrees well with the fact that the indenyl anion has a larger negative charge on the 1-carbon atom than on the 2-carbon atom. As mentioned before, the indene part of the transition state is probably very much like the indenyl anion.

A complete set of reasonable electronic parameters may be constructed if it is assumed that the stabilizing effect of the ground state (hyperconjugation) of the ring methylene group is equal to the stabilization between a methyl group and a double bond in the ground state. If this is true, we get the following values of the individual x-parameters:

$x^0_0 = -1.4$ kcal mol^{-1};
$x^0_1 = -1.4$ kcal mol^{-1}

$x'_1 = +0.6$ kcal mol^{-1};
$x'_2 = 0.0$ kcal mol^{-1}

Steric repulsion between the catalyzing base and the substrates, as judged from models, seems to be approximately the same for 1- and 2-substituted indenes. However, Plenat and Bergson found [9] that the relative catalytic effect of TEA was more sensitive to the nature of the substrates, *i.e.* the substituted indenes, than that of DABCO. For instance the ratio k_{DABCO}/k_{TEA} was found to be 19:1 for 1-methylindene and 66:1 for 1,2-dimethylindene. In this investigation we found the corresponding ratio for 2-methylindene to be 8:1 and for indene 4:1. The introduction of a substituent in the nucleus of indene should of course present increased interaction between the substrate and TEA and it is obvious that a 1-methylsubstituent is more hindering than a 2-methyl group. However, it can be concluded from the

above mentioned considerations, that it is not the 1- or 2-substituents separately which are causing the substrate sensitivity for TEA, but a combination as reflected in the 1,2-dimethylindene case.

From Table 2 it can be seen that the enthalpies of activation are about the same for 1-methyl- and 2-methylindene; 10.9 and 11.4 kcal mol^{-1} for the DABCO-catalyzed reactions and 9.6 and 9.8, respectively, for the TEA-catalyzed reaction. The activation enthalpies of the disubstituted indenes were significantly higher, 12.7 kcal mol^{-1} for 1,2-dimethylindene and 13.6 kcal mol^{-1} for 1,3-dimethylindene in the DABCO-catalyzed reaction. As expected, there is no difference in $\Delta S^{\ddagger}$ between 1-, 1,2- and 1,3-dimethylindene (−30, −28, and −28 e.u.). Much of the difference in reactivity between these substrates can thus be attributed to differences in $\Delta H^{\ddagger}$. For this reason, it seems confusing that $\Delta S^{\ddagger}$ for the DABCO and TEA-catalyzed isomerization of 2-methylindene is larger than the corresponding reactions with 1-methylindene as a substrate. Even if the experimental errors are large, it must be concluded that the lower reacting power of the 2-substituted compounds is probably due to these differences in activation entropy. The origin of this difference is not clear at present.

EXPERIMENTAL

Instrumental. Melting points and boiling points are uncorrected. The NMR-spectra were recorded in CCl_4 at 34 ± 1°C on a Varian A 60 D spectrometer. Preparative GLC of the indenes was performed on a Varian Aerograph Model 90-P4 with helium as carrier gas. The column used for purification was 0.6 m × 3/8″, packed with 20 % Apiezon L on Chromosorb W, 60/80 mesh. The purities of the indenes were checked on a Perkin-Elmer 900 gas chromatograph, equipped with a flame ionization detector, and with a 3 m × 1/8″ column, packed with 5 % Apiezon L on 100/120 mesh Varaport. The polarimetric measurements were made with a Perkin-Elmer polarimeter 141 M and the infra-red spectra with a Perkin-Elmer Model 237 spectrophotometer.

Syntheses

2-Alkylindanones were prepared according to Cologne and Weinstein [24] by condensation of the appropriate arylalkylketone with formaldehyde and subsequent dehydration of the ketols obtained with sulphuric acid. 2-Methylindanone-1: $b.p._{11} = 112-113°C$, $n_D^{22} = 1.5520$ (Lit.[24] $b.p._{15} = 120°C$, $n_D^{22} = 1.5511$). 2-Ethylindanone-1: $b.p._{11} = 125-126°C$, $n_D^{22} = 1.5465$ (Lit.[24] $b.p._{12} = 127°C$, $n_D^{22} = 1.5457$).

2-Alkyl-3-deuterioindenes (1) Alkylindanone (0.05 mol) in 50 ml of dry ether was slowly dropped into a solution of 0.03 mol of $LiAlD_4$ (Merck, deuterium content min. 99 %) in 20 ml ether. After refluxing for 2 h and after the usual work-up procedure,[5] the resulting indanols were dehydrated without further purification with 0.01 g of *p*-TsOH in 200 ml of dry benzene, using a Soxhlet extractor, filled with molecular sieves (Union Carbide, 5 Å). After half an hour of reflux the solution was chilled, extracted with 5 % aq. $NaHCO_3$ and then with a saturated NaCl solution and dried. The indenes were distilled in vacuum and purified by preparative GLC. The deuterium content in the 3-position was 97 ∓ 1 % as judged by the NMR-spectrum.

2-Methyl-3-deuterioindene (1a). Yield 68 % (calc. on the ketone) $b.p._{11} = 76-77°C$, $n_D^{22} = 1.5637$ (Lit.[25] gives for 2-methylindene $b.p._{2,3} = 64-65°C$, $n_D^{20} = 1.5645$). NMR: (Shifts in δ) 6.7 − 7.3, 4 H, complex; 3.11, 2 H, s (singlet); 2.09, 3 H, s.

2-Ethyl-3-deuterioindene (1b). Yield 76 % (calc. on ketone) $b.p._{11} = 82-85°C$, $n_D^{22} = 1.5590$ (Lit.[25] gives for 2-ethylindene $b.p._{1,1} = 62°C$, $n_D^{20} = 1.5598$). NMR: 6.7 − 7.3, 4 H, complex; 3.09, 2 H, s; 2.32, 2 H, q, $J = 7.5$ cps; 1.15; 3 H, t, $J = 7.5$ cps.

2-Alkyl-1-deuterioindenes (2). 2-Alkylindene [25] (0.02 mol) in 10 ml of dry ether was treated with 0.03 mol of butyllithium in ether solution at −30°C and held at this temperature for 1.5 h. D_2O, 5 ml (Norsk Hydro, 99.8 % D) was added with vigorous stirring during 5 min. The temperature was never allowed to exceed −15°C. After acidification with 2 M HCl, the ether layer was separated and the acidic phase extracted with ether. After drying of the extracts, the solvent was evaporated and the residue distilled in vacuum. The indenes were further purified by preparative GLC. No rearrangement occurred during the synthesis, since the deuterium content was more than 96 % in the 1-position as judged by the NMR-spectrum.

2-Methyl-1-deuterioindene (2a). Yield 82 %, $b.p._{10} = 74-75°C$, $n_D^{22} = 1.5643$. NMR: 6.7 − 7.3, 4 H, complex; 6.42, 1 H, t, $J = 2.5$ cps; 3.07, 1 H, complex; 2.08, 3 H, d, $J = 2.5$ cps.

2-Ethyl-1-deuterioindene (2b). Yield 77 %, $b.p._{10} = 81-83°C$, $n_D^{22} = 1.5592$. NMR: 6.7 − 7.3, 4 H, complex; 6.39, 1 H, t, $J = 2.5$ cps; 3.05, 1 H, complex; 2.30, 2 H, q, $J = 7.5$ cps; 1.15, 3 H, t, $J = 7.5$ cps.

An interesting fact is that in the spectra of 2-alkyl-3-deuterioindenes, the signal from the X-protons (methylene) appeared at a shift which was 0.04 ppm higher than the corresponding signal from the 1-deuterium-substituted

analogues. This effect was also demonstrated in the rearrangement, since at equilibrium, the amount of *1a* and *2a* (or *1b* and *2b*) could be seen as separate entities. This isotope effect is not unusual,[26] and still larger shifts are found in ^{19}F spectroscopy. Bovey [27] reports that in the spectrum of a 1:16 v/v mixture of cyclohexane and cyclohexane-d_{11}, the signal from the remaining H appeared at a shift 0.057 ppm from the sharp resonance of cyclohexane. The same effect, although to a lower extent, can be observed when the spectra from 1-deuterio- and 3-deuterioindenes are examined. The origin of this isotope effect is thought to result from differences in zero point vibrational functions, which are associated with different isotopic masses. A more extensive theoretical treatise is given elsewhere.[28]

(−)-3-Methyl-2-carbethoxy-indanone-1 (4). (−)-3-Methylindanone-1 (*3*) {$[\alpha]_D^{25} = +1.8°$, ($c = 31.6$, benzene)}, 10 g (69 mmol), obtained by ring-closure of (+)-β-phenyl-butyric acid, {$[\alpha]_D^{25} = +57.6°$ ($c = 2.8$, benzene)}[5] in 75 ml of dry benzene, was dropped into 6.5 g (270 mmol) of NaH (BDH, puriss) and 75 g (630 mmol) of diethyl carbonate in 50 ml of dry benzene at +65°C for 2 h, and maintained at that temperature for another hour. The solution was chilled to +10°C, acetic acid was added to pH 6, the mixture chilled to ±0°C and the pH adjusted to 1 with hydrochloric acid. The organic phase was extracted twice with 5 % $NaHCO_3$ and with NaCl aq. sat., After evaporation of the benzene, the residue was distilled under vacuum, yield: 83 % b.p.$_{0,5}$ = 131 − 133°C, $n_D^{22} = 1.5435$, $[\alpha]_D^{25} = -105°C$ ($c = 2.0$, benzene). NMR: About 30 % of the product was present as the enol. Shifts: 10.52, s; 6.7 − 7.3, complex; 4.23, q; 3.14, d; 1.21, d; 1.23, complex. IR: C=O, 1720 cm^{-1} (ester), 1670 cm^{-1} (ketone)

(−)-2,3-Dimethyl-2-carbethoxyindanone (5). (−)-3-Methyl-2-carbethoxyindanone-1, 12 g (0.054 mol) in 100 ml of dry ether was treated dropwise with 13.4 g (0.054 mol) of thallous ethoxide at room temperature. After 5 min, the thallium salt of 3-methyl-2-carbethoxy-indanone-1 precipitated as a yellow powder, which was filtered and washed with a small amount of ether. Yield: quantitative, m.p. 156 − 157°C. The salt was boiled under reflux with 50 g (0.350 mol) of methyl iodide (Kebo, puriss.). After filtration of the thallium(III), iodide obtained, the residue was distilled under vacuum. Yield: 87 %, $n_D^{23} = 1.5282$, b.p.$_1$ = 135 − 136°C, $[\alpha]_D^{25} = -67°$ ($c = 2.6$, benzene). NMR: 6.7 − 7.3, 4 H, complex; 3.52, 1 H, q; 3.86, 2 H, complex; 1.31, 3 H, d; 1.20, 6 H, complex. IR: C=O 1745 cm^{-1} (ester), 1690 cm^{-1} (ketone).

(−)-2,3-Dimethylindanone-1 (6). (−)-2,3-Dimethyl-2-carbethoxyindanone (*5*) 8 g (0.034 mol) was stirred at room temperature with 100 ml of 5 % NaOH for 24 h. To the homogeneous solution 100 ml of 4 M H_2SO_4 was added and the mixture refluxed for 2 h. After ether extraction and evaporation of the solvent the residue was distilled. Yield: 87 %, b.p.$_{10}$ = 118 − 119°C, $n_D^{22} = 1.5428$. (Lit.[9] gives for the racemic compound b.p.$_{13}$ = 121°C, $n_D^{17} = 1.5548$), $[\alpha]_D^{25} = +3.0°$ ($c = 10.0$ benzene) NMR: 6.7 − 7.3, 4 H, complex; 2.31, 1 H, p; 1.72, 1 H, 1.10, 3 H, d; 0.95, 3 H, d. IR: C=O 1725 cm^{-1} (ketone).

(+)-1,2-Dimethylindene (7). (+)-2,3-Dimethylindanone, 4.0 g (0.025 mol) was dissolved in 50 ml of methanol and sodium borohydride, 1.0 g (0.027 mol) in 75 ml 0.1 M NaOH was added slowly to the solution maintained at 20 − 25°C by external cooling. After evaporation of the solvent, 100 ml of water was added, the mixture extracted with ether, the ether phases dried over anhydrous $MgSO_4$ and the solvent evaporated. Without any further purification, the indanol was dissolved in 150 ml of dry benzene and dehydrated by the same route as the 2-alkyl-3-deuterioindenes. The crude indene was flash-distilled and purified *via* preparative GLC. Yield: 2.5 g, 69 %, b.p.$_{11}$ = 82 − 86°C, $n_D^{22} = 1.5655$ (lit. gives for the racemic compound b.p.$_{13}$ = 86 − 87°C, $n_D^{25} = 1.5642$). $[\alpha]_D^{25} = +115°$ ($c = 2.0$, benzene). NMR: 6.6 − 7.3, 4 H, complex; 6.25, 1 H, p; 3.01, 1 H, p; 1.96, 3 H, q; 1.18, 3 H, d.

Kinetics

The kinetic experiments were performed in a thermostat having an accuracy of ±0.05°C. Diazabicyclooctane (KEBO), m.p. 156 − 158°C, was recrystallized twice from hexane (spectrograde). Triethylamine (Merck) was distilled over sodium. Pyridine (Mallinckrodt) was dried over calcium hydride and distilled and degassed with oxygen-free N_2. Stock solutions of the aliphatic amines were prepared and added to the substrate, such that the indene concentration was 2 M. At specific intervals, samples were withdrawn from the reaction bottle, quenched with dilute hydrochloric acid and the indenes extracted with carbon tetrachloride. The samples were diluted with carbon tetrachloride to give concentrations approximately 2 M in indene. The NMR-spectra were recorded and the average of at least three integrals of A- and X-protons was recorded. The internal standard was the CH_3-group of 2-methylindene or the $-CH_2-$group in 2-ethylindene. About 15 observations during 3 halflives were recorded. The rate constants were calculated by means of a least-squares plot. The isomerizations were studied at 20.0, 30.0, and 40.0°C.

In a test run the isomerization of 1-methylindene and 3-deuterioindene, respectively, with DABCO as a catalyst was studied. These runs were undertaken at 30.0°C. The internal standard was *t*-BuOH, 0.1 M, which has a very small retarding effect on the reaction rate.

The polarimetric measurements were performed with 0.5 M optically active 1,2-dimethyl-

indene and inactive 1,2-dimethylindene added so that the total concentration was 2 M. The mutarotation was followed by means of an automatic data collecting system, equipped with a paper tape punch. About 200 observations were made over at least 3 half-life times. The rate constants were calculated *via* a least-squares plot performed by the computer programme PROGAEXP.[14]

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Specific Heat Measurements on Lysozyme, Chymotrypsinogen, and Ovalbumin in Aqueous Solution and in Solid State

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Specific heats on solid lysozyme, chymotrypsinogen, and ovalbumin with different amount of water have been measured as a function of the water content. The $\bar{c}p°$ values obtained from extrapolation to zero water content are 1.192 ± 0.005 (lysozyme), 1.223 ± 0.004 (chymotrypsinogen) and 1.231 ± 0.005 (ovalbumin) in J K^{-1} g^{-1}. The specific heat for the water associated with the proteins is 30 % larger than the value for pure water.

Specific heat measurements were also carried out on aqueous protein solutions at different concentrations in order to obtain partial specific heat values at infinite dilution. The values found are 1.494 ± 0.007, 1.529 ± 0.015, and 1.534 ± 0.014 in J K^{-1} g^{-1} for lysozyme, chymotrypsinogen, and ovalbumin, respectively.

Specific heat values were calculated for solid lysozyme, chymotrypsinogen and insulin from the sum of the heat capacity values of the amino acids, corrected for the heat capacity contributions for the formation of the peptide bonds. The calculated values are within a few percent of the experimentally obtained values.

A similar analysis of the results from specific heat measurements on aqueous protein solutions indicates that a substantial part of the non-polar amino acid side groups are solvated by water.

The biochemical properties of protein are to a large extent governed by the mutual interaction between protein and water. One of the most important factors which stabilizes native proteins seems to be the interactions between water and the non-polar groups of the protein. Kauzmann [1] proposed that the stabilization effect is caused by the entropy gain when non-polar groups are taken out of contact with water to the inside of the protein. It was assumed that the hydrophobic side chains are buried in the interior of the protein. Klotz,[2] on the other hand, pointed out that the favourable enthalpy contribution from the solvation of non-polar groups could be an important stabilization factor. X-Ray investigations on several proteins have shown that nearly all ionic side-chains are exposed to solvent, and a substantial number of hydrophobic groups are accessible to solvent.[3]

There is an increasing interest for a quantitative characterization of these interactions and thermodynamic methods seem to be among those which are best suited for the purpose. From experiments with low molecular weight compounds it has been shown that hydrophobic groups in contact with water are associated with very large apparent heat capacity values. It is believed that the "excess" heat capacity is due to enthalpy of melting of water structures formed under the influence of the hydrophobic groups. Recent model compound experiments [4,5] indicate that the heat capacity values can be accounted for by simple additivity rules.

The analysis of the heat capacity of a protein in terms of the contributions from amino acid side groups, the effect of solvation and the polypeptide back bone can be shown to give information about the distribution of the non-polar groups between the interior and surface of a protein. Unfortunately, there are only a few globular proteins for which absolute specific heats are measured in aqueous solution or in the solid state.[6-9] Furthermore, most of the available heat capacity values are not precise enough to attempt to predict structural features.

The long range goal of this work is to make

correlations between heat capacities and structural features for proteins and protein constituents. Here we present a first exploratory study on the specific heats for a few globular proteins. The measurements have been made by a novel double drop heat capacity calorimeter.[10]

Specific heat measurements were made on ovalbumin, chymotrypsinogen, and lysozyme in dilute aqueous solutions at different concentrations. From these measurements partial specific heats at infinite dilution, $\bar{c}p^{\circ}_2$, were derived for the proteins. Measurements were also made on the proteins in their solid states at different water contents.

The observed cp° values of the solid proteins are compared with calculated cp values and the $\bar{c}p^{\circ}_2$ values obtained in aqueous solution are used to estimate the per cent of solvated hydrophobic residues which are solvated on proteins in aqueous solution.

EXPERIMENTAL PROCEDURE AND CALCULATIONS

Materials. Lysozyme (LYSF OCC, salt free) was obtained from Worthington Biochemical Corp. Ovalbumin was obtained from Miles-Servac (PTY) Ltd. Chymotrypsinogen (3× crystallized, salt free) was obtained from Nutritional Biochemical Corp. All proteins were used without further purification. Glass distilled water was used with dissolved gases removed by boiling.

Calorimetry. A newly developed double drop calorimetric method was used.[10] The calorimeter consists of two main parts: (1) a "furnace" for temperature equilibration of the sample ampoule and reference ampoule and (2) a receiver twin calorimeter of the heat conduction type. The calorimetric unit was kept at about 29.7°C and the furnace at 20.3°C. The ampoules were equilibrated in the furnace for about 30 min, after which they are dropped into the receiver calorimetric unit and the difference between the heat quantities transferred by the two ampoules measured. From the difference obtained with the sample ampoule filled and empty, and the measured temperature difference ($\pm 2 \times 10^{-4}$ °C) between the furnace and calorimeter, the mean specific heat (cp) for the temperature interval 20.3 – 29.7°C can be calculated.

Calibration of the calorimeter was performed with water. Results from test experiments indicate systematic errors to be less than 0.1 %.[10]

Heat capacity studies on proteins performed with scanning calorimeters [9,11] show that the partial specific heat values vary almost linearly with the temperature in the interval here concerned. Therefore the specific heat values given in the present study may be assumed to refer to the mean temperature 25°C. Variations in the mean temperature were small, ±0.01°C, and any corrections to 25°C were insignificant. The only correction applied on the experimental specific heat values was the effect caused by the displaced air,[12] which was of the same order of magnitude as the precision of the measurements.

Uncertainties given for the cp values are twice the standard deviation of the mean $s = 2\langle\delta^2\rangle^{\frac{1}{2}}/\{n(n-1)\}^{\frac{1}{2}}$. The partial cp values were calculated by using the linear regression analytical method.[13]

Procedure. All aqueous protein solutions were prepared from dry proteins, which had been stored in vacuum ($< 10^{-3}$ mmHg) over P_2O_5, at room temperature, for at least 4 days. The concentrations were determined on the weight basis. During the preparation of the solutions care was taken to avoid unwanted hydration of the dry proteins. Even short exposure of the proteins to the air gives errors in the partial specific heat of the proteins of the order of a few percent. All manipulations with the dry proteins were therefore performed under dry nitrogen atmosphere.

About 0.6 g of the protein solution was filled into the ampoule immediately after preparation. The sample ampoule and the reference ampoule, which in the present study was always empty, were placed in the calorimeter. The order of selection of the concentrations studied was random. 4 – 8 consecutive determinations were performed at each concentration.

Various desired water contents for the hydration of the solid proteins were obtained by placing the dry proteins in evacuated vacuum desiccators over aqueous sulfuric acid of the appropriate concentration. The proteins were allowed to equilibrate for at least 24 h. The hydrated samples (0.2 – 0.05 g) were then filled into an ampoule, which was sealed by a plunger system. The plunger could be pressed against the proteins to achieve a minimum of vapor space without compression of air. After 4 – 8 consecutive cp measurements on each sample, the water contents were determined by weight difference before and after drying in an oven at atmospheric pressure and 105°C for 24 h.

Calculations. Specific heats were measured for the aqueous protein solutions at several concentrations and for the solid proteins at several water contents. From the measured cp values as a function of the concentration the contributions to the total cp from water and protein were calculated. The relationship between the partial specific heats is given by eqn. 1 (*cf.* Ref. 7)

$$(1 + W_2)cp = \bar{c}p_1 + W_2\bar{c}p_2 \qquad (1)$$

where W_2 is the quotient between the masses of the solute and the solvent ($W_2 = m_2/m_1$). Index 1 stands for the solvent and index 2 for the solute. In the solid samples the solvent is the protein and the solute is the water. cp is the measured specific heat, and $\bar{c}p_1$ and $\bar{c}p_2$ are the partial specific heats for the solvent and solute, respectively. Eqn. 1 shows that a plot of $(1+W_2)$cp against W_2 gives the $\bar{c}p_1$ value as the ordinate intercept and the $\bar{c}p_2$ value as the slope.

RESULTS

Measurements on lysozyme in aqueous solution were made at 14 different concentrations from 1.11 to 27.12 weight % lysozyme. The results are summarized in Table 1. The pH in this concentration range varied between 4.56 and 4.58. In Fig. 1a the results are plotted as described above. The experimental points were best fitted using two straight lines with a break between 9.5 and 10.1 weight %. According to eqn. 1 the partial specific heats for lysozyme and water are constant on both sides of the 10 % region. At that concentration there is a 2.1 % increase of the $\bar{c}p_2$ value and an increase of 0.05 % for the $\bar{c}p_1$ values.

The same effect occurs for aqueous ovalbumin solutions, which were measured in the range 0.58 – 12.44 weight % ovalbumin (Fig. 1b). The partial cp values are constant up to 4.3 – 4.8 %, after which an increase of 1.4 % and 0.04 % occurs for ovalbumin and water, respectively.

Table 1. Specific heat for aqueous lysozyme solutions at different concentrations and calculated apparent specific heat [a] for lysozyme.

Weight-% lysozyme	cp/J K^{-1} g^{-1}	ϕ_{cp_2}/J K^{-1} g^{-1}
1.11	4.1497 ± 0.0005	1.470 ± 0.045
2.45	4.1135 ± 0.0004	1.473 ± 0.016
3.63	4.0818 ± 0.0005	1.476 ± 0.013
4.79	4.0499 ± 0.0004	1.466 ± 0.009
6.11	4.0154 ± 0.0004	1.489 ± 0.006
6.60	4.0014 ± 0.0005	1.476 ± 0.007
9.47	3.9253 ± 0.0004	1.492 ± 0.004
10.11	3.9128 ± 0.0004	1.538 ± 0.004
13.08	3.8322 ± 0.0004	1.528 ± 0.003
17.18	3.7263 ± 0.0004	1.540 ± 0.002
21.90	3.6001 ± 0.0004	1.533 ± 0.002
27.12	3.4606 ± 0.0003	1.528 ± 0.001

[a] $\phi_{cp_2} = \dfrac{Cp - m_1\bar{c}p^\circ_1}{m_2}$; where Cp is the total heat capacity and $\bar{c}p^\circ_1$ is the specific heat for pure liquid water.

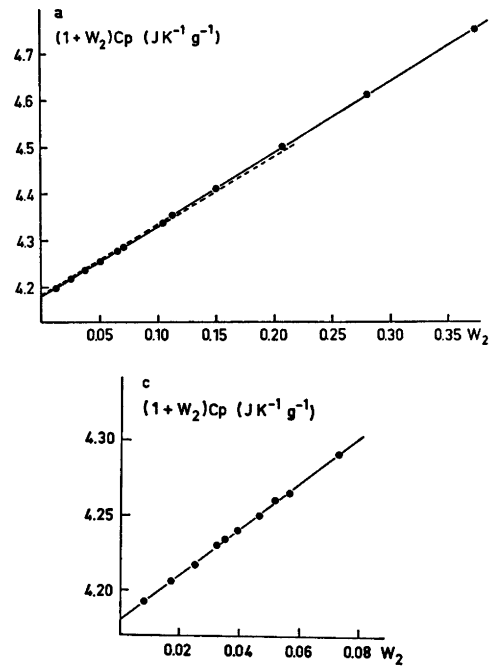

Fig. 1. Result of specific heat measurements on aqueous protein solutions as a function of the concentration: (a) lysozyme, (b) ovalbumin, (c) chymotrypsinogen.

In the measured concentration range there was a decrease of the pH from 6.3 to 6.1.

Fig. 1c shows the results from cp determinations on aqueous chymotrypsinogen in the interval 0.78 – 6.79 weight %, with a pH change from 4.06 to 4.10. In this region the $\bar{c}p$ values were constant, within the experimental errors.

Results from aqueous protein measurements extrapolated to zero concentration and the values found in the literature are summarized in Table 2.

Measurements on solid protein samples were carried out at six different water contents on each protein. Fig. 2 a, b, and c show the results for lysozyme, ovalbumin, and chymotrypsinogen, respectively. It is seen that the three curves show the same characteristic pattern: the experimental results from samples with less than 5 % and more than 15 % water fall on the same straight line, but the values between these limits fall below the extrapolated lines. The deviation from linearity is more pronounced for ovalbumin (*ca.* 3 %), than for lysozyme and chymotrypsinogen (*ca.* 2.0 – 1.5 %). Partial specific heats for the three proteins are the same on both sides of this interval.

The partial specific heat values extrapolated to zero water content from the solid protein experiments together with values found in the literature are summarized in Table 3.

Table 2. Partial specific heats of infinite aqueous dilution of lysozyme, chymotrypsinogen and ovalbumin at 25°C.

Protein	This study Water $\bar{c}p°_1$/J K^{-1} g^{-1}	Protein $\bar{c}p°_2$/J K^{-1} g^{-1}	Lit. Protein $\bar{c}p°_2$/J K^{-1} g^{-1}
Lysozyme	4.1792 ± 0.0004	1.494 ± 0.007	1.30 ± 0.04[a]
Chymotrypsinogen	4.1808 ± 0.0006	1.529 ± 0.015	1.60 ± 0.05[b]
Ovalbumin	4.1778 ± 0.0004	1.534 ± 0.014	1.91 ± 0.05[c]
			1.66 ± 0.09[d]

[a] Ref. 11. [b] Ref. 9. [c] Ref. 6. [d] Ref. 7. (mean temp. 11°C).

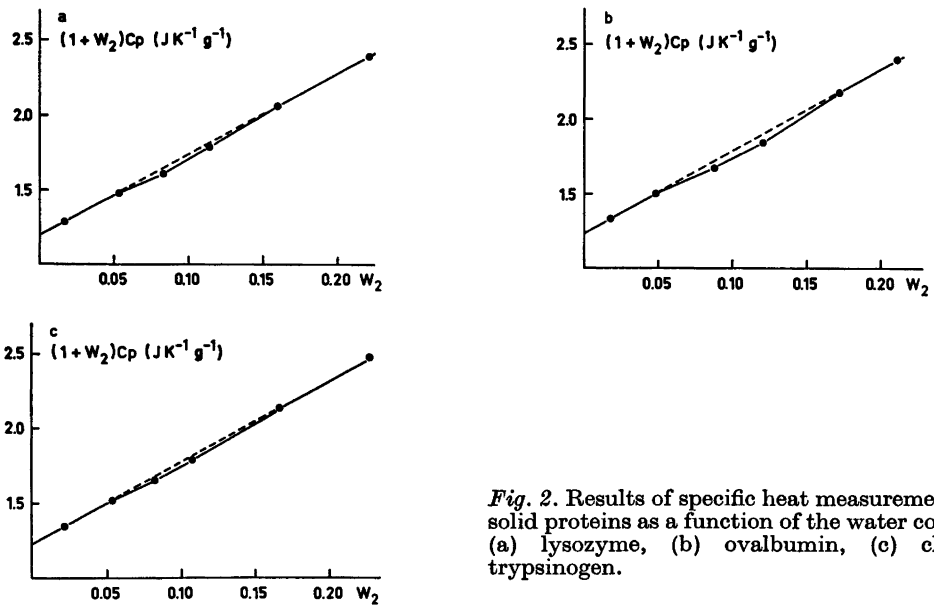

Fig. 2. Results of specific heat measurements on solid proteins as a function of the water content: (a) lysozyme, (b) ovalbumin, (c) chymotrypsinogen.

Table 3. Partial specific heats at zero water contents of solid lysozyme, chymotrypsinogen and ovalbumin at 25°C.

Protein	This study Protein $\bar{c}p°_1$/J K^{-1} g^{-1}	Water $\bar{c}p°_2$/J K^{-1} g^{-1}	Lit. Protein $\bar{c}p°_1$/J K^{-1} g^{-1}
Lysozyme	1.192 ± 0.005	5.42 ± 0.03	
Chymotrypsinogen	1.223 ± 0.004	5.46 ± 0.03	1.293 ± 0.003[a]
Ovalbumin	1.231 ± 0.005	5.53 ± 0.04	1.97 ± 0.08[b]
			1.18 ± 0.25[c]

[a] Ref. 8. [b] Ref. 6. [c] Ref. 7.

DISCUSSION

Hutchens *et al.*[8] suggested that it is possible to calculate the standard entropies for proteins from their amino acid composition and gave entropy calculations on solid proteins. The specific heats for the solid proteins (Table 3) and for the crystalline amino acids [14] on the average differ only slightly from each other, which seems to support the idea that heat capacities can also be calculated from the amino acid composition. With use of eqn. 2

$$n \text{ amino acids (c)} \rightarrow \text{protein (s)} + (n-i)H_2O \text{ (l)} \quad (2)$$

where n is the total number of amino acids and i is the number of polypeptide chains in the protein, it is possible to calculate the contributions from the peptide bonds to the total heat capacity of the protein. If ΔCp stands for the average molar heat capacity contribution from one peptide bond, then the ΔCp can be written by use of eqn. 2 as

$$\Delta C\text{p} = C\text{p}°(\text{protein}) + (n-i)C\text{p}°(H_2O) - \sum_i^n C\text{p}_i(\text{amino acids})/(n-i) \quad (3)$$

ΔCp values for three dipeptides, lysozyme, and chymotrypsinogen were calculated by use of eqn. 3. The heat capacity values for the amino acids and the dipeptides were taken from Ref. 14, where the data for L-Lys and L-His were estimated from the values given for L-Lys.HCl and L-His.HCl by subtracting 34.14 J K^{-1} mol^{-1}, which is the difference between D-Glu.HCl and L-Glu. The amino acid compositions for lysozyme and chymotrypsinogen given in Ref. 15 were used. The ΔCp values calculated in this study and the corresponding entropy data by Hutchens [8] are summarized in Table 4. In both cases a relative constant value for ΔCp or ΔS can be ascribed to the formation of the peptide bond. This points out that it may be possible to calculate the heat capacity for a solid protein from its amino acid composition with an accuracy of a few percent. Rearranging eqn. 3, using the ΔCp = 40.6 J K^{-1} mol^{-1} calculated from the two proteins and the Cp° = 75.3 J K^{-1} mol^{-1} for water leads to eqn. 4

Table 4. Calculated heat capacity and entropy changes [a] for forming peptide bond in the solid state at 25°C.

	ΔCp[b]	ΔS[b]
Lysozyme	40	
Chymotrypsinogen	43	38
Insulin		39
Gly-Gly	41	43
Ala-Gly	36	51
Leu-Gly	32	36

[a] Values taken from Hutchens *et al.*[8] [b] In J/K × (peptide bond).

$$c\text{p}°(\text{protein}) = \sum C\text{p}(\text{amino acid}) - (n-i)34.7 \text{ (J K}^{-1}\text{ g}^{-1})/\text{M} \quad (4)$$

The eqn. 4 was used to calculate the specific heat for insulin from its amino acid composition. The value obtained was 1.213 J K^{-1} g^{-1} compared with the measured value of 1.255.[8] This agreement is not surprising because if one calculates specific heat using eqn. 4 for all the various polypeptides made up of single amino acid units, the mean value falls within 1.2 ± 0.2 J K^{-1} g^{-1}. From this it can be assumed that the specific heat for most solid proteins is close t 1.2 J K^{-1} g^{-1}.

The specific heat for water bound on the solid proteins (Table 3) is about 30 % higher than for pure liquid water. This large value is surprising, because the specific heat of pure water is by itself "abnormally" high, caused by the gradual rupture of the hydrogen bonds with increasing temperature. The qualitative explanation could be that the water bound to the protein makes double hydrogen bonds at specific hydrophilic sites on the protein, such as was found in the collagen-water system by NMR-studies,[16] and that the rupture of the hydrogen bonds between water and protein falls off more rapidly than in pure liquid water with increasing temperature.

The specific heat decrease in the middle adsorption range (Figs. 2a, b, c) is probably due to specific heat change of the bound water, since from the argument above the specific conformation in the solid protein does not seem to affect the heat capacity values significantly. According to the adsorption mechanism proposed by Berlin *et al.*[17] the water is bound in double layers in the middle range due to the saturation of the specific water sites. This could lead to the decrease of the specific heat of bound water to a value closer to that of pure liquid water. The adsorption of more water was proposed to induce a conformational change which leads to an increase of available water sites, and the specific heat of water therefore increases to the same value as that found at low water content.

Partial specific heat of the protein in aqueous solution. In Fig. 1a, b, and c it can be seen that the partial specific heats are constant up to a rather high concentration. No obvious explanation can be offered for the abrupt changes in the c_p values for lysozyme and ovalbumin in the middle range. For the following discussion the $\bar{c}p^\circ$ values are obtained from the data below the breaking points (Table 3).

The derived $\bar{c}p_2^\circ$ values could be affected due to association reactions, which have been found to occur at higher pH from several sedimentation and chromatographic investigations on lysozyme [18-24] and chymotrypsinogen.[25-27] The effect on C_p caused by the association can be separated in two components: (1) direct changes in the specific heat caused by aggregation, and (2) change in the equilibrium of the association reaction induced by the temperature change. The effect 1 can be assumed to decrease the total specific heat due to the hydrophobic character found for the association reaction, whereas effect 2 always causes a positive c_p effect since an increase of temperature must cause a shift in equilibrium in the endothermic direction, requiring a positive absorption of heat. Since the observed $\bar{c}p_2$ is independent of concentration it is reasonable to assume that effects 1 and 2 are too small to be significant, and the observed $\bar{c}p_2^\circ$ values are equal to the true inifinitely dilute value.

The most striking result from the c_p measurements is the large Δc_p for the transferring of a solid protein to aqueous solution. The Δc_p°(solv) values for the proteins are 0.302 ± 0.008, 0.306 ± 0.015, and 0.303 ± 0.015 J K^{-1} g^{-1} for lysozyme, chymotrypsinogen, and ovalbumin, respectively, or an increase of 25 % in c_p for the process of solvating a protein. It is seen that nearly identical Δc_p°(solv) values were obtained for the three proteins.

Calculation of partial specific heats of proteins. In this section an attempt is made to analyse the obtained $\bar{C}p^\circ_2$ values by use of model compound data and simple additivity rules. In simple terms the different contributions to the $\bar{C}p^\circ_2$ values can be separated into several groups and are tabulated in Table 5.

A. Contributions from the back bone. From the observed independency of the heat capacities of the solid proteins on degree of hydration, it is assumed that the C_p contributions from the back bone do not change with the solvation of the protein. The C_p° for the back bone is estimated by using eqn. 4 on n glycine residues. The ΔC_p for solvating the terminal amino and carboxylic acid is taken to be zero.[4]

B. Contributions from solvated polar groups. All polar groups are assumed to be solvated.[3] The $\bar{C}p^\circ_2$ values for alcohol, amino, carboxylic, and amide groups given in Ref. 4 are used. The $\bar{C}p^\circ_2$ value for guanidine and imidazole groups are estimated by simple additivity rules from the values of the functional groups in Ref. 4. The phenolic group on tyrosine is estimated from values given in Ref. 28.

C. Contributions from non-polar groups. This group includes all the non-polar amino acid side chains, indole, mono- and disulfide groups, and

the non-polar parts of the polar side chains. The $Cp°_2$ values from these groups can be written as follows

$$\bar{C}p°_2 = Cp° + \alpha \Delta Cp(solv) \quad (5)$$

where $Cp°$ value is calculated from solid amino acid values [14] by subtracting the value for glycine, $\Delta Cp(solv)$ is the change when the non-polar groups are transferred to aqueous solution and α is the degree of solvation of the non-polar groups ($\alpha = 1$, when complete solvation). The ΔCp values were calculated for the different species through simple additivity rules from values given in Refs. 4, 14, 28. The ΔCp values for mono- and disulfide groups are not possible to estimate, but as the groups are probably not hydrated, the ΔCp is assumed to be zero. The ΔCp for the NH in indole is estimated to be zero.[4]

D. Contributions from ionisation. The $\bar{C}p°_2$ values for the polar groups all refer to the non ionic forms.[4] It is known that ionisation reactions occur with a decrease in the heat capacity.[29] Corrections for the ionisation of groups in the dissolved proteins were estimated from the expected pK values for the different side groups, the pH of the solution, and a mean ΔCp for ionisation reactions given in Ref. 30. It was assumed that lysozyme has 25 ionic groups at pH = 4.6 and chymotrypsinogen 27 at pH = 4.1.

The estimated contributions to the total $\bar{C}p°_2$ are summarized in Table 5.

The $\bar{C}p°_2$ can be written as the sum of all the different contributions

$$\bar{C}p°_2 = Cp(backbone) + \bar{C}p°_2(polar) + Cp° (non\text{-}polar) + \alpha \Delta Cp(solv) + \Delta Cp(ion) \quad (6)$$

The eqn. 6 with $\alpha = 1$, assuming all of the non-polar groups solvated, is used to calculate the theoretical heat capacities for completely solvated lysozyme and chymotrypsinogen (Table 6). From Table 6 it is seen that the calculated values exceed the experimentally obtained values with 70 – 80 %. This result suggests that the actual degree of solvation, α, is

Table 5. Contributions from the different groups to the total heat capacity of a protein in aqueous solution.

A. *Contributions from the back bone* $Cp°$ (back bone)				
$Cp°$(back bone) = n99.20 – (n – i)34.7 (J K^{-1} mol^{-1})				Eqn. 4
B. *Contributions from solvated polar groups* $\bar{C}p°_2$(polar)				
Alcohol	$\bar{C}p°_2$(polar) =	64	(J K^{-1} mol^{-1})	Ref. 4
» (phenol.)		–30		28[a]
Amino		58		4
Carboxylic		87		»
Amido		86		»
Guanidino		140		Estimated
Imidazole		110		»
C. *Contributions from non-polar groups,* $\bar{C}p°_2 = Cp°(non\text{-}polar) + \alpha \Delta Cp(solv)$				
Non cyclic aliphatic (CH_2 increment)	$Cp°(non\text{-}polar)$ =	23.5	(J K^{-1} mol^{-1})	Ref. 14[a]
Cyclic aliphatic (» »)		17.3		»
Aromatic (–CH=, »)		13.5		»
Benzene		80.8	(J K^{-1} mol^{-1})	Ref. 14[a]
Indole		115.9		»
Monosulphide		114.3[b]		»
Disulphide		17.4[b]		»
Non cyclic aliphatic (CH_2 increment)	$\Delta Cp(solv)$ =	65.7	(J K^{-1} mol^{-1})	Ref. 4,14[a]
Cyclic aliphatic (» »)		54.5		Estimated
Aromatic (–CH=, »)		43.2		Ref. 4,14,28[a]
D. *Contribution from ionisation* ΔCp(ion)				
	$\Delta Cp(ion)$ =	–167	(J K^{-1} mol^{-1})	Ref. 30

[a] Calculated from values given in the references. [b] Simple additivity rules do not seem to be applied for the mono and disulphide groups. However the errors for these values are compensated in the final calculation.

Table 6. Total contributions from the different species to the heat capacity (J K^{-1} mol^{-1}) of lysozyme and chymotrypsinogen.

	Lysozyme	Chymotrypsinogen
Observed, $\bar{C}p^\circ_2$	21 385	39 243
Calculated contributions from		
Back bone, Cp°(back bone)	8 354	15 835
Polar groups, Cp°_2(polar)	5 289	8 040
Non-polar groups, Cp°(non-polar)	6 927	12 867
Solvation of non-polar groups, ΔCp(solv)	18 669	35 613
Ionisation, ΔCp(ion)	−4 175	−4 509
Complete solvated, $\bar{C}p^\circ_2$(solv)($\alpha = 1$)	35 064	67 846
The degree of solvation, α.	0.27	0.20

much less than 1. The difference between the calculated ($\alpha = 1$) and the observed heat capacity values can be used to estimate the degree of hydration of the non-polar groups from

$$\alpha = 1 - \frac{\bar{C}p^\circ_2(\text{calc},\alpha=1) - \bar{C}p^\circ_2(\text{obs})}{\Delta Cp(\text{solv})} \qquad (7)$$

Values for α were calculated for lysozyme and chymotrypsinogen and the results are summarized in Table 6. For lysozyme the value for α was found to be 0.27. This value agrees with the proposed solvation picture given by Klotz.[3] The value of $\alpha = 0.26$ for lysozyme obtained from static accessibility calculation from X-ray data made by Lee and Richards [31] seems to be surprising since the solvation of polar groups is calculated to be only 0.49, whereas in above calculation a value of 1 has been assumed. The value of α obtained for chymotrypsinogen in this study is 0.20 which is smaller than found for lysozyme, which seems to be reasonable from the fact that larger molecules can easily bury greater fractions of the non-polar groups.

With the above method it is also possible to estimate the change of solvation upon denaturation. The ΔCp values found for the thermal denaturation of lysozyme [11] and chymotrypsinogen [9] indicate that the solvation increases to the values 0.35 and 0.34 for the respective proteins.

Finally, the values calculated above for the degree of solvation, α, can also be compared with the values derived from the very approximate α_i values given by Tanford.[32] Tanford gives values of the degrees of solvation of 0.3 for native and 0.4 for incomplete (thermal) denaturation of small protein molecules. The above results confirm these estimates.

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Intramolecular Cyclizations of Thioureas Derived from Sulphoraphene: a Case of Asymmetrically Induced Additions to Vinylic Sulphoxides*

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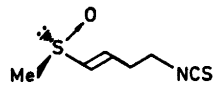
Sulphoraphene *1*, isolated from radish seeds, yields a thiourea *3a* on controlled reaction with ammonia. Upon more severe treatment, *3a* undergoes intramolecular cyclization to the epimeric $(R)_S$-4-methylsulphinylmethyl-tetrahydropyrimidine-2-thiones, *4a* and *4b*. The absolute configurations of the latter are established by stereospecific synthesis of one of the reduced isomers, *5b*, starting from (*R*)-β-methionine *6*. The degree of asymmetric induction in the cyclization of *3a* is determined and the mechanism discussed. The *N*-phenyl (*3b*) and *N*-methyl thiourea (*3c*) undergo similar cyclizations.



1

Me S(O)CH=CH $[CH_2]_2$ $NHCSNH_2$

2

Scheme 1.

Sulphoraphene ((*R*)-4-methylsulphinyl-3(*E*)-butenyl isothiocyanate) *1*,[1-3] derivable, by enzymic hydrolysis, from a glucosinolate (*cf.* Ref. 4) present in seeds of radish (*Raphanus sativus* L.),[1] possesses the notable property of being the first naturally derived compound that is optically active solely by virtue of an asymmetric sulphur atom. Upon prolonged treatment with alcoholic ammonia at 37°, sulphoraphene was converted by Schmid and Karrer,[1] its discoverers, into a levorotatory, crystalline compound, $C_6H_{12}N_2OS_2$, m.p. 219 – 220°, formulated as "sulphoraphene thiourea" *2*. We have investigated the structure and formation of this product and present the results.

When a chloroform solution of sulphoraphene, produced by enzymic hydrolysis of the glucosinolate fraction from radish seed extracts, was treated with ammonia-saturated methanol for 4 h at room temperature, the thiourea *3a*, m.p. 88 – 89°, was formed in an unexceptional reaction. Its composition and spectroscopical properties, including NMR-signals from its two vinylic protons, were in accord with the linear thiourea-structure *3a*.

Prolonged treatment of *3a*, or sulphoraphene itself, with methanolic ammonia, followed by repeated recrystallization of the product mixture, afforded an apparently homogeneous substance, possessing the properties previously attributed to "sulphoraphene-thiourea"[1] and, in fact, indistinguishable from the latter on comparison.* The composition, chemical properties, and spectroscopic characteristics all support the structure *4a*, or *4b*, for this product. With the multiple purpose of substantiating the proposed

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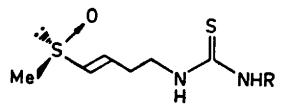
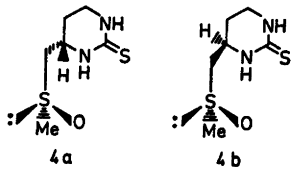
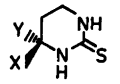


3a: R=H
3b: R=Ph
3c: R=Me



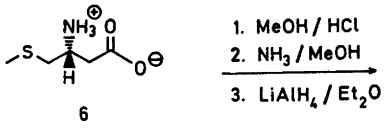
4a 4b



5a: X=H; Y=MeSCH$_2$
5b: X=MeSCH$_2$; Y=H

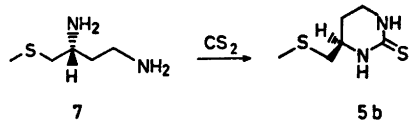
Scheme 2.

structure, establishing its absolute configuration, and assessing the degree of asymmetric induction in the cyclization reaction, the cyclic sulphoxide was reduced to the monochiral sulphide *5a*, the enantiomer of which was synthesized, in a stereospecific fashion, from (*R*)-*β*-methionine *6* through the sequence of reactions shown in Scheme 3.



6

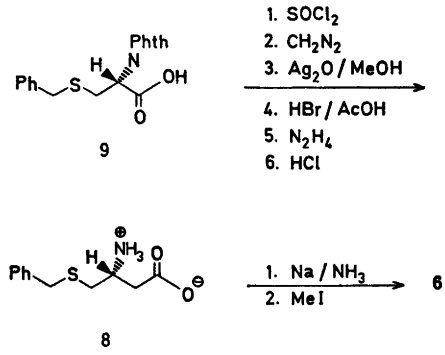
1. MeOH / HCl
2. NH_3 / MeOH
3. $LiAlH_4$ / Et_2O



7 5b

Scheme 3.

(*R*)-*β*-Methionine *6*, in its turn, was synthesized by Arndt-Eistert homologization [5,6] of (*R*)-*N*-phthaloyl-*S*-benzylcysteine [7] *9*, followed by dephthaloylation to (*R*)-*S*-benzyl-*β*-homocysteine [6] *8*, debenzylation, and, finally, S-methylation:



1. $SOCl_2$
2. CH_2N_2
3. Ag_2O / MeOH
4. HBr / AcOH
5. N_2H_4
6. HCl

9

1. Na / NH_3
2. MeI

6

8

Scheme 4.

Production of (*R*)-*N*-phthaloyl-*S*-benzylcysteine *9* by phthaloylation of (*R*)-*S*-benzylcysteine, in acceptable yield and with stereochemical integrity, is not straightforward, however. The proposed application of *o*-carbethoxythiobenzoic acid for this purpose [9] failed in our hands, whereas heating of *S*-benzylcysteine with phthalic anhydride at 110° for 30 min [7,10] led to low yields of slightly racemized product. Adoption of the Nefkens procedure,[11] however, involving *N*-carbethoxyphthalimide, permitted the preparation, in 90 % yield and high optical purity, of amorphous *9*, easily converted into its crystalline methyl ester. The chain-lengthening *9*→*8* proceeded through a sequence of unexceptional steps, essentially according to directions in the literature.[6,12] Further processing of *8* into (*R*)-*β*-methionine *6*,[8] and thence into its methyl ester hydrochloride,[13] went smoothly, as did the ammonolysis of the latter, followed by $LiAlH_4$-reduction to the diamine *7*, characterized as its dipicrate. An attempt to circumvent the described sequence by subjecting the hitherto unknown methyl ester hydrochloride of (*R*)-*S*-benzyl-*β*-homocysteine to treatment with Na/NH_3, followed by MeI, in order to arrive directly at (*R*)-*β*-methionine amide, proved of no avail.

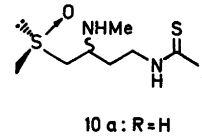
Known methods for converting 1,3-diamines, such as *7*, into tetrahydropyrimidine-2-thiones include thermolysis at 125 – 160° of dithiocarbamates, formed upon reaction with CS_2,[14,15] or,

alternatively, heating of the diamines at high temperature with thiourea [16] or thiocyanic acid.[17] In attempts to achieve ring-formation under milder conditions the reaction of aliphatic 1,3-diamines with thiocarbonyl chloride, a reaction apparently without literature precedent, was studied. Despite extensive variation of reaction conditions, yields never exceeded 27 % of the desired, cyclic products. On this background, it was unexpected to find the dithiocarbamate of (*R*)-1,3-diamino-4-methylthiobutane *7* undergoing quantitative and facile ring-closure in ethanol suspension at 25° to supposedly optically pure (*R*)-4-methylthiomethyl-tetrahydropyrimidine-2-thione *5b*, $[\alpha]_D$ −168°. The enantiomeric relationship of the latter to a repeatedly recrystallized, dextrorotatory, cyclic sulphide-thiourea of natural derivation was established by rotation measurements and mirror-image CD curves. On admixture, equal proportions of the enantiomers formed a lower-melting racemic compound.

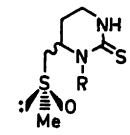
A preparation of the cyclic sulphoxide, with $[\alpha]_D$ −65°, produced from sulphoraphene according to literature directions,[1] afforded on reduction an analytically pure, non-fractionated, cyclic sulphide-thiourea with the rotation value $[\alpha]_D$ +145°, consequently representing an optical purity of 86 %. Assuming optical purity of naturally derived sulphoraphene *1*, and steric stability of the chiral S-atom during treatment with ammonia, the observed rotation signifies the composition 93 % R_SS_C- (*4a*) and 7 % R_SR_C-isomer (*4b*) for the thiourea with $[\alpha]_D$ −65°. The previously reported rotation, $[\alpha]_D$ −72°,[1] hence represents a 88:12 composition of the same two diastereoisomers.

The rotation, $[\alpha]_D$ +24°, of the cyclic sulphide thiourea, obtained upon quantitative reduction of a non-fractionated mixture of cyclic sulphoxides, $[\alpha]_D$ −119°C, reveals an asymmetric induction of 14 % in the cyclization of the linear sulphoxide thiourea *3a*, with the (R_SS_C)-isomer *4a* being the predominant one. The combined experimental data permit calculations of the rotation values: $[\alpha]_D$ −54° and $[\alpha]_D$ −205° for the pure (R_SS_C)- (*4a*) and (R_SR_C)-isomer, *4b*, respectively.

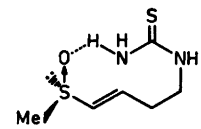
The observed cyclization is hardly trivial. Intramolecular nucleophilic addition of a thiourea grouping across the double bond should expectedly yield 2-amino-1,3-thiazine-derivatives rather than, as observed, pyrimidine-2-thiones. Addition-elimination reactions, involving ammonia, can be ruled out in view of the following observations: (i) identical cyclization takes place thermally, in the presence or absence of ethanol, but considerably faster in soda glass than in Pyrex glass: (ii) cyclization proceeds rapidly in aqueous base; (iii) methylamine, instead of ammonia, causes *competitive* formation of linear adducts, *10a*, and *non-methylated*, cyclic diastereomers *11a*. A controlling experiment revealed no aptitude for the linear sulphoxide thiourea to react with extraneous thiourea.



10 a : R = H
10 b : R = Me



11 a : R = H
11 b : R = Ph
11 c : R = Me



12

Scheme 5.

A base-catalyzed, intramolecular attack of the terminal nitrogen at the vinylic β-carbon atom, accompanied or followed by protonation of the resulting α-sulphinylcarbanion, hence seems to be best in accord with the experimental results. It deserves attention that no cyclization is observed when the linear thiourea is treated with ammonia in chloroform solution. Hydrogen bonding, possibly *via* solvent bridges, between the sulphoxide and the reacting NH_2-grouping, as suggested in *12*, may conceivably be instrumental in facilitating and governing the cyclization reaction.

Noticeable stereoselectivity in kinetically controlled, nucleophilic additions to vinylic sulphoxides is not without precedent. Thus, additions of piperidine,[18] or diethyl malonate and ethyl acetoacetate [19] to vinylic sulphoxides have been shown to proceed with a considerable degree of asymmetric induction. Additional re-

sults from this laboratory [20] confirm and extend these results.

Cyclizations, as described above, also occur with terminally substituted thioureas. Thus, the phenylthiourea *3b*, obtained, as described,[1] from sulphoraphene *1* and aniline, undergoes cyclization on prolonged exposure to aniline in ethanol at 37° to give a mixture of diastereomers *11b*, $[\alpha]_D$ +12°, from which a supposedly homogeneous isomer, $[\alpha]_D$ +22°, was obtained after repeated recrystallizations. A similarly composed mixture was rapidly produced on heating the thiourea *3b* in ethanol at 80° in a soft glass vessel; in Pyrex glass, the reaction proceeded at a greatly diminished rate. With methylamine, under carefully controlled conditions, sulphoraphene affords the expected *N*-methylthiourea *3c*. This, on treatment with aqueous base or ethanolic ammonia, undergoes cyclization to a mixture of diastereomers, *11c*, from which an apparently homogeneous isomer could be obtained after several recrystallizations. Exposed to methylamine for a prolonged period of time, the *N*-methylthiourea affords, as with other bases, a mixture of two cyclized epimers *11c*, but in addition, another product mixture, *10b*, arising from competitive addition of methylamine across the double bond of the linear thiourea.

The observed reactions provide interesting synthetic perspectives which are presently being pursued in this laboratory.

EXPERIMENTAL

Melting points are uncorrected and determined in capillary tubes in a heated bath. Paper chromatography (PC) was conducted by the descending technique on Schleicher and Schüll paper 2043b, with the upper layer of the system BuOH:EtOH:H_2O (4:1:4) as the mobile phase.

Rotations are measured on a Perkin-Elmer 141 polarimeter; CD curves on a Jouan CD-185 Dicrographe. UV-Spectra are recorded on a Perkin-Elmer 402 spectrophotometer, IR spectra (in KBr when not otherwise indicated) on a Perkin-Elmer 421 grating instrument, and mass spectra on the Perkin-Elmer 270 mass spectrometer at 70 eV. ^{1}H NMR-Spectra are determined at 60 MHz or 100 MHz on Varian instruments, with TMS, or, for D_2O-solutions, the Na-salt of DSS as internal standards. ^{13}C NMR-Spectra are measured as proton-noise decoupled spectra on a Bruker WH-90 instrument with Fourier Transform pulse technique, and are reported as δ-values in ppm downfield from TMS.

Microanalyses were performed by Mr. G. Cornali and his staff.

Sulphoraphene 1. Commercial radish seeds (66 g) were finely ground and defatted by extraction with two 2 l portions of hot petroleum ether. The glucoside fraction was removed by boiling the residue with two 2 l portions of 70 % MeOH. MeOH was removed from the filtrate by concentration *in vacuo*, and the aqueous solution was diluted with 1 l of H_2O, filtered through Hyflo-Supercel, and extracted once with ether (200 ml) and once with $CHCl_3$ (200 ml). The organic phases were discarded. Residual organic solvents were removed *in vacuo*, and the aqueous solution was buffered at pH 6.5 by adding a saturated Na_2HPO_4 -solution. Enzymic hydrolysis was accomplished by adding a crude cell-free myrosinase solution (18 ml), produced from yellow mustard, and a few mg's of ascorbic acid. After being kept at 40° for 3.5 h, the solution was extracted with five 150 ml portions of $CHCl_3$. The combined extracts (800 ml), containing sulphoraphene *1*, were dried over Na_2SO_4 and used for production of the various thioureas described in the sequel.

(R)-1-(4-Methylsulphinyl-3(E)-butenyl)-thiourea 3a. An aliquot of the $CHCl_3$-solution of sulphoraphene (300 ml) was treated with MeOH (12 ml), saturated at 0° with NH_3, for 4 h at 25°. The solution was concentrated in vacuum to an oily residue.

Paper chromatography revealed the contents in this solution of one major (R_F 0.56) and two minor (R_F 0.51 and 0.39) thioureas, the latter of which represents the cyclized product(s) described below. The thiourea with R_F 0.51, present only in trace amounts, is believed to represent the saturated counterpart of the thiourea derived from sulphoraphene, *i.e.* (*R*)-1-(4-methylsulphinylbutyl)-thiourea, on the basis of coinciding chromatographic behaviour in three solvent systems with an authentic specimen. Undoubtedly, this thiourea derives from sulphoraphane originating from trace amounts of the saturated glucosinolate, known from other sources,[21] in radish seeds.

The crude thiourea mixture was chromatographed on a silica gel column (60 g), with $CHCl_3$, containing a gradient of EtOH from 0 to 10 %, as the eluent. The thiourea-containing fractions, revealed by the production of a blue colour with Grote's reagent, were combined and evaporated to dryness (118 mg). The crystalline residue was recrystallized twice from MeOH:Et_2O to give an analytical specimen of *3a* as colourless crystals, homogeneous on chromatography, m.p. 88–89°; $[\alpha]_D^{22}$ −70° (*c* 0.7, H_2O) (Found: C 37.28; H 6.34; N 14.27. Calc. for $C_6H_{12}N_2OS_2$: C 37.47; H 6.29; N 14.57). Spectroscopic characteristics: UV: λ_{max} (EtOH) 243 nm (ε 15 000), 205 nm (13 740); IR: 3210 (vs), 3110 (vs), 1645 (vs,) 1540 (vs), 1450 (m), 1410 (m), 1350 (m), 1318 (s), 1295 (s), 1270 (s), 1195 (s), 1130 (s), 1060 (w), 1030 (vs), 963 (m), 950 (s), 818 (m), 765 (s), and 690 cm^{-1} (s). MS: *m/e*

192 (M^+). ^{1}H NMR ,in $(CD_3)_2SO$: δ 2.4 (2 H, m), 2.61 (3 H, s), 3.4 (2 H, m), 6.35 (1 H, dt, J 16 and 7 Hz), 6.75 (1 H, d, J 16 Hz), and 7.1–7.9 ppm (3 H, exchangeable).

In keeping with its structure as a vinylic thioether, *3a* decomposes readily in acid to give MeSH.

C-Epimers of 4-((R)-methylsulphinylmethyl)-tetrahydropyrimidine-2-thione, 4a and 4b. A solution of the linear thiourea *3a* (55 mg) in EtOH (5 ml), to which was added a saturated EtOH-solution of NH_3 (3 ml), was kept for 8 days at 40°, when PC revealed virtually complete transformation of *3a* (R_F 0.56) into a new type of product (R_F 0.39). Trace amounts of starting material and more polar by-products were removed by chromatography on a silica gel column (10 g), with EtOAc:MeOH (1:1) as the eluent. The chromatographically homogeneous epimer mixture (40 mg), $[\alpha]_D^{23}$ −119° (*c* 1.1, H_2O), was subjected to five recrystallizations from MeOH:Et_2O to give what is supposedly the almost homogeneous (R_SS_C)-epimer *4a*, m.p. 223–224°, $[\alpha]_D^{25}$ −61° ±4° (*c* 0.4, H_2O). (Found: C 37.63; H 6.34; N 14.31; S 33.02. Calc. for $C_6H_{12}N_2OS_2$: C 37.47; H 6.29; N 14.57; S 33.34). Spectroscopic characteristics, UV: λ_{max}(EtOH) 247 nm (ε 15 500), 208 nm (11 100); IR: 3300 (s), 3200 (vs), 1550 (vs), 1510 (vs), 1460 (m), 1430 (s), 1355 (s), 1335 (s), 1320 (s), 1260 (m), 1230 (m), 1195 (vs), 1180 (s), 1105 (w), 1065 (m), 1025 (m), 998 (vs), 963 (s), 938 (m), 920 (w), 892 (w), 872 (w), 832 (w), and 705 (br.) cm^{-1} MS: *m/e* 192 (M^+). ^{1}H NMR, in $(CD_3)_2SO$: δ 1.9 (2 H, m) (C-5), 2.61 (3 H, s), 2.95 (2 H, d, J 6.5 Hz) (−S(O)CH_2)−, 3.2 (2 H, m) (C-6), 3.75 (1 H, m) (C-4), and 7.8–8.1 ppm (2 H, exchangeable).

No methanethiol was produced on heating the epimer mixture with acid.

In contrast to the above described behaviour, a *chloroform* solution of the linear thiourea *3a*, saturated with NH_3, underwent no changes within 30 days at 25°, as estimated from PC.

Though unchanged in aqueous solution after 4 weeks, the thiourea *3a* undergoes quantitative cyclization in less than 30 min in 0.5 M NaOH at 25° to a mixture of the epimers, *11a*.

In ethanolic $MeNH_2$, *3a* reacts to a mixture of products within 1 h at 25°. Separation by preparative TLC on silica gel (eluent MeOH) affords a more lipophilic fraction (60 %), m.p. 195–203°, identified as the mixture *11a*, and a more polar, basic component (40 %), yielding a red colouration with ninhydrin. This very hygroscopic material possesses spectroscopical characteristics supporting its identity as a mixture of adducts, *10a*. UV: λ_{max}(EtOH) 245 nm ($\varepsilon \sim 10^4$); ^{1}H NMR (in CD_3OD): δ 1.8–2.0 (2 H, m) (−C−CH_2−C), 2.41 and 2.43 (3H, 2s) (CH_3−NH−C−, in the epimers, *10a*), 2.73 (3 H, s) [MeS(O)], 2.8–3.2 (2 × 2 H, m) [−S(O)-CH_2− and −CH_2−NH−], 3.5–3.7 (1 H, m) [−C*H*(NHMe)−], and 4.73 ppm (*ca.* 3 H, s) (−NH and −NH_2) (the cyclized product *11c*, containing the Me−N−CS-grouping, should exhibit CH_3−N-absorption at δ 3.0–3.5 ppm [22]). The mixture of adducts remained unchanged on standing at 40° in ethanolic methylamine for 8 days, excluding its role as an intermediate in the production of the cyclic products.

Heating *3a* to 90° for 86 h in a capillary, made of soft glass, brings about extensive cyclization to *11a*. In Pyrex glass, very little cyclization is observed. Heating in EtOH at 80°, in soft glass, quantitatively converts *3a* into *11a* in the course of 63 h.

(R)-S-Benzyl-N-phthaloylcysteine methyl ester. *(R)-S*-Benzylcysteine (22.6 g) and Na_2CO_3. $10H_2O$ (32.7 g) were dissolved in hot water (175 ml). After cooling to 25°, *N*-carbethoxyphthalimide [11] (26 g) was added. After 1 h, the solution was acidified with 6 N HCl, when a colourless syrup separated. Next day, $CHCl_3$ (400 ml) was added, and unreacted *S*-benzylcysteine (2 g) was removed by filtration. The filtrate was extracted with three 75 ml portions of water to remove most of the unreacted urethan. After drying and removal of $CHCl_3$, a colourless syrup remained, consisting of *(R)-S*-benzyl-*N*-phthaloylcysteine *9* (34.7 g, 90 % yield, on the basis of non-recovered starting material) with a maximum content of 10 % urethan according to ^{1}H NMR-analysis. This product, $[\alpha]_D^{25}$ −154° (*c* 1, MeOH) [reported:[7] $[\alpha]_D^{25}$ −167° (*c* 0.6, MeOH)], was used for the subsequent synthesis without further purification.

The above acid (66 mg), dissolved in Et_2O (3 ml), was treated with excess CH_2N_2. Next day, the solvent was removed and the crystalline methyl ester was recrystallized thrice from MeOH before analysis, m.p. 66°, $[\alpha]_D^{24}$ −162°. (*c* 0.8, C_6H_6). (Found: C 63.81; H 4.92; N 3.83. Calc. for $C_{19}H_{17}NO_4S$: C 64.21; H 4.82; N 3.94).

(R)-S-Benzyl-β-homocysteine 8. The above cysteine derivative *9* was converted into its acid chloride, $[\alpha]_D^{22}$ −140° (*c* 1.2, C_6H_6) [reported: $[\alpha]_D^{16}$ −136° [7]], and thence into the corresponding diazoketone, $[\alpha]_D^{23}$ −188° (*c* 0.3, C_6H_6) [reported for a benzene solvate: $[\alpha]_D^{16}$ −170° (*c* 0.4, C_6H_6) [7]], essentially as described.[6,7,12] Again, further processing of the latter into *(R)-S*-benzyl-*N*-phthalimido-homocysteine methyl ester, a syrup with $[\alpha]_D^{25}$ −59° (*c* 2, C_6H_6) [reported: m.p. 67°, $[\alpha]_D^{20}$ −80° (*c* 1.12, C_6H_6) [6]], the corresponding free acid, also a syrup, $[\alpha]_D^{22}$ −83° (*c* 2.3, C_6H_6) [reported: $[\alpha]_D^{14}$ −78° (*c* 1.8, C_6H_6) [6]], and *S*-benzyl-*β*-homocysteine *8*, m.p. 182–183° (dec.), $[\alpha]_D^{23}$ −58° (*c* 1.1, 1 N HCl) [reported: m.p. 173° (dec.),[6] $[\alpha]_D^{19}$ −57° (*c* 1.19, 1 N HCl),[6] m.p. 171–174° (dec.),[23] $[\alpha]_D^{23}$ −64° (*c* 1.16, 1 N HCl)[23]], took place with little deviation from the reported procedures.

(R)-S-Benzyl-β-homocysteine methyl ester hydrochloride. The amino acid above was converted into its methyl ester hydrochloride on treatment with anhydrous HCl in MeOH in the usual way. After two recrystallizations from MeOH/Et_2O an analytical sample was obtained,

m.p. 116–117°, $[\alpha]_D^{25}$ −37° (*c* 0.5, MeOH). (Found: C 52.00; H 6.64; N 11.68. Calc. for $C_{12}H_{18}NO_2SCl$: C 52.25; H 6.58; N 11.62).

(R)-β-Methionine amide hydrochloride. (*R*)-*S*-Benzyl-β-homocysteine, described above, was converted into (*R*)-β-methionine as reported,[8] in 79 % yield. Esterification with MeOH and anhydrous HCl yielded the hygroscopic (*R*)-β-methionine methyl ester hydrochloride, m.p. 86–87°, $[\alpha]_D^{25}$ −14.5° (*c* 0.8, H_2O) [reported: m.p. 88–90°, $[\alpha]_D$ −14.3° (*c* 5, H_2O) [13]]. On treatment of the ester hydrochloride (730 mg), dissolved in anhydrous MeOH (40 ml) saturated at 0° with dry NH_3, for 14 days at 5°, a quantitative yield of (*R*)-β-methionine amide hydrochloride was obtained. An analytical specimen was obtained by recrystallization from MeOH/Et_2O, m.p. 161–163°, $[\alpha]_D^{25}$ −2° ± 0.5° (*c* 1.1, MeOH), −17° (*c* 0.5, 2 N NH_3). (Found: C 32.52; H 7.05; N 14.48; S 17.56. Calc. for $C_5H_{13}N_2OSCl$: C 32.52; H 7.10; N 15.17; S 17.36).

(R)-1,3-Diamino-4-methylthio-butane 7. To the above amide hydrochloride (128 mg), suspended in anhydrous Et_2O (50 ml), was added $LiAlH_4$ (390 mg), and the mixture was refluxed for 26 h, when an additional amount of $LiAlH_4$ (100 mg) was added; the refluxing was continued for 45 h. After cooling, water-saturated ether, followed by a saturated solution of KNa-tartrate, were added to the reaction mixture. The combined ether extracts were dried over KOH. On evaporation, the diamine was obtained as a colourless syrup.

For characterization, the amine was converted into its crystalline dipicrate, recrystallized, before analysis, from MeOH/Et_2O, m.p. 185–186° (dec.), $[\alpha]_D^{25}$ +2.9° (*c* 0.6, MeOH), $[\alpha]_{546}^{25}$ +5.6° (*c* 0.6, MeOH). (Found: C 34.42; H 3.45; N 18.83; S 5.80. Calc. for $C_{17}H_{20}N_8O_{14}S$: C 34.46; H 3.40; N 18.91; S 5.41).

(R)-4-Methylthiomethyl-tetrahydropyrimidine-2-thione 5b. The above diamine, resulting from the reduction of β-methionine amide hydrochloride (118 mg), was dissolved in EtOH (5 ml). The solution was slowly added to a mixture of CS_2 (3 ml) and EtOH (5 ml). The suspension of the dithiocarbamate was stirred at 25° for 24 h when no more precipitate was present. Evaporation to dryness gave a crystalline residue, which, after treatment with charcoal in $CHCl_3$, was recrystallized twice from MeCN to give an analytical specimen, m.p. 178–179°, $[\alpha]_D^{25}$ −168° (*c* 0.5, $CHCl_3$). (Found: C 40.97; H 6.72; N 15.99; S 36.26. Calc. for $C_6H_{12}N_2S_2$: C 40.88; H 6.86; N 15.89; S 36.38). Spectroscopic characteristics: λ_{max}(EtOH) 249 nm (ε 14 800), λ_{max} (MeCN) 255 nm (ε 17 300), 222 nm (sh.); ^1H NMR in $CDCl_3$: δ 1.8–2.2 (2 H, m), 2.15 (3 H, s), 2.5–2.7 (2 H, m), 3.3–3.8 (2+1 H, m), and 6.9 (2 H, "s", exchangeable) ppm. ^{13}C NMR, in $CDCl_3$: δ 15.7 (CH_3-S-), 25.5 ($-C-CH_2-C$), 39.2 (or 39.7) ($-S-CH_2-$), 39.7 (or 39.2) ($-NH-CH_2$), 49.2 ($N-C(H)-C$), and 176.8 ($-N-CS-N$) ppm.

Reduction of cyclic sulphoxide(s) 11a. A cyclic sulphoxide fraction (21 mg), $[\alpha]_D^{23}$ −65° (*c* 1.0, H_2O), obtained by ammonia-induced cyclization of *3a* as described above, was dissolved in AcOH (7 ml). Solid $NaHCO_3$ (1 g) was added (to dispel dissolved O_2) and a 0.11 N $TiCl_3$-solution (5 ml) was added. After standing at 80° for 90 min, excess $TiCl_3$ was removed with 0.5 N $Fe^{III}NH_4$-sulphate (5 ml). Water was added to a volume of 30 ml and pH was adjusted to 7 with solid Na_2CO_3. The organic product was removed by extraction with $CHCl_3$ (3 × 30 ml). After drying, the combined extracts were evaporated to give a crystalline product (17 mg, 90 %), $[\alpha]_D^{27}$ +145° (*c* 0.7, $CHCl_3$), which, according to TLC, was homogeneous. After recrystallization from MeOH a product was obtained with m.p. 176–178°, $[\alpha]_D^{27}$ +142 (*c* 1.0, $CHCl_3$). On comparison, its MS-, ^1H NMR- and IR-spectrum (in $CDCl_3$) proved indistinguishable from those of the above described synthetic compound *5b*.

Upon two recrystallizations from MeCN, the reduced product afforded a crystalline fraction, m.p. 178–179°, $[\alpha]_D^{25}$ + 168° (*c* 0.6, $CHCl_3$), supposedly representing the homogeneous (*S*)-enantiomer *5a*.

On measuring the CD-curves in MeCN, the following $\Delta\varepsilon$-values were obtained:

λ nm	(*R*)-isomer *5b*	(*S*)-isomer *5a*
206	−8.3	+8.8
228	+1.1	−1.7
249	−1.2	+0.8
273	+0.33	−0.41

On mixing equal amounts of the enantiomers in $CHCl_3$ and recrystallizing the residue from MeCN, the racemic compound was obtained, m.p. 142–143°, $[\alpha]_D \sim 0°$.

Cyclization of the linear N-phenylthiourea 3b. When the phenylthiourea *3b* (34 mg), obtained from sulphoraphene and aniline,[1] was left in ethanol (5 ml), saturated with NH_3, for 24 h, it was converted into a chromatographically homogeneous, new product, $[\alpha]_D^{25}$ +12° (*c* 0.4, $CHCl_3$), which could be recrystallized from EtOH/Et_2O to constant rotation, m.p. 208–209°, $[\alpha]_D^{25}$ +22° (*c* 0.5, $CHCl_3$). (Found: C 53.66; H 6.24; N 10.19; S 23.74. Calc. for $C_{13}H_{16}N_2OS_2$: C 53.78; H 6.02; N 10.45; S 23.93). Spectroscopic data: λ_{max}(EtOH) 253 nm (ε 7 000), 238 nm (sh); ^1H NMR, in $CDCl_3$: δ 2.1–2.7 (2 H, m), 2.54 (3 H, s), 3.00 (2 H, "d"), 3.3–3.7 (2 H, m), 4.2–4.6 (1 H, m), 7.3–7.5 (5 H, m), and 7.9 (1 H, "s", exchangeable) ppm; MS: 268 (M^+).

The stability to alkali, blue colour with Grote's reagent, and absence of IR-absorption in the 1600–1650 cm^{-1} range,[24] all are in agreement with the structure *11b* for the cyclization product rather than the isomeric 1,3-thiazine isomer.

Heating an EtOH-solution of *3b* in a soda-glass tube at 40° brings about a slow conversion

into cyclic products; after 10 days, non-cyclized thiourea remains. At 80°, however, the cyclization reaction is complete within less than 24 h, whereas, in borosilicate glass, the greater part of the linear thiourea *3b* is unchanged after 5 days under the same conditions.

Cyclization of the linear N-*methylthiourea 3c.* To a $CHCl_3$-solution (45 ml) of sulphoraphene, arising from 32 g of radish seeds, 12 drops of a saturated solution of $MeNH_2$ in EtOH were gradually added, in the course of 3 h. The residue from evaporation of the mixture was chromatographed on a silica gel column, using first EtOAc:MeOH (9:1), later MeOH as the eluent. The Grote-positive fractions were evaporated to dryness to give a colourless syrup, homogeneous on PC (R_F 0.68) and giving the ^1H NMR-spectrum expected for the linear *N*-methylthiourea *3c*: (in $CDCl_3$): δ 2.4 – 2.8 (2 H, m), 2.70 (3 H, s), 3.02 (3 H, d), 3.74 (2 H, "q"), 6.4 – 6.6 (2 × 1 H, m) and 7.1 (2 H, "s", exchangeable) ppm.

The thiourea (57 mg) was kept at 20° for 48 h in EtOH, saturated with NH_3. The solid residue proved homogeneous on PC (R_F 0.48), After three recrystallizations from EtOH, a colourless, crystalline product (16 mg) m.p. 232 – 238°, $[\alpha]_D \sim -87°$ (*c* 0.2, MeCN), was obtained, giving a typical blue Grote-reaction and exhibiting spectroscopic characteristics in accord with the cyclic structure *11c*. UV: λ_{max}(EtOH) 248 nm (ε 10 000); MS: *m/e* 206 (M^+); IR: no absorption within the range 1610 – 1630 cm^{-1}; ^1H NMR: (in CD_3CN + 25 % D_2O): δ 1.9 – 2.3 (2 H, m) 2.71 (3 H, s) 3.0 – 3.5 (2 × 2 H, m), 3.39 (3 H, s), and 3.9 – 4.3 (1 H, m) ppm.

A similar product was obtained when the linear thiourea *3c* was kept at 25° in 0.5 N NaOH for 1 h.

When treated with ethanolic $MeNH_2$ at 25° for 2 h, the methylthiourea *3c* (23 mg) afforded two Grote-positive products, which were separated on a silica gel column, with MeOH as an eluent. The fastest moving component (11 mg) proved identical with the cyclic product(s) *11c* discussed above. The slower-moving product (4 mg) possessing an R_F-value of 0.39 in PC, was identified as the adduct(s) *10b*, mainly on the basis of its UV-spectrum, λ_{max}(EtOH) 243 nm, typical for a non-cyclic thiourea, its MS (*m/e* 223, M^+), and IR-spectrum. On prolonged treatment with $MeNH_2$ in EtOH, the adduct showed no signs of undergoing reaction as estimated from chromatographic analysis.

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Conformations and ^{1}H NMR Spectra of 5-Alkoxy-, 5-Hydroxy-, and 5-Amino-Δ^2-1,2,3-triazolines

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^{1}H NMR spectroscopy (and in a single case X-ray results) shows that 5-alkoxy- and 5-hydroxy-Δ^2-1,2,3-triazolines prefer an envelope conformation with the hetero substituent at C-5 pseudo-axial at the flap and the N-1 substituent pseudoequatorial, probably due to the anomeric effect.

1-(*p*-Nitrophenyl)-5-amino-Δ^2-1,2,3-triazolines with $R^5 = H$ prefer the same conformation, but for $R^5 \neq H$ there seems to be more than one important conformation. In aminotriazolines steric effects, rather than the anomeric effect, seem to be dominating.

5-Amino-Δ^2-1,2,3-triazolines unsubstituted in the amino group have been prepared for the first time.

During ^{1}H NMR investigations on 5-hydroxy- and 5-amino-Δ^2-1,2,3-triazolines we observed that the chemical shifts of protons and methyl groups at C-4 depend in a characteristic manner on the orientation relative to the hetero substituent (X) at C-5. This was extensively used by us for assignments of relative configurations at C-4 and C-5 in both 5-hydroxy- and 5-amino-Δ^2-1,2,3-triazolines,[1-3] and subsequently by other authors [4] in some 5-dialkylamino-Δ^2-1,2,3-triazolines.

For all hydroxytriazolines and most aminotriazolines one observes that a *cis* * oriented proton (or methyl group) at C-4 resonates at lower field than that in *trans*. This may be due to a non-planar conformation of the triazoline ring. Thus with Δ^1-pyrazolines ring-puckering has been assumed to explain chemical shift differences for protons or methyl groups adjacent to the N=N double bond.[5-7] These differences have been proposed to stem from the different positions of the protons in the strongly anisotropic field due to the N=N bond.[8] The anisotropy of single bonds (C−C, C−H, and C−N) cannot be ignored, however, and we prefer to consider only the sum of these contributions, the total anisotropy of the ring. Quantitative calculations on this [9] (*cf.* Refs. 10−12) seems unjustified in view of the difficulties encountered even in simple hydrocarbons. The total effect seems to be consistent; a pseudo-axial proton or methyl group resonates at higher field than a pseudo-equatorial (henceforth abbreviated to axial and equatorial). By analogy the chemical shift differences of C-4 groups in Δ^2-1,2,3-triazolines might be due to folding of the ring system so as to shield the axial relative to the equatorial group.

A near planar ring probably represents an energy maximum due to eclipsing of the C-4 and C-5 substituents; however, the eclipsing energy may be smaller than in straight-chain hydrocarbons, since the small ring angles at C-4 and C-5 must result in larger distances between the interfering substituents. The preference of nitrogen (N-1) for being in the sp^3 state, rather than in the sp^2 state,[13] would further destabilize the planar structure. The planar conformation of cyclopentene represents an energy barrier of 0.6 kcal/mol [14] between the two equivalent folded conformations, but the barrier might well be higher in the triazolines.

Four non-planar ring conformations are possible (Fig. 1), and at room temperature they might equilibrate so fast as to give time-

* Used without specification *cis* and *trans* refer to position relative to X.

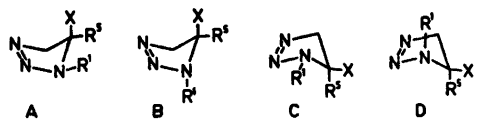

Fig. 1.

averaged NMR spectra. The factors that determine the preferred conformation may be divided into electronic effects, steric effects, and hydrogen bonding. The electronic effects include stabilization by conjugation in the triazene part of the ring. This does not necessarily involve strong preference for a planar arrangement of the ring atoms and R^1; but is should favor A and C over B and D, since in the lone-pair orbital of N-1 the *p* orbital component that is in a position to overlap with the N-2 *p* orbital is largest when the lone-pair is axial.[13]

The electronegativity of R^1 determines the electron density in the N-1 lone-pair orbital, which in turn must influence the degree of conjugation within the triazene part (*cf.* Ref. 15) and possibly also the interaction with X. This latter interaction is reminiscent of the anomeric effect (rabbit ear effect, dipolar effect [16–19]) and would act so as to favor conformation A. Other authors [20] have also suggested the operation of an anomeric effect between a hydroxy group and a nitrogen atom of a five-membered heterocylic ring. n-π Repulsive forces [21] between X and the N=N π-system would tend to place X equatorially.

Steric effects must play an important role. The interactions of the C-5 substituents with the N=N π-system are probably smaller than with the C-4 substituents, and the axial position of the larger C-5 group may thus be favored.

Interaction of R^1 with the C-4 substituents would be expected to favor an equatorial position of R^1, whereas its interaction with the C-5 substituents would favor an axial position, particularly if both C-5 groups are very bulky.

Hydrogen bonding between X and the N=N π-system [22] would favor an axial position of X.

The data used to solve the conformational problem include vicinal coupling constants, chemical shifts, and one X-ray structure. Most NMR data have been gathered from the literature; only the section on aminotriazolines contains mostly new NMR data.

5-Alkoxy-Δ^2-1,2,3-triazolines. The conformation of the triazoline ring in 5-alkoxytriazolines may be inferred from vicinal coupling constants. Table 1 reveals that the *cis* and *trans* coupling constants are very constant, being 7.0–7.5 Hz and 2.0–2.8 Hz, respectively. The small value of the *cis* coupling constant is not compatible with a planar triazoline ring, since J^0 would then be expected to be 13 Hz or larger, based on the *cis* coupling constant in 1-phenyl-4-carbomethoxy-Δ^2-1,2,3-triazoline (J_{cis} = 13.03 Hz, J_{trans} = 9.87 Hz [25]). Further, in a non-planar structure (Fig. 1) the alkoxy group cannot take the equatorial position (as in C or D), since this should give rise to a *trans* coupling constant much larger than 3 Hz, the dihedral angle being considerably larger than 120°. The alkoxy group must therefore occupy the axial position, and one or both of such conformations (A or B) predominate.

Three alkoxytriazolines deserve special mention. With Ic (Table 1) there is no *trans* coupling on which to base assumptions as to the

Table 1. ^{1}H NMR data of 1-(*p*-nitrophenyl)-5-alkoxy-Δ^2-1,2,3-triazolines ($CDCl_3$).

	R^4_{cis}	R^4_{trans}	R^5	X	R^4_{cis}	R^4_{trans}	J_{cis} [a]	J_{trans} [a]	Ref.
Ia	H	H	H	OEt	4.61	4.29	7.2	2.8	
Ib	H	H	H	OBu	4.65	4.25	7.0	2.7	23
Ic	Me	H	H	OPr	1.67	4.27	7.5		24
Id	H	Me	H	OPr	4.65	1.36		2.0	24
Ie	H	H	Me	OEt	4.72	4.19			23

[a] J_{cis} is the coupling constant between R^4_{trans}=H and R^5=H, J_{trans} that between R^4_{cis}=H and R^5=H.

conformation. The degree of ring folding seems nevertheless to be approximately the same in Ic as in Ia or Ib, as judged from the nearly identical *cis* vicinal coupling constants.

In Id conformation C would be expected on steric grounds, since here both R^1 and R^4_{trans}=Me are equatorial. However, even here X and consequently also R^4_{trans} must be preferentially axial, the *trans* coupling constant being only 2.0 Hz. Hence the axial position of R^4_{trans}=Me does not seem to be particularly unfavorable or, alternatively, the tendency of X to be axial must be very strong. The axial position of R^4_{trans} is best compatible with an equatorial position of R^1, *i.e.* Id presumably adopts conformation A.

In the triazoline [23] prepared from *p*-nitrophenyl azide and 3,4-dihydro-2H-pyran we have measured the coupling constants of R^4_{trans}=H with the two adjacent methylene protons of the tetrahydropyran ring to be 5.4 and 2.8 Hz. This indicates a *gauche* relationship to both methylene protons, which in turn implies that the oxygen atom at C-5 is axially oriented, if the tetrahydropyran ring is to adopt the usual chair conformation. The *cis* vicinal coupling constant between R^4_{trans}=H and R^5=H is 7.1 Hz, *i.e.* of the same magnitude as for the examples in Table 1.

Chemical shifts of the C-4 protons in the 1-*p*-nitrophenyltriazolines clearly distinguish a *cis* (4.61 – 4.72 ppm) from a *trans* proton (4.19 – 4.29 ppm).

In all discussed examples conformation A seems to predominate at equilibrium. Since the chemical shift between the C-4 protons is not diminished by the introduction of a methyl group at C-5 (Ie), conformation A seems to be favored by an anomeric effect rather than by steric effects involving the C-5 substituents. Unfortunately, two experiments designed to verify this were inconclusive. Thus the conformation (or predominating conformation) of Ia seemingly did not change on going from $CDCl_3$ to the much more polar solvent acetonitrile, the coupling constants remaining largely unaltered (7.9 and 2.9 Hz). Also, reduction of the nitro group in Ia to an amino group did not lead to a change in coupling constants or chemical shift difference between the C-4 protons (NMR data for the reduced compound: 4.42 and 4.10 ppm; 7.5 and 2.8 Hz). Both experiments suggest that the degree of folding of the triazoline ring remains the same.

5-Hydroxy-Δ^2-1,2,3-triazolines. An X-ray crystallographic investigation [26] on 1,4-dimethyl-5-ethyl-5-hydroxy-Δ^2-1,2,3-triazoline confirmed that there was a significant degree of conjugation within the triazene chain, as evidenced by a substantial shortening of the N-1···N-2 bond (1.357 Å as opposed to 1.44 Å for a normal N – N single bond [27]). In spite of this the triazoline ring was strongly puckered, the angle between the plane containing N-1, N-2, N-3, and C-4 and that defined by C-4, C-5, and N-1 being 27.5°. The molecule adopted conformation A, the OH group being axial and R^1 equatorial. Of course, the conformation is not necessarily the same in solution and crystal, even when there are no exceptionally strong interactions between the individual molecules. But it is presumably the more common and has often been assumed by other authors (*cf.* references given in Refs. 17 and 28).

Unfortunately, data for vicinal coupling constants in hydroxytriazolines are not available, since hydroxytriazolines with R^5=H are unknown. However, support for a puckered triazoline ring may be obtained indirectly from the coupling constants of R^4_{cis}=H in 4,5-tetramethylene-5-hydroxy-Δ^2-1,2,3-triazolines.[1,2] The cyclohexane ring is likely to adopt a chair conformation, thus excluding a planar structure of the triazoline ring. The chair conformation may be attained in two ways, as shown in Fig. 2. The coupling constants between R^4_{cis}=H and the adjacent methylene protons of the cyclohexane ring are 7.2 and 10.2 Hz for R^1 being methyl or benzyl; for R^1 being phenyl they are 7.2 and 8.5 Hz. These coupling constants may easily be interpreted in terms of conformation E and are incompatible with conformation F, which should give coupling constants below 6 Hz,[29] these dihedral angles being around 60°. Since R^1 (methyl or benzyl) has the same chemical shift in these molecules as in those where R^4_{trans} is

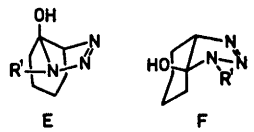

Fig. 2.

not part of a cyclohexane ring (the same applies to pyridine solvent shifts for R^1 and R^4_{cis}),[1,2] the conformation is probably the same in all these cases, *i.e.* puckered in such a way as to place the hydroxy group axially.

Also chemical shift data, and particularly their systematic variations with substituents, provide evidence for a non-planar conformation in solution. The effect on equal substituents at C-4 of changing R^1 (Table 2) would not be

Table 2. Chemical shifts of methyl groups at C-4 in 4,4-dimethyl-5-hydroxy-5-isopropyl-Δ^2-1,2,3-triazolines ($CDCl_3$).[1,2]

R^1	R^4_{cis}=Me	R^4_{trans}=Me
Me	1.52	1.13
Benzyl	1.55	1.21
p-NH_2-Ph	1.60	1.29
p-MeO-Ph	1.60	1.30
Ph	1.58	1.33
p-Br-Ph	1.58	1.33
p-NO_2-Ph	1.56	1.44

understandable on the basis of a planar arrangement of the ring and N-1···R^1 bond. R^4_{cis}=Me is seen to be largely unaffected, whereas R^4_{trans}=Me varies monotonically with the electronegativity of R^1. This variation is probably caused by electronic effects, possibly involving the N-1 lone-pair, since steric variations in R^1 are negligible, at least in the aromatic series.

The remarkably constant chemical shift of R^1=Me in 1-methyl-5-hydroxy-Δ^2-1,2,3-triazolines (3.26 ± 0.02 ppm, unless R^5 is particularly bulky (Bu^t) or anisotropic (Ph)[1,2] points to a constant conformation of the ring in these triazolines. Again, this conformation cannot be planar, since this would involve eclipsing of vicinal alkyl groups in the cases where R^4_{trans} is methyl. In addition, the insensitivity of the chemical shift of R^1 to variations in the C-4 substituents indicates that R^1 is well separated from these. This is also supported by the fact that the equilibrium position between diastereomeric hydroxytriazolines ($R^4_{cis} \rightleftharpoons R^4_{trans}$) is rather indifferent to the steric requirements of R^1.[1,2] Therefore R^1 must be equatorially disposed, and conformation A is most probable on the reasonable assumption that the OH group is axial.

The effect of varying the size of R^5 is shown in Table 3. The constant chemical shift of R^4_{cis}=Me is yet another indication that the triazoline ring has a constant conformation.

Table 3. Chemical shift variations in some 1,4-dimethyl-5-hydroxy-Δ^2-1,2,3-triazolines as a function of R^5.[1,2]

R^5	R^4_{cis}=Me	R^4_{trans}=H	R^1=Me
Me	1.44	3.63	3.27
Et	1.44	3.78	3.24
Pr^i	1.44	3.84	3.24
Bu^t	1.41	4.10	3.37

The fact that R^4_{trans}=H is influenced by the steric requirements of the proximate R^5 is quite reasonable (*cf.* Ref. 30).

In summary, conformation A seems to predominate with hydroxytriazolines in solution. We propose the anomeric effect to be responsible for this. The fact that R^5 is larger than the OH group does probably not stabilize conformation A. Intramolecular OH···π bonding[22] is also presumed to be of minor importance. The free OH stretching frequency is rather low (3580 − 3595 cm^{-1} in $CHCl_3$, as opposed to the normal 3625 cm^{-1} [22]), but this may well be due to the nitrogen atom attached to C-5. The intensity of the free OH band varies with the concentration, thus excluding any exceptionally strong OH···π interaction;[21] the puckering of the ring is probably too small.[22] The occasional appearance of the OH stretching frequency as a doublet or asymmetric band is probably better explained in terms of rotational isomerism around the C-5···O bond.[31]

With regard to the conformation at the N-1···R^1 bond, it was observed that the diastereotopic benzylic protons in 1-benzyl-5-hydroxy-Δ^2-1,2,3-triazolines appeared in two

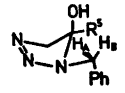

Fig. 3.

ranges, 4.6 – 4.7 ppm and 4.8 – 5.0 ppm, provided that $R^5 \neq Ph$. This is consistent with conformation A. Of the three possible rotational isomers the one with the benzylic phenyl group pointing away from R^5 and OH (Fig. 3) is most likely, and the observed shielding by $R^5 = Ph$ of the benzylic proton that normally resonates most downfield suggests that this is the one located nearest R^5, namely B. The normal shielding of proton A relative to proton B is most likely due to its position in a shielding region of the triazoline ring rather than to an effect from the benzylic phenyl group.[32] The pyridine induced solvent shifts of protons A and B[2] are also consistent with this model. Thus the shift of proton A is very constant for different R^5 groups, whereas the shift of proton B is strongly dependent on R^5, being small when R^5 is large (Ph or Bu^t).

5-Amino-Δ^2-1,2,3-triazolines. In order to elucidate the conformation of 5-aminotriazolines, we have prepared series of homologous triazolines where the C-4 and C-5 substituents are varied systematically while R^1 is kept constant (*p*-nitrophenyl). In this connection triazolines with an unsubstituted amino group have been prepared for the first time.

Pertinent ^{1}H NMR data, measured by us or gathered from the literature, are presented in Tables 4 and 5. Assignments of signals are based on trends within homologous series in conjunction with considerations on equilibria positions [1] as well as on absolute δ-values.

As with the alkoxytriazolines, the small *trans* coupling constants (3.0 – 3.4 Hz, Table 4) suggest that X is preferentially axial, even though this also places R^4_{trans} axially, irrespectively of its being H, Me, or Et. This is only compatible with R^1 being equatorially oriented, as in conformation A (*cf.* the section on alkoxytriazolines).

Informations may also be obtained from aminotriazolines fused with a cyclohexane ring. For $X = NH_2$ (IIIp, Table 7 in the experimental part) $R^4_{cis} = H$ couples with the adjacent methylene protons with identical coupling constants of 6.2 Hz; for $X = NMe_2$ (IIIq) these are 5.0 and 3.1 Hz. This suggests that, compared with the corresponding hydroxytriazolines, a larger fraction of molecules with equatorial X is present.

Chemical shifts also provide information on the conformation of aminotriazolines. δ-Values for C-4 protons in triazolines where $R^4_{cis} = R^4_{trans} = H$ (R^5 is an alkyl group) are particularly informative and have been summarized in Table 6. The rule that R^4_{cis} resonates at lower field than R^4_{trans}, generally followed by the hydroxytriazolines, is not valid for the aminotriazolines (see values marked * in Table 6).

If the difference in chemical shift of the C-4 protons were due to deshielding of $R^4_{cis} = H$ by the lone-pair of the amino group, as postulated by other authors,[4] assuming a planar geometry of the triazoline ring, it is difficult to explain why the *cis* proton resonates at highest field in the starred triazolines in Table 6. Also the fact that it is $R^4_{trans} = H$, and not $R^4_{cis} = H$, that suffers the greatest change when the size of the amino group is varied, remains unexplained. Thus for

Table 4. PMR data of 1-(*p*-nitrophenyl)-5-amino-Δ^2-1,2,3-triazolines where $R^4_{cis} = R^5 = H$.

	X	R^4_{trans}	R^1 *ortho*	R^1 *meta*	Me in X	R^4_{trans}	$R^4_{cis} = H$	$R^5 = H$	J_{cis} [a]	J_{trans} [a]	Remarks
IIa	NH_2	H									
IIb	NHMe	H									
IIc	NMe_2	H	7.60	8.30	2.12	4.02	4.55	5.05	9.8	3.3	*cf.* Ref. 4
IId	NH_2	Me									
IIe	NHMe	Me	7.46	8.25	2.01	1.34	4.47	4.74		3.0	(not pure)
IIf	NMe_2	Me [b]	7.56	8.24	2.12	1.31	4.55	4.51	[b]	3.4	*cf.* Ref. 4
IIg	NH_2	Et									
IIh	NHMe	Et	7.52	8.34	2.02		4.38	4.81		3.2	
IIi	NMe_2	Et					4.46			3	Ref. 4

[a] *cf.* note in Table 1. [b] 4.12 ppm for $R^4_{trans} = H$ and $J_{cis} = 9$ cps have been reported [4] for the diastereomeric form of IIf. We were unable to observe this form.

Table 5. ^{1}H NMR data for 1-(*p*-nitrophenyl)-5-alkyl-5-amino-Δ^2-1,2,3-triazolines (the C-4 groups are hydrogen or methyl). IIId–f were measured in mixture with structure–isomeric triazolines. IIIj–o were measured as mixtures of stereoisomeric triazolines.

	X	R^4_{cis}	R^4_{trans}	R^5	R^1 *ortho*	R^1 *meta*	Me in X	R^4=Me *cis*	R^4=Me *trans*	R^4=H *cis*	R^4=H *trans*	R^5	Remarks
IIIa	NH_2	H	H	Me	7.68	8.30				4.40	4.40	1.77	
IIIb	NHMe	H	H	Me	7.66	8.28	2.01			4.50	4.18	1.80	
IIIc	NMe_2	H	H	Me	7.90	8.31	2.17			4.68	3.94	1.74	*cf.* Ref. 4
IIId	NH_2	H	H	Et						4.34	4.46		
IIIe	NHMe	H	H	Et	7.61	8.25	2.04			4.40	4.25	0.87 (t)	
IIIf	NMe_2	H	H	Et	7.90	8.30	2.16			4.58	3.96	0.66 (t)	*cf.* Ref. 4
IIIg	NH_2	H	H	Pr^i	7.68	8.27				4.18	4.50	0.74 (d) 1.12 (d)	
IIIh	NHMe	H	H	Pr^i	7.65	8.30	2.02			4.27	4.27	0.78 (d) 1.12 (d)	
IIIi	NMe_2	H	H	Pr^i	7.76	8.25	2.21			4.37	4.18	1.00 (d) 0.98 (d)	*cf.* Ref. 4
IIIj	NH_2	Me H	H Me	Me	7.68	8.24		1.51	1.40	4.29	4.10	1.74 1.47	
IIIk	NHMe	Me H	H Me	Me	7.63	8.24	1.87 2.12	1.54	1.38	4.47	3.98	1.81 1.57	Ref. 1
IIIl	NMe_2	Me H	H Me	Me	7.79	8.20	2.16		1.37	4.56		1.52	
IIIm	NH_2	Me H	H Me	Et	7.85	8.45		1.50			4.35	0.89 (t)	
IIIn	NHMe	Me H	H Me	Et	7.64	8.30	1.89	1.53			4.11	0.93 (t)	
IIIo	NMe_2	Me H	H Me	Et	7.65 7.91	8.24	2.33 2.19	1.57?	1.57	4.57	4.18	0.59 (t)	*cf.* Ref. 39

Table 6. Chemical shifts for R^4_{cis}=H and R^4_{trans}=H in 1-(*p*-nitrophenyl)-5-alkyl-5-amino-Δ^2-1,2,3-triazolines (*cf.* Table 5).* Exceptions from the rule that R^4_{cis} resonates at lower field than R^4_{trans} (*cf.* text).

R^5	R^4	X=NH_2	X=NHMe	X=NMe_2
Me	*cis*	4.40*	4.50	4.68
	trans	4.40*	4.18	3.94
Et	*cis*	4.34*	4.40	4.58
	trans	4.46*	4.25	3.96
Pr^i	*cis*	4.18*	4.27*	4.37
	trans	4.50*	4.27*	4.18

R^5=Me one observes a 0.46 ppm upfield shift for R^4_{trans}=H but only a 0.28 ppm downfield shift for R^4_{cis}=H on proceeding from X=NH_2 to X=NMe_2. The presence of lone-pairs on X is seemingly not essential for this. Quite analogous effects are observed on increasing the size of R^5, the proton *trans* to R^5 here suffering a large upfield shift and the other a smaller downfield shift.

Deshielding of the *cis* proton by the lone-pair(s) on X is clearly inadequate to explain these observations, and, also, the effect of hydroxy or amino groups on vicinal protons is not at all clearcut. Admittedly, there are examples of even very large deshieldings of this sort, but this requires either extreme steric compression [33] or that a lone-pair points directly toward the affected proton.[34] In fact, if the hetero group is freely rotating, it is more common that the *trans* oriented proton resonates at lowest field.[20,35–38]

It is more likely that the observed chemical shift variations are the result of conformational

changes. Rotational isomerism about the C-5···X bond (*cf.* Ref. 4) is one possibility, which would, however, also fail to explain, at least in a straightforward manner, the large shieldings experienced by a proton at C-4, when the C-5 group *trans* to it increases in size. We favor an explanation based on a variation in the equilibrium composition of conformations A, B, C, and D, and propose that a large C-5 substituent prefers the axial to the equatorial position, because it then avoids being *gauche* to both C-4 protons (*cf.* Ref. 7). This forces the C-4 proton situated *trans* to the C-5 group with the largest steric requirements into the axial position, where it will be shielded by the triazoline ring.

This picture explains the tendency of aminotriazolines with $R^5=H$ to adopt conformation A, and also the "*trans* shielding effect" exerted by an alkyl as well as an amino group at C-5. The anomeric effect, which seemed to determine the conformation of alkoxy- and hydroxytriazolines, would not account for these effects.

For R^5 being an alkyl group we have not been able to conclude which are the participating conformers. A and C are the most likely candidates, but the coexistence of B or D is also possible in view of the increasing steric congestion along the N-1···C-5 bond with increasing size of C-5 substituents.

EXPERIMENTAL

^1H NMR spectra were recorded on a JEOL JNM-C-60HL instrument, using TMS as internal standard. Chemical shifts (in $CDCl_3$ at magnet temperature, unless otherwise stated) are given as δ-values. AB systems were treated in a second order manner.[41] δ-Values for *p*-nitrophenyl groups are just the centers of the characteristic 'doublets'.

1-(p-Nitrophenyl)-5-ethoxy-Δ^2-1,2,3-triazoline (Ia) was prepared by dissolving 1.64 g of *p*-nitrophenyl azide in 8 ml of ethyl vinyl ether. After 11 days the product had separated in 99 % yield. After recrystallization from ethyl acetate the m.p. of the slightly yellowish crystals was 135 °C. (Found: C 50.77; H 5.23; N 23.80. Calc. for $C_{10}H_{11}N_4O_3$: C 50.86; H 5.12; N 23.72).

1-(p-Aminophenyl)-5-ethoxy-Δ^2-1,2,3-triazoline was obtained in quantitative yield by catalytic hydrogenation of Ia (0.356 g in 3 ml of methanol) for 1 h at 2.5 atm H_2 pressure, using PtO_2 (3 mg) as the catalyst. After treatment with activated carbon and crystallization from ether, yellow crystals of m.p. 71 – 76 °C were obtained. (Found: C 58.26; H 6.63; N 27.35. Calc. for $C_{10}H_{14}N_4O$: C 58.24; H 6.84; N 27.17).

1-(p-Nitrophenyl)-4,5-tetrahydropyrano-Δ^2-1,2,3-triazoline was prepared according to the literature.[23]

5-Hydroxy-Δ^2-1,2,3-triazolines have been described previously.[1,2]

5-Amino-Δ^2-1,2,3-triazolines are prepared by adding either *ca.* 0.3 g of liquid ammonia and 2 g of anhydrous sodium sulfate, 1.5 ml of 40 % aqueous methylamine, or *ca.* 0.5 g of dimethyl-

Table 7. Data of preparative interest for new amino triazolines in Tables 4 and 5.

	R^4	R^5	X	Reaction time	Yield %	M.p. °C	Formula	Analyses Found C	H	N	Calculated C	H	N
IIh	Et	H	NHMe	6 d	100	108 – 109	$C_{11}H_{15}N_5O_2$	53.00	6.07	28.10	53.15	6.10	27.98
IIIa	H	Me	NH_2	3 d	25	123 – 124[a]	$C_9H_{11}N_5O_2$	48.86	5.01	31.66	48.72	5.24	31.82
IIIb	H	Me	NHMe	24 h	90	124 – 126	$C_{10}H_{13}N_5O_2$	51.06	5.57	29.77	51.01	5.53	29.77
IIIg	H	Pr^i	NH_2	7 d	–[b]	122 – 124[a]	$C_{11}H_{15}N_5O_2$	53.00	6.07	28.10	53.17	6.33	28.27
IIIh	H	Pr^i	NHMe	2 d	100	144 – 145	$C_{12}H_{17}N_5O_2$	54.74	6.51	26.60	54.70	6.51	26.67
IIIj	Me	Me	NH_2	2 d	–[b]	153 – 155	$C_{10}H_{13}N_5O_2$	51.06	5.57	29.77	51.23	5.70	29.72
IIIl	Me	Me	NMe_2	3 d[c]	–[b]	119 – 120	$C_{12}H_{17}N_5O_2$	54.74	6.51	26.60	54.97	6.62	26.54
IIIm	Me	Et	NH_2	4 d	80	131 – 132	$C_{11}H_{15}N_5O_2$	53.00	6.07	28.10	52.95	5.98	28.03
IIIn	Me	Et	NHMe	2 d	96	109 – 111	$C_{12}H_{17}N_5O_2$	54.74	6.51	26.60	54.86	6.60	26.84
IIIp	$-(CH_2)_4-$		NH_2	24 h	100	129 – 130	$C_{13}H_{15}N_5O_2$	55.16	5.79	26.80	55.23	5.86	26.94
IIIq	$-(CH_2)_4-$		NMe_2	2.5 h[c]	100	84 – 86	$C_{14}H_{19}N_5O_2$	58.12	6.62	24.21	58.18	6.63	24.01

[a] Ethyl acetate-pentane was used for recrystallization. [b] Two isomeric triazolines were formed due to enamine formation in two directions;[40] only the shown triazoline was isolated in a pure state. [c] The reaction was carried out in chloroform in place of ether.

amine to a solution of 0.82 g of *p*-nitrophenyl azide and the appropriate aldehyde (*ca.* 100 % excess) or ketone (*ca.* 20 % excess) in 7 ml of diethyl ether. The mixture is allowed to stand at room temperature for the specified time (Table 7) in a stoppered flask.

If the product separates during the reaction, it is just filtered off, washed with pentane, and recrystallized from methanol; if not, the product will crystallize on removal of the solvent from the organic phase and triturating the residue with pentane. Recrystallization is performed, using methanol as solvent, until a constant melting point is obtained. Sodium sulfate used in conjunction with ammonia is first removed by dissolving the product in chloroform and filtering. Yields (referring to unrecrystallized product), melting points, and analyses are given in Table 7. The triazolines are yellow to brown. 5-Methylaminotriazolines in particular are often unstable and decompose on standing at room temperature.

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The Reaction of 2-Phenyl-2-norbornene with Formaldehyde and Dimethylamine. Additional Evidence for the Occurrence of a 1,5-Hydride Shift during the Aminomethylation of a Strained Bicycloalkene Structure

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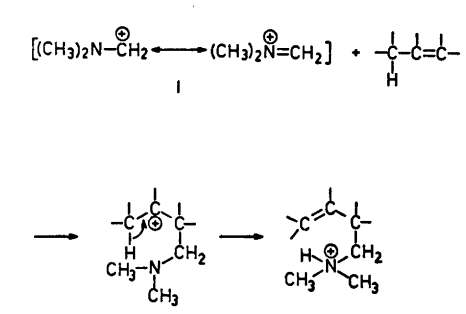
Reaction of 2-phenyl-2-norbornene, with formaldehyde and dimethylamine in acetic acid, produced a mixture of compounds best explained by a 1,5-hydride shift in one intermediate step. The compounds in question were *N*-methyl-endo-3-phenyl-exo-2-norbornanemethylamine, *N*,*N*-dimethyl-endo-3-phenyl-exo-2-norbornanemethylamine, 1,1′-bis(endo-3-phenyl-exo-2-norbornyl)-dimethylamine, 1,1′-bis(endo-3-phenyl-exo-2-norbornyl)trimethylamine and endo-3-phenyl-exo-2-norbornanecarbaldehyde. *N*,*N*-Dimethyl-1-phenyl-7-nortricyclenemethylamine and *N*,*N*-dimethyl-3-phenyl-2-norbornene-2-methylamine, formed presumably by the elimination of a proton from the intermediate carbonium ion during the aminomethylation of 2-phenyl-2-norbornene were also identified. In addition, exo-3-dimethylaminomethyl-endo-2-phenyl-exo-2-norbornanol, most likely formed by the addition of water to the same carbonium ion, was found. The normal aminomethylation product of endo-3-phenyl-exo-2-norbornanecarbaldehyde, 2-dimethylaminomethyl-endo-3-phenyl-2-norbonanecarbaldehyde, was identified.

Aminomethylation of an alkene with formaldehyde and a sec. amine usually gives unsaturated amines or saturated amino alcohols.[1] The reaction leading to the unsaturated amine is explained by the assumption that carbonium-immonium-ion I, produced from an amine and formaldehyde, is first added to the double bond, followed by a transfer of a proton from the carbon atom adjacent to the double bond, to the nitrogen atom (Scheme 1).[2] The tert. amines isolated from the aminomethylation products of norbornene are, however, saturated [3,4] with the primary products being the sec. amines II and III and the aldehyde IV.[5] Furthermore, mass spectra of the reaction products of norbornene, formaldehyde and hexadeuteriodimethylamine indicate that deuterium shifts from the dimethyl group of dimethylamine to the norbornene ring.



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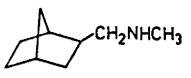

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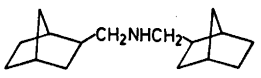

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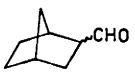

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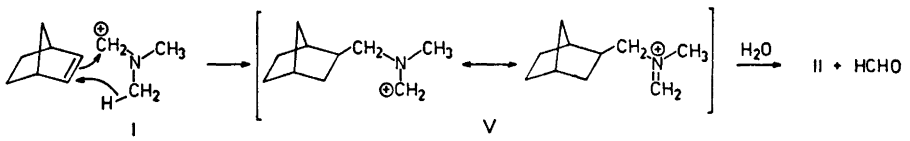
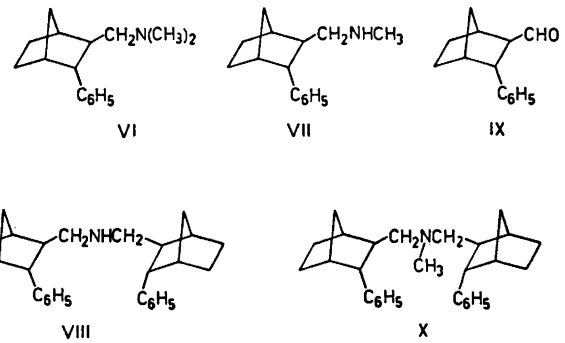


I V

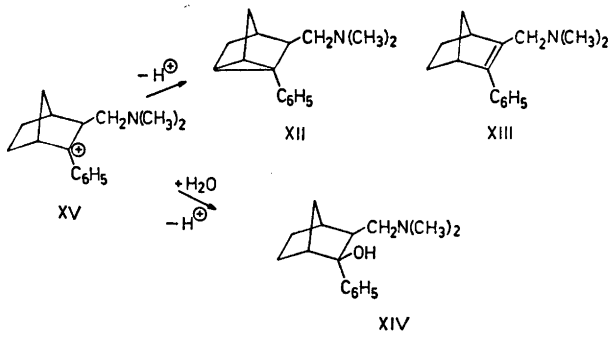
Scheme 2.



VI VII IX VIII X

Based on this finding, the intermediate steps in the reaction of norbornene with formaldehyde and dimethylamine must involve the addition of the carbonium-immonium-ion (I) to the double bond and the shift of hydride ion to the norbornane ring (Scheme 2). The similarly derived products formed in a reaction sequence involving a 1,5-hydride shift and subsequent hydrolysis are the amine III and the aldehyde IV. This is readily seen by assuming that in Scheme 2 the ion I is replaced by the ion V. Furthermore, our recent investigation [5] revealed that the tert. amines, referred to above, which have previously isolated following aminomethylation [3,4] are Eschweiler-Clarke methylation products produced from the amines II and III.

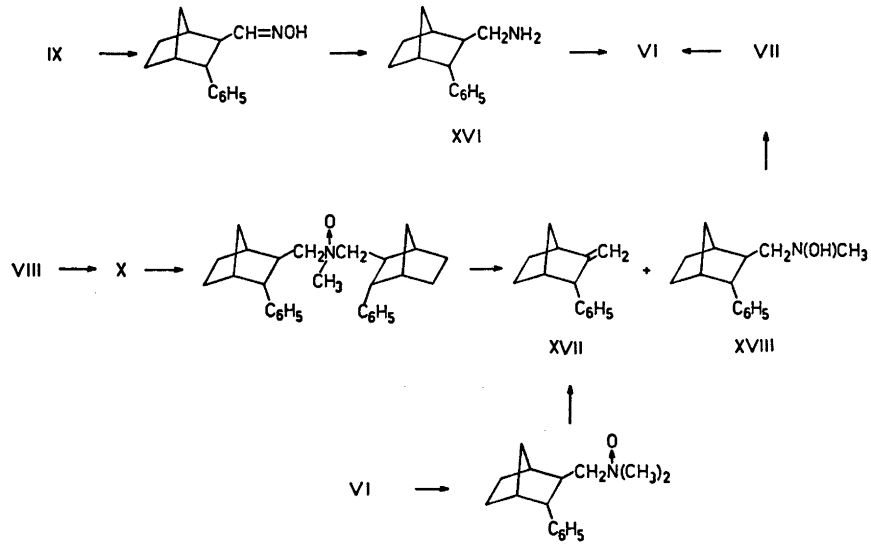
Since aminomethylation of 2-phenyl-2-norbornene yielded a saturated tert. amine as *N*,*N*-dimethyl-endo-3-phenyl-exo-2-norbornanemethylamine (VI), [6] it was of interest to determine if the phenyl substituted products VII, VIII and IX corresponding to the amines II and III and the aldehyde IV as well as X, the methylation product of VIII, are formed during the aminomethylation of 2-phenyl-2-norbornene. 2-Phenyl-2-norbornene was previously aminomethylated using a mol ratio of 1:4:1.5 (2-phenyl-2-norbornene:paraformaldehyde:dimethylamine), with dimethylamine in aqueous solution and the solvent, acetic acid, fortified by a catalytic amount of concentrated sulfuric acid. These conditions were changed so that the corresponding molar ratio was 1:1:1, dimethylamine was added as the hydrochloride, and the solvent was acetic acid without sulfuric acid. The previous reaction time of 16 h at reflux temperature, was decreased to 30 min at the same temperature. Dilution of the reaction mixture with an excess of water produced crystals which were collected by filtration and identified as the hydrochloride of VIII. The main product isolated by ether extraction of the acidic filtrate was the aldehyde IX. Neutralization of the acidic filtrate produced a mixture of six amines as indicated by gas chromatography. Three of these compounds were identified as the expected tert. amine VI, sec. amine VII and 2-dimethylaminomethyl-2-norbornanecarbaldehyde (XI). The latter is the conventional aminomethylation product of the aldehyde IX. Considering the results from the corresponding aminomethylation of norbornene,[5] the three remaining amines were unexpectedly a tricyclic amine XII, an unsaturated amine XIII, and an amino alcohol XIV. The amines XII and XIII are throught to be formed from the intermediate ion XV by the elimination of a proton and the amino alcohol XIV arises from the same ion by hydration (Scheme 3).



Scheme 3.

Since the structure of the tert. amine VI was previously verified [6] and its IR and NMR spectra were available, it appeared reasonable to identify VII, VIII and IX by converting them to VI. Therefore the aldehyde IX was converted to an oxime, which then was reduced with $LiAlH_4$ to the prim. amine XVI, and thereafter converted by methylation [7] to VI. The sec. amine VIII was methylated to X, which by Cope elimination [8] decomposed to a mixture of the known hydrocarbon XVII [9] and hydroxylamine XVIII. The reduction of XVIII with $LiAlH_4$ in ether produced the sec. amine VII, which by methylation yielded VI. The hydrocarbon XVII was prepared as a reference compound by Cope elimination. [9] The reactions which are proof for the structure of VII, VIII and XI are shown in Scheme 4. To confirm the structures of XII and XIII, the former was converted by Cope elimination to the tricyclic hydrocarbon XIX and its structure determined by mass and NMR spectra (Fig. 1). The latter (XIII) was hydrogenated to XX, which was converted by the Cope elimination to XVII (Scheme 5). The verification of the structure of XIV is based on comparison of the IR and NMR spectra of the compound prepared in another way.[10]

In another experiment with the molar ratio 1:4:2, 2-phenyl-2-norbornene, paraformaldehyde



Scheme 4.

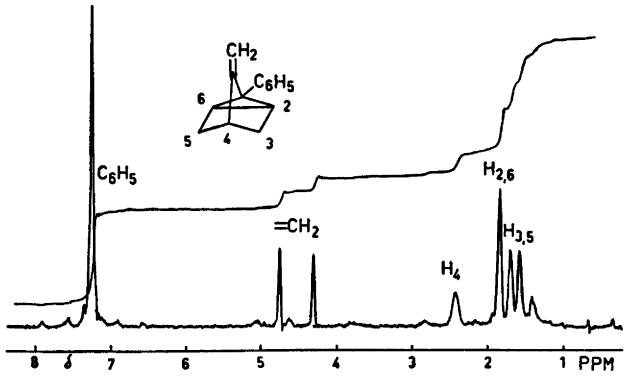
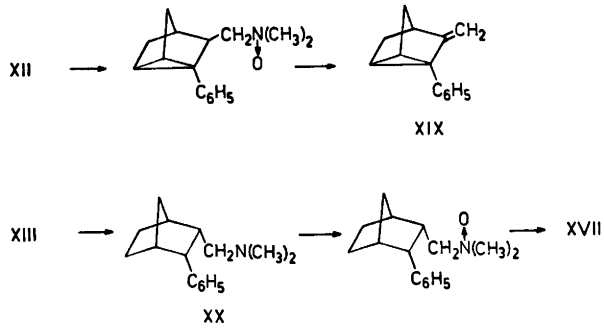


Fig. 1. NMR spectrum of 7-methylene-1-phenylnortricyclene (XIX).



Scheme 5.

and dimethylammonium chloride were heated in acetic acid for 18 hours. Due to the excess of paraformaldehyde and long reaction time, one would expect VII and VIII to methylate to VI and X, respectively, under these conditions. Dilution of the crude reaction mixture with water yielded a compound identified as the hydrochloride of X. Analysis of the other amines indicated that the tert. amine VI was one of the major components. Among the other products were XI, XII and XIII, but gas chromatography indicated that the amino alcohol XIV was not present in the mixture. To ascertain the behavior of XIV during the aminomethylation, it was heated in acetic acid for 20 h. The mixture thus produced contained the same components as found after the aminomethylation of 2-phenyl-2-norbornene and, in addition, 2-phenyl-2-norbornene, therefore one could conclude that the formation of XIV in the aminomethylation of 2-phenyl-2-norbornene is a reversible reaction.

The results of this investigation indicate that the reaction of 2-phenyl-2-norbornene with formaldehyde and dimethylamine occurs to some extent by a similar intramolecular 1,5-hydride shift as was found to be the case with norbornene.[4,5] However, a number of other products not formed from norbornene, are produced either by the elimination of a proton from the ion XV or by the addition of water to the ion XV. The 1,5-hydride shift can explain the formation of the amines VII and VIII and the aldehyde IX. VI and VIII are most likely the Eschweiler-Clarke methylation products of the amine VII and VIII, respectively. The amino aldehyde XI seems to be formed by direct aminomethylation of the aldehyde IX. Removal of a proton from the ion XV gives either a tricyclic amine XII or an unsaturated amine

XIII, and the addition of water to XV leads to the amino alcohol XIV.

In another investigation using the deuterated compounds, we will show that the Eschweiler-Clarke methylation of amines VII and VIII under the conditions of aminomethylation gives among the tert. amines VI and X also cleavage products whose existence is explained by the 1,3-hydride shift.[11]

EXPERIMENTAL

Reaction of 2-phenyl-2-norbornene with formaldehyde and dimethylammonium chloride in acetic acid (the shorter reaction time). 2-Phenyl-2-norbornene (0.2 mol) prepared by dehydration of the alcohol obtained from 2-norbornanone and bromobenzene [6] by Grignard reaction, paraformaldehyde (6.2 g, equivalent to 0.206 mol formaldehyde) and dimethylammonium chloride (0.2 mol) were heated in acetic acid (60 ml) at 115°C for 30 min. Water (300 ml) and ethyl ether (50 ml) were added to the reaction mixture and the VII-hydrochloride which formed was collected by filtration, recrystallized from ethyl alcohol (yield 11.0 g) and characterized as shown later.

The ether layer from the filtrate was separated and the acetic acid-water layer extracted three times with small amounts of ether. The combined ether solutions containing neutral reaction products were freed of acid by washing with a dilute potassium carbonate solution and water, and then dried with calcium sulphate. The acetic acid-water layer from the filtrate was made alkaline with solid potassium carbonate, the freed amines were extracted by ether and the ether extract dried with anhydrous potassium carbonate.

Evaporation of the ether extract of the neutral solution left a residue of 11.5 g, which was distilled in a vacuum. The first fraction, b.p. 105 – 115°C/0.05 Torr, yield 8.85 g, contained 13 % biphenyl and 8 % 1-phenylnortricyclene, which were transported as impurities from the starting material, and 79 % endo-3-phenyl-exo-2-norbornanecarbaldehyde (IX) (see the structure interpretation of IX on the following pages). Fraction 2, b.p. 120 – 220°C/0.05 Torr, yield 1.2 g, was not examined closer.

The ether extract containing free amines, was evaporated until dry and the residue distilled in a vacuum. The fraction boiling at 120 – 150°C/0.05 Torr, yielded 14.5 g and contained according to gas chromatography 6 % of the tricyclic amine XII, 12 % of the unsaturated amine XIII, 11 % of the tert. amine VI, 36 % of the sec. amine VII, 7 % of the amino aldehyde XI, and 28 % of the amino alcohol XIV. The structure interpretations of these compounds are presented in a separate chapter.

Reaction of 2-phenyl-2-norbornene with an excess of formaldehyde and dimethylammonium chloride in acetic acid (the longer reaction time). 2-Phenyl-2-norbornene (0.25 mol), paraformaldehyde (33 g, equivalent to 1.1 mol formaldehyde) and dimethylammonium chloride (0.5 mol) were heated in acetic acid (170 ml) at 110 – 120°C for 18 h. Water (1500 ml) and ether (200 ml) were added to the reaction mixture and the X-hydrochloride, which formed, collected by filtration and recrystallized from ethyl alcohol (yield 6.6 g). The neutral products and amines were isolated as described earlier.

The neutral products (15.0 g), which boiled at 120 – 150°C/0.1 Torr contained endo-3-phenyl-exo-2-norbornanecarbaldehyde (IX) as a main component. Other components in this fraction (more than ten by gas chromatography) were not indentified.

The isolated free amines were distilled (b.p. 95 – 98°C/0.04 Torr, yield 20.6 g) and analyzed by gas chromatography. The distillate contained 8.5 % of the tricyclic amine XII, 15 % of the unsaturated amine XIII, 76 % of the tert. amine VI and 0.5 % of the amino aldehyde XI. *N,N*-Dimethyl-endo-3-phenyl-exo-2-norbornanemethylamine (VI) was isolated by using preparative gas chromatography, and its NMR and IR spectra were compared to the corresponding spectra of VI, which were available from the synthesis of VI by known methods.[6]

Endo-3-phenyl-exo-2-norbornanecarbaldehyde (IX) and its structure interpretation. The aldehyde obtained in the aminomethylation of 2-phenyl-2-norbornene (shorter reaction time) was purified according to Frisch [12] by its bisulphite adduct. From 6 g of the adduct was recovered 3 g of IX, b.p. 111 – 112°C/0.2 Torr, n_D^{20} 1.5547, $C_{14}H_{16}O$ (200.27). Mass spectrum: M^+ *m/e* 200 (23 %) with the base peak at *m/e* 43 (100 %). NMR spectrum (in CCl_4): δ 9.68 (1 H) doublet, $J = 1 - 2$ cps (formyl proton), 7.18 (5 H) singlet (phenyl protons), 3.60 and 3.50 (1 H) two doublets (C-3 exo-proton), 2.70 – 2.38 (3 H) a broad signal (C-2 and the bridgehead protons) and 1.77 – 1.07 ppm (6 H) a broad signal (the other protons). In the IR spectrum (in CCl_4) there was a C=O absorption at 1722 cm^{-1}. The semicarbazone of IX melted at 184 – 186°C (recrystallized from ethyl alcohol). To further confirm the structure of IX, it was converted to an oxime, which was reduced without purification with $LiAlH_4$ in ether to endo-3-phenyl-exo-2-norbornanemethylamine (XVI). XVI was distilled in a bulb tube oven at 110 – 113°C/0.02 Torr. $C_{14}H_{19}N$ (201.30). Mass spectrum: M^+ *m/e* 201 (1 %) with the base peak at *m/e* 31 (100 %). NMR spectrum (in C_6D_6): δ 7.22 (5 H) a singlet (phenyl protons), 2.60 (1 H) a broad signal having a shoulder (C-3 exo-proton), 2.47 (1 H) a doublet, $J = 7$ cps (methylene protons of the aminomethyl group), 2.30 and 2.12 (2 H) two broad signals (the bridgehead protons), 1.84 – 1.52 (1 H) three broad signals (C-2 endo proton) and 1.52 – 0.88 ppm (6 H) the other protons of

the norbornane structure. In the IR spectrum (on film) weak NH_2 absorptions at 3380 and 3470 cm^{-1} were seen. The amine XVI was methylated with formaldehyde in formic acid as described by Cope *et al.*[7] to a tert. amine, whose NMR and IR spectra were similar to those of the previously mentioned *N,N*-dimethyl-endo-3-phenyl-exo-2-norbornanemethylamine (VI).

N-Methyl-endo-3-phenyl-exo-2-norbornanemethylamine (VII) and exo-3-dimethylaminomethyl-endo-2-phenyl-exo-2-norbornanol (XIV). The previously described amine mixture (9.5 g) obtained by aminomethylation of 2-phenyl-2-norbornene (shorter reaction time) and aqueous potassium hydroxide (20 %, 60 ml) were placed in a flask and to this mixture was added gradually *p*-toluenesulphonyl chloride (2.8 g). The contents of the flask were heated for half an hour and the reaction products extracted by ether. The ether solution was extracted with dilute aqueous hydrochloric acid and water, then dried with calcium sulphate. After evaporation of the solvent, the viscous residue (3.4 g) was recrystallized from ethyl alcohol. *N*-Methyl-endo-3-phenyl-exo-2-norbornanemethylamine *p*-toluenesulphonamide, $C_{22}H_{27}NSO_2$ (369.50) mass spectrum M^+ *m/e* 369, was hydrolyzed according to Snyder *et al.*[13] by heating in 47 % hydrobromic acid in the presence of phenol. *N*-Methyl-endo-3-phenyl-exo-2-norbornanemethylamine (VII), b.p. 119 – 121°C/0.03 Torr, n_D^{20} 1.5493, $C_{15}H_{21}N$ (215.33) was obtained in 76 % yield and showed the follwing spectroscopic properties: mass spectrum *m/e* 215 (2 %) with the base peak $CH_2N^+{\cdot}H=CH_2$ at *m/e* 44 (100 %); NMR spectrum (in C_6D_6): δ 7.22 (5 H) a singlet (phenyl protons), 2.67 (1 H) a multiplet (C-3 exo-proton), 2.50 – 2.05 (7 H) (in this region the methyl protons appeared as a singlet and the bridgehead and methylene protons of the methylaminomethyl group as a complex signal) and 2.00 – 1.00 ppm (6 H) the other protons of the norbornane structure. Treatment of VII with formaldehyde in formic acid produced *N,N*-dimethyl-endo-3-phenyl-exo-2-norbornanemethylamine (VI). The unreacted amines extracted by dilute hydrochloric acid, were extracted into ether after addition of solid potassium carbonate. The ether solution was dried with anhydrous potassium carbonate, evaporated and the residue (6.4 g) distilled with collection at 110 – 150°C/0.1 Torr. The distillate was dissolved in petroleum ether at room temperature, cooled in acetone-dry ice, and the precipitated exo-3-dimethylaminomethyl-endo-2-phenyl-exo-2-norbornanol (XIV) collected. NMR and IR spectra of XIV were comparable to the spectra of the reference compound, prepared by a known method.[10] After five recrystallizations, XIV melted at 65 – 66°C (reported [10] m.p. 59.5 – 61.0°).

Endo-3-phenyl-2-dimethylaminomethyl-2-norbornanecarbaldehyde (XI). The aminoaldehyde was isolated from the mixture of amines obtained from aminomethylation of 2-phenyl-2-norbornene (shorter reaction time), by preparative gas chromatography. The reference compound was prepared by heating endo-3-phenyl-exo-2-norbornanecarbaldehyde (IX) (2g), paraformaldehyde (0.3 g) and dimethylammonium chloride (0.85 g) in 3 ml acetic acid for 10 min. The product XI was distilled in a bulb tube oven at 110°C/0.1 Torr yielding 0.15 g. $C_{17}H_{23}NO$ (257.36). Mass spectrum: M^+ *m/e* 257 with the base peak at *m/e* 58 (100 %). NMR spectrum (in C_6H_6): δ 9.57 (1 H) a singlet (formyl proton), 7.23 (5 H) a multiplet (phenyl protons), 3.20 (1 H) a doublet, $J=3$ cps (C-3 exo-proton), 2.89 and 2.27 (2 H) $J=13$ cps (the AB-system of methylene protons of the dimethylaminomethyl group), and 2.57 – 0.50 (14 H) including at 2.48, one of the bridgehead protons as a broad signal and at 2.17 ppm, the methyl protons of dimethylaminomethyl group as a singlet.

Structure evaluation of N,N-*dimethyl-1-phenyl-7-nortricyclenemethylamine (XII). 7-Methylene-1-phenylnortricyclene (XIX).* The amine mixture (7 g) obtained by aminomethylation of 2-phenyl-2-norbornene (longer reaction time) was converted with hydrogen peroxide to amine oxides, which were decomposed by heating under reduced pressure. The products were dissolved in ether and purified by removing the unreacted amines from the ether solution with aqueous hydrochloric acid. After drying and removing the ether, the residue was distilled at 124 – 126°C/8 Torr (2.0 g) yielding two components (14:86), which were isolated by preparative gas chromatography. The main component was endo-3-phenyl-2-methylenenorbornane (XVII) whose mass spectrum confirms the molecular formula of XVII and whose NMR spectrum is identical to that reported for XVII.[9] The minor component was 7-methylene-1-phenylnortricyclene (XIX), $C_{14}H_{14}$ (182.25), according to the NMR spectrum (Fig. 1). Mass spectrum: M^+ *m/e* 182 (100 %).

Structure evaluation of N,N-*dimethyl-3-phenyl-2-norbornene-2-methylamine (XIII).* N,N-*Dimethyl-endo-3-phenyl-endo-2-norbornanemethylamine (XX).* An aliquot (6.4 g) of the crude amine mixture produced in the aminomethylation (longer reaction time) of 2-phenyl-2-norbornene was hydrogenated in 100 ml methanol with Pd/C as a catalyst. During distillation the products boiled at 115 – 117°C/0.3 Torr (5.15 g). From this mixture, *N,N*-dimethyl-endo-3-phenyl-endo-2-norbornanemethylamine (XX) (19 mg) was isolated, $C_{16}H_{23}N$ (229.35), purity 98.5 %, m.p. 59 – 61°C by preparative gas chromatography. Mass spectrum: M^+ *m/e* 229 with the base peak at *m/e* 58 (100 %). NMR spectrum (in C_6D_6): δ 7.22 (5 H) a singlet (phenyl protons), 3.10 (1 H) C-3 exo-proton, 2.53 – 1.20 (17 H) including at 2.01 ppm the methyl protons of the dimethylaminomethyl group as a singlet. To confirm the structure, the sample used for NMR spectrum (17 mg) was decomposed *via* an amine oxide to a hydrocarbon (8.5 mg),

which was identified as endo-3-phenyl-2-methylenenorbornane (XVII) by its mass spectrum.

1,1'-Bis(endo-3-phenyl-exo-2-norbornyl)dimethylamine (VIII). The slightly water soluble amine hydrochloride produced by aminomethylation of 2-phenyl-2-norbornene (shorter reaction time) was heated with aqueous potassium carbonate (20 %, 150 ml) and the freed amine separated and purified *via* the sulphonamide as described earlier for VII. Hydrolysis of sulphonamide (10 g) yielded 1,1'-bis(endo-3-phenyl-exo-2-norbornyl)dimethylamine (VIII) (6.7 g, 87 %) which was distilled in a bulb tube oven at 225°C/0.02 Torr. $C_{28}H_{35}N$ (385.57), n_D^{20} 1.5773. Mass spectrum: M^+ *m/e* 385 (1 %) with the base peak at *m/e* 31 (100 %). NMR spectrum (in C_6D_6): δ 7.20 (10 H) a singlet (phenyl protons) and the other protons 2.80 – 1.00 (25 H) in this region the *N*-methylene protons show as a doublet ($J = 7$ cps) at 2.46 ppm. In the IR spectrum (as film) there is a weak absorption at 3380 cm^{-1}. Methylation of VIII with formaldehyde in formic acid produced 1,1'-bis(endo-3-phenyl-exo-2-norbornyl)trimethylamine (X), whose structure is explained below.

1,1'-Bis(endo-3-phenyl-exo-2-norbornyl)trimethylamine (X) and its structure. The slightly water soluble hydrochloride (6.0 g) obtained by aminomethylation of 2-phenyl-2-norbornene (longer reaction time) was freed from its salt with potassium carbonate as described above for VIII and the product 1,1'-bis(endo-3-phenyl-exo-2-norbornyl)trimethylamine (X) distilled in a bulb tube oven at 225°C/0.03 Torr. n_D^{20} 1.5698. $C_{29}H_{37}N$ (399.59). Mass spectrum: M^+ *m/e* 399 (1 %), M – 171 (an abstraction of endo-3-phenyl-exo-2-norbornyl radical) *m/e* 228 (5 %) and the base peak at *m/e* 58 (100 %). NMR spectrum (in C_6D_6): δ 7.21 (10 H) a singlet (phenyl protons), 2.82 – 2.48 (2 H) a multiplet (C-3 exo-protons) and 2.37 – 0.90 ppm (25 H) the other protons as two multiplets. Amine X (0.25 g) was converted with hydrogen peroxide to an amine oxide (yield 0.3 g), which decomposed by heating under reduced pressure to a neutral compound and an amine. These compounds were readily separated by solvent extraction. The neutral product (0.05 g) endo-3-phenyl-2-methylenenorbornane (XVII) was obtained in 25 % yield and its NMR spectrum agreed with that of the reference compound mentioned earlier. The isolated amine was crystallized from petroleum ether to give 0.06 g *N*-methyl-endo-3-phenyl-exo-2-norbornanemethylhydroxylamine (XVIII) in 22 % yield. M.p. 96 – 98°. $C_{15}H_{21}ON$ (231.33). Mass spectrum: M^+ *m/e* 231 (3 %), $CH_3N^{+\cdot}(OH)=CH_2$ *m/e* 231 (3 %) with the base peak at *m/e* 44 (100 %). NMR spectrum (in C_6D_6): δ 7.26 (5 H) a singlet (phenyl protons), 2.92 – 2.05 (9 H) a complex signal group, which included a distinct singlet of methyl protons at 2.52 and a broader signal at 1.84 – 1.05 ppm (7 H). The reduction of XVIII with $LiAlH_4$ in dioxane produced *N*-methyl-endo-3-phenyl-exo-2-norbornanemethylamine (VII), which was identified by gas chromatography and by its mass spectrum.

Decomposition of exo-3-dimethylaminomethyl-endo-2-phenyl-exo-2-norbornanol (XIV) in acetic acid. The amino alcohol XIV (0.545 g, 2.22 mmol) was heated in acetic acid under reflux for 20 h. To the reaction mixture dilute hydrochloric acid (10 ml) and ether (5 ml) were added, and the crystals which formed separated by filtration, then recrystallized from ethanol (yield 0.063 g). The product was identified by IR spectrum as a mixture of 1,1'-bis(endo-3-phenyl-exo-2-norbornyl)dimethylamine (VIII) and 1,1'-bis(endo-3-phenyl-exo-2-norbornyl)trimethylamine (X) hydrochlorides. From the filtrate the neutral products and amines were isolated by the conventional treatment of solvents. Gas chromatography of the neutral portion (0.17 g) yielded 2-phenyl-2-norbornene (17 %), endo-3-phenyl-exo-2-norbornanecarbaldehyde (IX) (71 %) and three unidentified compounds. 2-Phenyl-2-norbornene and IX were separated by distillation in a bulb tube oven under reduced pressure and their structures were confirmed by IR spectra. Gas chromatography revealed that the amine portion consisted of *N,N*-dimethyl-1-phenyl-7-nortricyclenemethylamine (XII) (2 %), *N,N*-dimethyl-3-phenyl-2-norbornene-2-methylamine (XIII) (7 %), *N,N*-dimethyl-endo-3-phenyl-exo-2-norbanenemethylamine (VI) (17 %), *N*-methyl-endo-3-phenyl-exo-2-norbornanemethylamine (VII) (28 %), the starting material XIV (43 %) and an unidentified amine (3 %).

Apparatus. The gas chromatographic analyses were performed with a Perkin-Elmer chromatograph model F 30 using a two meter long column (10 % Silicone Gum Rubber SE-30 on Chromosorb W) and a flame ionization detector. The analysis was programmed from 170°C to 220°C, the initial time was 5 min, and the heating rate 10 degree/min. An Autoprep model A-700 gas chromatograph manufactured by Wilkens Instrument & Research, Inc., and a 5 m long preparative column (20 % Silicone Gum Rubber SE-30 on Chromosorb W) were used for the chromatographic preparative separations at 210°C with helium as a carrier gas. The instrument was equipped with a hot wire detector. The IR spectra were carried out which a Perkin-Elmer model 457 double beam spectrophotometer, the mass spectra were recorded on a Hitachi Perkin-Elmer RMU 6 E double focusing mass spectrometer, and the NMR spectra on a Varian T 60 spectrometer with tetramethylsilane as the internal standard.

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3,3-Dialkylindolin-2-ones and 3,3-Dialkylisoindolin-1-ones. 2. Hofmann and Lossen Degradation of 4,4-Dialkyl-1,3-dioxo-1,2,3,4-tetrahydroisoquinolines (4,4-Dialkylhomophthalimides). A Mechanistic Study

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The Hofmann rearrangement reaction of 4,4-dialkylhomophthalimides and the Lossen rearrangement of 4,4-dialkyl-*N*-tosyloxyhomophthalimides afford indolinone or isoindolinone derivatives, or mixtures of both, depending on the nature of the 4-substituents. "Bulky" substituents give rise to isoindolinones, "small" substituents afford indolinones, and "medium sized" substituents give mixtures of both, though the actual size has less significance than their spatial geometry. The mechanisms of the reactions involved are discussed.

In a previous paper [1] we reported on the formation of 3,3-disubstituted indolinones and isoindolinones from 4,4-disubstituted homophthalimides * on treatment with alkali and sodium hypochlorite solution. Two reaction methods were used: in one (A), the homophthalimide derivative was treated with 2 M sodium hydroxide solution for various times prior to treatment with hypochlorite, and in the other (B), the alkaline homophthalimide solution was treated *immediately* with the hypochlorite. The products formed consisted of indolinone or isoindolinone derivatives, or mixtures of both, depending on the nature of the starting material and on the experimental conditions employed. Working with 4,4-tetramethylenehomophthalimide, it was concluded that in reaction A, the homophthalimide slowly underwent hydrolytic ring-opening to give a mixture of the two theoretically possible "amic" acids which, upon treatment with hypochlorite, rapidly underwent rearrangement to afford the indolinone and isoindolione derivatives.

Formation of the indolinones in method B, however, seems to rule out a corresponding mechanism initiated by a hydrolytic step, as the indolinones were formed at a much more rapid rate than hydrolysis of the imide. Consequently, a different reaction mechanism seems to be operating in method B, and it is the purpose of the present paper to report on the attempts made to elucidate this mechanism.

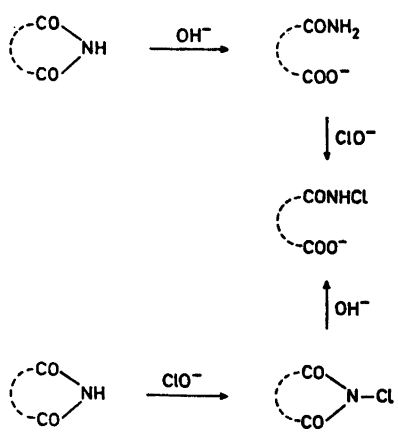
* As the correct names of these compounds are very cumbersome, they are designated as derivatives of homophthalimides in the following discussion.

DISCUSSION

The Hofmann amide degradation is generally believed to proceed through the following steps:[2]

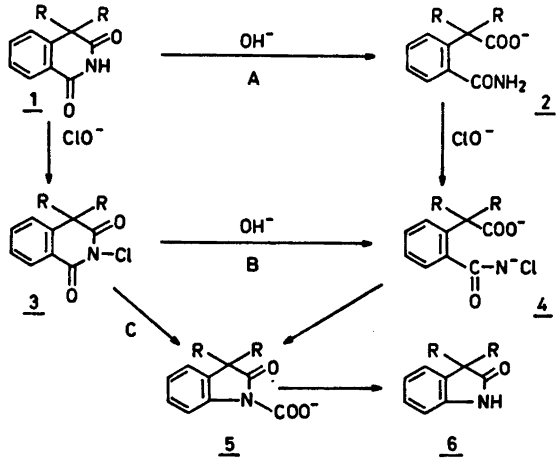
$$\mathrm{R-CONH_2} \xrightarrow{\mathrm{OHal^-}} \mathrm{R-CONHHal} \xrightarrow{\mathrm{OH^-}}$$
$$\mathrm{R-CON^-Hal} \rightarrow \mathrm{R-N=C=O} \rightarrow$$
$$\mathrm{R-NHCOOH} \rightarrow \mathrm{R-NH_2 + CO_2}$$

Obviously, the reaction requires an ionizable hydrogen atom at the amide nitrogen of the haloamide, that is, the reaction can proceed only with *primary* amides. Many imides, especially phthalimides, do however undergo this reaction which has usually been explained by an initial hydrolytic ring-opening, either of the free imide [2] or of the *N*-chloro derivative formed from this,[3] followed by rearrangement of the haloamide formed:

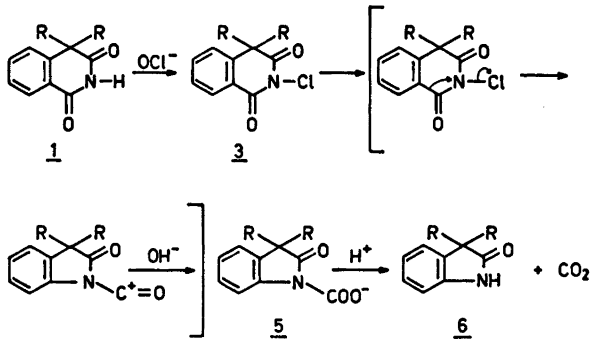


(For alternative hypotheses see Ref. 4, 5). Our results with 4,4-tetramethylenehomophthalimide, although only qualitative, clearly demonstrate that the hydrolysis of this imide is a slow reaction requiring several hours at room temperature to proceed to any considerable extent. In sharp contrast to this is the rapid formation of the indolinone derivative from this imide on reaction with alkaline sodium hypochlorite solution: almost 90 % of the indolinone derivative is obtained after a reaction time of less than 15 min. at room temperature. It can therefore be concluded that at least in this case hydrolysis of the free imide (Scheme 1, route A) is not the initial step but that the reaction is initiated by *N*-chlorination of the imide. The *N*-chloro derivative *3* might then either undergo hydrolysis to the *N*-chloro primary amide *4* (Scheme 1, route B) or rearrange directly (Scheme 1, route C). The direct rearrangement C may conceivably proceed either without participation of OH^- ions according to Scheme 2, or by the "concerted" mechanism outlined in Scheme 4. Whatever the operating mechanism may be, the carbamic acid derivative *5* is an intermediate, since its sodium salt was isolated on cooling the reaction mixture and characterised by conversion to the methyl ester with dimethyl sulphate.[1]

The Scheme 2 mechanism appeared at first sight to be a very plausible alternative, its greatest merit being the fact that it obviates the necessity for hydrolytic ring-opening of the imide. In order to test the validity of this mechanism, the reaction was carried out in the presence of $H_2{}^{18}O$: non-incorporation of ^{18}O into the indolinone formed would strongly support the proposed mechanism. MS measurements showed that formation of the indolinone *6* was accompanied by incorporation of ^{18}O. As, however, independent experiments with heavy water showed that whereas *5* did not exchange ^{18}O with the medium, the imide *1* did so very rapidly, this observation was without meaningful interpretation. Consequently, the *N*-chloro derivative *3* was prepared separately and made to react with sodium hydroxide solution. It was found that already



Scheme 1.



Scheme 2.

after about ten minutes, an 80 % yield of the indolinone *6* could be isolated after acidification of the reaction mixture, and that it contained incorporated ^{18}O to the extent required by 100 % participation of OH^- ions from the medium. Although a rapid exchange of oxygen in the chloroimide followed by rearrangement according to Scheme 2 is not entirely ruled out by these experiments this apparently means that the *N*-chloro derivative *3* rearranges either *via* a conventional hydrolysis reaction (Scheme 1 route B) or by way of the "concerted" reaction (Scheme 4) as all efforts to effect the rearrangement with for example silver salts in an inert solvent have failed so far. That rapid hydrolysis of *3* is not inconceivable may be deduced from the fact that *N*-methoxy-4,4-tetrahydrohomophthalimide, which both sterically and electronically closely resembles the *N*-chloro analogue *3*, is virtually completely hydrolysed by 1 M sodium hydroxide in 50 % aqueous dioxane at room temperature in less than 5 min. (The resistance offered by the imide *1* to alkaline hydrolysis must apparently by ascribed to the effect exerted by its anion, which would tend to repel the approach of OH^- ions from the medium). Since no experiments could be devised to distinguish between these two mechanisms (Scheme 1, route B and Scheme 4), the choice was finally made on the basis of indirect evidence derived from the Lossen reaction, as discussed further on.

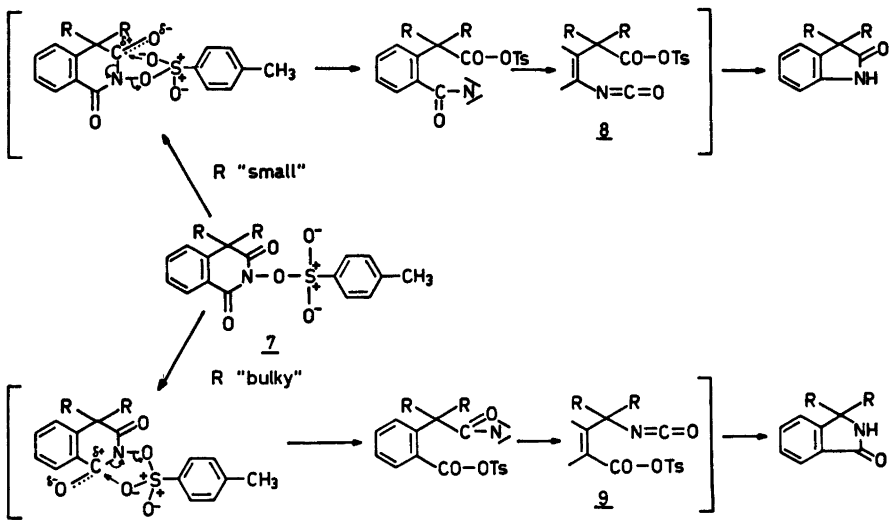
Both of the above rearrangement mechanisms would also explain the role played by the 4-substituent in governing the course of the reaction: increasing "bulkiness" of the substituent would shift attack from the more electrophilic but more hindered aliphatic carbonyl group to the less electrophilic but less hindered aromatic one, thus favouring transition from indolinone to isoindolinone formation. This is in complete alignment with the finding that the 4,4-dimethyl-, 4,4-tetramethylene-, and 4,4-pentamethylenehomophthalimides gave high yields of the corresponding indolinones, 4,4-diallylhomophthalimide gave moderate yields of both indolinone and isoindolinone derivatives, and 4,4-diethylhomophthalimide afforded solely the isoindolinone derivative in moderate yield. The 4,4-dipropyl- and 4,4-dibutylhomophthalimides did not react at all, indicating that here the steric effect extends to block reaction even at the imide nitrogen or aromatic carbonyl group.

A semi-quantitative indication of the relative degrees of reactivity of the carbonyl groups in the various 4,4-dialkylhomophthalimides could be obtained by determining the rate of ^{18}O incorporation. It can be seen from Table 4 that the velocity of exchange decreases in the series

$$R = -(CH_2)_4- \gg C_2H_5-;$$
$$CH_2=CHCH_2- > n\text{-}C_3H_7-$$

This is in agreement with the found reactivities of the homophthalimides with respect to the Hofmann rearrangement reaction.

In order to obtain further insight into the mechanism of the Hofmann reaction, the Lossen rearrangement of the corresponding *N*-tosyloxyhomophthalimide derivatives *7* was studied, as it is known that these two reactions

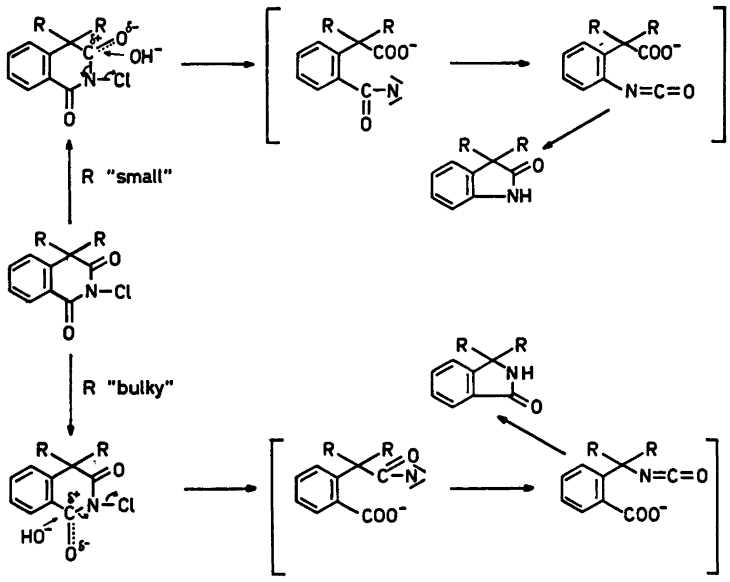


Scheme 3.

proceed according to the same mechanism. It was found that when the *N*-tosyloxy derivatives of homophthalimide substituted with the 4,4-dimethyl-, 4,4-tetramethylene-, 4,4-diallyl-, 4,4-diethyl-, and 4,4-dibutyl groups were warmed in aqueous dioxane containing potassium carbonate, the first two members of the series afforded the corresponding indolinone derivatives, the allyl derivative gave a mixture of indolinone and isoindolinone, the ethyl derivative gave only isoindolinone, and the butyl derivative also afforded small amounts of the isoindolinone. It is obvious that the substituent in the 4-position has a profound directive influence on the course of the reaction. Also, since the electronic effects of the methyl group must be very similar to that of the ethyl group, this influence must be steric rather than electronic in nature. Furthermore, experiments in heavy water revealed that *incorporation of ^{18}O occurred neither in the indolinones nor in the isoindolinones formed* which means that in this case the attacking species is not an OH^- ion and the reaction does not involve hydrolytic opening of the imide ring. An attractive mechanism reconcilable with this finding would be the one outlined in Scheme 3, since this would explain in a satisfactory manner the directive influence of the 4-substituent. This mechanism is entirely analogous to the one proposed for the Hofmann reaction, with the difference that the leaving group here is a toluene sulphonate instead of a chloride ion and the attacking species is an intramolecular sulphonyl oxygen instead of an external hydroxyl ion. The increasing steric hindrance exerted by the 4-substituent with increasing bulk would progressively shift the attack from the aliphatic to the aromatic carbonyl group, resulting in formation of indolinone or isoindolinone as the case may be.

As was mentioned above in the discussion of the Hofmann rearrangement, the experiments devised cannot distinguish between a reaction involving normal hydrolysis of the *N*-chlorohomophthalimide (Scheme 1, route B) and a "concerted" reaction not involving a distinct hydrolytic step (Scheme 4). However, if we accept the Lossen mechanism as a justifiable model for the closely related Hofmann reaction, we would favour the entirely analogous "concerted" mechanism as outlined below (Scheme 4) in preference to the stepwise hydrolysis and subsequent rearrangement.

It was a fortuitous circumstance for the elucidation of the mechanism of the Lossen rearrangement that in these compounds the unique structural constellation of the postulated mixed anhydride derivatives *8* and *9* imparted to these intermediates a much greater tendency to ring-closure than to reaction with the



Scheme 4.

environmental water, for otherwise correct interpretation of the heavy water experiments would not have been possible.

EXPERIMENTAL

All melting points were taken with a Heræus Fus-O-Mat apparatus. Mass spectra were determined on an LKB 9000 instrument. Microanalyses were carried out by Prof. K. J. Karrman, University of Lund, Lund, Sweden.

N-Chloro-4,4-tetramethylenehomophthalimide and its Hofmann rearrangement. A mixture of 4,4-tetramethylenehomophthalimide (16 g; 0.147 mol) and *tert*-butyl hypochlorite (16 g; 0.147 mol) was stirred in a waterbath at 60°C for 3 h, by when a clear orange solution had formed. Excess hypochlorite was removed in vacuum and the residual oil was recrystallized from 175 ml of diisopropyl ether, giving 15.3 g (83 %) of white crystals, m.p. 85 – 94°C. An analytically pure sample melted at 90 – 94°C. (Found: C 62.8; H 4.86; N 5.60; O 12.8; Cl 13.9. $C_{13}H_{12}ClNO_2$ requires: C 62.5; H 4.85; N 5.61; O 12.8, Cl 14.2).

The above chloro derivative (1.25 g; 0.005 mol) was treated with ice-cold 2 M sodium hydroxide solution (10 ml; 0.02 mol) and stirred at room temperature for 10 min. Acidification with acetic acid afforded 730 mg (78 %) of 3,3-tetramethyleneindolin-2-one, m.p. 113°C.

When the rearrangement reaction was carried out in a medium containing 20 atom-% of $H_2{}^{18}O$, the indolinone isolated contained about 9 % of the labelled isomer of M.W. 189.

N-Chloro-4,4-diethylhomophthalimide and its Hofmann rearrangement. A mixture of 4,4-diethylhomophthalimide (10.9 g; 0.05 mol) and *tert*-butyl hypochlorite (10 ml; 0.092 mol) in dry benzene (50 ml) was stirred at room temperature overnight. Removal of volatile material in vacuum afforded 12.4 g (99 %) of a thick oil which slowly crystallized on standing. An analytical sample recrystallized from petroleum ether melted at 51 – 56°C. (Found: C 61.6; H 5.5; Cl 14.8; N 5.5; O 12.9. $C_{13}H_{14}ClNO_2$ requires: C 62.0; H 5.6; Cl 14.1; N 5.6; O 12.7).

The above chloro derivative (2.5 g; 0.01 mol) was warmed and stirred with 2 M sodium hydroxide solution (20 ml; 0.04 mol) for 45 min, the reaction mixture was cooled, acidified with acetic acid and the product collected, giving 1.4 g (74 %) of 3,3-diethylisoindolin-1-one, m.p. 166 – 169 °C.

General method for the preparation of 4,4-dialkyl-N-hydroxy-homophthalimides. Sodium (2.5 g; 0.11 mol) was added to dry methanol (50 ml) and when reaction was complete the solution was cooled and treated with a hot solution of hydroxylamine hydrochloride (7.7 g; 0.11 mol) in water (5 ml). To the stirred solution was added the appropriate substituted homophtalic acid (or, preferably, the anhydride, obtained by heating the acid) and the mixture was refluxed for 4 to 6 h, then stirred at ambient temperature overnight. Dilute hydrochloric acid was added, the solution taken to dryness

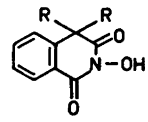


Table 1. Data for 4,4-dialkyl-*N*-hydroxyhomophthalimides.

R	M.p. °C	Yield %	Recryst. solv. [a]	Formula	Analysis Calc. C	H	N	O	Found C	H	N	O
CH_3	82	95	DIP/PET	$C_{11}H_{11}NO_3$	64.4	5.4	6.8	23.0	64.4	5.4	6.8	23.5
C_2H_5	128–130	69	DIP	$C_{13}H_{15}NO_3$	66.9	6.5	6.0	20.6	67.1	6.5	5.7	20.7
C_4H_9	184–186	70	MET	$C_{17}H_{15}NO_3$	70.6	8.0	4.8	16.6	68.7	8.0	4.7	16.1
$CH_2=CHCH_2$	123–128	93	DIP/PET	$C_{15}H_{15}NO_3$	70.0	5.9	5.4	18.7	70.0	5.9	5.4	18.9
$-(CH_2)_4-$ (–R–R–)	116–118	82	CHL/PET	$C_{13}H_{13}NO_3$	67.5	5.7	6.1	20.8	67.2	5.7	6.0	20.9

[a] DIP = Diisopropyl ether, PET = petroleum ether, MET = methanol, and CHL = chloroform.

under vacuum, the residue taken up in hot acetone, the sodium chloride filtered off and the filtrate taken to dryness, giving an oil which became crystalline on cooling. The data of the derivatives thus prepared are presented in Table 1.

General method for the preparation of 4,4-dialkyl-N-tosyloxyhomophthalimides. A solution made up from the appropriate 4,4-dialkyl-*N*-hydroxyhomophthalimide (0.05 mol), DMF (200 ml) and 1 M sodium hydroxide solution (50 ml; 0.05 mol) was added dropwise at room temperature to a solution of tosyl chloride (10 g; 0.052 mol) in chloroform (150 ml). The mixture was stirred at room temperature for 2 to 20 h, the layers were separated and the aqueous phase was extracted with chloroform. The combined chloroform phase was washed with water, with dilute hydrochloric acid and finally with sodium carbonate solution. After drying, the solvent was removed, giving an oil which generally crystallized on standing. The data of the derivatives thus prepared are presented in Table 2.

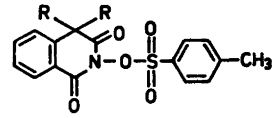
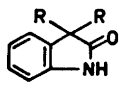
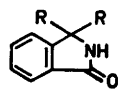


Table 2. Data for 4,4-dialkyl-*N*-tosyloxyhomophthalimides.

R	M.p. °C	Yield %	Recryst. solv.[a]	Formula	Analysis Calc. C	H	N	O	Found C	H	N	O
CH_3	135	50 [b]	AC/DIP	$C_{16}H_{17}NO_5S$	60.2	4.8	3.9	8.9	60.1	4.9	3.9	8.9
C_2H_5	132–133	75	MET	$C_{20}H_{21}NO_5S$	62.0	5.5	3.6	8.3	61.8	5.4	3.6	8.2
C_4H_9	94–98	90	MET	$C_{24}H_{29}NO_5S$	65.0	6.6	3.2	7.2	64.8	7.0	3.2	7.3
$CH_2=CHCH_2$	64–68	73	MET	$C_{22}H_{21}NO_5S$	64.2	5.1	3.4	7.8	64.3	5.2	3.4	7.8
$-(CH_2)_4-$ (–R–R–)	118–120	65	MET	$C_{20}H_{19}NO_5S$	62.3	5.0	3.6	8.3	62.6	5.0	3.7	8.1

[a] AC = acetone, MET = methanol, and DIP = diisopropyl ether. [b] Also obtained 36 % yield of 3,3-dimethylindolin-2-one.

Table 3. Lossen rearrangement of 4,4-dialkyl-*N*-tosyloxyhomophthalimides.

R	% Products isolated 		Remarks
CH_2	68	–	
C_5H_8	–	58	Reaction in $H_2{}^{18}O$: 2 % ^{18}O incorporated.
C_4H_9	–	26	M.p. 81 – 84 °C. Product isolated by silica-gel column chromat. Found: C 77.7; H 9.5; N 5.8; O 7.0. $C_{13}H_{23}NO$ requires C 78.3; H 9.5; N 5.7; O 6.5.
$CH_2=CHCH_2$	47	31	Products separated by silica-gel column chrom.
$-(CH_2)_4-$ (–R–R–)	74	–	Reaction in $H_2{}^{18}O$: < 1 % ^{18}O incorporated.

General procedure for the Lossen rearrangement reaction. A mixture made up from the 4,4-dialkyl-*N*-tosyloxyhomophthalimide (0.01 mol), dioxane (10 ml), potassium carbonate (1.5 g; 0.011 mol) and water (5 ml) was stirred in an oil-bath (80 °C) overnight. Volatile material was removed under vacuum, the residue was treated with water, extracted with chloroform, the organic extract was washed with water, dried (K_2CO_3), the solvent was removed and the residual product was recrystallized. Pertinent data of the products obtained are presented in Table 3. In the experiments with isotope labelling, water containing 20 atom-% of $H_2{}^{18}O$ was used.

In the reaction with the dibutyl derivative, the product was characterised as isoindolinone by the NMR spectrum, in which the aromatic H-atoms appear at τ 2.0 – 2.6 in contrast to their appearance at τ 2.8 – 3.0 in the indolinones. In addition, the UV-spectrum in methanol exhibits a peak at 225 nm, as did also other isoindolinones, in contrast to the peak appearing at 247 nm in the indolinones (*cf.* Ref. 1).

N-Methoxy-4,4-tetramethylenehomophthalimide. A mixture of *N*-hydroxy-4,4-tetramethylenehomophthalimide (23.1 g; 0.1 mol), potassium carbonate (13.8 g; 0.1 mol) and water (100 ml) was warmed until a clear solution had been obtained when dimethyl sulphate (10.3 ml; 0.11 mol) in acetone (50 ml) was added dropwise. After stirring in the water-bath for 2.5 h, an additional 10 ml of dimethyl sulphate was added, whereafter the mixture was stirred in the water-bath for a further 4 h. After standing at room temperature overnight, most of the acetone was removed by distillation, causing a heavy oil to separate out. This was washed with water by decantation whereupon it solidified, giving 22.4 g (91.5 %) of white crystals, m.p. 75 – 80 °C. Recrystallization from methanol gave white crystals m.p. 77 – 81°C. (Found: C 68.6; H 6.1; N 5.7; O 19.7. $C_{14}H_{15}NO_3$ requires: C 68.6; H 6.1; N 5.7; O 19.6).

α,α-Diallylhomophthalic acid. Ethyl *o*-carbethoxyphenyl acetate (59 g; 0.25 mol) in DMF (100 ml) was added dropwise at room temperature under vigorous stirring to a mixture of allyl bromide (72 ml; 0.8 mol) and sodium hydride (50 % in oil; 30 g; *ca.* 0.7 mol) in DMF (400 ml). The stirred mixture was warmed on the water bath (*ca.* 70 °C) for 5 h, then stood at room temperature overnight. The inorganic salt was filtered off, the filtrate was taken to dryness under vacuum, the residual oil was treated with 5.5 M sodium hydroxide solution (100 ml) and the mixture refluxed overnight. After dilution with water, the solution was extracted with chloroform, the aqueous phase was treated with dilute hydrochloric acid to incipient turbidity (pH *ca.* 5), the solution was stirred with decolourising carbon, filtered through a Celite pad, and the filtrate strongly acidified with hydrochloric acid. The resulting viscous oil was extracted into chloroform, the extract was washed with brine, dried (Na_2SO_4) and taken to dryness under vacuum, giving a crystalline residue weighing 51 g (78.5 %), m.p. *ca.* 140 °C. Fractional crystallization from a mixture of chloroform-ether-petroleum ether 1:1:1 afforded 27.7 g (42 %) of the diacid, m.p. 139 °C dec., and 20.0 g (30.3 %) of a colourless oil which was identified by NMR as practically pure anhydride. (Found: C 69.0; H 6.2; O 24.5.

$C_{16}H_{16}O_4$ requires: C 69.2; H 6.2; O 24.6 %).

Experiments with ^{18}O-labelling. ^{18}O-Labelling of 4,4-dialkylhomophthalimides. The homophthalimide derivative (0.1 mmol) and sodium hydroxide (0.25 mmol) in 0.1 ml of water containing 20 atom-% of $H_2{}^{18}O$ was allowed to stand at room temperature for various times, then acidified with acetic acid and the precipitated imide isolated. The amount of incorporated ^{18}O was determined by MS. The results are presented in Table 4.

Table 4. ^{18}O-Exchange of 4,4-dialkylhomophthalimide (*1*).

R	Reaction time	Content of labelled isomer %
$-(CH_2)_4-$	12 min	11
	25 min	20
	4 h	20
	96 h[a]	30
C_2H_5	24 h	10
$CH_2=CHCH_2$	24 h	10
C_3H_7	24 h	4–5

[a] Also contains 5 % of isomer M. W. 219.

^{18}O-Exchange of N-carboxy-4,4-tetramethyleneindolin-2-one. The sodium salt of the carbamic acid derivative *5*[1] (26 mg; 0.1 mmol) was dissolved in a solution of sodium hydroxide (9.1 mg; 0.23 mmol) in 0.2 ml of water containing 10 atom-% of $H_2{}^{18}O$. After 3 h at room temperature the solution was warmed on a water bath for 10 min, cooled, acidified with dilute acetic acid and the resulting 4,4-tetramethyleneindolin-2-one was isolated. MS measurements showed that it contained practically no incorporated heavy oxygen.

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Algal Carotenoids. IX.* Absolute Configuration of β,ε-Caroten-2-ol, β,β-Caroten-2-ol, and β,β-Carotene-2,2′-diol

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The previous tentative stereochemical assignments 2*R*,6′*R* for β,ε-caroten-2-ol (*1a*), 2*R* for β,β-caroten-2-ol (*2a*), and 2*R*,2′*R* for β,β-carotene-2,2′-diol (*3*) have been confirmed.

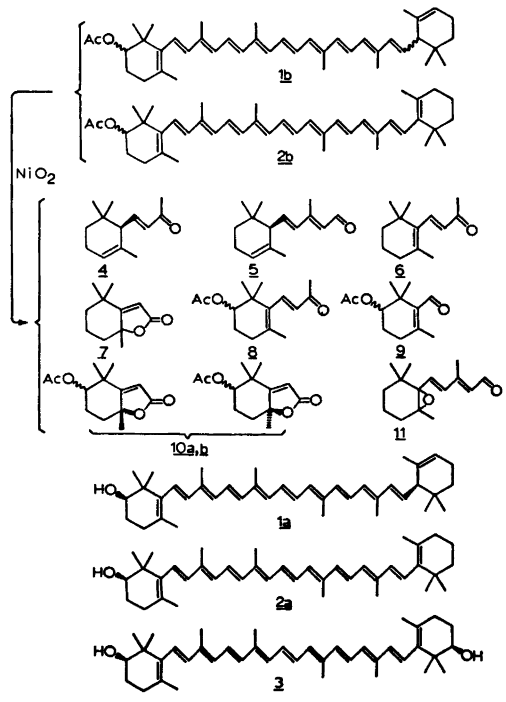
Oxidative (NiO_2) degradation of a mixture of β,ε-caroten-2-yl acetate (*1b*) and β,β-caroten-2-yl acetate (*2b*) gave *ca.* 50 components (GLC), among which (+)-α-ionone (*4*), the ε-apo-carotenal *5*, β-ionone (*6*), dihydroactinidiolide (*7*), 2-acetoxy-β-ionone (*8*), 2-acetoxy-β-cyclocitral (*9*), the *cis-trans* isomeric acetylated lactones *10a,b* and presumably the artefact epoxide *11* were identified from GLC, UV, MS, and CD data.

Whereas isolation of (+)-α-ionone (*4*) defined the 6′*R* configuration of *1a*, the 2*R* configuration followed from application of the modified Horeau method to *2a* and previous CD correlations.

For convenience carotenoid numeration has been used.

Recently we have isolated the first carotenoids possessing 2-hydroxylated β-rings, namely β,ε-caroten-2-ol (*1a*), β,β-caroten-2-ol (*2a*) and β,β-carotene-2,2′-diol(*3*) from the green alga *Trentepohlia iolithus* (L.) Wallroth.[1] Whereas complete spectral characterization defined the constitutions involved, the chemical reactions of these carotenoids have subsequently been studied.[2]

As to their absolute configurations the fact that β,β-carotene-2,2′-diol (*3*) and zeaxanthin [= (3*R*,3′*R*)-β,β-carotene-3,3′-diol] exhibited very similar CD curves of opposite sign was rationalized employing Mills' rule.[3] Regarding both *3* and zeaxanthin as 4-hydroxylated cyclohexene derivatives with preferred pseudo-equatorial hydroxy groups, 2*R* and 3*R* configuration, respectively, represent opposite half-chair conformations with opposite optical rotation. 2*R*-Stereochemistry for the mono-ol *2a* then followed from CD correlation. Moreover, by using the additivity hypothesis of Klyne and co-



Scheme 1.

* Part VIII. *Phytochemistry. In press.*

workers [4] 2*R*,6′*R* stereochemistry was inferred for *1a*; see Scheme 1.

Since the application of Mills' rule may lead to erroneous results,[5] it was necessary to check the validity of Mills' rule in this case. Furthermore, independent confirmation of the 6′*R* stereochemistry of *1a* was desirable.

RESULTS AND DISCUSSION

Since β,ε-caroten-2-ol (*1a*) and β,β-caroten-2-ol (*2a*) can only be separated with difficulty,[1] oxidative degradation of a mixture of *1a* and *2a* was effected in the Swiss laboratory by the procedure worked out for lutein (= β,ε-carotene-3,3′-diol).[6]

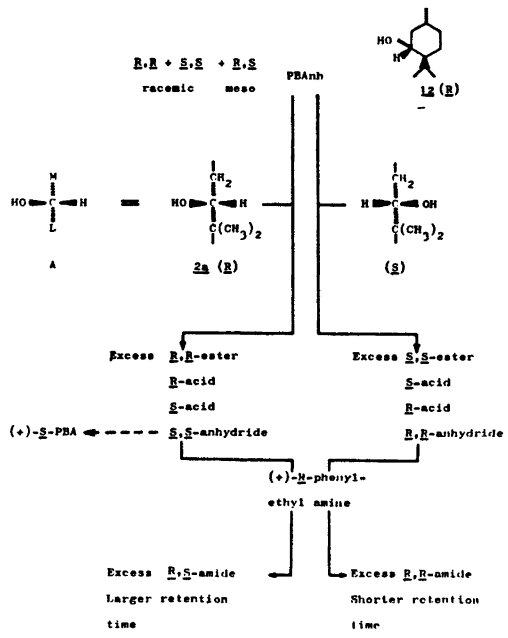
The hydroxy groups were protected by acetylation and a *ca.* 1:1 mixture of semicrystalline *1b* and *2b*, Scheme 1, was oxidized with NiO_2 to give approximately 50 components by GLC. By preparative GLC and subsequent UV, MS, and CD characterization, the following compounds were identified:

(+)-(6*R*)-α-Ionone (*4*) with predicted GLC, UV, and MS properties had opposite Cotton effect to that of (−)-α-ionone (6*S*).[6,7] The ε-apo-11-carotenal (*5*) had GLC, UV,[8] and MS properties consistent with structure *5*. 6*R*-Stereochemistry followed from positive CD 298 nm (curve conform to but bathochromically shifted relative to that of *4*).

β-Ionone (*6*), inseparable from authentic β-ionone, had MS properties as predicted. Dihydroactinidiolide (*7*) [9] with GLC, UV, and MS data consistent with this assignment, was according to the CD data, a racemic mixture. Both *6* and *7* are considered derived from the unsubstituted β-rings and offer no stereochemical information. The same is true for a minor component, from MS alone tentatively identified as the epoxide *11* and considered to be an artefact caused by the oxidation reagent.

Three compounds derived from the 2-acetylated β-end groups were isolated, namely 2-acetoxy-β-ionone (*8*), 2-acetoxy-β-cyclocitral (*9*), and a presumed *cis-trans* isomeric mixture of the acetylated lactones *10a,b*. Again the identifications were based on GLC, UV, and MS data. The CD spectrum of 2-acetoxy-β-cyclocitral (*9*) was analogous to and hypsochromically displaced by *ca.* 20 nm relative to that of the analogue *8* (both resembling that of *2a* itself). Were comparison of the CD spectra of *8* and of 2-acetoxy-β-ionone of known configuration possible, stereochemical conclusions could be made on this basis.

However, evidence for the 2*R* configuration for *2a* was obtained independently using the modified Horeau method.[10] Briefly the Horeau method [11–14] is based on the partial resolution of racemic and meso α-phenylbutyric anhydride (PBAnh) by means of an optically active alcohol, which reacts preferentially with one diastereomer, followed by hydrolysis and optical rotation measurement of the unreacted α-phenylbutyric acid (PBA), Scheme 2.



Scheme 2.

When the isolated PBA is dextrorotatory the alcohol in concern has configuration A; L(arge) and M(edium) refer to the space requirements of the substituents. In the modified version,[10] Scheme 2, the excess enantiomer of the PBAnh is reacted with an optically active primary amine [(+)-*R*-α-phenylethyl amine] followed by quantitative separation of the diastereomeric amides by GLC. Since the chiral amine does not react equally fast with the two enantiomeric PBAnh, parallel experiments with an achiral secondary alcohol like cyclohexanol must be performed.

In the original modification Brooks and Gilbert [10] used a different column for the GLC separation. The relative retention time for the two diastereomeric amides were therefore checked with (−)-*R*-menthol (*12*), corresponding to configuration A, Scheme 2, and consequently providing excess *R,S*-amide.

Three separate experiments with *β,β*-caroten-2-ol (*2a*) each resulted in excess formation of the *R,S*-amide using achiral cyclohexanol in the reference reaction. Lower optical yields in the experiments with *β,β*-caroten-2-ol (*2a*) than for (−)-*R*-menthol (*12*) are explained by the lower solubility of *2a*.

Configuration A, corresponding to *β,β*-caroten-2*R*-ol (*2a*) is thus proved in agreement with the previous tentative assignment.[1] 2*R*-Configuration for *3a* and *1a* follows from the CD correlations already made.[1]

Furthermore it follows from this independent determination of the absolute configuration of *β,β*-caroten-2-ol (*2a*) that 2-acetoxy-*β*-ionone (*8*), 2-acetoxy-*β*-cyclocitral (*9*), and the diastereomeric lactones *10a,b* (Scheme 1) all have the 2*R* configuration.

The fact that the 6′*R* configuration of *1a* corresponds to that of *ε*-rings in related carotenoids isolated from higher plants, suggests a common enzymatic cyclization mechanism.

The implications of the 2*R* stereochemistry here established for the 2-hydroxy-*β*-type carotenoids for the stereochemistry of 2-isopentenyl substituted carotenoids (C_{50}) will be treated elsewhere.[15]

EXPERIMENTAL PART

Materials. Mixed crystalline mono-ols (*1a* and *2a*) were obtained from *Trentepohlia iolithus* after chromatography of the saponified mono-ester fraction as described previously.[1]

Crystalline *β,β*-caroten-2-ol (*2a*) was obtained after chromatography of the mixed mono-ols on magnesia.[1]

Solvents used were of analytical grade.

Instruments. UV spectra (ethanol, if not stated otherwise) were recorded on a Beckman Acta III spectrometer and CD spectra (ethanol, if not stated otherwise) on a Roussel-Jouan Dichrographe model 185 with a xenon high pressure lamp. Due to the small samples these spectra are qualitative only.

Mass spectra were obtained on a GLC/MS Varian MAT CH5 instrument.

Preparative GLC was effected on a Perkin Elmer 900 gas chromatograph with packed glass column 2.5 m/3 mm type SP 1000 (4 % Carbowax 20 M, treated with *p*-nitroterephthalic acid on Chromosorb 100 − 120 mesh) programmed at 160 − 240°C, 5°/min, He-flow 65 ml/min.

Retention times cited below were for an analytical glass capillary column Ucon HB 5100 + H_3PO_4 20 m/0.32 mm, programmed from 80 to 180°C, 2°/min, 0.4 atm. pressure.

For the Horeau experiments a glass capillary column OV 101 + FFAP 23 m/0.3 mm, isotherm, 160°C, 0.5 atm. pressure was employed.

Oxidative degradation

Acetylation. 1a and *2a* (140 mg, 43:57) in pyridine (25 ml) was acetylated quantitatively with acetic anhydride (5 ml) for 24 h at room temperature; yield 139 mg (90 %) *1b* and *2b*.

Degradation. The mixed acetates (*1b* and *2b*, 139 mg) dissolved in ether-benzene (1:1, 100 ml) and NiO_2[16] (8 g) were stirred mechanically at room temperature until decolouration occurred (36 h). The filtrate was concentrated to dryness; yield 28 mg comprising *ca.* 50 components by analytical GLC.

Retention times by analytical GLC were: *α*-ionone (*4*) 10.9 min, *β*-ionone (*6*) 14.3 min, 2-acetoxy-*β*-cyclocitral (*9*) 23.1 min, dihydroactinidiolide (*7*) 25.3 min, *ε*-apo-11-carotenal (*5*) 29.3 min, 2-acetoxy-*β*-ionone (*8*) 37.6 min, and the isomeric lactones *10a,b* 46.8 and 47.1 min.

(+)-(6R)-α-Ionone (4), yield *ca.* 0.3 mg, inseparable from authentic *α*-ionone by co-chromatography, had *m/e* 192 (M), 177 (M − CH_3), 43 (CH_3CO); UV λ_{max} 227 nm; CD (hexane, nm, relative intensities) 368 (−1.0), 352 (−3.5), 337 (−5.0), 324 (−5.3), 315 (−4.0), 244 (+43.5).

(−)-*α*-Ionone (6*S*) for comparison had CD (methylcyclohexane: isopentane 1:3, nm, $\Delta\varepsilon$) 372 (+0.19), 353 (+0.61), 338 (+0.92), 325 (+0.93), 312 (+0.71), in alcohol also 243 (−17.33).

ε-Apo-11-carotenal (*5*) had *m/e* 218 (M); UV λ_{max} 286 nm, reported [8] 286 nm in ethanol: CD (nm, strong) 298 (+), 273 (+), 205 (end absorption +).

β-Ionone (*6*), inseparable from authentic *β*-ionone, had *m/e* 192 (M), 177 (M − CH_3), 43 (CH_3CO).

Dihydroactinidiolide (*7*) had *m/e* 180 (M), 165 (M − CH_3); UV λ_{max} 225 nm, reported [9] 241 nm, solvent unknown; no CD (racemic).

2-Acetoxy-β-ionone (*8*) had *m/e* 250 (M), 235 (M − CH_3), 190 (M − AcOH), 175 (M − CH_3 − AcOH), 43 (CH_3CO); UV λ_{max} 286 nm, reported [17] for *β*-ionone (*6*) 295 nm in ethanol; CD (nm, weak) 370 − 320 (+), 290 − 260 (−), 250 (0), 240 − 200 (end absorption +).

2-Acetoxy-β-cyclocitral (*9*) had *m/e* 210 (M), 195 (M − CH_3), 150 (M − AcOH); UV λ_{max} 243 nm, reported [18] for *β*-cyclocitral 247.5 nm in ethanol; CD (nm) *ca.* 363 (−), 342 (0), *ca.* 315

(+), 282 (0), 250–240 (–), 233 (0), 200 (end absorption +).

Isomeric lactones 10a,b had *m/e* 238 (M), 178 (M–AcOH), 43 (CH_3CO); UV λ_{max} 218 nm, *cf. 7*.

Epoxidic aldehyde 11 had *m/e* 234 (M), 219 (M–CH_3), 191 (M–CH_3–CO→oxepinium ion, $m^*=156$).

Modified Horeau experiments

Procedure by Brooks and Gilbert [10] was used directly for (–)-menthol (*12*) and the first experiment with β,β-caroten-2-ol (*2a*); in the latter case in a heterogeneous system due to low solubility.

α-Phenylbutyric anhydride was prepared as described by others.[19,20]

(–)-R-*Menthol* [10] (*12*). The retention time found for *R*-α-phenylethyl- *R*-α-phenylbutyramide was 7.0 min and for *R*-α-phenylethyl-*S*-α-phenylbutyramide 8.1 min. Standard reaction with cyclohexanol gave the ratio *R,S*-amide:*R,R*-amide = 1:(1.115 ± 0.007). Reaction with *12* gave ratio *R,S*-amide:*R,R*-amide = 1:(0.866 ± 0.015); corrected value by comparison with standard reaction 1:0.866/1.115 = 1:0.775.

β,β-Caroten-2-ol (*2a*). (i) *2a* (4.8 mg) in pyridine (14 μl) and PBAnh (6 μl) was heated in a sealed Pyrex tube at 40–45°C for 1 3/4 h. (+)-*R*-α-phenylethyl amine (6 μl) was added at room temperature, the mixture shaken for 15 min and transferred to ethyl acetate (3–4 ml) for GLC.

Found for standard reaction with cyclohexanol (1 μl = 1 mg) ratio *R,S*-amide:*R,R*-amide 1:(1.115 ± 0.007).

Found for *2a* ratio *R,S*-amide:*R,R*-amide 1:(0.95 ± 0.02); corrected ratio 1:0.85.

(ii) Procedure as for (i) but using *2a* (2.9 mg), pyridine (21 μl, all carotenoid dissolved) PBAnh (3.6 μl) and (+)-*R*-α-phenylethyl amine (3.6 μl).

Found for standard reaction with cyclohexanol (0.6 μl) ratio *R,S*-amide:*R,R*-amide 1:(1.085 ± 0.01).

Found for *2a* ratio *R,S*-amide:*R,R*-amide 1:(0.945 ± 0.007); corrected ratio 1:0.87.

(iii) Procedure as for (i) but using *2a* (2.4 mg), pyridine (14 μl, all carotenoid dissolved), PBAnh (3 μl) and (+)-*R*-α-phenylethyl amine (3 μl).

Found for standard reaction with cyclohexanol (0.5 μl) ratio *R,S*-amide:*R,R*-amide = 1:(1.120 ± 0.011).

Found for *2a* ratio *R,S*-amide:*R,R*-amide = 1:(0.928 ± 0.008); corrected ratio 1:0.83.

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The Reduction of Lignin Model Compounds and Spruce Dioxane Lignin by the Birch Reaction

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Several phenyl methyl ethers and spruce dioxane lignin were reduced by treatment with sodium in liquid ammonia in the presence of ethanol (Birch reduction). The product mixtures were resolved by vapor phase chromatographic techniques and the reduction products identified using combinations of NMR, IR, UV and mass spectrometric methods.

The aromatic moiety in 3,4-dimethoxyphenyl (veratryl) structures was reduced to a mixture of the corresponding unconjugated dihydro and tetrahydro derivatives. In addition, a methoxyl substituent was replaced by hydrogen, giving rise to 4-substituted di- and tetrahydroanisoles. In this instance both conjugated and unconjugated dienes were present with the latter greatly predominating. By contrast, reduction of a syringyl derivative, 3,4,5-trimethoxytoluene, resulted in the formation of the 1,4-dihydro derivative in 80 % yield as the only major product. With phenyl ethers containing a benzyl alcohol, α-keto or an α- or β-aryl ether substituent, reduction of these groups to the corresponding hydrocarbon took place and generally preceded reduction of the aromatic ring.

Reduction of spruce dioxane lignin with sodium in liquid ammonia under similar conditions resulted in the isolation of a material whose spectral properties were consistent with those of 2,5-dihydro-4-propylanisole.

Metal–liquid ammonia treatment of isolated lignins and softwood and hardwood meal has been used by Freudenberg,[1,2] Shorygina,[3–6] and Yamaguchi [7,8] and their co-workers, primarily as a means of studying lignin structure. As a consequence of this type of treatment, interunitary ether linkages were found to undergo hydrogenolysis yielding a variety of guaiacyl and syringylpropane monomeric fragments in which the propyl substituent exhibited varying degrees of reduction. The commonly observed net effect of the overall reaction is an increase in the hydroxyl content and a decrease in the methoxyl content of the lignin.

The discovery of Wooster, [9] subsequently exploited by Birch, [10–12] that the presence of a suitable proton source in the liquid ammonia solution promotes the reduction of benzenoid and other aromatic ring systems was particularly significant in the development of dissolving metal chemistry. In the application of dissolving metal reductions to lignin reported to date, no external proton source appears to have been added. There is, however, the strong possibility that lignin (in particular the phenolic hydroxyl and terminal methylol groups) may function as its own proton source in such reductions.

The extensive changes occurring when lignin is subjected to a dissolving metal reduction makes such a process theoretically attractive from a chemical modification viewpoint.[13] An examination of the literature indicates, however, a lack of systematic studies designed to describe the complete scope of possible transformations occurring when lignin is reacted under Birch reduction conditions. In attempting to fill this void, studies utilizing lignin model compounds appear to offer the best approach for isolating and subsequently cataloging the various reaction types in view of the complexity of the overall process.

Accordingly, the present investigation consisted mainly of an examination of the reactions of comparatively simple lignin model com-

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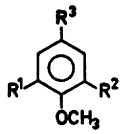
Table 1. Model compounds reacted with sodium in liquid ammonia.



Compound	R^1	R^2	R^3
I	H	H	H
V	H	OCH_3	H
VIII	H	OCH_3	CH_3
XV	OCH_3	OCH_3	CH_3
XVII	H	OCH_3	CH_2-O-(*o*-methoxyphenyl)
XVIII	H	OCH_3	$CH(OH)CH_3$
XXIII	H	OCH_3	$CH(OH)CH_2-O-$(*o*-methoxyphenyl)
XXIV	H	OCH_3	$COCH_3$
XXVI	H	OCH_3	$COCH_2-O-$(*o*-methoxyphenyl)
XXVII	H	H	$O-$(*p*-methoxyphenyl)

pounds with sodium-liquid ammonia solutions in the presence of an external proton source, ethanol; *i.e.*, with a Birch reduction system. The models selected for study (see Table 1) consisted solely of compounds whose phenolic rings were completely etherified. This arbitrary structural restriction was made on the basis of results from a previous study [10] where it was shown that with few exceptions [10,14,15] unetherified phenolic units were not reduced by metals in liquid ammonia.

The model compound study was augmented by a brief examination of the behavior of Norway spruce dioxane lignin in the same reduction system.

RESULTS AND DISCUSSION

Products formed from the reactions of the various models with sodium in liquid ammonia in the presence of ethanol are summarized in Table 2. Spectrometric data essential to the identification of the various products are compiled in Table 3.

Anisole (I). The reduction of anisole with Na/NH_3 in the presence of ethanol was previously reported by Birch [10] to yield 2,5-dihydroanisole (II) as the principal product. In a subsequent investigation, Benkeser *et al.* [16] presented evidence indicating the probable formation of two additional compounds, 2,3-dihydroanisole (III) and 3,4,5,6-tetrahydroanisole (IV). The yields of reaction products recorded in Table 2 agree closely with those reported by Benkeser [16] using either sodium or lithium as the

Table 2. Reaction products and product yields.

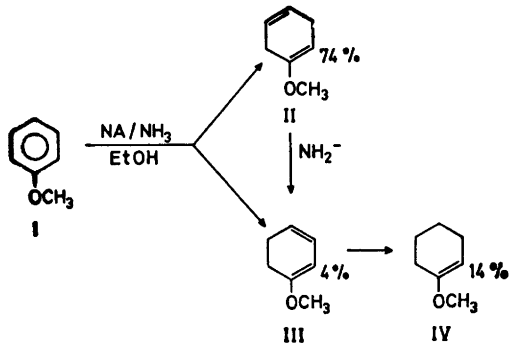
Starting material	Products	Yield, %
Anisole (I)	2,5-dihydroanisole (II)	74
	2,3-dihydroanisole (III)	4
	2,3,4,5-tetrahydroanisole (IV)	14
	phenol [a]	Trace
Veratrole (V)	3,6-dihydroveratrole (VI)	19
	2,5-dihydroanisole (II)	37
	2,3-dihydroanisole (III)	3
	3,4,5,6-tetrahydroveratrole (VII)	24
	2,3,4,5-tetrahydroanisole (IV)	9
	guaiacol [a]	7
4-Methylveratrole (VIII)	2,5-dihydro-4-methylanisole (IX)	25
	2,3-dihydro-4-methylanisole (X)	0.4
	3,6-dihydro-4-methylveratrole (XI)	57
	2,3,4,5-tetrahydro-4-methylanisole (XII)	1.9
	3,4,5,6-tetrahydro-4-methylveratrole (XIII)	6.6
	5-methylguaiacol (XIV)	3.3

Table 2. Continued.

Starting material	Products	Yield, %	
3,4,5-Trimethoxytoluene (XV)	1,4-dihydro-3,5-dimethoxytoluene (XVI)	80	
	di- and tetrahydroveratroles	Trace	
		0.5 h	4 h
3,4-Dimethoxy-α-(*o*-methoxyphenoxy)toluene (XVII)	2,5-dihydro-4-methylanisole (IX)	25	27
	2,3-dihydro-4-methylanisole (X)	< 0.1	< 0.1
	3,6-dihydro-4-methylveratrole (XI)	68	62
	2,3,4,5-tetrahydro-4-methylanisole (XII)	0.9	1.9
	3,4,5,6-tetrahydro-4-methylveratrole (XIII)	2.4	6.8
	5-methylguaiacol	[b]	1.9
	guaiacol [a]	[b]	~100
1-(3,4-Dimethoxyphenyl) ethanol (XVIII)	2,5-dihydro-4-ethylanisole (XIX)	46	
	3,6-dihydro-4-ethylveratrole (XX)	41	
	2,3,4,5-tetrahydro-4-ethylanisole (XXI)	1.7	
	3,4,5,6-tetrahydro-4-ethylveratrole (XXII)	3.7	
1-(3,4-Dimethoxyphenyl)-2-(*o*-methoxyphenoxy)ethanol (XXIII)	2,5-dihydro-4-ethylanisole (XIX)	50	
	3,6-dihydro-4-ethylveratrole (XX)	47	
	2,3,4,5-tetrahydro-4-ethylanisole (XXI)	1.3	
	3,4,5,6-tetrahydro-4-ethylveratrole (XXII)	5.6	
	guaiacol [a]	50	
Acetoveratrone (XXIV)	2,5-dihydro-4-ethylanisole (XIX) + 2,5-dihydro-3-ethylanisole (XXV)	50	
	3,6-dihydro-4-ethylveratrole (XX)	16	
	2,3,4,5-tetrahydro-4-ethylanisole (XXI)	1.0	
	3,4,5,6-tetrahydro-4-ethylveratrole (XXII)	1.7	
	polymer-like material	8.0	
3,4-Dimethoxyphenyl-2-*o*-methoxyphenoxy acetophenone (XXVI)	2,5-dihydro-4-ethylanisole (XIX) + 2,5-dihydro-3-ethylanisole (XXV)	57	
	3,6-dihydro-4-ethylveratrole (XX)	29	
	2,3,4,5-tetrahydro-4-ethylanisole (XXI)	1.1	
	3,4,5,6-tetrahydro-4-ethylveratrole (XXII)	2.9	
	guaiacol [a]	89	
4,4′-Dimethoxydiphenyl ether (XXVII)	2,5-dihydroanisole (II)	0.9	
	2,2′,5,5′-tetrahydro-4,4′-dimethoxydiphenyl ether (XXVIII)	87 [c]	

[a] Identified by comparison of gas chromatographic retention time with that of authentic material (anisole, phenol, or guaiacol) and estimated by comparison with peak areas corresponding to known amounts of the corresponding authentic substance. [b] Not measured. [c] Yield based on weight of neutral fraction which was almost pure XXVIII.

metallic component. The principal steps in the overall reaction are outlined below.



The comparatively low yield of 2,3-dihydroanisole (III) undoubtedly reflects the facile reduction of the conjugated diene system to the tetrahydro derivative.

As indicated by the data in Table 2, reductive cleavage resulting in the formation of phenol occurred to less than 0.1 %. In the absence of ethanol under otherwise identical conditions, the yield of phenol was 27 %. Partial conversion (isomerization) of II to III probably from the action of the amido ion formed in the equilibrium reaction

$$^{-}OC_2H_5 + NH_3 \rightleftharpoons NH_2^{-} + C_2H_5OH$$

on the former structure.[17,18]

Veratrole (V). Veratrole was previously reported by Birch [12] to yield a mixture of 2,5-dihydroanisole (II) and 3,6-dihydroveratrole (VI) with the former predominating. In the present study, the same two products were obtained together with other compounds listed in Table 2. The total yield of products (~99 %) accounts essentially for all of the starting material.

The nature of the products indicates that two main competing reactions occur: (1) replacement of one methoxyl group with a hydrogen resulting ultimately in the formation of anisole reduction products and (2) reduction to di- and tetrahydroveratrole. The fact that the ratios of the three anisole derivatives parallel those of the corresponding products resulting from direct treatment of anisole indicates that the methoxyl group is replaced by a hydrogen before the aromatic nucleus is reduced.

The detection of a significant amount (7 %) of guaiacol indicates the occurrence of a competing ether cleavage reaction. The high yield of guaiacol compared to that of phenol is consistent with the substituent effect noted by Birch.[12]

4-Methylveratrole (VIII). The two principal products detected in the reaction mixture, 2,5-dihydro-4-methylanisole (IX) and 3,6-dihydro-4-methylveratrole (XI), together account for 82 % of the starting material (Table 2).

The position of the methoxyl group relative to the methyl group in the anisole derivatives was established by comparison of the IR and NMR spectra of 2,3,4,5-tetrahydro-4-methylanisole with those of the isolated products. The NMR spectrum of the conjugated compound 2,3-dihydro-4-methylanisole (X), which shows two vinyl protons as two doublets (*cf.* Table 3), further reinforces the validity of the structure assignment.

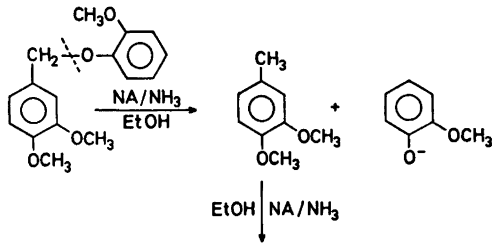
Spectra of the phenolic compound (XIV) suggested the presence of a methylguaiacol. Comparison of the IR and NMR spectra of this compound with those of authentic 4- and 5-methylguaiacol (XIV) clearly indicated that it was the latter isomer.

3,4,5-Trimethoxytoluene (XV). The reaction mixture consisted of only one major product, 1,4-dihydro-3,5-dimethoxytoluene (XVI), formed in 80 % yield and of trace amounts of di- and tetrahydroanisoles and veratroles. Failure of the 1,4-dihydro derivative to undergo further reduction is attributed to the difficulty in conjugating double bonds which involves displacement of one double bond from a position in which it is conjugated with the unshared electron pair of the oxygen of a methoxyl group. Benzoic acid derivatives typically give rise to 1,4-dihydro compounds under Birch conditions, and the previously reported [19] conversion of 3,4,5-trimethoxybenzoic acid to 1,4-dihydro-3,5-dimethoxybenzoic acid by such treatment is particularly relevant to the case at hand.

In an attempt to form a bromo derivative, 1,4-dihydro-3,5-dimethoxytoluene was reacted with bromine in CCl_4. The isolated product, 2,6-dibromo-3,5-dimethoxytoluene, ostensibly arose from a series of bromine additions and hydrogen bromide eliminations.

3,4-Dimethoxy-α-(o-methoxyphenoxy)toluene (XVII). Because of the small amount of material available for reaction, none of the products were isolated but were identified and estimated

by comparing retention times and peak areas with those of authentic materials isolated from the reduction of 4-methylveratrole (VIII). The yields of the various products obtained in the reduction of this ether are very nearly identical to those of the corresponding compounds resulting from the reduction of 4-methylveratrole (see Table 2). This suggests that the benzyl aryl ether linkage undergoes rapid hydrogenolysis generating guaiacol and 4-methylveratrole, which subsequently undergoes reduction to di- and tetrahydro anisoles and veratroles.



di- and tetrahydroanisoles and veratroles

That the hydrogenation of the benzyl aryl ether linkage is complete is indicated by the essentially quantitative recovery of guaiacol. The results in Table 2 also reveal that the overall reaction is virtually complete in 30 min or less and that the products thereafter undergo little additional change.

1-(3,4-Dimethoxyphenyl)ethanol (XVIII). Inspection of Table 2 indicates that the two dihydro products, XIX and XX, account for nearly 90 % of the starting material. While these two products were recovered from the product mixture, the tetrahydro compounds XXI and XXII were identified solely on the basis of similarities in the gas chromatographic patterns of the product mixture with those of veratrole and 4-methylveratrole. Quantitative analysis of XXI and XXII was accomplished by comparing their peak areas with that corresponding to a specified quantity of 3,6-dihydro-4-ethylanisole.

The general similarity of the product distribution to that noted above for veratrole and 4-methylveratrole leads to the conclusion that the starting material undergoes a stepwise reaction involving first hydrogenolysis of the benzyl alcohol group followed by reduction of the product, 4-ethylveratrole, by the usual pattern. The total absence of hydroxyl group characteristics in the various product fractions supports the conclusion that reduction of this alcohol to the corresponding hydrocarbon is essentially quantitative.

1-(3,4-Dimethoxyphenyl)-2-(o-methoxyphenoxy)ethanol (XXIII). The distribution of reaction products replicates in general the now familiar pattern observed for other veratryl derivatives in which the dihydroanisole and dihydroveratrole derivatives account for the bulk of the starting material. The observed reductive cleavage of the β-aryl ether group is especially significant since linkages of this type comprise a major portion of the interunitary linkages in lignin. In this regard, treatment of lignin with sodium in liquid ammonia has, in fact, resulted in the formation of monomeric fragments in which the β-carbon of the side chain was completely reduced as would be expected if hydrogenolysis of the β-aryl ether linkage had occurred.

The less-than-theoretical recovery of guaiacol (50 %) probably has no fundamental significance since the other cleavage product can be fully accounted for in terms of the partially reduced 4-ethylanisoles and 4-ethylveratroles. Failure to achieve the expected near-quantitative recovery of guaiacol is ascribed to mechanical losses (*e.g.*, volatilization) in the workup.

As in the previous instance, the reduction products were identified by their relative positions in the gas chromatogram of the product mixture and were estimated by comparing their areas with the peak area produced by injection of a known amount of 3,6-dihydro-4-ethylveratrole (XX).

Acetoveratrone (XXIV). Acetoveratrone reacted more vigorously and was converted to products which collectively had sustained a greater loss of methoxyl groups than the models discussed heretofore. These observations can be logically rationalized on the basis of the electron-withdrawing property of the aceto group which would have the effect of activating the ring toward attack by a solvated electron.

Chromatography of the product mixture on an OV-1 column (maintained at 90 °C) resulted in the collection of a fraction whose NMR spectrum indicated the presence of a nonconjugated dihydroanisole with the methoxyl group situated either *meta* or *para* to the side chain.

When the chromatographic separation was repeated on a preparative size Carbowax column maintained at a considerably higher temperature (160 °C), the NMR spectrum (vinyl proton region) of the fraction corresponding to the same peak suggested the presence of a mixture consisting of 2,5- and 2,3-dihydro-4-ethylanisole. Evidently isomerization of the unconjugated to the conjugated compound had occurred as a result either of the higher column temperature or as the result of reaction with the column itself.

Additional peaks in the NMR spectrum of the material isolated from the Carbowax column, and in particular a signal at δ 1.65 (doublet), suggest the presence of 2,3-dihydro-3-ethylanisole. If this compound was indeed present in the product mixture, it most probably can be considered as an artifact resulting from isomerization of 2,5-dihydro-3-ethylanisole on the chromatographic column. If 2,5-dihydro-3-ethylanisole is a product in the Birch reduction of acetoveratrone, the increased tendency for replacement of methoxyl at the *para* position would accord with the activating effect of the aceto group on this position. This would further imply that some replacement of the *para* methoxyl group occurred prior to reduction of the α-carbonyl group.

The foregoing observations cannot be taken as providing incontrovertible evidence for the presence of 2,5-dihydro-3-ethylanisole in the product mixture. Nevertheless. if present, this compound was not separated from the corresponding 4-ethyl isomer under the imposed chromatographic conditions. Accordingly, the value reported in Table 2 is the combined yield of both isomers.

3,4-Dimethoxyphenyl-2-(o-methoxyphenoxy)-acetophenone (XXVI). The reductive pattern of this compound was similar to that of acetoveratrone in that more than 50 % of the starting material was accounted for by the dihydroanisole derivatives (Table 2). The yield of 3,6-dihydro-4-ethylveratrole was, however, nearly double the value obtained for acetoveratrone. The reduction of the veratryl (3,4-dimethoxyphenyl) moiety was accompanied by the near quantitative cleavage of the β-aryl ether linkage as indicated by an 89 % recovery of guaiacol (Table 2). It is also noteworthy that, whereas reduction of acetoveratrone was accompanied by the formation of what appeared to be polymeric substances, material of this type was absent in the case at hand. This observation probably accounts for the difference in the total yield of recovered products in each instance.

4,4'-Dimethoxydiphenyl ether (XXVII). The diaryl ether linkage represented by the title compound was not extensively cleaved under Birch reduction conditions, and the sole cleavage product detected was 2,5-dihydroanisole in 0.9 % yield. The only other product identified was the tetrahydrodiphenyl ether (XXVIII) which accounted for nearly 90 % of the starting material.

The effect of para substituents on the cleavage of diaryl ether linkage in Na/NH_3 solutions has been studied by Sartoretto and Sowa.[30] These authors found that methoxyl groups situated *para* to the ether linkage retarded the cleavage reaction. Further reduction of partially reduced diaryl ether XXVIII in all likelihood is retarded since both double bonds in each ring contain oxygen-bearing (electron-releasing) substituents.

Dioxane lignin. In order to enhance the probability of obtaining monomeric fragments with partially reduced phenolic rings, the dioxane lignin was pretreated with borohydride to remove carbonyl groups followed by methylation with diazomethane to convert the phenolic hydroxyl groups to methyl ethers. The modified lignin was dissolved in liquid ammonia and reacted with sodium (Na/phenylpropane, ~20/1) in the presence of ethanol. The reaction mixture was fractionated into neutral, acidic and dioxane-soluble components as described in the Experimental section.

The neutral fraction was separated by vapor phase chromatography into two components which were collected and subjected to spectroscopic analysis. The NMR and mass spectra of the component with the longer retention time were consistent with the patterns expected for 2,5-dihydro-4-propylanisole (see XXIX in Table 3). The assignment of the methoxyl *para* to the propyl group is arbitrary but considered justifiable in the light of the results of the model studies and since the influence of the α-carbonyl groups on cleavage of the *para*-situated methoxyl had been eliminated by prior reduction.

The amount of 2,5-dihydro-4-propylanisole (XXIX) was estimated from peak area measure-

Table 3. NMR, IR, UV and mass spectral properties of reaction products.

Product	NMR, δ[a]	IR[b], cm^{-1}	Other (UV or mass spectra)
II	($CDCl_3$) 5.71 (s, 2 H), 4.67 (bs, 1 H), 3.53 (s, 3 H), 2.76 (s, 4 H)	3010(m), 2920(m), 2810(m), 1680(s), 1210(s), 1175(s), 1160(s)	UV: λ_{max}, 211 nm
III	(CCl_4) 5.82(q[c], 1 H, J = 6.0 Hz), 5.17–5.53 (m, 1 H), 4.86 (d, 1 H, J = 6.0 Hz), 3.52 (s, 3 H), 2.21 (s, 2 H), 2.19 (s, 2 H)	3030(m), 2930(s), 2810(s), 1640(s), 1580(s), 1250(s), 1210(s), 1155(s),	UV: λ_{max}, 269 nm
IV	($CDCl_3$) 4.54–4.73 (m, 1 H), 3.47 (s, 3 H), 1.90–2.03 (m, 4 H), 140–1.80 (m, 4 H)	2920(s), 2820(m), 1660(s), 1200(s), 1170(s), 1155(s)	–
VI	($CDCl_3$) 5.63(s[d], 2 H), 3.62(s, 2 H), 2.87(s, 4 H)	3020(m), 2920(m), 2810(s), 1700(3), 1210(s), 1150(m), 1115(m)	–
VII	($CDCl_3$) 3.57(s, 6 H), 1.97–2.28 (m, 4 H), 1.47–1.73(m, 4 H))	2910(s), 2820(m), 1680(m), 1210(s), 1150(m), 1110(m)	–
IX	(CCl_4) 5.27–5.46(m, 1 H), 4.42–4.63 (m, 1 H), 3.54(s, 3 H), 2.59(s, 4 H), 1.69(bs, 3 H)	2980(m), 2920(m), 2800(s), 1690(m), 1660(s), 1220(s), 1170(s), 1130(s)	–
X	(CCl_4) 5.51(bd, 1 H, J = 6.0 Hz), 4.77(d, 1 H, J = 6.0 Hz), 3.50(s, 3 H), 2.18(s, 4 H), 1.72(bs, 3 H)	2920(m), 2810(m), 1650(s), 1600(s), 1240(s), 1215(s), 1160(s)	UV: λ_{max}, 271 nm
XI	(neat) 5.15–5.36(m, 1 H), 3.54 (s, 3 H), 3.51(s, 3 H), 2.69(s, 4 H), 1.64(bs, 3 H)	2920(s), 2810(s), 1700(m), 1670(w), 1210(s), 1150(s), 1100(s),	–
XII	($CDCl_3$) 4.38–4.62(m, 1 H), 3.48 (s, 3 H), 1.80–2.33(m, 2 H), 145–1.80(m, 4 H), 0.96(d, 3 H, J = 4.5 Hz)	2910(s), 2820(m), 1720(m), 1660(s), 1220(s), 1200(s), 1170(s), 1150(s)	UV: λ_{max}, 209 nm
XIII	(CCl_4) 3.51(s, 6 H), 1.71–2.30 (m, 4 H), 1.47–1.71(m, 2 H), 0.92–1.10(m. 3 H)	2910(s), 2820(m), 1680(m), 1210(s), 1120(m)	UV: λ_{max}, 209 nm
XIV	($CDCl_3$) 6.5–7.0(m, 3 H), 5.84 (s, 1 H; exchanges with D_2O), 3.71(s, 3 H), 2.20(s, 3 H)	3500(s), 2920(m), 2820(m), 1590(s), 1500(s), 1230(s), 1205(s), 1170(m), 1140(m), 1125(s), 790(s), 760(s)	–
XVI	(neat) 4.45–4.62(m, 2 H), 3.46 (s, 6 H), 2.80–2.36(m, 1 H), 2.75 (bs, 2 H), 1.06(d, 3 H, J = 6.5 Hz)	2940(s), 2810(m), 1685(s), 1660(m), 1230(s), 1200(s), 1140(s), 1125(s)	–
XIX	(CCl_4) 5.27–5.52(m, 1 H), 4.47–4.65(m, 1 H), 3.50(s, 3 H), 2.50–2.88(m, 4 H), 2.03(q, 2 H, J = 6.0 Hz), 1.04(t, 3 H, J = 6.0 Hz)	2945(s), 2860(m), 2805(s), 1690(m), 1660(m), 1215(s), 1170(m), 1135(m),	Mass spectrum: Base peak *m/e* 109; parent peak, *m/e* 138 (75 %)
XX	(CCl_4) 5.18–5.38(m, 1 H), 3.57 (s, 3 H), 3.55(s, 3 H), 2.70(s, 4 H), 1.99(q, 2 H, J = 6.0 Hz), 1.02 (t, 3 H, J = 6.0 Hz)	2945(s), 2860(m), 2805(s), 1700(m), 1210(s), 1150(m), 1095(s)	Mass spectrum: Base peak *m/e* 139; parent peak, *m/e* 172 (10 %)

Table 3. Continued.

Product	NMR, δ	IR,[b] cm^{-1}	Other (UV or mass spectra)
XXVIII	($CDCl_3$) 4.87–5.18(m, 2 H), 4.40–4.70(m, 2 H), 3.50(s, 6 H), 2.81 (s, 8 H)	2920(m), 2880(m), 2820(m), 1670(s), 1210(s), 1160(s)	Mass spectrum: Base peak *m/e* 111; parent peak, *m/e* 234 (53 %)
XXIX	(CCl_4)5.30–5.50(m, 1 H), 4.44–4.67 (m, 1 H), 3.54(s, 3 H), 2.50–2.80 (m, 4 H), 1.75–2.30(m, 2 H), 1.25–1.75(m, 2 H), 0.78–1.13(m, 3 H)	–	Mass spectrum: Base peak *m/e* 109; parent peak, *m/e* 152 (78 %)
XXX	(CCl_4) 6.70–6.90(m, 3 H), 3.89(s, 3 H), 2.30–2.86(m, 2 H), 1.40–2.00(m, 2 H), 0.74–1.12(m, 3 H)	(CCl_4), 3550(s), 2945(s), 2920(s), 1605(m), 1505(s), 1275(s), 1245(m), 1220(m), 1165(m), 1140(m)	Mass spectrum: Base peak *m/e* 137; parent peak, *m/e* 166 (91 %)

[a] bs=broad singlet, bd=broad doublet. [b] Spectra measured as films except as otherwise indicated. [c] Further splitting observed. [d] Shoulders present.

ment to be ~1 % using 2-methoxy-4-propylphenol as a standard. This low yield is consistent with the expectation that the dihydro compound would arise only from those lignin monomer units which originally possessed free phenolic hydroxyl groups and which had no carbon-carbon bonds linking either the aromatic ring or the side chain to the adjoining unit. Based on the lignin structural scheme proposed by Freudenberg,[21] approximately only 5 % of the units in the polymer network meet these requirements.

The NMR spectrum of the neutral component with the lower retention time did not correspond to any of the spectra of the products identified in the model compound reductions.

The acidic fraction was several fold larger than the neutral fraction and may be accounted for by the hydrogenolytic conversion of phenolic ether groups to phenols which are stable to reduction under Birch conditions. Gas chromatographic analysis of the acidic fraction revealed the presence of only one peak. The material corresponding to this peak was collected (200 mg or 1.6 %) and subjected to IR, NMR, and mass spectroscopic analysis. Collectively, these spectra suggested that the material was a phenol with methoxyl and propyl groups attached to the ring. Since the IR spectrum did not correspond to that of authentic 4-propylguaiacol, the compound has been tentatively identified as 2-methoxy-5-propylphenol (XXX, Table 3). If this assignment is correct, the formation of this compound would be analogous to and consistent with the previously noted detection of 2-methoxy-5-methylphenol in the reduction of 4-methylveratrole.

Lignin-derived structures, such as the above, which contain a methoxyl substituent *para* to side chain, would necessarily have as their origin phenolic units which subsequently were converted to methyl ethers in the methylation pretreatment. This structural restriction accounts in part for the low recovery of XXX.

An NMR spectrum of the dioxane-soluble fraction displayed a broad band in the aromatic proton region but no pronounced signal in the vinyl proton region. This fraction appeared therefore to consist mainly of phenolic oligomers and was not investigated further.

Summary

1. Veratrole and its derivatives were reduced under Birch conditions to mixtures of di- and tetrahydroanisoles and veratroles. The dihydro-derivatives consisted mainly of the unconjugated type (1,4-addition) with much smaller amounts of conjugated cyclohexadiene derivatives being formed, either by 1,2-addition or from isomerization of unconjugated diene systems.

2. The relative proportion of the products varied somewhat from reaction mixture to reaction mixture in a manner which appeared to reflect differences in the electron-donating or electron-withdrawing characteristics of the sub-

stituent at the 1-position (*i.e.*, the side chain). The side chain substituent gave evidence of influencing not only the sites and degree of ring reduction but also the relative extents of replacement of the *meta*- and *para*-situated methoxyl groups as well.

3. Unlike the veratryl derivatives whose reduction gave rise to complex mixtures of partially reduced anisoles and veratroles, Birch reduction of 3,4,5-trimethoxytoluene afforded a comparatively simple product mixture consisting essentially of one product hydrogenated at the 1- and 4- positions with concomitant loss of the 4-methoxyl group.

4. Model compounds having α- and β-aryl ether substituents on the side chain were reduced in a stepwise fashion with cleavage of ether linkage preceding ring reduction. Hydrogenolysis in such cases was rapid and produced essentially quantitative yields of the phenol corresponding to the aryl substituent. Under the same conditions, methyl aryl ether groups were hydrogenolyzed to the corresponding phenols only to a slight extent, and the diphenyl ether linkage in 4,4′-dimethoxydiphenyl ether appeared entirely resistant to cleavage. In this latter case, the individual rings were each reduced to the dihydro stage in high yield.

5. Birch reduction of spruce dioxane lignin led to the formation of a neutral material in approximately 1 % yield which the available evidence strongly indicated to be 2,5-dihydro-4-propylanisole. Other evidence suggested that phenolic fragments in the monomer to oligomer range were produced as a consequence of hydrogenolysis of alkyl aryl ether linkages.

Conclusion

The present model compound study has shown that phenol ethers are extensively converted to cyclohexene and cyclohexadiene derivatives by reduction with sodium in liquid ammonia in the presence of a proton donor. Reduction of the aromatic ring is largely preceded by reduction of α-carbonyl groups and hydrogenolysis of benzyl alcohol and α- and β-aryl ether groups.

With respect to lignin itself, results of the current study lead to the conclusion that Birch reduction of phenolic ether moieties will occur only to a very limited extent since phenolic structures, preferentially formed through hydrogenolysis of phenyl alkyl ether units, generally are not reduced under Birch conditions. Some Birch reduction can be expected to take place, however, on those units having ether units more resistant to hydrogenolysis (*e.g.*, diphenyl ether units) and on phenolic units converted to methyl ethers prior to reduction as demonstrated by the present study.

EXPERIMENTAL

Synthesis of reactants. 3,4-Dimethoxy-α-(*o*-methoxyphenoxy)toluene (XVII) was prepared as described by Mikawa,[22] m.p. 77 – 78 °C [lit.[22] 78 – 79 °C]. 3,4,5-Trimethoxytoluene (XV) was obtained by a Clemmensen reduction [23] of syringaldehyde [24] followed by methylation with dimethyl sulfate and alkali, [25] b.p. 95 – 98 °C at 2 – 3 mmHg. NMR (neat): δ 6.43 (s, 2 H), 3.78 (s, 3 H), 3.73 (s, 6 H), 2.26 (s, 3 H).

Acetoguaiacone was methylated with dimethyl sulfate and alkali to produce acetoveratrone (XXIV), b.p. 135 – 136 °C at 2 – 3 mmHg. NMR ($CDCl_3$): δ 7.37 – 7.70 (m, 2 H), 6.76 – 7.00 (m, 1 H), 3.90 (s, 3 H), 2.55 (s, 3 H). Reduction of XXIV with sodium borohydride afforded 1-(3,4-dimethoxyphenyl)ethanol (XVIII), b.p. 105 – 110 °C at 2 – 3 mmHg. NMR (CCl_4): δ 6.82 (bs, 1 H), 6.70 (s, 2 H), 4.64 (q, 1 H, $J = 5.5$ Hz), 3.67 (s, 6 H), 3.59 (s, 1 H), 1.32 (d, 3 H, $J = 5.5$ Hz).

1-(3,4-Dimethoxyphenyl)-2-(*o*-methoxyphenoxy)ethanol (XXIII) was prepared by reduction of the corresponding ketone, [26] m.p. 133.5 – 134.0 °C [lit.[34] 133 – 134 °C]. 4,4′-Dihydroxydiphenyl ether was converted to the corresponding dimethyl ether (XXVII) by methylation with dimethyl sulfate and alkali, m.p. 100 – 102 °C [lit. [28] 102 °C].

Anisole, veratrole, and 4-methylveratrole were obtained commercially and used without further purification.

Synthesis of products. 2,3,4,5-Tetrahydroanisole (IV) was prepared by reaction of cyclohexanone with trimethyl orthoformate in the presence of mesitylene sulfonic acid as described by Lindsay and Reese.[29] Distillation of the product mixture through a Vigreux column yielded a fraction boiling at 139 – 142 °C [lit. [29] 138 – 142 °C] whose NMR and IR spectral characteristics (Table 2) were consistent with those expected for the compound. The same procedure was used to prepare 2,3,4,5-tetrahydro-4-methylanisole (XII) from 4-methylcyclohexanone; b.p. range of XII, 159 – 161.5 °C.

2,3-Dihydroanisole was obtained by two methods. The first consisted of treatment of 2-cyclohexen-1-one with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid as outlined by Nussbaum *et al.* [30] The product mixture was distilled through a Vigreux column

and the fraction boiling at 146 – 147 °C collected. Gas chromatographic analysis indicated that the product was approximately 80 % pure.

2,3-Dihydroanisole was also obtained by reacting a fraction comprised of a mixture of 2,3-dihydroanisole and 2,5-dihydroanisole with potassium *t*-butoxide [31] to isomerize the non-conjugated component. The fraction boiling at 142 – 145 °C was approximately 70 % pure as indicated by gas chromatographic analysis. This fraction was further purified by preparative gas chromatography (see Table 3 for NMR and IR characteristics).

3-Hydroxy-4-methoxytoluene (5-methylguaiacol) was prepared by subjecting isovanillin (3-hydroxy-4-methoxybenzaldehyde) to a Clemmensen reduction. The product was distilled under reduced pressure yielding an oil which solidified on cooling, m.p. 32 – 33 °C [lit. [12] 33 °C]. NMR ($CDCl_3$): δ 6.56 – 6.80 (bd, 3 H), 5.60 (s, 1 H), 3.67 (s, 3 H), 2.18 (s, 3 H).

4-Propylanisole was prepared by the catalytic hydrogenation of isoeugenol methyl ether.

Other products were obtained by preparative gas chromatography of the product mixtures and subsequently purified.

Bromination of 1,4-dihydro-3,5-dimethoxytoluene. 1,4-Dihydro-3,5-dimethoxytoluene (200 mg) was dissolved in CCl_4 and reacted at room temperature with a solution of bromine in the same solvent. After removal of solvent, a solid was obtained which after recrystallization from methanol/water melted at 170 °C. Yield, 88 mg, mass spectrum (70 eV): parent ion (p) and base peak at *m/e* 312, p + 2 and p – 2 peaks are 54 % of parent peak; NMR ($CDCl_3$): 6.46 (s, 1 H), 3.89 (s, 6 H), 2.62 (s, 3 H).

Reduction of model compounds. Approximately 400 ml of liquid NH_3 was distilled from a cylinder through a glass filter and condensed in a 1 l, three-necked, round-bottomed flask by means of a dry ice/acetone-cooled cold finger. The flask was immersed in a dry ice/acetone bath and fitted with a dropping funnel to which was attached a $CaCl_2$ drying tube.

The aromatic compound (0.2 mol) in ethanol* (1.0 mol) was added to the ammonia through the dropping funnel and the mixture heated to its reflux temperature by removal of the dry ice/acetone bath. Sodium (1.0 mol) was then added carefully in 0.5 – 1.0 g amounts; and when the addition was completed, the mixture was allowed to reflux for 4 h. Some additional ammonia was distilled into the flask during this period to maintain the level constant. Under these conditions, the starting compound was for all practical considerations completely consumed in all instances.

The reaction was quenched after replacing the cold finger with a stopper, by the slow addition of 100 ml of methanol through the dropping funnel. After most of the excess sodium had reacted (~ 30 min), 100 ml of water was added and the mixture was extracted with 5 × 50 ml of ether (neutral fraction); the water phase was acidified with concentrated HCl and extracted with 5 × 50 ml ether (acidic fraction).

Small samples of the neutral and acidic fractions were set aside for quantitative VPC analysis. The remaining portions of the two fractions were concentrated by distilling off the solvent through a Vigreux column at atmospheric pressure.

Preparation and modification of dioxane lignin. Dioxane lignin was prepared from 150 g of ethanol-benzene (1:1) extracted Norway spruce wood meal by treatment with a solution of 1,4-dioxane/water (9:1) containing 0.2 N HCl as described by Browning.[32] Yield, 22.8 g. Anal.: C 59.45; H 5.67; OCH_3 14.27.

To maximize the possibility of obtaining Birch reaction products, the dioxane lignin was first modified by reducing the carbonyl groups with $NaBH_4$ and etherifying the phenolic hydroxyl groups with diazomethane. Dioxane lignin (10.5 g) in 440 ml of $EtOH/H_2O$ (1:1) and 120 ml of 0.1 M NaOH was reacted with 6.4 g of $NaBH_4$ overnight.[33] The reaction mixture was acidified to pH 4 with hydrochloric acid and concentrated. Water was added and the resulting precipitate was centrifuged off and washed twice with water. Yield, 9.3 g. An IR spectrum of the product indicated that all but a trace of carbonyl had been removed.

The borohydride-reduced lignin (9.1 g) was partially dissolved in 200 ml of 1,4-dioxane and reacted twice with diazomethane over a 2 h period. The solution was concentrated to 200 ml and after removal of a small portion for IR and elemental analysis, was reacted directly with Na/NH_3 in the presence of ethanol. Anal.: C 62.08; H 5.90; OCH_3 16.51.

Birch reduction of modified dioxane lignin. Modified dioxane lignin dissolved in 1,4-dioxane (see previous section) was reacted for 4 h in the standard way using 400 ml of liquid ammonia, 23.0 g of sodium, and 46.0 g of ethanol. After the addition of methanol and water and evaporation of the ammonia, a brownish-yellow liquid remained which contained no undissolved material.

The reaction mixture was extracted with 5 × 50 ml of ether (neutral fraction) following which the water phase was acidified with concentrated hydrochloric acid. A brown precipitate formed upon acidification which did not dissolve when the mixture was extracted with 5 × 50 ml of $CHCl_3$ (acidic fraction). The polymer-like precipitate was filtered off and dissolved in 1,4-dioxane (dioxane-soluble fraction).

The amount of material in each fraction in solution was obtained by evaporating an aliquot of each solution to dryness and weighing the residue: neutral fraction, 0.35 g; acidic fraction, 1.85 g; and dioxane-soluble fraction, 3.45 g.

Separation of reaction products. Preliminary

* Dioxane (50 ml) was employed as a co-solvent to dissolve compounds XVII, XXIII, XXVI, and XXVII.

attempts to separate the product mixtures into their individual components by distillation through Vigreux and spinning band columns were generally unsuccessful. Chromatography on a silver nitrate-silica gel column [34] was similarly unproductive, seemingly as a result of reaction between the products and the column.

Satisfactory separation of the product mixtures was ultimately obtained using preparative-scale gas chromatography. The retention time data in these analyses were diagnostically useful for classifying peaks as corresponding to dihydro unconjugated, dihydro conjugated, and tetrahydro cyclohexyl ring systems.

Preparative-scale gas chromatography was performed on an F & M Model 776 gas chromatograph. A 220 × 1.8 cm stainless steel column was filled with 3 % Carbowax 20M-TPA on Chromosorb G (30/60). Column conditions: oven, 90 – 150 °C (isothermal): detector, 190 °C; injector, 170 °C; manifold, 180 °C; flow rate, 200 ml N_2/min. A 300 – 400 µl amount of 50 % (by volume) ether solution was injected.

The collected samples were injected on an analytical column to confirm purity and, if necessary, reinjected on the preparative column to effect greater purification. After separation and purification, the components in the product mixture were subsequently characterized by various combinations of IR, UV, NMR and mass spectrometry. In other instances (compounds XVII, XXIII, XXIV, and XXVI), identification and estimation were based solely on comparison of the retention time and peak area of the component with the corresponding parameters of a known standard.

The analytical instrumentation consisted of Varian Aerograph models 1520 and 1700 fitted with a 180 × 0.3 cm stainless steel column filled with 3 % Carbowax 20M-TPA on Chromosorb G (45/60). Column conditions: oven, 90 – 130 °C (isothermal); detector. 195 – 250 °C; injector, 150 – 200 °C; flow rate, 25 ml N_2/min. Products in the acetoveratrone and dioxane lignin reaction mixtures were separated and collected using an F & M Model 720 (thermal conductivity) instrument fitted with a 180 × 0.6 cm stainless steel column filled with 3 % OV-1 on Chromosorb G (45/60). Column conditions: oven, 90 – 160 °C (isothermal); detector, 120 – 200 °C; 110 – 180 °C; flow rate, 40 ml He/min.

For quantitative gas chromatographic analysis, standards were prepared from the collected or synthesized samples whenever possible and peak heights were compared to those of the corresponding peaks in the reaction mixture. Otherwise, peak areas were compared to those of a closely related compound with a similar structure. The column support in these cases was coated with Carbowax 20M-TPA (3 %).

Spectrometric analysis. Infrared spectra were recorded on a Perkin-Elmer grating infrared spectrophotometer. Liquid samples were measured as films; solids were either mixed with KBr and made into pellets or dissolved in chloroform or carbon tetrachloride. Mass spectra were obtained using a Perkin-Elmer Hitachi Model RMU-6E single-focusing spectrophotometer. Ultraviolet spectra were obtained on samples dissolved in 95 % ethanol using a Unicam Model SP-800A recording spectrophotometer.

NMR spectra were recorded on a Varian A-60 spectrometer using trimethylsilane as an internal standard. The spectra were obtained with the samples in the neat condition whenever possible, but usually the amount of collected sample was too small and the sample was dissolved in a solvent. The instability of some of the products in $CDCl_3$ resulted in the use of CCl_4 which, after first being washed with base, obviated this problem.

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Diels-Alder Reactions of 2,4-Cyclohexadienones. I. Structural and Steric Orientation in the Dimerisation of 2,4-Cyclohexadienones*

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Salicyl alcohol (*3a*) and its methyl homologues *3b*—*3e* on treatment with periodate give the Diels-Alder dimers *5a*—*e* of the initially formed spiro(oxirane-2,4-cyclohexadienones) *4a*—*e*. Similarly, dimers *2a*—*e* are formed from 2,4-cyclohexadienones *1a*—*e* ("*o*-quinols").

Cleavage of the oxirane rings in *5a*—*e* by hydrogen bromide gave the bis(bromohydrins) *6a*—*e*, which on treatment with Raney nickel were converted to the corresponding *o*-quinol dimers *2c* and *2e* or to the further hydrogenated compounds *8*, *10*, and *11*, the three latter products also being obtained on Raney nickel reduction of *o*-quinol dimers *2a*, *2b* and *2d*, respectively.

Since the complete structure of dimer *5a* has been determined by X-ray crystallographic analysis of its bis(bromohydrin) *6a*, the final structure of *o*-quinol dimer *2a* now is established as well. Furthermore, the results of the Raney nickel reductions show that the Diels-Alder dimerisations of the spiro(oxirane-2,4-cyclohexadienones) *4b*—*e* and those of *o*-quinols *1b*—*e* are analogous with regard to regiospecificity and stereochemical specificity.

Recent X-ray analysis [16] elucidated the structures of *2b* and *2d* and showed these to be analogous to the structure of *2a* and, furthermore, photochemical work [14] proved that dimer *2e* is analogous to *2a* with regard to *endo* configuration and structural orientation. From the structural correlations revealed by the Raney nickel reductions it can be concluded that the orientation and the stereochemistry thus established for *o*-quinol dimers *2b*, *2d*, and *2e* are characteristic for the spirooxirane dimers *5b*, *5d*, and *5e* as well.

The steric arrangements of the *tert*-carbinol and oxirane groups in the two types of dimers can be ascribed to steric approach control in the dimerisation. Reasons for the regiospecificity in the dimerisation of 2,4-cyclohexadienones are briefly discussed.

In earlier communications [1-4] it has been shown that periodate oxidation of methyl homologues of *o*-cresol gives 6-hydroxy-6-methyl-2,4-cyclohexadienones ("*o*-quinols", *1b*—*1e*) which undergo rapid Diels-Alder reaction to give the *o*-quinol dimers *2b*—*2e*. The corresponding dimer (*2a*) of unsubstituted *o*-quinol (*1a*) has been obtained on acid hydrolysis of the acetate of *1a*.[5] Contrary to the free *o*-quinols, the acetates are comparatively stable at room temperature.

The aim of the present study was primarily to elucidate the structures of *o*-quinol dimers *2a*—*2e*.

Dimerisation of *o*-quinols *1a*—*e* formally offers several possibilities with regard to the structure of the products. Firstly, in the molecule acting as dienophile either the α,β or the γ,δ double bond of its conjugated carbonyl system may participate in the reaction. As observed earlier in similar cases,[6] the γ,δ double bond reacts specifically (see for instance, Refs. 2 and 3 and the review article, Ref. 7). Furthermore, there are the following alternative possibilities:

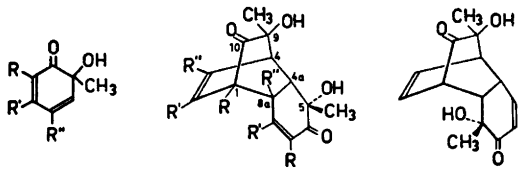
(*a*) The dimers may have *endo* or *exo* configuration.

(*b*) Due to the presence of an asymmetric C-atom in the monomers four isomeric dimers with different steric arrangements at C-atoms 5 and 9 are conceivable.

(*c*) There are two possible structural orientations of the diene and dienophile moieties, as illustrated by formulae *2a* and *2a'*.

Although the alternatives involved in items *a*—*c* make the formation of sixteen isomeric dimers (D,L pairs) possible, in each case only one dimeric product has been obtained. The

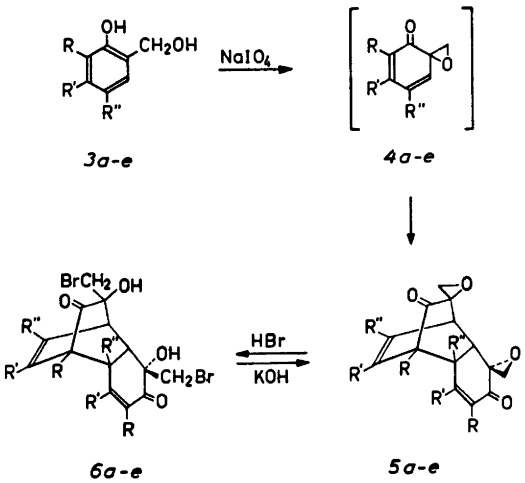
* Part XI in the series "Periodate Oxidation of Phenols". Preliminary communication, see Part X.[1]



1a-g *2a-g* *2a'*

1a, *2a* R = R′ = R″ = H
1b, *2b* R = CH_3; R′ = R″ = H
1c, *2c* R′ = CH_3; R = R″ = H
1d, *2d* R″ = CH_3; R = R′ = H
1e, *2e* R = R″ = CH_3; R′ = H
1f = *1b*, F instead of OH; *2f* = *2b*, F instead of OH
1g = *1b*, Cl instead of OH; *2g* = *2b*, Cl instead of OH

Chart 1.



3a-e *4a-e* *6a-e* *5a-e*

3a, *4a*, *5a*, *6a* R = R′ = R″ = H
3b, *4b*, *5b*, *6b* R = CH_3; R′ = R″ = H
3c, *4c*, *5c*, *6c* R′ = CH_3; R = R″ = H
3d, *4d*, *5d*, *6d* R″ = CH_3; R = R′ = H
3e, *4e*, *5e*, *6e* R = R″ = CH_3; R′ = H
7a-e = *6a-e*, OAc instead of OH

Chart 2.

dimerisation of the *o*-quinols (*1a-e*) thus proceeds with a very high degree of stereochemical selectivity (items *a* and *b*) and regioselectivity (item *c*). No further isomers could be detected by NMR analysis and thin layer chromatography of the crude reaction products. For simplicity, it will therefore be assumed in the following that the dimerisations are stereospecific and regiospecific.

By degrading the dimer of *o*-quinol *1a* to 1,7-dimethyl-2-naphthol, Metlesics and Wessely [5] proved the structural orientation in this dimer to be that given in formula *2a* rather than *2a'*. Experimental evidence for the *endo* configuration and the steric arrangements at C-5 and C-9, however, was still lacking.

Periodate oxidation of salicyl alcohol (*3a*) was found [8] to result in the formation of spiro(oxirane-2,4-cyclohexadienone) *4a* which rapidly dimerised to give the bis(spirooxirane) *5a*. Treatment of this dimer with hydrogen bromide afforded a bis(bromohydrin), for which structure *6a* was established by X-ray crystallography.[9] This also proved structure *5a* for the parent dimer.[8]

Reductive opening of the oxirane rings in *5a* or reductive debromination of *6a* could be expected to give the *o*-quinol dimer *2a*, provided that the latter had the same structural and steric arrangements as established for *5a* and *6a*. Attempts to perform these conversions by treating *5a* with $LiAlH_4$ or $NaBH_4$ or by treating *6a* or the corresponding bis(iodohydrin) with hydrogen in the presence of Pt and Pd catalysts as well as with various other reductants remained without success. Raney nickel in boiling ethanol, however, effected the conversion of the bromomethyl groups in *6a* into methyl groups with simultaneous hydrogenation of the C=C and C=O double bonds present to give the tetrahydroxy compound *8*. The same reduction product was obtained on similar treatment of *o*-quinol dimer *2a*. This constitutes unambiguous proof of structure *2a* and shows that the Diels-Alder dimerisation of *o*-quinol *1a* is completely analogous to that of spirooxirane *4a* with regard to both regiospecificity and stereospecificity.

Treatment of *8* with chromic acid in the presence of manganese(II) nitrate [10] resulted in rapid cleavage of the 1,2-diol groups to give the dicarboxylic acid *9*. The ease with which this cleavage takes place tends to indicate *cis* configuration for both 1,2-diol groups (*cf.* Ref. 11).

It was further of interest to examine whether the similarity in structural and steric characteristics thus established for the pair *5a* and *2a* is true also for other pairs of the two kinds

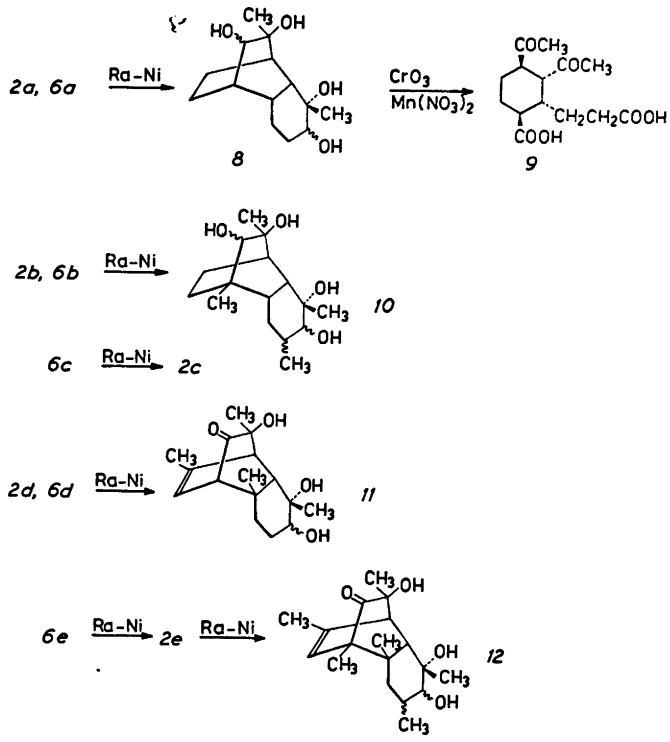


Chart 3.

of dimers. The bis(bromohydrins) (*6b – 6e*) obtained from the methyl-substituted dimers *5b – 5e* as well as the corresponding *o*-quinol dimers (*2b – 2e*) were therefore treated with Raney nickel (Chart 3).

The bis(spirooxiranes) *5b – 5d* were prepared by periodate oxidation of the *o*-hydroxybenzyl alcohols *3b – 3d*, again only one isomer being obtained from each of the alcohols. Treatment of the dimers *5b – 5d* with hydrogen bromide provided the bis(bromohydrins) *6b – 6d*, which, when treated with methanolic potassium hydroxide, regenerated the bis(spirooxiranes) *5b – 5d*. Dimer *5e* and its bis(bromohydrin) *6e* have been described earlier.[8] The *o*-quinol dimers *2b*, *2d*, and *2e*, which are formed on periodate oxidation of 2,6-dimethyl-, 2,4-dimethyl-, and 2,4,6-trimethylphenol *via* the short-lived *o*-quinols *1b*, *1d*, and *1e*, respectively, have also been reported.[2,3] Dimer *2c* has now been prepared similarly from 2,5-dimethylphenol.

Whereas the periodate oxidation of 2,6- and 2,4-dimethylphenol as well as of 2,4,6-trimethylphenol is rapid, the phenols being consumed within a few minutes and the corresponding dimers being obtained in fair yields, 2,5-dimethylphenol reacts sluggishly, unconsumed phenol still being found after 1 h. In addition to the expected dimer *2c* (see Exptl.), the reaction mixture contained substantial amounts of 2,5-dimethyl-1,4-benzoquinone as well as a monocarboxylic acid formed from *2c* by ketol cleavage, as will be further described in a following paper.

Since periodate consumption by *o*-cresol is still slower than by 2,5-dimethylphenol, periodate oxidation of the former phenol cannot be used for the preparation of dimer *2a*, which, however, is available *via* the *o*-quinol acetate (*1a*, OAc instead of OH).[5]

Raney nickel reduction of bis(bromohydrins) *6b – 6e* revealed that these compounds and, consequently, the bis(spirooxiranes) *5b – 5e* as well have the same structural and steric arrangements as the corresponding *o*-quinol dimers *2b – 2e*. Thus, bis(bromohydrin) *6b* as well as *o*-quinol dimer *2b* gave the octahydro derivative *10* of dimer *2b*. Product *10* was obtained in fair yields, indicating high stereoselectivity of the Raney nickel reduction.

From the reaction mixture obtained on Raney

nickel reduction of *6c*, the corresponding *o*-quinol dimer *2c* could be isolated and, similarly, *6e* was reduced to *2e*. In the last-mentioned case, prolonged reduction converted both *6e* and *2e* into the partially hydrogenated *o*-quinol dimer *12*. Finally, a similarly hydrogenated *o*-quinol dimer (*11*) was obtained from both bis(bromohydrin) *6d* and *o*-quinol dimer *2d*.

It is noteworthy that hydrogenation of *o*-quinol dimer *2e* in acetic acid, using PtO_2 as catalyst (3 atm, 48 h), yielded a tetrahydro compound, m.p. 213 – 214°,[13] not identical with *12*, m.p. 240 – 242°, obtained with Raney nickel. The UV spectra (ethanol) of these compounds indicate that the homoconjugated carbonyl system is intact in both cases. The spectra exhibit the expected transannular charge transfer bands at 205 nm (log $\varepsilon = 3.61$) and 208 nm (log $\varepsilon = 3.65$), respectively, and enhanced $n \rightarrow \pi^*$ absorptions at 310 nm (log $\varepsilon = 2.25$) and 308 nm (log $\varepsilon = 2.35$), respectively. Apparently, the two compounds are stereoisomers arising by hydrogenation of the α,β-conjugated carbonyl system of *2e*. This indicates different stereochemical selectivity of the two hydrogenation systems used.

From the results of reduction summarized in Chart 3 it can be concluded that the dimerisation of *o*-quinols *1a – 1e* involves the same structural and steric orientation as the dimerisation of the correspondingly substituted spiro(oxirane-2,4-cyclohexadienones) *4a – 4e*. However, since only the structure of dimer *5a* was completely known,[8,9] the reduction experiments only established the complete structure of *o*-quinol dimer *2a* (structural orientation as in formula *2a* rather than *2a'*, *endo* configuration and steric arrangements at C-5 and C-9 as shown by formula *2a*).

Further experimental data are now available which indicate that the structures of *o*-quinol dimers *2b – 2e* as well as those of the bis(spirooxiranes) *5b – 5e* are analogous to those of *2a* and *5a*, respectively.

NMR spectra of dimers *2d* and *2e* clearly show the presence of the vicinal hydrogen atoms at positions 4 and 4a. In the NMR spectrum (DMSO-d_6) of *2d* H-4 gives rise to a triplet at δ 2.96, whereas the signal for H-4a is a doublet at δ 2.72 ($J_{4,4a} = J_{4,2} = 2.2$ Hz). Similarly, the NMR spectrum ($CDCl_3 + D_2O$) of *2e* shows H-4 as a triplet at δ 3.15 and H-4a as a doublet at δ 2.84 ($J_{4,4a} = J_{4,2} = 2.0$ Hz). This proves the structural orientation of dimers *2d* and *2e* to be of type *2a* rather than *2a'*. The same orientation then must be true for the bis(spirooxiranes) *5d* and *5e*, and has already been proven for the last-mentioned dimer by similar NMR analysis.[8] Furthermore, Becker [14] concluded from the photochemically induced intramolecular cycloaddition of *o*-quinol dimers *2b*, *2d*, and *2e* (*cf.* also Ref. 15) that these dimers had *endo* configuration and a structural orientation analogous to *2a*. Finally, recent X-ray crystallographic analysis completely established the structures of *o*-quinol dimers *2b* and *2d*,[16] revealing that the steric orientations at C-5 and C-9 in these two dimers are the same as found for dimers *2a* (Ref. 1 and present paper) and *5a*.[9] The correlations between the *o*-quinol dimers and the bis(spirooxiranes) reported above then imply that the complete structures of bis(spirooxiranes) *5b* and *5d* are as depicted in Chart 2.

Experimental proof is still lacking for the structures of *o*-quinol dimer *2c* and its bis(spirooxirane) counterpart *5c* as well as for the steric arrangements at carbon atoms 5 and 9 of the dimer pair *2e* and *5e*. It seems justified, however, to assume that the structural features of these dimers are analogous to those discussed above. Chemical evidence for the stereochemistry at C-5 and C-9 of the *o*-quinol dimers will be given in a forthcoming paper.

It is likely that the structural and steric specificity found in the dimerisation of *o*-quinols and spiro(oxirane-2,4-cyclohexadienones) is valid for the dimerisation of other 2,4-cyclohexadienones as well. On the basis of dipole moment measurements, the dimer of 2,6,6-trimethyl-2,4-cyclohexadienone [17] has been assigned the *endo* form as well as a structural orientation corresponding to that of the dimers discussed in the present paper. Furthermore, chemical and spectroscopic data indicated that the steric arrangement at C-5 of the dimers *2f* and *2g* of the fluorinated [18] and chlorinated [19] 2,4-cyclohexadienones *1f* and *1g* is analogous to that found in dimers *2a* and *5a* as well as in the pairs *2b*, *5b* and *2d*, *5d*.

To summarize, it can be concluded that the rapid Diels-Alder dimerisation of *o*-quinols *1a – e* as well as that of the spiro(oxirane-2,4-cyclohexadienones) *4a – e:*

(*a*) follows the *endo* rule,

(*b*) is stereospecific, the CH_3 groups at C-5 and C-9 of dimers *2a – e* as well as the CH_3

groups of the oxirane rings in dimers *5a–e* being oriented away from the reaction center, which indicates steric approach control,[20] and

(c) is regiospecific, the diene and the dienophile moieties being oriented to each other as illustrated by formula *2a* rather than *2a'*.

The factors responsible for the regiospecificity of Diels-Alder reactions involving unsymmetrical dienes and dienophiles so far are not well understood (for reviews, see Refs. 21–25). Neither steric interactions nor polar factors have been found to be of decisive importance. Although Diels-Alder reactions now are generally considered to be concerted $4\pi+2\pi$ cycloadditions, it has been pointed out that the preferred structural orientation is "consistent with reaction through the transition state in which build up of diradical character is best accomodated".[25] It is easily seen that diradicals which, in a formal sense, could be regarded as intermediates in the formation of 2,4-cyclohexadienone dimers of type *2a* are favoured, by greater resonance stabilisation, over those which would lead to the isomers of type *2a'*.

The view that Diels-Alder reactions may involve a diradical-like transition state has found support in a very recent theoretical study on the regioselectivity of concerted cycloadditions.[26]

EXPERIMENTAL

Ultraviolet spectra were recorded on a Cary Model 14 spectrophotometer; IR and NMR spectra were obtained using Beckman 9A and Varian A-60 instruments, respectively. Chemical shifts are given in δ(ppm) units, TMS being used as internal standard. Melting points are uncorrected.

o-*Quinol dimers 2a, 2b, 2d,* and *2e* were prepared according to Refs. 5, 3, 2, and 3, respectively, and *bis(bromohydrins) 6a* and *6e* were obtained according to Ref. 8.

1,4a,5,8a-Tetrahydro-5,9-dihydroxy-2,5,8,9-tetramethyl-1,4-ethanonaphthalene-6,10(4H)-dione (2c).*,** A solution of sodium metaperiodate (54 g, 0.25 mol) in water (1.1 l) was added to a solution of 2,5-dimethylphenol (13.2 g, 0.10 mol) in water (3.3 l). After 1 h, unconsumed periodate was destroyed by addition of ethylene glycol (10 g), a small amount of amorphous material was filtered off and the solution was extracted with six 250 ml portions of methylene chloride. The dried extract on evaporation under vacuum left a deep red semi-solid residue. The solution of the latter in ethanol was treated with sulphur dioxide, and, after addition of sodium bisulfite, was concentrated under vacuum, water being added repeatedly. The aqueous mixture containing a crystalline deposit was made alkaline by addition of sodium bicarbonate and extracted with methylene chloride. Undissolved crystalline material was collected and identified as *2,5-dimethylhydroquinone*, m.p. 210°; yield, 13 %. The extract, after washing with aqueous bicarbonate and water, drying and evaporation, gave an oil, from which on treatment with ether (5 ml) almost colourless crystals of *2c* deposited (yield, 12 %). After recrystallisation from ethanol, m.p. 200–201°. (Found C 69.39; H 7.53. Calc. for $C_{16}H_{20}O_4$: C 69.54; H 7.30.) IR (KBr): ν_{max} (cm^{-1}) 1660 (conj. CO), 1725 (CO), 3450 (OH). UV (abs. ethanol), λ_{max} (nm) and log ε values: 210, 3.93 (charge transfer band, homoconjugated CO system); 233, 3.98 (conj. CO); 305, 2.45 (homoconj. and conj. CO). NMR (DMSO-d_6): δ 1.10 and 1.18 (singlets, 3 H each, 2 CH_3), 1.53 and 1.95 (doublets, 3 H each, 2 olefinic CH_3); 2.80–3.35 (4 H, 4 CH), 4.76 and 5.45 (singlets, 1 H each, 2 OH), 5.60–6.00 (2 H, 2 olefinic H).

If the deep red semi-solid residue obtained above before the SO_2 treatment was extracted with hot benzene, the extract gave *2,5-dimethyl-1,4-benzoquinone*, m.p. 122°.

Diacetate of 2c. From *2c* by treatment with the $Ac_2O/HClO_4$ reagent according to Ref. 28. M.p. 190–191° after recrystallisation from ethanol. (Found: C 66.84; H 6.58; CH_3CO 23.10. Calc. for $C_{20}H_{24}O_6$: C 66.65; H 6.71; CH_3CO 23.89.)

o-*Hydroxybenzyl alcohols* 3b, 3c, *and* 3d. 2-Hydroxy-3-methylbenzyl alcohol (*3b*) and 2-hydroxy-5-methylbenzyl alcohol (*3d*) were prepared from the corresponding methylphenol, trioxane and boric oxide (B_2O_3) according to Ref. 12, whereas a slight modification of the method described there was used for 2-hydroxy-4-methylbenzyl alcohol (*3c*). The orthoborate obtained in 50 % yield from *m*-cresol (27 g), boric oxide (8.75 g) and trioxane (7.5 g) was collected and added to a stirred mixture of toluene (50 ml) and ice water (50 ml), and the mixture was made alkaline by addition of 18 g of a 30 % aqueous sodium hydroxide solution. The aqueous phase was washed with diisopropyl ether, then was made slightly acidic with aqueous H_2SO_4 and extracted with ether. The extract yielded a crystalline product (yield, 30 %), which was recrystallised from chloroform and from benzene. M.p. 107–108° (Lit.[27] m.p. 111°).

Dimeric spiro(oxirane-2,4-cyclohexadienones) 5b, 5c, *and* 5d. A solution of sodium metaperiodate (11.75 g, 0.055 mol) in water (250 ml)

* Nomenclature according to *Chem. Abstr. 76* (1972) 1445 CS.

** Preparation based on experiments carried out by Dr. Britt Berggren and tekn.lic. Ingrid Jansson.

was added to a solution of the *o*-hydroxybenzyl alcohol (*3b*, *3c*, and *3d*, respectively, 6.90 g, 0.050 mol) in water (700 ml). The mixture was kept in the refrigerator for 24 h. The almost colourless precipitate formed was recrystallised from chloroform.

1',4',4'a,8'a-Tetrahydro-1',7'-dimethyl-dispiro-[oxirane-2,5'(6'H)-[1,4]ethanonaphthalene-9',2''-oxirane]-6',10'-dione (5b).* From *3b* and periodate. Yield of crude product, 74 %. M.p. 206 – 207°. (Found: C 70.61; H 5.92. Calc. for $C_{16}H_{16}O_4$: C 70.57; H 5.92.) IR (KBr): ν_{max} (cm^{-1}) 1696 (conj. CO), 1731 (CO), 3076 (oxirane-CH_2). NMR (DMSO-d_6): δ 1.35 (s, 3 H, CH_3), 1.76 (t, 3 H, olefinic CH_3), 2.40 – 3.20 (7 H, 2 CH_2 and 3 CH), 5.80 and 6.48 (doublets, 1 H each, 2 olefinic H), 6.66 (m, 1 H, olefinic H).

1',4',4'a,8'a-Tetrahydro-2',8'-dimethyl-dispiro-[oxirane-2,5'(6'H)-[1,4]ethanonaphthalene-9',2''-oxirane]-6',10'-dione (5c). From *3c* and periodate. Yield of crude product, 71 %. M.p. 222.5 – 223.5°. (Found: C 70.42; H 5.99. Calc. for $C_{16}H_{16}O_4$: C 70.57; H 5.92.) IR (KBr): ν_{max} (cm^{-1}) 1698 (conj. CO), 1731 (CO), 3048 (oxirane-CH_2). NMR (DMSO-d_6): δ 1.71 and 2.09 (doublets, 3 H each, 2 olefinic CH_3), 2.71 and 2.93 (doublets, 1 H each, AB system with $J = 6.8$ Hz, oxirane-CH_2), 3.02 (broadened singlet, 2 H, oxirane-CH_2), 3.52 (broad, 2 H, 2 CH), 6.01 – 6.27 (2 H, olefinic H). Signals arising from 2 H are partially hidden by the signal due to incompletely deuterated DMSO.

1',4',4'a,8'a-Tetrahydro-3',8'a-dimethyl-dispiro[oxirane-2,5'(6'H)-[1,4] ethanonaphthalene-9',2''-oxirane]-6',10'-dione (5d). From *3d* and periodate. Yield of crude product, 87 %. M.p. 217.5 – 218.5°. (Found: C 70.44; H 5.98. Calc. for $C_{16}H_{16}O_4$: C 70.57; H 5.92.) IR (KBr): ν_{max} (cm^{-1}) 1698 (conj. CO), 1727 (CO), 3056 (oxirane-CH_2). NMR (DMSO-d_6): δ 1.34 (s, 3 H, CH_3), 1.80 (d, 3 H, olefinic CH_3), 2.23 (d, 1 H, CH), 2.74 and 3.00 (doublets, 1 H each, AB system with $J = 6.5$ Hz, oxirane-CH_2), 2.99 and 3.18 (doublets, 1 H each, AB system with $J = 6.2$ Hz, oxirane-CH_2), 5.78 (m, 1 H, H-2'), 6.09 and 6.60 (doublets, 1 H each, H-7' and H-8', $J = 10$ Hz). Signals from 2 H are hidden by the signal for incompletely deuterated DMSO.

Bis(bromohydrins) 6b, 6c *and* 6d. Aqueous hydrobromic acid (7.40 g of 66 % HBr solution, 0.060 mol HBr) was added dropwise to a solution of bis(spirooxirane) (*5b*, *5c*, and *5d*, respectively, 6.80 g, 0.025 mol) in dioxane (750 ml). After 8 h at room temperature the solution was brought to dryness under vacuum.

5,9-Bis(bromomethyl)-1,4a,5,8a-tetrahydro-5,9-dihydroxy-1,7-dimethyl-1,4-ethanonaphthalene-6,10(4H)-dione (6b). From *5b* and HBr. The solid residue obtained gave colourless crystals (64 %) from ethanol, m.p. 185.5 – 186.5°. (Found: C 44.47; H 4.11; Br 36.43. Calc. for $C_{16}H_{16}O_4Br_2$: C 44.27; H 4.18; Br 36.81.) IR (KBr): ν_{max} (cm^{-1}) 1688 (conj. CO), 1713 (CO), 3439 and 3483 (OH). NMR (DMSO-d_6): δ 1.26 (s, 3 H, CH_3), 1.78 (broadened singlet, 3 H, olefinic CH_3), 2.70 – 3.80 (7 H, 2 CH_2 and 3 CH), 5.37 and 6.20 (singlets, 1 H each, 2 OH), 5.62 (dd, 1 H, olefinic H), 6.14 (d, 1 H, olefinic H), 6.40 (m, 1 H, olefinic H).

5,9-Bis(bromomethyl)-1,4a,5,8a-tetrahydro-5,9-dihydroxy-2,8-dimethyl-1,4-ethanonaphthalene-6,10(4H)-dione (6c). From *5c* and HBr. The acetone solution of the light yellow oily residue was concentrated to a volume of 15 ml. The crystalline product deposited (63 %), after recrystallisation from benzene and from acetone, had m.p. 174 – 175° and contained 1 mol of acetone per mol. (Found: C 46.01; H 4.79; Br 32.71. Calc. for $C_{16}H_{16}O_4Br_2.C_3H_6O$: C 46.36; H 4.97; Br 32.47.) IR (KBr): ν_{max} (cm^{-1}) 1698 (conj. CO), 1719 (CO), 3440 (OH). NMR (DMSO-d_6): δ 1.58 (d, 3 H, olefinic CH_3), 1.98 (broadened singlet, 3 H, olefinic CH_3), 2.09 (s, 6 H, acetone), 2.87 – 3.70 (8 H, 2 CH_2 and 4 CH), 5.18 and 6.17 (singlets, 1 H each, 2 OH), 5.80 (broadened doublet, 1 H, olefinic H), 5.98 (broadened singlet, 1 H, olefinic H).

5,9-Bis(bromomethyl)-1,4a,5,8a-tetrahydro-5,9-dihydroxy-3,8a-dimethyl-1,4-ethanonaphthalene-6,10(4H)-dione (6d). From *5d* and HBr. The solid residue was recrystallised from ethanol to give *6d* (88 %), m.p. 211 – 212°. (Found: C 44.56; H 4.25; Br 36.54. Calc. for $C_{16}H_{16}O_4Br_2$: C 44.27; H 4.18; Br 36.81.) IR (KBr): ν_{max} (cm^{-1}) 1673 (conj. CO), 1730 (CO), 3394 and 3482 (OH). NMR (DMSO-d_6): δ 1.37 (s, 3 H, CH_3), 1.73 (d, 3 H, olefinic CH_3), 2.84 and 2.92 (doublets, 1 H each, 2 CH), 3.33 (t, 1 H, CH), 3.34 and 3.57 (doublets, 1 H each, AB system with $J = 11$ Hz, CH_2), 3.65 (s, 2 H, CH_2), 5.36 and 6.24 (singlets, 1 H each, 2 OH), 5.55 (d, further split by allylic coupling, 1 H, H-2), 6.05 and 6.40 (doublets, 1 H each, H-7 and H-8, $J = 10$ Hz).

The bis(bromohydrins) *6b* – *6d* on treatment with a 2.4-fold excess of 0.1 M methanolic KOH during 10 min regenerated the corresponding bis(spirooxiranes) *5b* – *5d* (yields, 70 – 90 %), identified by m.p., mixed m.p. and IR spectra.

Diacetates 7b, *7c* and *7d* were obtained by dissolving the bis(bromohydrins) (*6b*, *6c*, and *6d*, respectively, in the EtOAc-Ac_2O-$HClO_4$ reagent [28] and working up the reaction mixture after 15 min. Recrystallisation from ethanol gave colourless products.

Diacetate 7b. From *6b* (68 %), m.p. 165 – 166°. (Found: C 46.34; H 4.36; Br 31.41. Calc. for $C_{20}H_{22}O_6Br_2$: C 46.35; H 4.28; Br 30.84.) IR (KBr): ν_{max} (cm^{-1}) 1653 (C=C), 1709 (conj. CO), 1741 – 1750 (CO and ester-CO).

Diacetate 7 c. From *6c* (70 %), m.p. 156 – 157°. (Found: C 46.53; H 4.33; Br 30.05. Calc. for $C_{20}H_{22}O_6Br_2$: C 46.35; H 4.28; Br 30.84.) IR (KBr): ν_{max} (cm^{-1}) 1623 (C=C), 1700 (conj. CO), 1740 and 1756 (CO and ester-CO).

Diacetate 7 d. From *6d* (65 %), m.p. 147.5 –

* Nomenclature according to *Chem. Abstr. 76* (1972) 1319 CS.

148.5°. (Found: C 46.44; H 4.35; Br 30.89. Calc. for $C_{20}H_{22}O_6Br_2$: C 46.35; H 4.28; Br 30.84.) IR (KBr): ν_{max} (cm^{-1}) 1630 (C=C), 1718 (conj. CO), 1738 and 1759 (CO and ester-CO).

Reductions with Raney nickel

Perhydro-2,3,5,6-tetrahydroxy-3,5-dimethyl-1,4-ethanonaphthalene (8). *(a) From bis(bromohydrin)* 6a. The stirred solution of *6a* containing 0.5 mol of dioxane per mol (1.015 g) in 95 % ethanol (125 ml) was refluxed for 5 h with Ra-Ni "W-6"[29] (10 g) and then filtered through celite and evaporated to dryness under vacuum. The solid residue was treated with boiling acetone (30 ml), which dissolved the major part of the product. The hot solution was filtered and, on cooling, gave colourless crystals (yield, 6 %), m.p. 251 – 252°, which rose to 253 – 254° on recrystallisation from methanol-acetone. (Found: *M*, by mass spectrometry, 256.1689. Calc. for $C_{14}H_{24}O_4$: *M*, 256.1674.) IR (KBr) ν_{max} (cm^{-1}) 3400 and 3450 (broad, OH), The NMR spectrum (CD_3OD) shows two singlets at δ 1.26 and 1.33 (3 H each, 2 CH_3) and signals between δ 1.40 and 3.67 (14 H, 6 CH and 4 CH_2). There were no signals due to olefinic protons.

(b) From o-*quinol dimer* 2a. The procedure was similar to that described under (a). From the cooled acetone solution a crude crystalline product of m.p. 210 – 215° (15 %) was obtained. Two recrystallisations from methanol-acetone gave pure *8*, m.p. 252 – 253°, identical with the product obtained according to (a) by mixed m.p., IR, NMR, and mass spectra.

2,3-Diacetyl-6-carboxycyclohexanepropanoic acid (9). To the solution of compound *8* (484 mg) in a mixture of acetic acid (7 ml), water (0.75 ml) and 1.5 ml of an aqueous solution of manganese(II) nitrate (500 g $Mn(NO_3)_2$/l), after cooling to 10°, a solution of chromic acid (0.8 g of CrO_3 in 2.3 ml of water) was added in portions during a period of 3 min. The solution was again cooled to 10° and conc. H_2SO_4 (0.45 ml) was added during 1 min. The reaction mixture was kept at 30° for 8 min and after addition of water (30 ml in portions) neutralised with aqueous bicarbonate and finally extracted with ethyl acetate. The extract gave a yellow oil which on treatment with a small amount of ether provided crystals, m.p. 179 – 180° (yield, 54 %) after recrystallisation from acetone. (Found: *M*, by mass spectrometry, 284.1274. Calc. for $C_{14}H_{20}O_6$: *M*, 284.1260.) IR (KBr): ν_{max} (cm^{-1}) 1702 and 1742 (CO and COOH), 2400 – 3600 (COOH). The NMR spectrum (DMSO-d_6) shows 2 singlets at δ 2.10 and 2.12, due to 2 CH_3CO.

Perhydro-2,3,5,6-tetrahydroxy-1,3,5,7-tetramethyl-1,4-ethanonaphthalene (10). *(a) From bis(bromohydrin)* 6b. The stirred solution of *6b* (1.085 g) in 95 % ethanol (110 ml) was refluxed for 7 h with Ra-Ni (19 g). Removal of the solvent from the filtered solution gave a semi-solid product, which was extracted with boiling acetone (30 ml). After cooling to 0° the mixture was filtered and the filtrate concentrated to a volume of 10 ml. Addition of hexane until beginning precipitation, followed by cooling in the refrigerator, provided *10* (25 %), m.p. 199 – 200° after recrystallisation from acetone. (Found: C 67.33; H 9.91. Calc. for $C_{16}H_{28}O_4$: C 67.57; H 9.92.) IR (KBr): ν_{max} (cm^{-1}) 3275, 3370, 3412, 3494 and 3519 (OH). The NMR spectrum (DMSO-d_6) indicates the presence of 4 OH (δ 3.51, 3.92, 4.14 and 4.22) and shows no absorption due to olefinic H.

(b) From o-*quinol dimer* 2b. Treatment of *2b* (1.90 g) with Ra-Ni (15 g) in ethanol (2.5 h reflux) and work-up as above. The semi-solid residue after treatment with 25 ml of a chloroform-hexane mixture (2:1) gave crystals of *10* (48 %), m.p. 200 – 201° after recrystallisation from ethyl acetate and from acetone. Identical with the product obtained according to (a) by mixed m.p., IR and NMR spectra.

o-*Quinol dimer* 2c *from bis(bromohydrin)* 6c. The stirred solution of *6c* (2.17 g) in 95 % ethanol (130 ml) was refluxed with Ra-Ni (6 g) for 1.5 h. The filtered solution on evaporation gave a light yellow oil which was chromatographed on a silica gel column using acetone-hexane (2:1) as solvent. Dimer *2c* (R_F = 0.65), m.p. 199 – 200° (from ethanol), was obtained in 15 % yield and shown to be identical with the product prepared by periodate oxidation of 2,5-dimethylphenol (p. 469) by mixed m.p., IR and NMR spectra.

1,4,4a,5,6,7,8,8a-Octahydro-5,6,9-trihydroxy-3,5,8a,9-tetramethyl-1,4-ethanonaphthalen-10-one (11). *(a) From bis(bromohydrin)* 6d. The stirred solution of *6d* (2.17 g) in 95 % ethanol (130 ml) was refluxed for 2 h with Ra-Ni (20 g), then filtered and evaporated giving a semi-crystalline residue which on treatment with a mixture of chloroform (10 ml) and acetone (10 ml) left crystals of *11* (22 %), m.p. 240 – 241° after recrystallisation from acetone. (Found: *M*, by mass spectrometry, 280.1676. Calc. for $C_{16}H_{24}O_4$: *M*, 280.1674.) IR (KBr): ν_{max} (cm^{-1}) 1718 (CO), 3272, 3480, and 3536 (OH). NMR (DMSO-d_6): δ 1.11 (s, 6 H, 2 CH_3), 1.18 (s, 3 H, CH_3), 1.30 – 2.10 (4 H, 2 CH_2), 1.87 (d, 3 H, olefinic CH_3), 2.27, 2.32, and 3.36 (doublets, 1 H each, 3 CH), 3.62 and 5.08 (singlets, 1 H each, 2 OH), 4.52 (d, 1 H, sec. OH), 5.44 (d, further split by allylic coupling, 1 H, olefinic H). The signal for 1 CH is hidden by the signal for incompletely deuterated DMSO.

(b) From o-*quinol dimer* 2d. The stirred solution of *2d* (1.90 g) in 95 % ethanol (125 ml) was refluxed for 3 h with Ra-Ni (15 g), then filtered and evaporated to dryness. The solid residue on recrystallisation from chloroform gave *11* (39 %), m.p. 239 – 240° after further recrystallisation from acetone. The product was identical by mixed m.p., IR and NMR spectra with *11* obtained according to (a).

o-Quinol dimer 2e *from bis(bromohydrin)* 6e. Compound *6e* (1.386 g) was treated for 4 h with Ra-Ni (9 g) in refluxing 95 % ethanol (140 ml). The filtered solution was brought to dryness, leaving crude crystalline *2e*, m.p. 170 – 175° (67 %). After recrystallisation from acetone and from benzene, the product had m.p. 181 – 182° and was identical by mixed m.p., IR and NMR spectra with *2e* prepared by periodate oxidation of mesitol.

1,4,4a,5,6,7,8,8a-Octahydro-5,6,9-trihydroxy-1,3,5,7,8a,9-hexamethyl-1,4-ethanonaphthalen-10-one (12). *o*-Quinol dimer *2e* (912 mg) was treated for 5 h with Ra-Ni (18 g) in refluxing 95 % ethanol. The filtered solution was concentrated under vacuum to a volume of 10 ml. After 2 h at 0° the crystalline product deposited was collected and recrystallised from 95 % ethanol, m.p. 240 – 242° (35 %). (Found: *M*, by mass spectrometry, 308.1996. Calc. for $C_{18}H_{28}O_4$: *M*, 308.1987) IR (KBr): ν_{max} (cm^{-1}) 1710 (CO), 3270, 3530 (OH). NMR (DMSO-d_6): δ 0.92 (s, 6 H, 2 CH_3), 1.05, 1.13, and 1.26 (singlets, 3 H each, 3 CH_3), 1.89 (d, 3 H, olefinic CH_3), 1.60 – 3.52 (partly overlapped by H_2O signal, 6 H, CH_2 and 4 CH), 3.60 (s, 1 H, OH), 4.58 (d, 1 H, *sec.* OH), 5.09 (broad singlet, 2 H, OH and olefinic H). UV data, see p. 468.

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Mercuric Mercury and Methylmercury Complexes of Glutathione

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The preparation, isolation and characterization of mercury(II) complexes of glutathione from alcoholic aqueous media is described. With mercury(II) chloride, a complex having the composition of a double salt in the solid state, $(C_{10}H_{16}O_6N_3S)_2Hg_3Cl(OH)(HCl)_2$, was obtained which on dissolution in water showed similar ^{13}C NMR spectral features as the complex, $(C_{10}H_{15}O_6N_3S)Hg.H_2O$, obtained with mercury(II) acetate. By comparison with the complex, $C_{10}H_{16}O_6N_3SHgCH_3$, obtained with methylmercury chloride, these complexes appear to have 1:1 inner salt (or chelate) structures in solution. Evidence from ^{13}C NMR Fourier transform spectroscopy suggests that the co-ordination requirements of (mercuric) mercury bonded to sulphur are satisfied by chelation of the glycyl peptide nitrogen of glutathione. The results are discussed in relation to earlier polarographic and alkalimetric investigations and recent NMR studies of metal complexation by glutathione.

Glutathione, a true peptide, is found in the erythrocytes of whole blood and has a number of functions including protection of hemoglobin against oxidation by hydrogen peroxide. It acts as a prosthetic group of glyceraldehyde-phosphate dehydrogenase and as a coenzyme of glyoxalase. It is also believed that glutathione functions as a "sulfhydryl-preserver" in maintaining certain proteins (as sulfhydryl-containing enzymes) in the reduced state which is essential for their activity.[1] Although the binding of mercury to human erythrocytes has been subjected to considerable investigation,[2] little is known concerning the detailed molecular nature of binding. The binding is generally attributed to sulfhydryl groups of hemoglobin, glutathione and of stromal groups.[3]

In a polarographic study of the reaction of mercuric mercury (acetate and chloride) with glutathione (RS), Stricks and Kolthoff[4] obtained indirect evidence for the formation of three compounds with mercury: $Hg(RS)_2$, $Hg_2(RS)_2$, and $Hg_3(RS)_2$ in the absence of chloride and in the pH range between 3 and 9. In the presence of much chloride ion, the formation of the complexes $Hg_2(RS)_2$ and $Hg_3(RS)_2$ is suppressed by formation of the complex $HgCl_4^{2-}$. Similar conclusions were reached by Kapoor, Doughty and Gorin[5] in their alkalimetric titration study of the reaction of $HgCl_2$ with glutathione, however, with one exception, no precipitates were isolated and characterized. Kapoor *et al.*[5] obtained a precipitate whose mercury content was found to be 49 % by bringing a 3:2 $HgCl_2$/glutathione mixture to pH 5. No other analyses were obtained on this substance and no structural features were proposed for it.

Recently Fuhr and Rabenstein[6] reported on the nature of mercurial binding to glutathione in mercury(II) nitrate solutions using ^{13}C NMR spectroscopy, and Simpson, Hopkins and Haque[7] reported on binding of methylmercury chloride to the model peptide, *N*-acetyl-L-cysteine, by means of a ^1H NMR study. In this paper, the isolation of mercury complexes of glutathione with mercury(II) acetate, mercury(II) chloride, and methylmercury chloride is reported together with the use of Fourier Transform ^{13}C NMR spectroscopy for their characterization.

RESULTS AND DISCUSSION

In contrast to the dilute aqueous conditions employed in polarographic and titrimetric investigations,[4,5] mercury complexes were pre-

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pared and isolated in this study from alcoholic aqueous media moderately concentrated with reactants. Complete elemental analyses were obtained to establish empirical formulae. Recent work [8] in the characterization of hydrated mercury complexes of cysteine methyl ester serves to emphasize the value of complete elemental analysis including analysis for oxygen. FT ^{13}C NMR spectroscopy has been valuable for elucidating mercurial binding and configuration in isolated mercury complexes of methionine [9] and of cysteine, *S*-methyl cysteine and cysteine methyl ester.[10]

The mercury(II) glutathionate complex prepared from mercury(II) acetate is devoid of acetate groups as shown by infrared spectra, microanalysis, and ^{13}C NMR spectra. Microanalysis provided elemental ratios in excellent agreement with the composition $C_{10}H_{17}O_7N_3SHg$ (M. W. 524.14) which could be formulated in terms of either a hydroxy complex, $(C_{10}H_{16}O_6N_3S)Hg(OH)$, or as a hydrated inner salt (or chelate), $(C_{10}H_{15}O_6N_3S)Hg.H_2O$. The consideration of a hydroxy complex is not unreasonable in view of crystallographic work by Johansson [11] demonstrating a basic salt structure for mercury(II) perchlorate. Similarly, Björnlund [12] has established that the compound previously formulated as $HgO.Hg(BrO_3)_2.H_2O$ is, in reality, $Hg(OH)BrO_3$. Unfortunately, the three complexes reported here were shown to be amorphous by X-ray diffraction. Attempts to grow crystals at moderate temperature (120 °C) from mercury-sulphur containing complexes in the presence of their mother liquors resulted in complete degradation,[8] and attempts to obtain crystals by solute diffusion [13] were unpromising and thwarted by oxidation of glutathione.

Microanalysis and infrared spectra showed the methylmercury(II) glutathionate complex, obtained using methylmercury chloride, to be anhydrous and to have the composition $C_{11}H_{19}O_6N_3SHg$ and not be in the form of a hydrochloride salt. In this complex, the methylmercury group is bonded only to the sulphur of glutathione and is free of any other intramolecular interaction in solution. This conclusion is consistent with the nature of the 1:1 complex formed by CH_3Hg^+ and *N*-acetyl-L-cysteine [7].

In the case of the complex obtained using a 1:1 molar ratio of glutathione and mercury(II) chloride, complexation was found to be more complicated. Examination of the elementary stoichiometric ratios revealed a chlorine to mercury ratio of 1.5 to 1.0 and provided the basis for consideration of the mixed complex, $[(C_{10}H_{16}O_6N_3S)HgCl.HCl][(C_{10}H_{16}O_6N_3S)Hg(OH).HCl].H_2O$. From the calculated composition: C 20.75; H 3.22; O 19.36; N 7.26; S 5.54; Hg 34.67; Cl 9.19 %, monohydration was rejected on the basis of the unacceptable deviation from the oxygen analyses. The hydrogen analysis is not a sufficiently sensitive indicator for hydration in complexes of high formula weight. The % drying loss is not reliable by itself as an indicator of hydration and is best supported by oxygen analysis. The best fit of the analytical data for the complex of glutathione obtained with $HgCl_2$ was provided by a basic chloride, dihydrochloride formulation: $(C_{10}H_{16}O_6N_3S)_2Hg_2Cl(OH)(HCl)_2$.

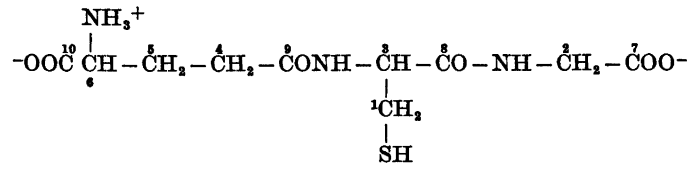
For the complex obtained with glutathione and mercury(II) chloride, it would appear that precipitation of this complex in ethanolic media occurs as a double salt as suggested by elemental analysis. Upon dissolution in water, the double complex must dissociate and behave as a simple 1:1 mercury glutathionate species like that obtained using mercury(II) acetate because its solutions provide similar ^{13}C NMR spectra.

The complexes described above cannot simply be monomeric forms of the $Hg_2(SR)_2^{2-}$ complexes proposed by Kapoor *et al.* [5] The latter were indicated at pH 7 after the consumption of one mol of base per mol of glutathione. Kapoor *et al.* consider that the second equivalent of mercury in the bis complexes co-ordinates with two amino groups made available by neutralization of the ammonium ions. The complexes reported here were isolated under more acidic conditions and would not be expected to show co-ordination by free amino groups. Infrared spectra support this interpretation by the similarity of their broad N–H stretching regions with that of glutathione.

STRUCTURAL EVIDENCE BY ^{13}C NMR

The ^{13}C NMR chemical shift evidence (Table 1) shows that in aqueous solution the glutathione complexes obtained with mercury(II) acetate and mercury(II) chloride are very similar in their structural and electronic features. As it was necessary to examine the mercury(II)

Table 1. ^{13}C NMR chemical shifts (ppm rel. TMS) for D_2O solutions of glutathione and its complexes obtained with mercury(II) acetate, methylmercury chloride, and mercury(II) chloride.



Complex	M. Conc.[a]	pD	1	2	3	4	5	6	7	8	9	10
Free	0.20	3.4	26.3	42.4	56.4	32.1	26.9	54.6	172.8	174.0	174.0	175.3
$Hg(OAc)_2$	0.19	2.0	34.1	43.3	55.7	32.1	26.3	54.0	171.9	173.1	174.7	175.2
CH_3HgCl[b]	0.21	2.2	29.1	42.1	57.5	32.1	26.3	53.7	172.8	173.3	173.5	175.0
$HgCl_2$[c]	0.18	2.3	33.5	42.5	56.7	32.5	26.7	54.5	172.1	–	173.3	175.2

[a] Relative to glutathione; [b] $\delta_{CH_3Hg} = 10.8$ ppm; [c] Measured at 90 °C, all other samples at 37 °C.

glutathionate hydrochloride complex at 90 °C in order to have all the sample in solution, the chemical shifts for this complex may change by approximately 1.0 ppm due to the temperature effect.* Both mercuric complexes show a pronounced downfield shift (~8 ppm) of the methylene carbon bearing the sulphur indicating mercurial bonding to sulphur as generally surmised. A downfield shift of 4.4 ppm (based on noncomplexed glutathione at pD 7.0) was reported by Fuhr and Rabenstein [6] for the cysteinyl methylene carbon of glutathione complexing with mercury(II) nitrate. Jung *et al.* [14] reported a downfield shift of 13 ppm for the same methylene carbon when glutathione was converted to its oxidized (S – S) form.

It is proposed that the co-ordination requirements of mercury(II) ion in our solvated mercuric complexes are satisfied by chelation to a deprotonated peptide nitrogen analogous to that reported by Rabenstein [15] for bis(glycylglycinato)cobaltate (III). Crystal structures of metal-peptide complexes [16] would appear to rule out consideration of chelation to oxygen following lactam-lactim enolization. Two possibilities exist for such peptide nitrogen coordination with either of the amide linkages flanking the methine carbon (C – 3) (see Table 1). Chelation involving the C-9 linkage would result in a 5-membered ring but should produce practically the same perturbation of C-3 as would be expected in the 6-membered ring involving chelation by the C-8 linkage. The fact that C-4 shows essentially the same frequency in the mercuric complexes both at 37 and 90 °C relative to glutathione together with the fact that C-2 for the $Hg(OAc)_2$ complex shows a downfield shift at 37 °C relative to both glutathione and its methylmercury derivative provide evidence for chelation by the C-8 peptide nitrogen. The distinction between perturbations to C-2 and C-4 appears to disappear at 90 °C but the nature of complexation could also be altering. Additional evidence in support of this chelated structure is provided by reference to the methylmercury derivative whose C-2 and C-4 chemical shifts at 37 °C are essentially the same as those for glutathione.

The chemical shifts for C-8 and C-9 of the complexes are assigned on a provisional basis because the differences are relatively small and because the parent frequencies are identical. The small downfield shifts for C-8 and C-9 of the methylmercury derivative of glutathione appear consistent with CH_3Hg bonding to

* The temperature effect was verified by examining solutions of glutathione and of the complex prepared from mercury(II) acetate at 90 °C. The ^{13}C frequencies observed for carbons numbered 2 – 6 in these two samples were essentially the same at those observed for the $HgCl_2$ complex of glutathione at 90 °C. The C-1 frequency of glutathione was shifted downfield by approximately 0.5 ppm at 90 °C confirming that the larger downfield shift occurring with complexation is due to mercurial bonding to sulphur. The carbonyl frequencies were not observed at 90 °C due to increased relaxation times. Prolonged examination at 90 °C was found to be detrimental to these samples, glutathione undergoing oxidation and the complex prepared from mercury(II) acetate discolouring intensely.

sulphur with no other mercurial interaction in solution with other parts of the substrate molecule. The ^{13}C chemical shifts and assignments are consistent with those reported by Jung *et al.* [14] for glutathione and its oxidized form. Some variation in frequency is to be expected between corresponding carbon nuclei of the different complexes due to differences in dipolar and ionic interactions, hydrogen bonding and pH; however, these effects for the carbonyl ^{13}C frequency have been estimated by Maciel and Natterstad [17] to be of the order of 1 ppm.

In their study of the complexation of glutathione by Cd^{2+} and Zn^{2+}, Fuhr and Rabenstein [6] concluded from changes in the Cys-CONH carbon frequency that some binding by these ions might be occurring to the glycyl peptide linkage in addition to the sulphur atom. With mercury(II) nitrate, it was concluded that binding is exclusively to the sulphydryl group at mercury to glutathione ratios up to 0.5 with formation of a 1:2 complex. By comparison with our data (Table 1) which shows deshielding of the cysteinyl methylene carbon of glutathione by 7.8 and 2.8 ppm for complexation with mercury(II) acetate and methylmercury chloride, respectively, it is noteworthy that the deshielding produced by complexation with mercury(II) nitrate (4.4 ppm) is more similar to that obtained with methylmercury chloride. It is not clear from the mercury(II) nitrate study whether nitrate is completely dissociated from mercury or if complexation involves $^{+}HgNO_3$ and 1:1 complex formation. Complexation of glutathione by mercury(II) acetate unquestionably involves loss of the acetate groups.

EXPERIMENTAL

A Varian XL-100-NMR spectrometer was used to obtain ^1H noise decoupled 25.2 MHz Fourier transform ^{13}C NMR spectra of approximately 0.2 M deuterium oxide solutions of glutathione and of its mercury complexes. Frequencies were measured relative to dioxane and converted to the TMS scale ($\delta_{TMS} = \delta_{dioxane} + 67.4$ ppm).[18] An accumulation time of approximately one hour was required to accumulate 1000 free induction decays (FID) each of 0.4 s with a pulse delay of 3.6 s. A pulse width of 80 μs was used. Off-resonance decoupling was employed to verify the chemical shifts and assignments reported by Jung *et al.* [14] for glutathione.

Microanalyses were performed by Alfred Bernhardt, Mikroanalytisches Laboratorium, 5251 Elbach über Engelkirchen, Fritz-Pregle-Strasse 14 – 16, W. Germany. Appropriate separatory processes were employed to eliminate interference of mercury with the C, H, and Cl determination. Sulphur was analyzed by a reductive process.

Mercury(II) chloride complex of glutathione, $(C_{10}H_{16}O_6N_3S)_2Hg_2Cl(OH)(HCl)_2$. Glutathione (0.768 g, 0.0025 mol, Sigma, reduced form) was dissolved in warm (50 °C) 95 % ethanol (10 ml) with magnetic stirring by adding just sufficient water (8 ml) for dissolution. A solution of mercury(II) chloride (0.679 g, 0.0025 mol, BDH Analar) in 95 % ethanol (10 ml) was added dropwise to the stirred glutathione solution. Precipitation occurred immediately, but the solid readily dissolved with stirring except near the end of the addition when a flocculent precipitate persisted. When the last few drops of $HgCl_2$ solution were added, the mixture formed a white slurry. When a white gum appeared after 5 min of stirring and cooling, the mixture was treated with water. Immediate clarification of the supernatant occurred, and the mixture was treated periodically with water (total 20 ml) until all gum had dissolved with stirring at room temperature. Finely divided solid was obtained from the mixture after refrigeration for 4 h. The solid was collected, washed with 95 % ethanol and dried over NaOH *in vacuo*. The yield of product was 1.03 g or 73 %. The product was found to be appreciably soluble in water. IR spectra (4000 – 600 cm^{-1}) appeared to be little different from glutathione, and X-ray diffraction patterns showed the product to be amorphous. (Found: C 21.25; H 3.41; O 18.47, 18.66, 18.62; N 7.20; S 5.58; Hg 34.84, 34.04; Cl 9.09, 9.07, 9.12; and drying loss (50 °C/HV), 3.11 %. $(C_{10}H_{16}O_6N_3S)_2Hg_2Cl(OH)(HCl)_2$ (M.W. 1139.26) requires C 21.08; H 3.10; O 18.26; N 7.38; S 5.63; Hg 35.22; Cl 9.34.)

Mercury(II) glutathionate monohydrate, $(C_{10}H_{15}O_6N_3S)Hg.H_2O$. Glutathione (0.768 g, 0.0025 mol, Sigma, reduced form) was dissolved at room temperature in water (20 ml) and 95 % ethanol (10 ml), then the solution was warmed to 40 °C. To this solution, a warm (40 °C) solution of mercury(II) acetate (0.797 g, 0.0025 mol, Merck) in methanol (10 ml) was added dropwise with magnetic stirring. Precipitation occurred immediately and persisted gradually producing coagulated spherical masses. Towards the end of the addition a milky suspension was obtained. On cooling to room temperature, the mixture lost its stickiness and the solid could be easily pulverized in the presence of the supernatant liquid. When the solid was found to be extremely difficult to filter with suction due to clogging of the paper, it was collected by slow filtration using a fluted paper. The very finely divided, powdery solid was washed with acetone and dried over NaOH *in vacuo*. The yield was 0.97 g or 75 %. The solid is insoluble in water at room temperature, but it dissolves readily on

addition of a drop of 12 M HCl or trifluoroacetic acid. Infrared spectra showed the absence of acetyl C=O stretching, otherwise, the spectral features (4000 – 600 cm^{-1}) are very similar to those of glutathione. X-Ray diffraction patterns showed the solid to be amorphous. (Found: C 23.05; H 3.48; O 21.36; N 7.96; S 6.14; Hg 38.23; drying loss (50 °C/HV), 3.23. ($C_{10}H_{16}O_6N_3S$); $Hg.H_2O$ (M.W. 524.14) requires C 22.90; H 3.27; O 21.37; N 8.02; S 6.12; Hg 38.27, and drying loss, 3.44 %).

Methylmercury glutathionate, ($C_{10}H_{16}O_6N_3S$)-$HgCH_3$. CAUTION: Use well-ventilated hood and exercise precautions with alkyl mercury.[19]

A solution of glutathione (0.768 g, 0.0025 mol, Sigma reduced form) was prepared in water (10 ml) with slight warming and then diluted with 95 % ethanol (10 ml). A saturated solution of methylmercuric chloride (0.627 g, 0.0025 mol, Alfa Inorganics) was prepared in 95 % ethanol (40 ml). The mercurial solution was added quickly since no precipitation or "tail-effect" formed during addition. The small portion of undissolved CH_3HgCl was transferred to the reaction mixture during washing with ethanol. No precipitation occurred following the addition nor on standing of the mixture.

After treating the mixture with acetone until turbid and refrigerating it, a flocculent white solid was collected, washed with acetone and dried over NaOH *in vacuo* (Fr. A, 0.212 g). The mother liquor was reduced to about 10 ml by rotary evaporation, then it was treated with diethyl ether to obtain turbidity – some acetone had to be added to maintain miscibility – however, an oil formed. Slight warming and addition of ethanol produced no beneficial effect. On standing overnight, the syrupy residue hardened to a gum. The mixture was then heated until the gum dissolved. When the solution had cooled to room temperature ether was added slowly and periodically over a 4 h period to maintain a light turbidity. Solid (Fr. B, 0.445 g) was then obtained following refrigeration. The mother liquor, on evaporation in a petri dish in a fume hood, gave a hard, glassy residue (Fr. C, 0.358 g). Fractions A and B contracted in volume appreciably during drying *in vacuo* (20 mmHg) at room temperature, but there appeared to be no evidence for sublimation. Each fraction was found to be amorphous by X-ray diffraction, and infrared spectra were rather similar to spectra of glutathione. (Found: C 25.40; H 3.98; O 18.33; N 7.88; S 6.05; Hg 38.47. $C_{11}H_{19}O_6N_3SHg$ (M.W. 521.96) requires C 25.31; H 3.69; O 18.39; N 8.05; S 6.14; Hg 38.45.)

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Short Communications

The Relevance of Base Catalysis in Aromatic Nucleophilic Substitution to C—X Bond Cleavage

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Lamm and Lammert [1] have recently criticized the "absence of element effect" criterion for assessing whether or not bond-breaking occurs during the rate-determining step of nucleophilic substitution reactions. According to this criterion, originated by Bunnett, Garbisch and Pruitt [2] and initially applied to S_NAr reactions, if several substrates whose leaving group first atoms represent different elements react with a common nucleophilic reagent at nearly the same rate, scission of the bond to the leaving group does not occur during the rate-determining step. (A proviso, however, is that if rate-determining scission of the C–X bond had made little progress at the transition state, a sameness of rate might nevertheless result.)

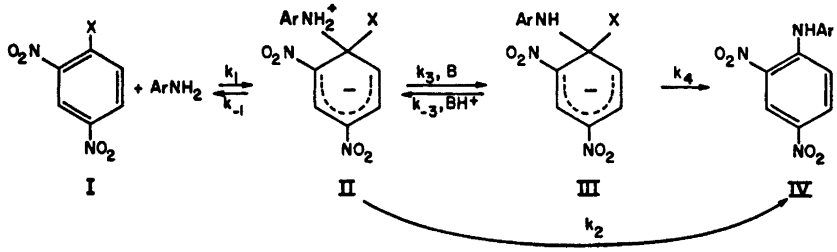
Bernasconi and Zollinger [3] reported in 1966 that the reaction of 1-chloro-2,4-dinitrobenzene with *p*-anisidine in benzene solution is strongly catalyzed by DABCO (1,4-diaza[2.2.2]bicyclooctane). The rate law has a prominent third-order term, first order in substrate, first order in anisidine and first order in DABCO. Lamm and Lammert extended study of that process to embrace also the leaving groups Br, $PhSO_2$, and $MeSO_2$. Among these four, whose first atoms represent three elements, the maximum difference in the third-order rate constants was 9-fold, rather greater than the maximum difference of 1.3-fold for reactions of the same substrates (excepting the methylsulfonyl compound) with piperidine in methanol,[2] but nevertheless a small difference.

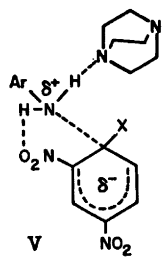
After Bernasconi and Zollinger, Lamm and Lammert took the third-order term to represent base catalysis. They accepted base catalysis as evidence that the rate-determining step is, in their words, "the second reaction step, involving cleavage of the C–X bond." From their results they drew the inference that a small element effect is of limited validity as a criterion of mechanism in the sense mentioned.

The reservations of Lamm and Lammert, if well grounded, would have important implications regarding the question of whether nucleophilic aromatic substitution and the analogous nucleophilic vinylic substitution [4] are one- or multi-step processes. Thus, in vinylic substitutions [4] retention of stereochemistry argues for a concerted process [5] while the near absence of a leaving group element effect indicates a multi-step mechanism. It is important to know whether the element effect criterion has general validity.

The work of Lamm and Lammert unfortunately does not constitute a definitive test of the criterion. The crucial point is that although a valid third-order term in the rate law, as described, does indicate the presence of a DABCO molecule in the rate-determining transition state, it does not indicate the structure of the transition state or the function of the DABCO molecule in it. There are at least two reasonable structures for the transition state which do not involve scission of the C–X bond.

First, it is conceivable that the initial (k_1) step is rate-limiting, with DABCO present in the transition state interacting by hydrogen-bonding with a rather acidic hydrogen of the anisidine amino moiety. In the transition state (V), one of the amino hydrogens would hydrogen-bond intramolecularly to the *o*-nitro





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group [6] and the other to a DABCO molecule. The feasibility of this model depends on the relative magnitudes of the decreases in $T\Delta S^{\ddagger}$ and in $\Delta H^{\ddagger}$ implied by incorporation of a DABCO molecule. The loss in $T\Delta S^{\ddagger}$ is likely to be about 5 kcal [7] and the strength of the hydrogen bond might well be of similar or greater magnitude; DABCO is quite basic, the amino hydrogens are likely to be quite acidic (because the transition state is probably attained late in the attachment of anisidine nitrogen to aromatic carbon) and the hydrogen bond is expected to be a strong one. (The fact that the reaction of I (X = Cl) with butylamine in benzene is merely second order,[8] which might seem to contradict this model, could be rationalized by postulating for a primary amine of high nucleophilicity an early transition state, in which the amine hydrogens would not be very acidic.)

A second alternative takes account of good evidence [9] that S_NAr reactions of amine nucleophiles may involve three steps: Initial formation of zwitterionic σ-complex II, deprotonation of II by a base to form anionic σ-complex III, and finally leaving group expulsion. Lamm and Lammert discussed it, however, as a two-step process. It is possible that formation of II is a mobile equilibrium and that the deprotonation (k_3) step is rate-limiting. The DABCO molecule would be present in the rate-determining transition state, taking the proton, but there would be no scission of the C–X bond. This model would require that expulsion of the leaving group from III be much faster than reprotonation to II.

There are now known several examples of relatively slow reactions, in water solution, that have rate-determining proton transfer steps.[10] Rate-determining proton transfer steps are even more likely in benzene because hydrogen-bonded networks of solvent to acidic or basic sites, which expedite proton transfer,[11] are not possible.

Because there are plausible alternative interpretations of the results of Lamm and Lammert, they do not constitute a serious challenge to the validity of the element effect as a criterion of mechanism.

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A Novel Fragmentation in the Mass Spectra of Some 3-(2-Hydroxyphenyl)-4-oxo-3,4-dihydroquinazolines

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In our current study on metabolites of the hypnotic drug methaqualone [2-methyl-4-oxo-3-(2-tolyl)-3,4-dihydroquinazoline] we reported the mass spectra of its monohydroxy derivatives.[1] We found that the fragmentation of these compounds was dominated by loss of the

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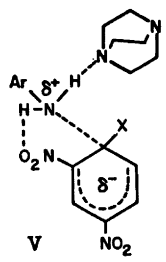

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group [6] and the other to a DABCO molecule. The feasibility of this model depends on the relative magnitudes of the decreases in $T\Delta S^{\ddagger}$ and in $\Delta H^{\ddagger}$ implied by incorporation of a DABCO molecule. The loss in $T\Delta S^{\ddagger}$ is likely to be about 5 kcal [7] and the strength of the hydrogen bond might well be of similar or greater magnitude; DABCO is quite basic, the amino hydrogens are likely to be quite acidic (because the transition state is probably attained late in the attachment of anisidine nitrogen to aromatic carbon) and the hydrogen bond is expected to be a strong one. (The fact that the reaction of I (X = Cl) with butylamine in benzene is merely second order,[8] which might seem to contradict this model, could be rationalized by postulating for a primary amine of high nucleophilicity an early transition state, in which the amine hydrogens would not be very acidic.)

A second alternative takes account of good evidence [9] that S_NAr reactions of amine nucleophiles may involve three steps: Initial formation of zwitterionic σ-complex II, deprotonation of II by a base to form anionic σ-complex III, and finally leaving group expulsion. Lamm and Lammert discussed it, however, as a two-step process. It is possible that formation of II is a mobile equilibrium and that the deprotonation (k_3) step is rate-limiting. The DABCO molecule would be present in the rate-determining transition state, taking the proton, but there would be no scission of the C–X bond. This model would require that expulsion of the leaving group from III be much faster than reprotonation to II.

There are now known several examples of relatively slow reactions, in water solution, that have rate-determining proton transfer steps.[10] Rate-determining proton transfer steps are even more likely in benzene because hydrogen-bonded networks of solvent to acidic or basic sites, which expedite proton transfer,[11] are not possible.

Because there are plausible alternative interpretations of the results of Lamm and Lammert, they do not constitute a serious challenge to the validity of the element effect as a criterion of mechanism.

Acknowledgement. We thank Professor C. F. Bernasconi for discussions.

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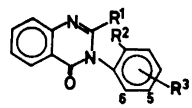
Table 1. Chemical and mass spectral data of the investigated compounds.



No.	R^1	R^2	R^3	Yield[a] %	M.p. °C	Prominent peaks (rel. int. %)
1[b]	CH_3	OH	6-CH_3	94	227.5–228.5	M^+ = 266(36), M^+ – 15(100), M^+ – 42(59)
2	H	OH	6-CH_3	48	203–205	M^+ = 252(100), M^+ – 17(76), M^+ – 28(16)
3	H	OCH_3	6-CH_3	53	133–133.5	M^+ = 266(96), M^+ – 1(61), M^+ – 17(39), M^+ – 31(100)
4	C_2H_5	OH	6-CH_3	68	130–132	M^+ = 280(40), M^+ – 15(12), M^+ – 29(100), M^+ – 56(40)
5	C_2H_5	OCH_3	6-CH_3	54	173.5–174	M^+ = 294(36), M^+ – 15(18), M^+ – 29(100), M^+ – 31(64)
6	H	OH	6-OH	40	152–155	M^+ = 254(100), M^+ – 17(51), M^+ – 28(42)
7	H	OCH_3	6-OCH_3	51	163–165	M^+ = 282(68), M^+ – 31(100)
8	CH_3	OH	6-OH	52	183–186	M^+ = 268(40), M^+ – 15(12), M^+ – 42(100)
9	CH_3	OCH_3	6-OCH_3	35	212–215	M^+ = 296(100), M^+ – 15(50), M^+ – 31(83)
10	C_2H_5	OH	6-OH	45	273–277	M^+ = 282(39), M^+ – 29(32), M^+ – 56(100)
11	C_2H_5	OCH_3	6-OCH_3	10	228–231	M^+ = 310(77), M^+ – 29(100), M^+ – 31(91)
12	CH_3	OH	5-CH_3	54	236–237	M^+ = 266(78), M^+ – 15(60), M^+ – 42(100)

[a] After recrystallization from ethanol or aqueous ethanol. [b] This compound has been described earlier.[1,2]

group in position 2, giving rise to the base peak. In the spectrum of one of these compounds, 3-(2-hydroxy-6-methylphenyl)-2-methyl-4-oxo-3,4-dihydroquinazoline (1), an unexpected and prominent peak at $M^+ - 42$ was observed. High resolution measurement and a metastable peak verified that the corresponding transition was due to the expulsion of a ketene moiety from the molecular ion. However, according to the literature no reasonable explanation for this unusual fragmentation seems to be available. In order to elucidate the mechanism the shift technique was used. Thus a number of suitably substituted 4-oxo-3,4-dihydroquinazolines were synthesized and their mass spectra evaluated. The data are collected in Table 1. In analogy to the behaviour of the monohydroxy derivatives of methaqualone, important peaks corresponding to loss of the 2-substituent appear, *i.e.* $M^+ - C_2H_5$ in the spectra of 4, 5, 10, and 11 and $M^+ - CH_3$ in the spectra of 8, 9, and 12.

All compounds having R^2 = OH (Table 1) also fragment through another important route, related to the loss of ketene from compound 1. Thus the spectra of compounds 2 and 6 exhibit a prominent peak at $M^+ - CO$, compounds 8 and 12 at $M^+ - CH_2CO$ and compounds 4 and 10 at $M^+ - CH_3CHCO$.

These data indicate the origin of the atoms involved. The fragmentation implies interaction between the group at C-2 in the quinazoline ring and the phenolic group R^2. For the case when ketene is lost, the reaction may tentatively be formulated as outlined in Fig. 1.

The mechanism of the fragmentation can be concerted or involve a skeletal rearrangement.

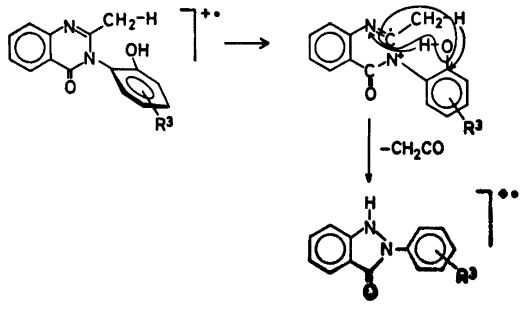


Fig. 1.

In compounds having a 2,6-disubstituted aromatic ring at N-3 this ring and the oxoquinazoline nucleus obviously are not coplanar. This implies that an initial cleavage of the quinazoline ring must occur in order for the interaction to take place (*cf.* Fig. 1). However, on the basis of the present evidence it is not feasible to speculate further on the details of the fragmentation. The driving force can be assumed to originate in the liberation of a neutral fragment. Besides, an energetically favourable steric arrangement must take place in order to make the reaction possible. This assumption is supported by the fact that only compounds having a hydroxyl group in *ortho*-position (R^2 = OH) are exposed to the fragmentation.[1]

Experimental. Melting points were determined in open capillary tubes in an electrically heated

metal block, using calibrated Anschütz thermometers. Infrared spectra were run in KBr discs using a Perkin-Elmer Infracord Model 157G with a grating monochromator. Infrared spectra were routinely recorded and are in agreement with the expected structures. Mass spectra were recorded on an AEI MS-30 mass spectrometer. The ionizing energy was maintained at 70 eV and the temperature of the source at 200°C.

Synthesis. Compounds having a methoxy group (*i.e.* compounds No. 3, 5, 7, 9, and 11) were prepared as follows. *N*-Formylanthranilic acid, *N*-acetylanthranilic acid, or *N*-propionylanthranilic acid (0.01 mol) was condensed with 2-methoxy-6-methylaniline [3] or 2,6-dimethoxyaniline [3] (0.01 mol) in the presence of phosphorus trichloride (0.005 mol) as earlier described.[2] Compounds 2, 4, 6, 8, 10, and 12 were prepared from the corresponding methoxy derivatives by boiling with an excess of 48 % hydrobromic acid for 1.5 h.

Acknowledgement. This work is supported by the Swedish Medical Research Council (Project No. 03X-3760 and 70E-3743).

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On the Stereochemistry of the Interaction between Nucleic Acids and Basic Protein Side Chains

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Interactions between nucleic acids and basic proteins play an important role in cell chemistry and it would appear to be of interest to establish the stereochemistry of these interactions at the atomic level. We thought that some information might be obtained by X-ray analysis of single crystals of salts between phosphate diesters and various organic bases simulating arginine and lysine side chains. Such complexes may serve as models for the contacts occurring in nucleoproteins between phosphate groups and basic amino acids. The compounds studied are the diethyl phosphates of propylguanidine (I), arginine (II) and putrescine (III). A good model should preferably contain no hydrogen bond forming groups other than those present at the contacts, and compound (II) is therefore less satisfactory than the others. The crystal structures of the three compounds have been reported elsewhere.[1-3] In the present note the patterns of hydrogen bonding will be discussed and related to the structure of complexes between DNA and polyarginine, protamine, and polylysine.

The bonding between arginine and phosphate diesters. Arginine forms salts with phosphate diesters and bonding occurs between $(RO)_2PO_2^-$ and the guanidinium cationic group $-(NH)C(NH_2)_2^+$. In Fig. 1 the surroundings of the guanidinium group in model compounds (I) and (II) are shown. Extensive hydrogen bonding occurs, the stereochemistry of which may be described as follows:

(1) The guanidinium group forms five N−H···O hydrogen bonds to oxygen atoms in neighbouring molecules. These bonds are not far from linear and lie roughly in the plane of the guanidinium group, as is to be expected. The tendency for this general pattern of hydrogen bonding is also evident in crystal structures of inorganic salts of arginine.[4] In the present structures, especially (I), in which the guanidinium group is bonded only to diester phosphate groups, some additional characteristic stereochemical features are observed:

(2) One of the five hydrogen bonds both in (I) and (II) involves an ester oxygen atom and is much weaker (length 3.08 Å and 3.09 Å, respectively) than the others, which lie within the normal range for N−H···O bonds (mean length about 2.85 Å). The direction of this bond is roughly that of the bisecting line of the P−O−C angle. Such a hydrogen bond has apparently not been observed previously.

(3) Four of the hydrogen bonds occur in two pairs of nearly parallel bonds to oxygen atoms in the same anionic group. The N···N distances in the guanidinium group (*ca.* 2.3 Å) are not far from the O···O distances in phosphates (*ca.* 2.5 Å) and carboxylates (*ca.* 2.25 Å), making it stereochemically favourable for pairs of nearly parallel bonds to be formed. The pairs are of two types, a "strong" pair involving two normal bonds to the two *oxo* oxygen atoms in the same phosphate group, and a "weak" pair of one normal and one weak bond to one *oxo* and one ester oxygen atom, respectively. The pairs are at an angle of about 120° with one another. In compound (II) the "strong" pair is formed to the carboxyl group rather than to the phosphate, but the "weak" pair exists in both structures. It may be concluded that a guanidinium group may form a strong link between two phosphate diester groups by pairs of hydrogen bonds, and that

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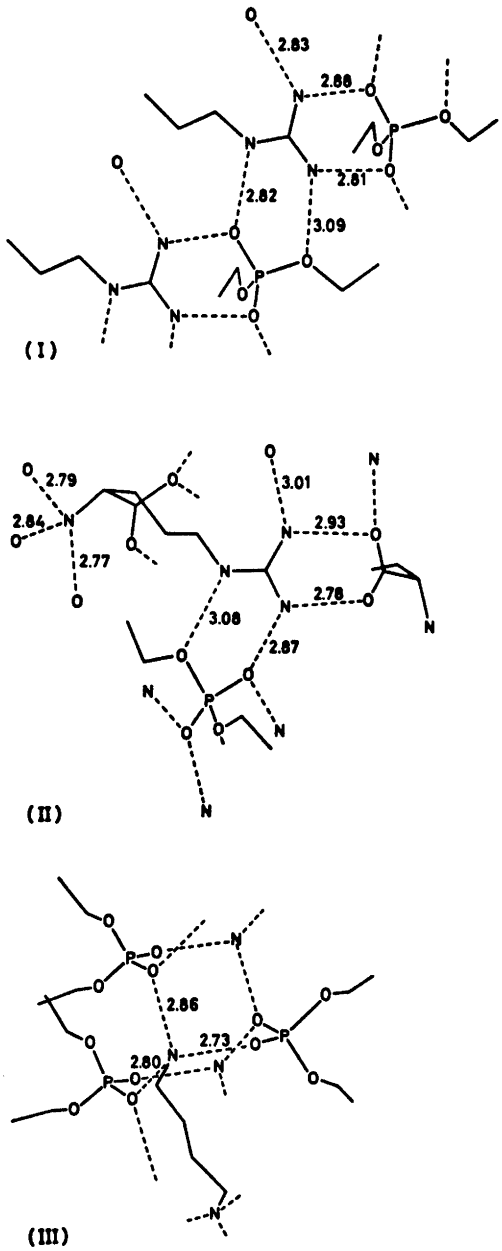

Fig. 1. Hydrogen bonds (broken lines) in diethyl phosphates of propylguanidine (I), arginine (II), and putrescine (III). Hydrogen atoms are not shown.

a phosphate diester group may be linked to two arginines, as shown by structure (I).

(4) The angles between the N–H···O bonds and the adjacent P–O (*oxo*) bonds lie in the range 108°–137°, the mean value being 123°. The hydrogen bonds of the "weak" pairs nearly lie in the O–P–O plane of the acceptor oxygen atoms, whereas the bonds of the "strong" pair form an angle of about 25° with it. In other structures this angle may be greater, but it seems likely that the general direction of any pair will be so as to point away from the oxygen atoms to which they are not bonded.

The stereochemical features described above in (1)–(4) are likely to persist at contacts between phosphate diesters and arginines in general and should be taken into account when formulating structural models of nucleoproteins and related complexes at the atomic level.

As an example, the hydrogen bonding in DNA-polyarginine and DNA-protamine complexes will be discussed on this basis. A general model of the structure of these complexes has been proposed, in which the polypeptide chain winds helically around the DNA molecule in the small groove.[6] The stereochemistry of the phosphate/guanidinium interactions was, however, not described. We have studied the stereochemical fit between DNA and polyarginine by wire models (scale 1 Å = 4 cm) using the coordinates of Arnott and Hukins [5] for B-DNA. We assume that the ten-fold symmetry is maintained in a general way on complex formation, as indicated by the X-ray fibre diagrams,[6,7] and that only minor modifications in the position and orientation of the phosphate groups occur. The different stable arginine conformations [4] were considered, and also the fact that the Cα carbon atoms have to lie less than about 10 Å from the ten-fold axis because of the symmetry (dipeptide length < 7.2 Å).

There are several feasible ways of linking the arginines to the phosphates in B-DNA by hydrogen bonds. In view of the bonding observed in the model compounds it appears, however, likely that both *oxo* oxygen atoms of the phosphates are engaged in the formation of a "strong" pair of hydrogen bonds to a guanidinium group, and satisfactory models can be built on this basis. The other pair from the guanidinium groups points away from the complex and may form cross-links to phosphates in the complementary helix in neighbouring complexes. This bonding pattern is similar to the one observed in compound (I). The distances between complexes in fibres of DNA-polyarginine and DNA-protamine [6,7] are consistent with the existence of such guanidinium bridges.

This bonding scheme is stereochemically compatible with placing the polypeptide chain in either of the DNA grooves, although the large groove appears to be the more favourable one. X-Ray evidence indicates, however, that the small groove is the site of binding in the DNA-protamine complex.[6,7]

It is also possible from a stereochemical point of view that the guanidinium groups link together consecutive phosphates along the DNA helices, as has been proposed.[7] The "strong" pair of hydrogen bonds can, however, in this case not be formed without strain, and we consider this possibility less likely than the one described above.

The bonding between lysine and phosphate diester. The $-NH_3^+$ group of protonated lysine would be expected to form, if possible, three hydrogen bonds in roughly tetrahedral arrangement, as observed in compound (III) (Fig. 1) and also in crystals of amino acids. The P–O···N angles in (III) lie in the range 110–135° and the mean length of the three bonds is 2.80 Å.

The oxygen atoms receiving hydrogen bonds from an NH_3^+ group are at distances of about 5 Å. The most favorable hydrogen bonding scheme in B-DNA-polylysine complexes would appear to be that the lysine NH_3^+ groups are linked to an *oxo* oxygen atom (O3) in one phosphate and, by a weak bond, to an *ester* oxygen atom (O4) in the next phosphate along the helix, these atoms being at a distance of 5.0 Å. *Oxo* oxygen atoms of consecutive phosphates seem to be too far apart. The third bond would then be formed to an *oxo* oxygen atom in the complementary helix in a neighbouring complex. The distance between complexes in fibres is consistent with this view.

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Algal Carotenoids. XI.* New Carotenoid Epoxides from *Trentepohlia iolithus*

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A recent re-examination of the carotenoids of the green alga *Trentepohlia iolithus*[1] demonstrated the natural occurrence of carotenoids containing 2-hydroxylated β-rings. Structures *1*, *2*, and *3* (Scheme 1) were established from full spectroscopic characterization. The absolute stereochemistry has subsequently been confirmed.[2]

We now report the natural occurrence of the epoxides *4a* and *7* (probably *7a*) of β,ε-caroten-2-ol (*1*) and of β,β-caroten-2-ol (*2*), comprising 0.2 % (0.4 mg) and 0.3 % (0.6 mg), respectively, of the total carotenoids of *T. iolithus*.

In order to include stereochemical considerations the partial synthesis of the 5,6-epoxides *4a* and *4b* from β,ε-caroten-2-ol (*1*, 3.6 mg) by *m*-chloroperbenzoic acid,[3] Scheme 2, will be discussed first. Reported data for relevant cyclohexene model substances reveal a directive effect of a hydroxy substituent, resulting in preferential epoxidation *cis* to the hydroxy substituent, explained by hydrogen bonding between the hydroxy group and the peracid.[4] Similar results are observed with an acetoxy substituent.[5] Thus zeaxanthin (β,β-caroten-3,3′-diol) diacetate gives preferentially *cis* products (*cis* relationship between the epoxy and acetoxy groups) on epoxidation.[5]

On epoxidation of β,ε-caroten-2-ol (*1*) two diastereomeric products *4a* (60 % of total) and *4b* (40 % of total) were obtained. Both *4a* and *4b* exhibited λ_{max} (acetone) 419, 441.5, and 470 nm and *m/e* 568 (M), fragment ions M–16, M–80, 181, and 221 consistent with carotenoid epoxides, as well as common fragment ions M–92, M–106, M–16–92, M–16–106, M–80–92 ascribed to eliminations from the polyene chain, combined with losses of 16 and 80 mass units.[6a] Relative yields and adsorptive properties support the stereochemistry assigned to the epoxidic products *4a* and *4b*, see Scheme 2. Thus *4a* was chromatographically less strongly retained than *4b*, compatible with intramolecular hydrogen bonding, and *4a* indeed showed the predicted hydrogen bonding in IR (predominantly associated hydroxyl at 3509 cm^{-1} as expected for conformation A Scheme 2, *cf.* Ref. 6b).

On furanoid rearrangement with hydrogen chloride in ether the epoxide *4a*, referred to

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Algal Carotenoids. XI.* New Carotenoid Epoxides from *Trentepohlia iolithus*

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A recent re-examination of the carotenoids of the green alga *Trentepohlia iolithus*[1] demonstrated the natural occurrence of carotenoids containing 2-hydroxylated β-rings. Structures *1*, *2*, and *3* (Scheme 1) were established from full spectroscopic characterization. The absolute stereochemistry has subsequently been confirmed.[2]

We now report the natural occurrence of the epoxides *4a* and *7* (probably *7a*) of β,ε-caroten-2-ol (*1*) and of β,β-caroten-2-ol (*2*), comprising 0.2 % (0.4 mg) and 0.3 % (0.6 mg), respectively, of the total carotenoids of *T. iolithus*.

In order to include stereochemical considerations the partial synthesis of the 5,6-epoxides *4a* and *4b* from β,ε-caroten-2-ol (*1*, 3.6 mg) by *m*-chloroperbenzoic acid,[3] Scheme 2, will be discussed first. Reported data for relevant cyclohexene model substances reveal a directive effect of a hydroxy substituent, resulting in preferential epoxidation *cis* to the hydroxy substituent, explained by hydrogen bonding between the hydroxy group and the peracid.[4] Similar results are observed with an acetoxy substituent.[5] Thus zeaxanthin (β,β-caroten-3,3′-diol) diacetate gives preferentially *cis* products (*cis* relationship between the epoxy and acetoxy groups) on epoxidation.[5]

On epoxidation of β,ε-caroten-2-ol (*1*) two diastereomeric products *4a* (60 % of total) and *4b* (40 % of total) were obtained. Both *4a* and *4b* exhibited λ_{max} (acetone) 419, 441.5, and 470 nm and *m/e* 568 (M), fragment ions M–16, M–80, 181, and 221 consistent with carotenoid epoxides, as well as common fragment ions M–92, M–106, M–16–92, M–16–106, M–80–92 ascribed to eliminations from the polyene chain, combined with losses of 16 and 80 mass units.[6a] Relative yields and adsorptive properties support the stereochemistry assigned to the epoxidic products *4a* and *4b*, see Scheme 2. Thus *4a* was chromatographically less strongly retained than *4b*, compatible with intramolecular hydrogen bonding, and *4a* indeed showed the predicted hydrogen bonding in IR (predominantly associated hydroxyl at 3509 cm^{-1} as expected for conformation A Scheme 2, *cf.* Ref. 6b).

On furanoid rearrangement with hydrogen chloride in ether the epoxide *4a*, referred to

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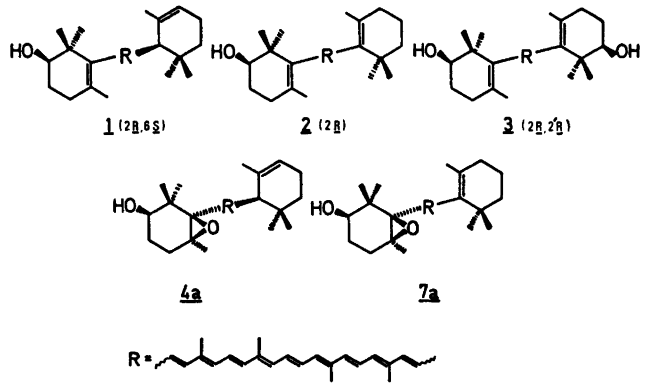
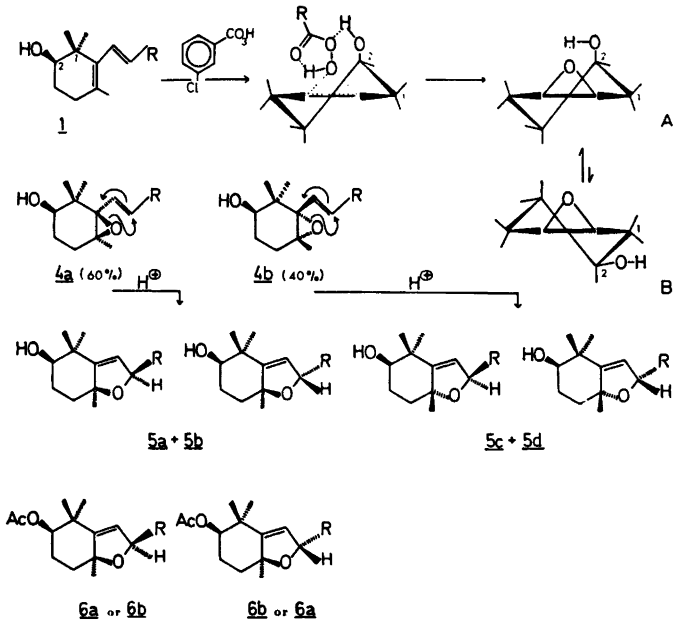


Scheme 1.



Scheme 2.

as *cis* epoxide, gave two products *5a* and *5b*, assumed to be C-8 epimers on the basis of the favoured mechanism for furanoid rearrangement, not altering the stereochemistry at C – 5,[7] Scheme 2. The epoxide *4b*, referred to as *trans* epoxide, likewise gave two furanoid products *5c* and *5d*. Relative R_F-values were *5a* > *5c* > *5b* = *5d*. However, in spite of identical R_F-values *5b* and *5d* are considered to be diastereomers. The furanoid products *5a*, *b*, *c*, *d* all had λ_{max} (methanol) at 396, 420, and 446 nm.

Separate acetylation of the furanoxides *5a* and *5b* was slow (17 h, 55 % conversion, typical of 2-hydroxy substitution [8]) and gave the monoacetates *6a* and *6b*, not separable in the systems tested. In general separation of the stereoisomeric epoxides and furanoxides was more difficult when acetylated.

Turning now to the naturally occurring epoxide *4a*, the natural epoxide had electronic spectrum, mass spectrum and R_F-value (co-chromatography) consistent with semisynthetic *4a* described above, and provided on standard acetylation slowly (*cf.* Ref. 8) a monoacetate (80 % conversion after 8 h) with unchanged electronic spectrum and *m/e* 610 (M), M – 16,

M−60, M−80. Furanoid rearrangement of *4a* gave two products with electronic spectra, mass spectra, and R_F-values (co-chromatography) as *5a* and *5b* described above. Separate acetylation of the furanoid products *5a* and *5b* gave 60 % conversion to the corresponding acetates, inseparable in our chromatographic systems and from *5a,b* derived from *1* in a special reaction discussed in the following paper.[8] On this basis it is concluded that the natural epoxide has structure *4a* (*cis*). It deserves comment that natural violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), has a *trans* relationship between the 5,6-epoxy bridge and the 3-hydroxy substituent.[4,7] However, the present epoxide *4a* and violaxanthin both have the same axial/equatorial relationship between the hydroxy substituent and the polyene chain, see Scheme 2 for alternative conformations A and B of *4a*.

The second naturally occurring epoxide *7* exhibited λ_{max} (ether) 423, 443, and 472 nm consistent with data reported for the mono-epoxide of β,β-carotene [9] and mass spectrum like *4a* except an (M−56−18) peak. Acetylation of *7* provided a monoacetate with unchanged electronic spectrum and mass spectrum as for *4a*-monoacetate with no significant RDA-fragmentation. Furanoid rearrangement of *7* gave two products both with λ_{max} (methanol) 404, 423, and 445 nm and *m/e* 568 (M), M−16, M−80, 221, inseparable from two furanoid products obtained from β,β-caroten-2-ol (*2*) in the reaction discussed in the following paper.[8] From the stereochemistry observed for the corresponding reaction of β,ε-caroten-2-ol (*1*) [8] and by analogy with the natural epoxide *4a*, the stereochemistry of *7a* (*cis*) is considered likely for the second epoxide *7* isolated from *T. iolithius*.

Methods commonly employed in this laboratory were used. Experimental details are given elsewhere.[10] The epoxides *4a* and *7* were readily separated from *1*, *2*, and *3* on magnesium oxide columns (benzene). R_F-values (Schleicher & Schüll No. 287 circular, kieselguhr paper, 1 % acetone in petroleum ether) were: *4a* (0.56), *4b* (0.35), *4a*-acetate (0.88), *5a* and *5b* (0.48 and 0.18), *5c* and *5d* (0.33 and 0.18), *6a* and *6b* (0.72), *7* (0.53), *7*-acetate (0.84), furanoid *7* (0.22 and 0.55), and furanoid *7*-acetates (0.74). Purification for mass spectrometry was achieved by TLC on kieselgel, 20 % acetone in petroleum ether).

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Algal Carotenoids. XII.* Chemical Reactions of Carotenoids with 2-Hydroxylated β-Rings

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Spectroscopic evidence alone was sufficient for the structural elucidation of the first carotenoids with 2-hydroxylated β-rings, namely β,ε-caroten-2-ol (*1a*), β,β-caroten-2-ol (*2a*), and β,β-carotene-2,2′-diol (*3*), Scheme 1, from the green alga *Trentepohlia iolithus*.[1,2] We now report a chemical characterization of carotenoids possessing this end group (*1a* and *2a*).

Models reveal steric hindrance of the 2-hydroxy-substituent of a β-ring. Lower reactivity than for analogous 3-hydroxy carotenoids was therefore predicted.

Standard acetylation [3] of β,β-caroten-2-ol (*2a*) was slower than for β,β-caroten-3-ol (*4a*): 50 % and 100 % conversion, respectively, to the corresponding acetates *2b* and *4b* after 3.5 h, see Fig. 1. The 2-hydroxy compound

* No. XI. *Acta Chem. Scand. B 28* (1974) 483.

M−60, M−80. Furanoid rearrangement of *4a* gave two products with electronic spectra, mass spectra, and R_F-values (co-chromatography) as *5a* and *5b* described above. Separate acetylation of the furanoid products *5a* and *5b* gave 60 % conversion to the corresponding acetates, inseparable in our chromatographic systems and from *5a,b* derived from *1* in a special reaction discussed in the following paper.[8] On this basis it is concluded that the natural epoxide has structure *4a* (*cis*). It deserves comment that natural violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), has a *trans* relationship between the 5,6-epoxy bridge and the 3-hydroxy substituent.[4,7] However, the present epoxide *4a* and violaxanthin both have the same axial/equatorial relationship between the hydroxy substituent and the polyene chain, see Scheme 2 for alternative conformations A and B of *4a*.

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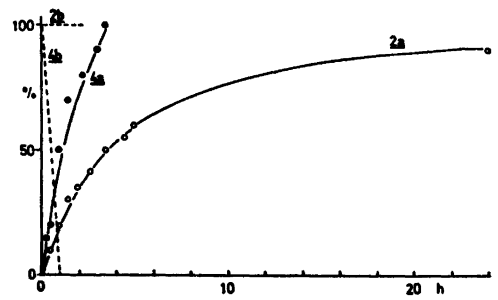


Fig. 1. Relative rates of acetylation of β,β-caroten-2-ol (*2a*), β,β-caroten-3-ol (*4a*), and relative rates of hydrolysis of β,β-carotenyl-2-acetate (*2b*) and β,β-carotenyl-3-acetate (*4a*).[17]

(*2a*) was chromatographically less strongly adsorbed than the 3-hydroxy analogue *3a* in agreement with the larger shielding of the hydroxy group in *2a*. However, no separation of the acetates *2b* and *4b* was achieved.

β,β-Caroten-2-yl acetate (*2b*) crystallized as red prisms, m.p. 81–83 °C, with predicted spectral properties (visible, IR, ^{1}H NMR). Alkali treatment of the acetates *2b* and *4b* (5 % KOH, −25 °C) resulted in complete hydrolysis of *4b* after 50 min when *2b* was not effected, see Fig. 1. However, *2b* was completely hydrolyzed after 60 min at 20 °C.

The low rate of acetylation was also observed for *1a*, which provided the acetate *1b* in 85 % yield after 16 h.

No difference in the rate of silylation of the 2-hydroxy compound *2a* and the 3-hydroxy compound *4a* was observed under standard conditions[3] at −30 °C. The trimethylsilyl ether *2c* (Scheme 1) was less polar than *4c*.

Comparative methylation of the 2-hydroxy compounds *1a* and *2a* and the 3,3′-dihydroxy compound *5a* (=zeaxanthin) was attempted by various modifications of Kuhn's method.[4–7] Abnormal products[8] were obtained using silver oxide;[4] *2a* also gave an abnormal product[8] with barium oxide.[5] By the CH_3I/BaO/DMF/DMSO modification[6,7] *1a* gave the methyl ether *1d*[8] and *2a* provided the methyl ether *2d*.[8] More satisfactory was methylation with methyl iodide and sodium hydride.[9,8] The 2-hydroxy compound *1a* gave the methyl ether *1d* (60 % of recovered carotenoid; 72 % total recovery) under conditions where the 3,3′-diol (*5a*) was quantitatively converted to the dimethyl ether *5d* (85 %) and monomethyl ether *5e* (15 %).[8] The methyl ether *1d* had *m/e* 566=M, M−15, M−56 (RDA-fragmentation of ε-ring[10]), M−92, M−106, M−158. All methyl ethers exhibited the same visible absorption as the parent alcohols.

The results discussed demonstrate the lower reactivity of the sterically hindered 2-hydroxy group in *1a* and *2a* than of the corresponding 3-hydroxy group in *4a* and *5a* as to acetylation, hydrolysis of the acetates and methylation. Differences in the rate of silylation, fast for both categories, were not established. Together with the higher R_F-values for the 2-hydroxy compounds their low rate of reaction may serve to distinguish carotenoids with 2-hydroxylated and 3-hydroxylated β-rings on the micro scale.

The green/blue colour reaction of the 2-hydroxy compounds *1a*, *2a*, and *3a* with hydrochloric acid,[10,1] rationalized by the isolation of a furanoid product of *1a* on treatment with 0.01 M HCl in chloroform-methanol,[11] prompted further investigation on the reaction leading to the partly characterized furanoxides.

The structures of the furanoid products obtained from the mono-ols *1a* and *2a* will first be discussed, Scheme 2. Under conditions to be described below two furanoxides *6a* and *6b* were obtained from *1a* and two furanoxides *7a* and *7b* from *2a*.

The furanoxides *6a* and *6b* were found identical (R_F-values, electronic and mass spectra as well as slow acetylation to the same acetates) with the furanoxides obtained from the so-called *cis* 5,6-epoxide (*cis* relationship between the epoxy and hydroxy substituent) *8* of *1a*. The location of the hydroxy group and the stereochemistry, including the C-8 epimeric nature of the two furanoxides follow therefrom. It is pointed out that the reaction X (Scheme 2) leading from *1a* to the furanoxides *6a*+*6b* must be stereospecific at C-5.

The furanoxides *7a* and *7b* from *2a* were found identical by the criteria mentioned above with the C-8 epimeric furanoid rearrangement products of natural 5,6-epoxy-5,6-dihydro-β,β-caroten-2-ol (*9*), tentatively considered to be a *cis* epoxide.[11] This would imply the same stereospecificity at C-5 in the reaction X leading from *2a* to *7a*+*7b*.

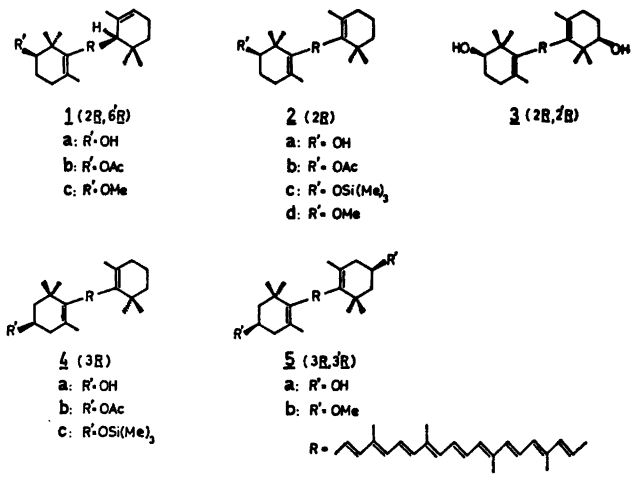
The nature of reaction X will now be considered. The presence of minor epoxides[11] as contaminants would offer an easy explanation. However, this possibility is ruled out from the ready separation of *1a* and *2a* from their 5,6-epoxides,[11] and from the yields obtained in reaction X (pigment recovery 15–30 %; furanoid products comprising 20–60 % of the recovered carotenoids).

A series of experiments designed to define the reaction are summarized:

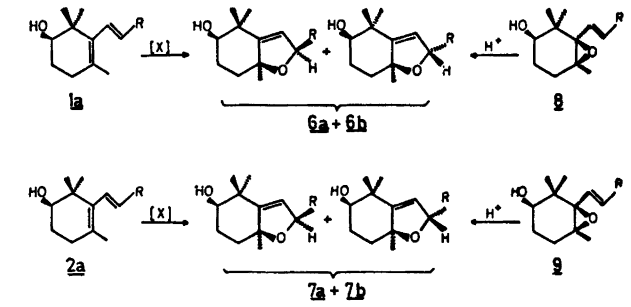
(a) Contrary to the previous assumption[1] hydrogen chloride is not the essential factor X, which is present in certain qualities of chloroform (Baker *p.a.* and a technical quality DAB 7, but not Merck *p.a.*) when chromatographed on alumina and stored at least 20 h before use.

(b) The reaction, formally requiring addition of an oxygen atom, was not prevented under presumed anaerobic conditions.

(c) No evidence for dichlorocarbene being involved was obtained. Thus treatment of *2a* with dichlorocarbene[12] gave no furanoid products, but two other products, one corre-



Scheme 1.



Scheme 2.

sponding to $C_{41}H_{56}Cl_2O$ (formal addition of dichlorocarbene) from mass spectral data.

(d) A free hydroxy group in 2-position is essential for the reaction. Thus no furanoid products were obtained from the acetate *1b* or the methyl ether *1d* with the 2-hydroxy function blocked. Moreover, no furanoid products were obtained from the 3,3'-dihydroxy compound *5a* or from β,β-carotene.

A free radical reaction of a phosgene peroxide complex,[13–16] formed on storage of chloroform after removal of the stabilizer, with participation of the 2-hydroxy group, providing the *cis* epoxide, subsequently rearranging to the furanoxides by traces of acids, or directly to the C_8-epimeric furanoxides, is considered.

Experimental details are given elsewhere.[17] R_F-Values (Schleicher & Schüll No. 287 circular, kieselguhr paper, percentage figure indicates acetone in petroleum ether) were: *1a* (2 %, 0.71), *1b* (1 %, 0.87), *1c* (1 %, 0.86), *2a* (2 %, 0.63), *2b* (1 %, 0.90), *2c* (0 %, 0.83), *2d* (2 %, 0.93), *4a* (1 %, 0.51), *4b* (1 %, 0.90), *4c* (0 %, 0.78), *5a* (10 %, 0.40), *5b*,[8] *6a*+*6b* (1 %, 0.48+0.18), *7a*-acetate = *7b*-acetate (1 %, 0.74), *7a*+*7b* (1 %, 0.55+0.22), *8* (1 %, 0.56), and *9* (1 %, 0.53).

Recommended procedure for preparation of furanoxides: To the 2-hydroxy carotenoid (0.05 – 1 mg) dissolved in *p.a.* chloroform (5 ml) is added Baker *p.a.* chloroform, chromatographed on Merck neutral alumina, activity grade 1, and stored under ordinary atmosphere for at least 20 h, (0.1 – 0.2 ml). A green colour spontaneously develops, turning yellow on ordinary work-up after a few minutes with ether and water.

Acknowledgement. G. N. was supported by a grant from the Norwegian Institute of Technology, University of Trondheim.

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Derivatives of Hydrazine. X. The Reaction between Thiophosgene and Semicarbazide or Thiosemicarbazide

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The reaction between thiophosgene and semicarbazide previously reported to give an *N*-isothiocyanatoamine has been shown by combined physical and chemical evidence to give instead bis(2-amino-1,3,4-oxadiazol-5-yl) trithiocarbonate. In the same way, the corresponding reaction between thiophosgene and thiosemicarbazide has been shown to give bis(2-amino-1,3,4-thiadiazol-5-yl) trithiocarbonate. A logical scheme to encompass the formation and reactions of these compounds is suggested.

The reaction between thiophosgene and hydrazine derivatives generally affords thiocarbonohydrazides. However, if carried out in aqueous hydrochloric acid other compounds, claimed by Beckett and Dyson [1] to be *N*-isothiocyanatoamines, are sometimes the main products. In the aromatic series it has previously been shown [2] that the product formed by the action of thiophosgene on 1,1-diphenylhydrazine is, in fact, 2-thiocyanatodiphenylamine formed by rearrangement of the very unstable *N*-isothiocyanatodiphenylamine. The reaction between thiophosgene and 1-methyl-1-phenylhydrazine proceeds in a similar way.[3] Aliphatic *N*-isothiocyanatoamines are also very unstable,[4] undergoing dimerisation [5] except when sterically hindered.[6] These results indicate that the products obtained by Beckett and Dyson, reported to be stable at room temperature, very probably do not contain the N—NCS grouping.

The present paper summarises the results obtained by reinvestigating the products from thiophosgene and semicarbazide (*1a*) or thiosemicarbazide (*1b*) in aqueous hydrochloric acid. They were formulated by Beckett and Dyson as *N*,*N*′-bisisothiocyanato-*N*,*N*′-biscarbamoyl-

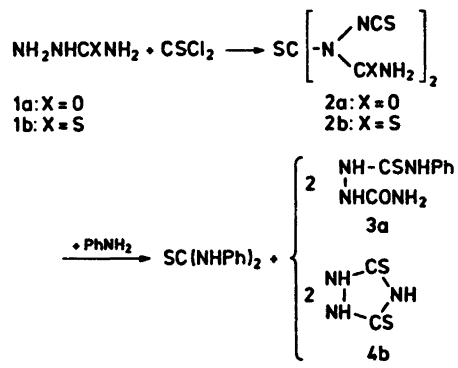


Fig. 1.

thiourea (*2a*) and the corresponding thiocarbamoyl derivative (*2b*) as shown in Fig. 1.

The structural proof for *2a* and *2b* was based on the reaction with aniline, which gave 1,3-diphenylthiourea and 1-(phenylthiocarbamoyl)semicarbazide (*3a*) or dithiourazole (*4b*), respectively. Since 4,4-diphenylsemicarbazide reacted analogously to *1a*, isomeric forms of *2a* and *2b* were not considered likely.

Using techniques not available at the time of the earlier work, we have reformulated the reaction between *1b* and thiophosgene as shown in Fig. 2. The reaction affords a compound $C_5H_4N_6S_5$ compatible with both structures *2b* and *6b*. The IR spectrum in KBr discs, however, is devoid of absorption in the region between 1700 cm^{-1} and 2700 cm^{-1}, excluding the presence of both the —NCS and the —SCN grouping.[3,4] Accordingly, formula *2b* cannot be correct for this compound. The reaction with aniline gave, in addition to 1,3-diphenylthiourea, a

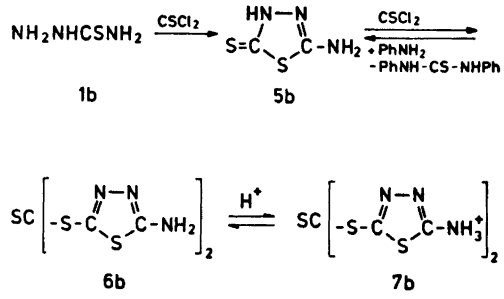
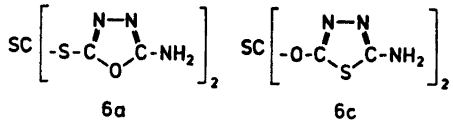


Fig. 2.

compound $C_2H_3N_3S_2$. This was formulated by Beckett and Dyson as dithiourazole (*4b*), but is now known to be, in fact, the isomeric 2-amino-1,3,4-thiadiazoline-5-thione (*5b*),[7,8] which can be prepared from thiosemicarbazide and carbon disulfide. Since identical products were obtained from the reaction between thiophosgene and *1b* or *5b*, these can be formulated as *6b*. The possibility that thiophosgene instead reacts with the amino group of *5b* giving a thiourea seems to be excluded by the observation that *1b* and thiophosgene in acetone give the dihydrochloride of *6b*, *7b*, with an IR spectrum showing only the expected changes from protonation of the amino groups. Furthermore, *6b* is a trithiocarbonate which is expected to react with aniline to give *5b* and 1,3-diphenylthiourea [9] whilst a thiourea is not expected to show this reaction.

The formation of *6b* from *1b* very probably proceeds *via* formation of *5b* as indicated in Fig. 2. The formation of *2b* or any other *N*-isothiocyanatoamine as an intermediate is considered very unlikely. First, if *2b* acts as an intermediate, a rearrangement to *6b* would involve simultaneous rearrangement of the thiocarbonyl group to the sulfur atoms of both NCS groups. Second, it is well known that the attack of N—NCS on thioamides affords nitriles,[10] and therefore a mechanism proceeding *via*, *e.g.*, $NH_2CSNHNCS$ is not expected.

Next the constitution of the reaction product from *1a* and thiophosgene was considered. A comparison of the physical and chemical properties with those of *6b* pointed to a similar structure. Thus, the IR spectra in KBr were almost identical in the range from 1000 – 4000 cm^{-1}, expect that the two strong bands at 1135 and 1500 cm^{-1} in *6b* were displaced to two doublets at 1170 + 1180 cm^{-1} and 1595 + 1608 cm^{-1}. Mass spectrometry established that pyrolysis of *6b* afforded substantial amounts of CS_2, while the product in question gave mixtures of CS_2 and COS with a percentage of COS ranging from 10 to 100, depending on the temperature and duration of the pyrolysis. Since elemental analysis established the formula $C_5H_4N_6O_5$ either structure *6a* or *6c* was possible.



The changes in the IR spectrum undoubtedly arise from the shift of two skeletal stretching frequencies, but both formulas are consistent with this result. However, the reaction with aniline gave 1,3-diphenylthiourea and 1-(phenylthiocarbamoyl)semicarbazide in confirmation of the results stated by Beckett and Dyson, while 1-(phenylcarbamoyl)thiosemicarbazide was not formed. This was definitely proved by comparison with authentic samples of both compounds, prepared from PhNCS and semicarbazide and from PhNCO and thiosemicarbazide, respectively. This leaves no doubt that the correct structure of the reaction product from thiophosgene and la is *6a*. (Of course, a "mixed" product, containing both the oxadiazolyl moiety of *6a* and the thiadiazolyl moiety of *6c*, is excluded by the same observation).

Further discussion of the structure of *6a* and *6b*, based on ^{13}C NMR spectroscopic investigations, will be presented in a forthcoming paper.

EXPERIMENTAL

Analyses were carried out at the Microanalysis Department of this laboratory. The melting points were determined in capillary tubes on a Büchi melting point apparatus and are not corrected. The infrared spectra were recorded on a Perkin Elmer Model 337 Grating Infrared Spectrophotometer. Mass spectra of the pyrolysis products from *6b* were obtained on a Finnigan 1015 S/L combined gas chromatograph-mass spectrometer.

1-(Phenylthiocarbamoyl)semicarbazide (3a). This compound was prepared following the directions given by Arndt *et al.*[11] The yield was 75 %, m.p. 201 – 202 °C. (Found: C 45.95; H 4.83; N 26.75; S 15.43. Calc. for $C_8H_{10}N_4OS$: C 45.71; H 4.80; N 26.66; S 15.86).

2-Amino-1,3,4-thiadiazoline-5-thione (5b). An authentic sample of this compound was prepared by Sandström's method.[8] The yield was 65 %, m.p. 234–235 °C. (Found: C 18.06; H 2.47; N 30.98. Calc. for $C_2H_3N_3S_2$: C 18.05; H 2.47; N 31.58).

Bis(2-amino-1,3,4-thiadiazol-5-yl) trithiocarbonate (6b). Method A. 2-Amino-1,3,4-thiadiazoline-5-thione (0.1 mol) was suspended in water (50 ml) and shaken with thiophosgene (0.05 mol) for 2 h. The precipitate was collected and dried. The compound was dissolved in dimethyl sulfoxide and reprecipitated with water. The yield was almost quantitative. M.p. 240–250 °C. (Found: C 19.80; H 1.37; N 26.91; S 52.10. Calc. for $C_5H_4N_6S_5$: C 19.47; H 1.31; N 27.28; S 51.95).

Method B. The directions given by Beckett and Dyson [1] were followed. The compound decomposed on attempted recrystallisation and was therefore purified by dissolution in dimethyl sulfoxide and reprecipitation with cold acetone. The analyses given below refer to the crude product. M.p. 240–250 °C decomp. The yield was 60 %. (Found: C 20.21; H 1.60; N 26.39; S 51.06). This compound was proved to be identical with the sample prepared as described under A by their IR and mass spectra and by mixture melting points.

*Bis(2-amino-1,3,4-thiadiazol-5-yl) trithiocarbonate dihydrochloride (*7b*).* 2-Amino-1,3,4-thiadiazoline-5-thione (0.1 mol) was dissolved in acetone (50 ml) and thiophosgene (0.05 mol) was added in one portion. The crystalline yellow precipitate was filtered off and dried. (Found: C 15.91; H 1.52; N 21.88. Calc. for $C_5H_6N_6S_5Cl_2$: C 15.73; H 1.59; N 22.04). On treatment with aqueous base, *6b* was liberated.

Bis(2-amino-1,3,4-oxadiazol-5-yl) trithiocarbonate (6a). The directions given by Beckett and Dyson [1] were followed. The compound could not be recrystallized, but it was reprecipitated from dimethyl sulfoxide and water. M.p. 185–194 °C decomp. The yield was 25 %. (Found: C 21.56; H 1.53; N 29.95; S 34.23. Calc. for $C_5H_4O_2S_3$: C 21.73; H 1.46; N 30.42; S 34.81).

1-(Phenylcarbamoyl)thiosemicarbazide. This compound was prepared following the directions given by Freund and Schander.[12] The yield was 70 %, m.p. 216–217 °C. (Found: C 45.66; H 4.85; N 26.81. Calc. for $C_8H_{10}N_4OS$: C 45.71; H 4.80; N 26.66).

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Syntheses in the Camphor Series. Alkylation of Quinones with Cycloalkyl Radicals. Attempted Syntheses of Lagopodin A and Desoxyhelicobasidin

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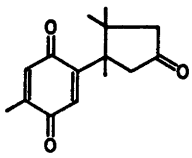
In an attempt to synthesize lagopodin A and desoxyhelicobasidin, a new synthesis of (3*R*)-3-carboxy-3,4,4-trimethylcyclopentanone and (1*R*)-1,2,2-trimethylcyclopentanecarboxylic acid was worked out together with several other reactions in the camphor series. For the same purpose alkylation of quinones with cycloalkyl radicals derived from cycloalkane carboxylic acids by decarboxylation with silver ion sand peroxydisulphate was investigated and several cycloalkyl derivatives were prepared. It was not possible to add the sterically hindered trimethyl cyclopentyl radicals to quinones. The structure *22* is suggested for a camphorlactone claimed to be *21*.[8]

In previous papers [1,2] we have described the alkylation of quinones with radicals from the decarboxylation of carboxylic acids with silver ions and peroxydisulphate (Eqns. 1 and 2)

$$Ag^+ + S_2O_8^{2-} \rightarrow Ag^{2+} + SO_4^{\cdot -} + SO_4^{2-} \quad (1)$$

$$Ag^{2+} + RCOOH \rightarrow R^{\cdot} + CO_2 + H^+ + Ag^+ \quad (2)$$

In the present work the alkylation of quinones with cycloalkylradicals was investigated in order to examine the possibility of synthesizing the naturally occurring terpenoid quinones lagopodin A [3] *1* and desoxyhelicobasidin [4] *2* by this method.



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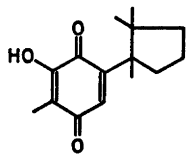

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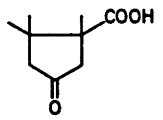
During the search for a convenient synthesis of the carboxylic acids *3* and *4*, required for this purpose, some other useful reactions in the



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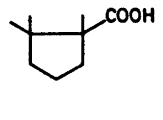

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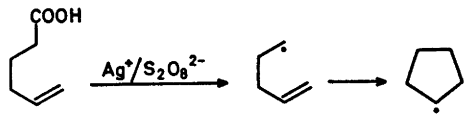
camphor series were discovered. These are discussed in the section containing the syntheses of *3* and *4*.

RESULTS

Alkylation of 1,4-naphthoquinones 5. The alkylation of 1,4-naphthoquinone *5* and 2-methyl-1,4-naphthoquinone *6* with cycloalkylradicals (C_3-C_6) gave the corresponding cycloalkylquinones in fair yields (Table 1).

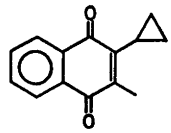
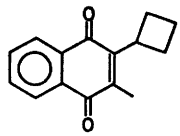
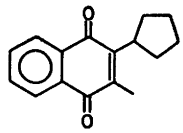
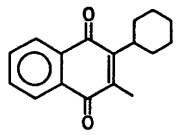
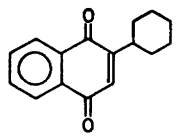
The possibility of generating cyclopentylradicals indirectly by the cyclisation of an open chain unsaturated radical (Eqn. 3) was also examined.



(3)

When 5-hexenoic acid was decarboxylated in the presence of 2-methyl-1,4-naphthoquinone, 2-methyl-3-(4-pentenyl)-1,4-naphthoquinone *12* was isolated. Only traces of the cyclopentylquinone *9* could be detected.

Table 1. Alkylation of quinones with cycloalkyl radicals.

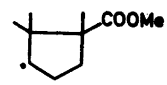
Quinone	Carboxylic acid	Product	Yield %	m.p.
6	Cyclopropane-	7 	37	82-83°
6	Cyclobutane-	8 	56	59-60°
6	Cyclopentane-	9 	42	96-97°
6	Cyclohexane-	10 	46	78-79°
5	Cyclohexane-	11 	55	87-88° (lit.[5] 87-88)

Synthesis of 3 and 4. Since the published syntheses of the optically active [3] or the racemic[6] *3* are rather complicated, a new route to *3* and *4* in their optically active forms was investigated.

Oxidative decarboxylation of (1*R*)-*cis*-camphoric acid-1-methylester *13* with lead tetraacetate in the presence of cupric ions by the method of Kochi [7] gave (1*R*)-1,2,2-trimethylcyclopent-3-ene-carboxylic acid methylester *14*.

Alkaline hydrolysis of *14* gave the corresponding carboxylic acid *15* which was hydrogenated catalytically to *4* (1*R*-configuration).

The oxidative decarboxylation of *13* was also performed by the use of silver ion-peroxydisulphate-cupric ions in water-acetonitrile in the same yield but with higher conversion of *13*. In this reaction the presence of pyridine proved essential for the oxidation of the intermediate radical *16* to *14* by the cupric ions. When the reaction was carried out in the absence of pyridine, a mixture of *14* and the saturated ester *17* (Scheme 1) was formed.



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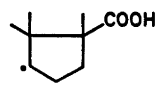
To investigate the selectivity of the oxidative decarboxylation between secondary and tertiary carboxyl groups with both lead tetraacetate and silver ion-peroxydisulphate, camphoric acid was decarboxylated by both oxidants under conditions similar to those in the syntheses of *14*.

Two radicals, *18* and *19*, result from the decarboxylation of the secondary and the tertiary carboxylic group, respectively.



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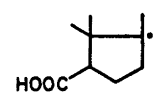

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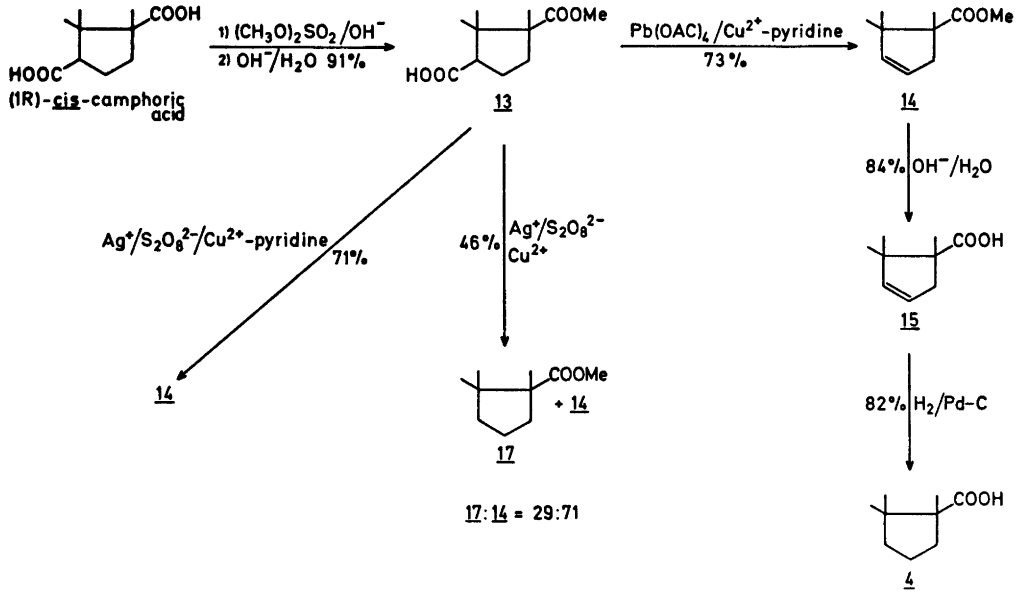
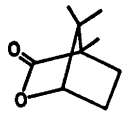


Scheme 1.

The main products in both reactions were the lactones *20* and *21* formed from *18* and *19*, respectively, probably by internal ligand-transfer,



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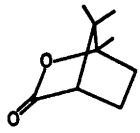

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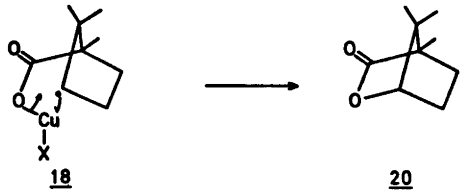
and analogously *19*→*21*.



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The expected products, cyclopentenoic acids (formed analogously to *14*) were only present in small amounts.

These results show that the tertiary carboxylic group is decarboxylated much faster than the secondary by both reagents.

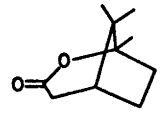
The spectra and physical data of *21* were very different from those published by Hayashi *et al.*[8] for a compound claimed to be *21*. Their data

Table 2. Ratio[a] of products from decarboxylation of camphoric acid.

Oxidant	*20* (%)	*21* (%)	total yield (%)
$Pb(OAc)_4$	17	83	64
$Ag^+/S_2O_8^{2-}$	5	95	57

[a] Determined by GLC.

are in better agreement with the structure *22*, also considered by these authors. A comparison of the two sets of data is given in Table 3.



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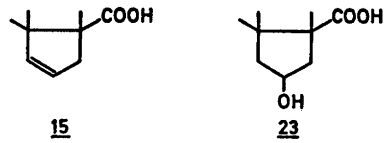
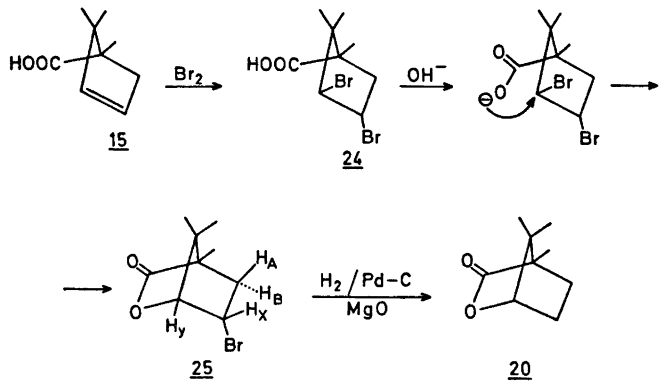
Attempts to bring about the conversion *15*→*23* by hydroboration of *15* or its tetrabutyl-



15 23 (5)

Table 3. Comparison of the physical data of *21* and *22*.

	Compound *21*	Compound of Hayashi *et al.*[8] *22*
IR	1775 cm^{-1} (5-membered lactone), supports *21*	1730 cm^{-1} (6-membered lactone), supports *22*
NMR	see Experimental, supports *21*	integral of methyl signals: other protons = 9:7, supports *22*
MS	no peaks beyond 154 (M^+ for *21*). Fragmentation pattern very similar to *20*, supports *21*	peak at 168 (M^+ for *22*). Fragmentation pattern very different from that of *20*, supports *22*
Analysis	see Experimental, supports *21*	supports both *21* and *22*
M.p.	113 – 115 °C	156 – 157 °C



15 24 25 20

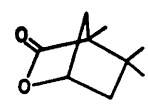
Scheme 2.

ammonium salt with disiamylborane, followed by oxidation with hydrogen peroxide by the method of Brown,[9] were unsuccessful.

Bromination of *15* gave a dibromoderivative *24* which, when treated *in situ* with aqueous sodium carbonate, gave a bromolactone *25*. Catalytical hydrogenation of *25* gave *20* which on hydrolysis and oxidation would lead to an isomer of the desired keto acid *3*.

The *endo*-configuration of *25* was deduced as follows: The carboxylate ion in the dibromacid *24* substitutes the bromine atom in *trans* position, and since the addition of bromine to the double bond proceeds in a *trans* fashion, the bromine of *25* is located *cis* to the lactone bridge (Scheme 2).

Treatment of *15* with *p*-toluenesulphonic acid in refluxing toluene gave the desired lactone *26*, together with a minor amount (13 %) of *20*. It proved impossible to separate *20* from *26*



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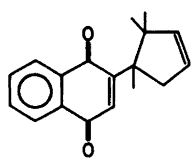
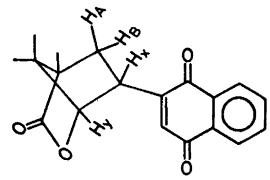
by other means than preparative GLC so the mixture was hydrolyzed and the (1*R*)-*cis*-4-hydroxy-1,2,3-trimethylcyclopentanecarboxylic acid *23* was purified by crystallization from water.

Oxidation of *23* by chromic acid in a 2-phase system gave *3* in good yield.

Attempted syntheses of lagopodin A and desoxyhelicobasidin 2. As a model of the synthesis of desoxyhelicobasidin we used naphthoquinone with *4* but without success, partly because of the low solubility of *4* in water and aqueous acetonitrile which caused formation of a 2-phase system on addition of peroxydisulphate



27



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and thereby ineffective decarboxylation of *4*. The same reaction using *4* as its silver- or tetrabutylammonium salt or *4* together with a weak base like 2,6-lutidine, gave no alkylated naphthoquinone, although *4* was consumed in these reactions.

To overcome the solubility problem, we tried to carry out the alkylation with *15* instead of *4* but in this reaction the simple alkylated naphthoquinone *27* was not obtained.

Instead *28* was formed as the only isolable quinone product in a yield of 41 % (apparently only the *endo*-isomer).

The *endo* structure was preferred for the following reason: The NMR coupling constants, of the H_A, H_B, and H_x protons, of *28* are in reasonably good agreement (Table 4) with those of the bromolactone *25* which for reasons already mentioned should be the *endo*-isomer.

We are not, however, able to find sufficient NMR data of related structures in the literature to justify a definite assignment.

Table 4. ^1H Coupling constants (Hz) of *25* and *28*.

Compounds	J_{AB}	J_{AX}	J_{BX}	J_{XY}
25	−15.2	10.1	4.0	2.0
28	−13.9	10.7	5.1	1.7

Two mechanisms, 5A and 5B, for the formation of *28* can be formulated, see below.

In view of the small gain in energy by cyclization of *29* compared with the greater amount of energy liberated by a decarboxylation, *30* is preferred to *29* as intermediate.

The mechanism B has also been proposed by Moriarty [10] for the oxidation of various alkenecarboxylic acids by lead tetraacetate.

Attempts to alkylate toluquinone with *3* to give lagopodin A and isomers also failed, indicating that the steric hindrance of the tertiary cycloalkyl radicals in the alkylation step is of such a magnitude that the fast oxidation of these radicals by Ag^{2+} or $SO_4^{\cdot-}$ prevails.

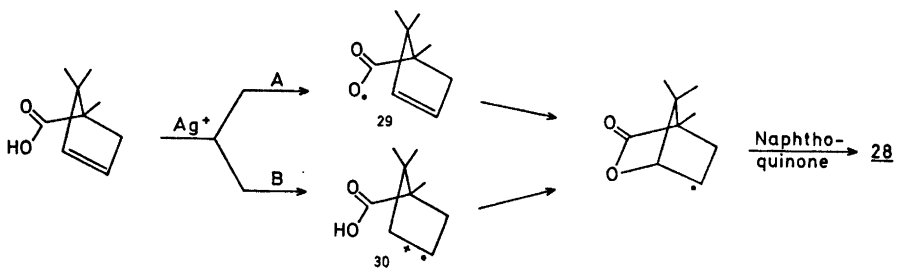
In agreement with this alkylation with 1-methylcyclohexanecarboxylic acid also gave very poor yields.

EXPERIMENTAL

Melting points are uncorrected. *NMR* spectra were recorded on a Varian A-60, *IR* spectra on a Perkin-Elmer Infracord, and *UV* spectra on a Perkin-Elmer 402 spectrometer. Analytical GLC was performed with a Perkin-Elmer F11 chromatograph. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. Calculation of *NMR* shifts and coupling constants was made by means of the LAOCN-3 programme.

Alkylation of quinones. Details of the reaction have been described earlier.[1]

General procedure. To a vigorously stirred water-acetonitrile solution of the quinone, the



carboxylic acid, and silver nitrate at 60 – 65 °C an aqueous solution of ammonium peroxydisulphate was added during 45 min. After a further 10 min stirring at 60 – 65 °C, the mixture was cooled to room temperature and extracted with ether. The ether phase was washed with aqueous $NaHCO_3$ until neutral, dried, and evaporated. The crude products were chromatographed, either on TLC plates (silica gel Merck); procedure A: eluent 20 % ether in petrol ether; procedure B: eluent CH_2Cl_2 or on a column of neutral aluminia; procedure C: eluent CH_2Cl_2.

2-Cyclopropyl-3-methyl-1,4-naphthoquinone, 7. To 2-methyl-1,4-naphthoquinone (1.72 g, 0.01 mol), cyclopropanecarboxylic acid (1.29 g, 0.015 mol), and silver nitrate, 0.5 g, in acetonitrile (25 ml) and water (60 ml) was added ammonium peroxydisulphate (3.42 g, 0.015 mol) in water (15 ml). Chromatography (procedure B) of the crude product gave *7*, 780 mg (37 % based on the quinone), m.p. 82 – 83 °C (from CH_3OH). (Found: C 78.6; H 5.82. Calc. for $C_{14}H_{12}O_2$: C 79.2; H 5.70). UV (EtOH) λ_{max} nm (log ε): 246 (4.21), 251 (4.21), 274 (4.10), 333 (3.5). IR (CCl_4): cm^{-1} 1660(s), 1595 (m). NMR (CCl_4): δ 0.8 – 1.3 (4 H, m), 1.3 – 2.0 (1 H, m), 2.20 (3 H, s), 7.4 – 8.1 (4 H, m).

2-Cyclobutyl-3-methyl-1,4-naphthoquinone, 8. To 2-methyl-1,4-naphthoquinone (0.86 g, 0.005 mol), cyclobutanecarboxylic acid (0.75 g, 0.0075 mol), and silver nitrate (0.5 g) in acetonitrile (20 ml) and water (20 ml) was added ammonium peroxydisulphate (1.71 g, 0.0075 mol) in water (10 ml). Chromatography (procedure B) of the crude product gave *8*, 630 mg (56 % calculated on the quinone), m.p. 59 – 60 °C (from methanol). (Found: C 79.7; H 6.46. Calc. for $C_{15}H_{14}O_2$: C 79.6; H 6.24). UV (EtOH): λ_{max} nm (log ε) 245 (4.23), 251 (4.22), 274 (4.17), 332 (3.4). IR (CCl_4): cm^{-1} 1660(s), 1595(m). NMR (CCl_4): δ 1.7 – 2.8 (6 H, m), 2.07 (3 H, d, $J = 1$), 3.2 – 4.0 (1 H, m), 7.5 – 8.1 (4 H, m). On irradiation of the multiplet at 3.2 – 4.0 ppm, the doublet at 2.07 ppm collapsed into a singlet.

2-Cyclopentyl-3-methyl-1,4-naphthoquinone, 9. To 2-methyl-1,4-naphthoquinone (0.86 g, 0.005 mol), cyclopentanecarboxylic acid (0.86 g, 0.0075 mol), and silver nitrate (0.5 g) in acetonitrile (70 ml) and water (20 ml) was added ammonium peroxydisulphate (1.71 g, 0.0075 mol) in water (10 ml). Chromatography (procedure B) of the crude product gave *9*, 510 mg (43 % based on the quinone), m.p. 96 – 97 °C (from CH_3OH). (Found: C 79.6; H 6.77. Calc. for $C_{16}H_{16}O_2$: C (79.6; H 6.71). UV (EtOH): λ_{max} nm (log ε) 246 (4.24), 250 (4.24), 269 (4.16), 276 (4.18), 332 (3.5). IR (CCl_4): cm^{-1} 1660(s), 1595(m). NMR (CCl_4): δ 1.3 – 2.2 (8 H, m), 2.17 (3 H, s), 2.9 – 3.5 (1 H, m), 7.5 – 8.1 (4 H, m).

2-Cyclohexyl-3-methyl-1,4-naphthoquinone, 10. The general procedure was followed. To 2-methyl-1,4-naphthoquinone (1.72 g, 0.01 mol), cyclohexanecarboxylic acid (1.92 g, 0.015 mol) and silver nitrate (0.5 g) in acetonitrile (40 ml) and water (40 ml) was added ammonium peroxydisulphate (3.42 g, 0.015 mol) in water (20 ml). Chromatography (procedure A) gave *10*, 1.16 g (46 % calculated on the quinone), m.p. 78 – 79 °C. (Found: C 80.0; H 7.24. Calc. for $C_{17}H_{18}O_2$: C 80.3; H 7.15). UV (EtOH): λ_{max} nm (log ε) 246 (4.28), 250, sh, 269, sh, 275 (4.11), 331 (3.5). IR (CCl_4): 1660(s), 1595(m). NMR (CCl_4): δ 1.0 – 2.2 (10 H, m), 2.17 (3 H, s), 2.4 – 3.1 (1 H, m), 7.5 – 8.1 (4 H, m).

2-Cyclohexyl-1,4-naphthoquinone, 11. To 1,4-naphthoquinone (1.58 g, 0.01 mol), cyclohexanecarboxylic acid (1.92 g, 0.015 mol), and silver nitrate (0.5 g) in acetonitrile (22 ml) and water (20 ml) was added ammonium peroxydisulphate (3.42 g, 0.015 mol) in water (15 ml). Chromatography (procedure A) of the crude product gave *11*, 1.32 g (55 % based on the quinone), m.p. 87 – 88 °C (lit.[5] 87 – 88 °C). UV (EtOH): λ_{max} nm (log ε) 248 (4.25), 253 (4.24), 267 (4.17), 334 (3.5). IR (CCl_4): cm^{-1} 1665(s), 1615(m), 1595(m). NMR (CCl_4): δ 0.9 – 2.4 (10 H, m), 2.4 – 3.2 (1 H, m), 6.61 (1 H, d, $J = 1$), 7.5 – 8.2 (4 H, m).

3-Methyl-2-(4-pentenyl)-1,4-naphthoquinone, 12. To 1-methyl-1,4-naphthoquinone (1.72 g, 0.01 mol), 5-hexenoic acid (1.71 g, 0.015 mol), and silver nitrate (0.5 g) in acetonitrile (25 ml) and water (25 ml) was added ammonium peroxydisulphate (3.42 g, 0.015 mol) in water (15 ml). Chromatography (procedure B) of the crude product gave *12*, 920 mg (38 % based on the quinone), m.p. 46 – 47 °C. (Found: C 79.6; H 6.75. Calc. for $C_{16}H_{16}O_2$: C 80.0; H 6.71). UV (EtOH): λ_{max} nm (log ε) 249 (4.19), 267 (4.15), 273 (4.15), 333 (3.4). IR (CCl_4): cm^{-1} 1660(s), 1620(m), 1595(m). NMR (CCl_4): δ ~1.5 (2 H, distorted quintet, $J = 7$), 2.1 (2 H, b, t, $J = 7$), 2.10 (3 H, s), 2.5 (2 H, d, t, $J_1 = 8$, $J_2 = 7$), 4.8 – 5.3 (2 H, m), 5.5 – 6.2 (1 H, m), 7.5 – 8.1 (4 H, m). Only traces of *9* could be detected by TLC.

2-(endo-5-(1,7,7-Trimethyl-2-oxo-3-oxa)-bicyclo[2.2.1]heptyl)-1,4-naphthoquinone, 28. To 1,4-naphthoquinone (0.79 g, 0.005 mol), *15* (0.93 g, 0.006 mol), and silver nitrate (0.5 g) in acetonitrile (15 ml) and water (20 ml) was added ammonium peroxydisulphate (1.30 g, 0.0057 mol) in water (8 ml). Chromatography (procedure C) gave *28*, 640 mg (41 % calculated on the quinone), m.p. 132 – 133 °C (after recrystallization from methanol and from cyclohexane). $[\alpha]_D^{25} = -158°$ (ethanol, $c = 0.5$) (Found: C 72.9; H 5.79. Calc. for $C_{19}H_{18}O_4$: C 73.5; H 5.84). UV (EtOH): λ_{max} nm (log ε) 248 (4.21), 254 (4.21), 264 (4.09), 339 (3.4). IR (CCl_4): cm^{-1} 1795(s), 1665(s), 1625(m), 1600(m). NMR ($CDCl_3$): δ 1.09 (3 H, s), 1.15 (3 H, s), 1.17 (3 H, s), 1.71* (1 H, A part of ABXYZ system), 2.32* (1 H, B part), 3.84* (1 H, X part), 4.50 (1 H, d, Y part, $J_{XY} = 1.7$), 6.82 (1 H, d, Z part, $J_{XZ} = 1.5$), 7.6 – 8.2 (4 H, m) $J_{AB}^* = -13.9$, $J_{AX}^* = 10.7$, $J_{BX}^* = 5.1$. The signal at δ 3.84, which appeared as four triplets, was changed

* Calculated values.

into four doublets, $J = 1.7$, by irradiation of the doublet at 6.82.

Dimethyl-(1R)-cis-camphorate was prepared by the method of Riedel [11] in a yield of 97 %. NMR (CCl_4): δ 0.72 (3 H, s), 1.18 (3 H, s), 1.21 (3 H, s), 1.3 – 2.9 (5 H, m), 3.65 (6 H, s).

(1R)-cis-Camphoric acid-1-methylester, 13. 123 g (0.54 mol) of dimethyl-(1*R*)-*cis*-camphorate and potassium hydroxide (0.54 mol) were dissolved in methanol (800 ml) and water (400 ml) and left at room temperature for 3 days. The solution was refluxed for 30 min and the methanol was distilled off. The aqueous phase was washed with ether, acidified with conc. HCl and extracted with CH_2Cl_2. Drying and evaporation of the CH_2Cl_2 phase gave 108 g (94 %) of almost pure *13*, m.p. 85 – 86 °C (from petrol ether) (lit.[12] 86 – 87 °C) $[\alpha]_D^{25} = +43°$ (ethanol $c = 2$). IR (CCl_4): cm^{-1} 2500 – 3200(s), 1735(s), 1705(s). NMR (CCl_4): δ 0.83 (3 H, s), 1.20 (3 H, s), 1.28 (3 H, s), 1.3 – 3.0 (5 H, s), 3.67 (3 H, s), 11.7 (1 H, s).

Decarboxylation of (1R)-cis camphoric acid-1-methylester, 13 *with lead tetraacetate. Preparation of 15.* A mixture of 47 g (0.22 mol) of *13*, lead tetraacetate (111 g, 90 % pure, 0.22 mol), neutral cupric acetate (7.2 g), and pyridine (11 g) in benzene (450 ml) was refluxed with stirring for 2 h. The reaction mixture was filtered, washed with dilute nitric acid to remove pyridine and copper salts and extracted with aqueous potassium carbonate until neutral. On acidification of the alkaline washings, 13 g of *13* were recovered. The benzene phase was dried and distilled. The fraction boiling at 180 – 186 °C was (1*R*)-1,2,2-trimethylcyclopent-3-ene-1-carboxylic acid methylester, *14*, 19.5 g (73 % based on converted *13*). (For use in the hydrolysis it is only necessary to distil off the benzene). $n_D^{22} = 1.4501$, $[\alpha]_D^{25} = +113°$ (ethanol, $c = 1$). IR (CCl_4): cm^{-1} 1735(s), 1620(w). NMR (CCl_4): δ 0.88 (3 H, s), 1.13 (3 H, s), 1.18 (3 H, s), 2.0 (1 H, distorted d, $J \sim 17$), 3.2 (1 H, distorted d, $J \sim 17$), 3.66 (3 H, s), 5.3 (1 H, m), 5.5 (1 H, m).

Decarboxylation of 13 *with* $Ag^+/S_2O_8^{2-}$. To a stirred mixture of *13* (21.4 g, 0.1 mol) cupric sulphate (6 g), silver sulphate (4 g), and pyridine (6 ml) in water (150 ml) and acetonitrile (100 ml) at 60 – 70 °C was added ammonium peroxydisulphate (30.6 g, 0.13 mol) in water (50 ml) during 1 h while the pH was kept at 6 – 7 by addition of 4 M sodium hydroxide. After a further 10 min stirring, 4 M sulphuric acid (100 ml) was added and the mixture was cooled and extracted three times with petrol ether. The organic phase was dried and the petrol ether was distilled off together with some acetonitrile leaving almost pure *14* (11.8 g, 70 %) containing less than 1 % of *17* (GLC, 5 % SE-30, using authentic samples as references). In the absence of pyridine it was necessary to use more acetonitrile (140 ml) and less water (90 ml) to bring *13* in solution, and the crude product was very impure and had to be distilled before GLC analysis. The fraction, boiling at 106 – 130 °C/45 mm, (7.8 g, 46 %) was collected and found to contain *14* (71 %) and *17* (29 %) by GLC.

1,2,2-Trimethylcyclopent-3-ene-1-carboxylic acid, 15. A solution of *14* (7.7 g, 0.046 mol) and potassium hydroxide (35 g, 0.63 mol) in methanol (170 ml) and water (15 ml) was refluxed for 20 h. The methanol was distilled off, the residue was dissolved in water and washed with ether. The aqueous phase was cooled to 0 °C, acidified with conc. HCl, and the precipitated (1*R*)-1,2,2-trimethylcyclopent-3-ene-1-carboxylic acid *15* filtered, washed with cold water, and dried. Yield: 5.9 g (84 %), m.p. 157 – 158 °C (from formic acid or aqueous formamide or after sublimation at 120 °C/10 mm, lit.[13] 157 – 159.5 °C). $[\alpha]_D^{25} = +122°$ (ethanol, $c = 1$). IR ($CHCl_3$): cm^{-1} 2500 – 3500(s), 1705(s), 1625(w). NMR (CCl_4): δ 1.02 (3 H, s), 1.17 (3 H, s), 1.27 (3 H, s), 2.0 (1 H, distorted d, $J = 17$), 3.2 (1 H, distorted d, $J = 17$), 5.3 (1 H, m), 5.5 (1 H, m), 11.9 (1 H, s).

Decarboxylation of (1R)-cis-camphoric acid by lead tetraacetate. Preparation of 20 and 21. Camphoric acid (10 g, 0.05 mol), lead tetraacetate (27.8 g, 90 % pure, 0.055 mol), neutral cupric acetate (2 g), and pyridine (3 ml) were refluxed in benzene (200 ml) for 3 h. The mixture was washed with 30 % nitric acid, water, and aqueous sodium carbonate, successively, dried, and evaporated *in vacuo* at room temperature leaving 4.9 g (64 %) of a mixture of *20*, 17 % and *21*, 83 % (GLC). On acidification of the sodium carbonate phase, *ca.* 1.5 g of acidic material – mainly camphoric acid – was obtained.

Decarboxylation of (1R)-cis-camphoric acid with Ag^+*/persulphate.* Camphoric acid (10, g, 0.05 mol), cupric sulphate (2 g), silver sulphate (2 g), pyridine (3 ml), water (200 ml), and acetonitrile (20 ml) were stirred at 60 – 65 °C while 13.68 g (0.06 mol) of ammonium peroxydisulphate in water (50 ml) were added during 70 min. During the addition, the pH was kept at 5 – 7 by addition of diluted NaOH. After a further 10 min stirring at 60°C, 2 M H_2SO_4 (50 ml) was added and the mixture was extracted with ether. The ether extract was washed with aqueous sodium carbonate, dried, and evaporated to give 4.4 g (57 %) of a mixture of *20* and *21* (5:95, GLC). Small amounts of acidic material could be obtained on acidification of the carbonate phase. Preparative GLC 10 % PEG) of the mixture gave *21*, m.p. 113 – 115 °C $[\alpha]_D^{25} = +12.6°$ (ethanol, $c = 0.5$). (Found: C 70.0; H 9.10. Calc. for $C_9H_{14}O_2$: C 70.1; H 9.15). IR (KBr): cm^{-1} 1775(s). NMR (CCl_4): δ 0.95 (3 H, s), 1.06 (3 H, s), 1.29 (3 H, s), 1.5 – 2.1 (4 H, m), 2.28 (1 H, distorted dd). MS Mass % of base peak): 154 (5) M^+, 139 (5), 126 (31), 111 (27), 108 (26), 95 (100).

Lactonisation of 1,2,2-trimethylcyclopent-3-ene-carboxylic acid, 15. Preparation of 26 and 20. 15 (8.55 g, 0.056 mol) and *p*-toluenesulfonic acid (4.3 g, 0,0027 mol) were refluxed for 20 h in

toluene (70 ml). The mixture was extracted with aqueous sodium carbonate. On acidification of the carbonate washing, *15* (1.35 g) was recovered by extraction with CH_2Cl_2. The toluene phase was dried and evaporated *in vacuo* at room temperature. The residue was sublimated at 115 °C/10 mm to give 4.08 g (57 %) of a mixture of (1*R*)-*cis*-4-hydroxy-1,2,2-trimethylcyclopentane carboxylic acid lactone, *26*, and the isomeric 3-hydroxy carboxylic acid lactone, *20*, ratio 87:13 (GLC, 10 % PEG). This mixture could not be separated by crystallization. The *IR* spectrum of the mixture was almost identical with the spectrum of *26*, published by Faigle and Karrer.[14] NMR (CCl_4) of *26*: (after substraction of the signals from 20) δ 0.97 (3 H, s), 1.07 (3 H, s), 1.10 (3 H, s), 1.7 (2 H, m), 1.9 (2 H, m), 4.6 (1 H, m).

(1R)-cis-4-Hydroxy-1,2,2-trimethylcyclopentanecarboxylic acid, 23. The lactone mixture of *20* and *26* (4 g) was refluxed for 20 h with 40 g KOH in 160 ml of methanol and 80 ml of water. The methanol was evaporated and the aqueous phase washed with ether, acidified with conc. HCl and extracted with several portions of CH_2Cl_2. The CH_2Cl_2 phase was dried and evaporated and the residue recrystallized from water giving pure *23* (2.95 g, 66 %), m.p. 199 – 200 °C (lit.[15] 200 °C). $[\alpha]_D^{25} = +14°$ (ethanol, $c = 4$). IR (KBr): cm^{-1} 3400(s), 2500 – 3100(s), 1700(s). NMR (D_2O, K_2CO_3): δ 0.93 (3 H, s), 0.98 (3H, s), 1.01 (3 H, s), 1.38 – 2.37 (4 H, m), 4.07 – 4.50 (1 H, m).

(3R)-3-Carboxy-3,4,4-trimethylcyclopentanone, 3. To *23* (1.5 g, 0.087 mol) dissolved in a 2-phase system consisting of ether (20 ml), water (10 ml), and dimethoxyethane (3.5 ml) was added at 25 °C a mixture of $Na_2Cr_2O_7.2H_2O$ (1.6 g, 0.054 mol), and conc. H_2SO_4 (1 g) in water (15 ml) with vigorous stirring during 15 min. After further 4 h of stirring, the organic layer was separated and the aqueous phase extracted several times with ether. The combined organic phases were dried and evaporated to give *3* (1.20 g, 81 %), m.p. 220 – 221 °C (from H_2O (lit.[3] 221 °C). $[\alpha]_D^{25} = +23°$ (ethanol, $c = 2$). IR ($CHCl_3$): cm^{-1} 2500 – 3500(s), 1745(s), 1700(s). NMR ($CDCl_3$): δ 1.14 (3 H, s), 1.21 (3 H, s), 1.35 (3 H, s), 2.20 and 3.01 (2 H, AB system, $J_{AB} = 19$), 2.36 (1H, b , s), ~11 (1 H, b, s).

(1R)-1,2,2-Trimethylcyclopentanecarboxylic acid, 4. 15 (10.0 g, 0.065 mol) in 100 ml alcohol was hydrogenated over 5 % Pd/C (0.6 g) for 20 h at room temperature and 1 atm. The solution was filtered and evaporated and the residue recrystallized from acetonitrile to give *4* (8.3 g, 82 %), m.p. 191 – 192 °C (lit.[16] 192 – 193 °C). $[\alpha]_D^{25} = +20°$ (ethanol, $c = 1$). IR (CCl_4): cm^{-1} 2500 – 3300(s), 1700(s). NMR (CCl_4): δ 0.98 (3 H, s), 1.07 (3 H, s), 1.18 (3 H, s), ~1.6 (5 H, m), 2.0 – 2.7 (1 H, m), 11.77 (1 H, s).

Methyl-(1R)-1,2,2-trimethylcyclopentanecarboxylate, 17. To *4* (4.68 g, 0.03 mol) and sodium hydroxide (1.6 g, 0.039 mol) in 20 ml of water at 40 °C was added dimethyl sulphate (3.4 ml, ~0.036 mol). The temperature was kept at 60 °C for 1 h and excess of NaOH was added. The product *17* was extracted with ether and distilled (3.6 g, 71 %), b.p.$_{760}$ 176 – 180 °C. $n_D^{24} = 1.4448$. $[\alpha]_D^{25} = +8.4°$ (ethanol, $c = 3$). IR (film): cm^{-1} 1740(s). NMR (CCl_4): δ 0.81 (3 H, s), 1.01 (3 H, s), 1.10 (3 H, s), 1.6 (5 H, m), 2.0 – 2.7 (1 H, m), 3.59 (3 H, s).

(1R,3S)-3-Bromo-cis-4-hydroxy-1,2,2-trimethylcyclopentanecarboxylic acid lactone, 25. To *15* (10.0 g, 0.065 mol) in chloroform (30 ml) kept below 5 °C was added bromine (11.5 ml, 0.072 mol) during 1 h. After the addition, the mixture was stirred at 5 °C for 30 min. The $CHCl_3$ was evaporated and the residue was stirred vigorously with 10 % Na_2CO_3 (120 ml) for 1 h. Extraction of the mixture with methylene chloride, drying, and evaporation gave 13.8 g of a crystalline product which was recrystallized from petrol ether (b.p. 60 °C) to give pure bromolactone *25* (11.9 g, 72 %), m.p. 90.5 – 91.5 °C. (Found: C 46.5; H 5.75; Br 34.2. Calc. for $C_9H_{13}O_2Br$: C 46.4; H 5.62; Br 34.3). $[\alpha]_D^{25} = -71°$ (ethanol, $c = 1$). IR (CCl_4): cm^{-1} 1795(s). NMR (CCl_4): δ 1.01 (3 H, s), 1.04 (3 H, s), 1.09 (3 H, s), 1.82* (1 H, A part of ABXY system), 2.60* (1 H, B part), 4.26* (1 H, X part), 4.53* (4 H, Y part). $J_{AB}^* = -15.2$. $J_{AX}^* = 4.0$. $J_{BX}^* = 10.1$. $J_{XY}^* = 2.0$.

Catalytic hydrogenation of bromolactone, 25. 25 (2.34 g, 0.01 mol) in methanol (20 ml) was hydrogenated over 1 g of 5 % Pd/C and 4 g of MgO (1 atm. 25 °C) until slightly more than 0.01 mol of hydrogen was consumed (28 h). The solution was filtered, diluted with water (100 ml) and extracted 5 times with methylene chloride. The organic phase was washed with aqueous K_2CO_3, dried, and evaporated and the residue sublimated at 100 °C/10 mm to give *20*, 910 mg (59 %), m.p. (from ether) 162 – 164 °C (lit.[17] 165 – 167 °C). The IR spectrum was identical with that published by Faigle and Karrer,[14] $[\alpha]_D^{25} = -19°$ (ethanol, $c = 2$). NMR (CCl_4): δ 0.92 (3 H, s), 0.98 (3 H, s), 1.01 (3 H, s), 1.4 – 2.1 (4 H, m), 4.23 (1 H, m). MS: Mass (intensity – % of base peak): 154 (2) M^+, 140 (2), 126 (10), 111 (10), 108 (15), 97 (18), 95 (100).

* Calculated values.

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Derivatives of Hydrazine. XI. Rotational Isomerism of Methyl Diselenocarbamates and Diselenocarbazates

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The preparation of methyl diselenocarbamates and diselenocarbazates carrying one or more *N*-alkyl substituents is described. The ^{1}H NMR spectra of these compounds are reported and signals assigned to *Z* and *E* isomers arising from rotational isomerism about the central C–N bond. The assignments are primarily based on multiplicity, position, and solvent shifts of the signals compared with results obtained from analogous compounds with known configuration. The *Z*–*E* isomer ratios are briefly discussed.

During work on derivatives of dithio- and diselenocarbazic acids,[1–4] we became interested in the conformational properties of diselenocarbazic esters. These compounds, in analogy with, *e.g.*, amides [5] and hydrazides,[6,7] may exhibit *Z*–*E* isomerism arising from hindered rotation around the central N^2–C bond. Dipolar structures and selenol forms are also possible in analogy with observations reported for related sulfur compounds.[2,8]

This paper describes a study, primarily by means of ^{1}H NMR spectroscopy, on the rotational isomerism of methyl N^2-methyldiselenocarbazate (*1*), methyl N^2,N^3-dimethyldiselenocarbazate (*2*), and methyl N^2,N^3,N^3-trimethyldiselenocarbazate (*3*) in CCl_4, $CDCl_3$, and nitrobenzene. The compounds prepared for this study, including the three *N*-alkyl-*N*-methyldiselenocarbamates (*4*–*6*) used as reference substances, are listed in Table 1.

The ^{1}H NMR spectra of the compounds *1*–*6* are given in Table 2. The signals arising from the methyl groups (N^2–CH_3, N^3–CH_3, and Se–CH_3) as well as the N^3–H protons can generally be identified by their integrated intensities, multiplicities and relative positions. Furthermore, the signals from the Se–CH_3 groups were characterized by ^{77}Se satellites (J_{Se-C-H} = *ca.* 14 Hz), and in many cases the identity was also established by recording the spectra of the corresponding Se–CD_3 compounds. This procedure was necessary in cases where the chemical shifts of the Se–CH_3 groups were close to those of N^3–CH_3 groups. In the case of *2* the signals from the N^3–CH_3 group collapsed into sharp peaks on addition of D_2O.

With the sole exception of *3* each *N*-methyl group gave rise to two NMR signals when the spectra were recorded at room temperature. Since none of the compounds *1*–*6* carry hydrogen at the N^2 atom, dipolar and selenol forms are not possible. Accordingly the occurrence of two signals from each group must be due to the presence of anisochronous CH_3 groups arising from hindered rotation about the central N^2–C bond. The NMR spectrum of *3* was recorded in a range of solvents and in a temperature range from −55 °C to +140 °C, but it was impossible to detect more than one signal from each group. Since the rotational barrier around the N^2–C bond cannot be much different in the compounds *1*–*3* this is taken as evidence for *3* existing exclusively in one isomeric form.

In order to decide which signals arise from the *Z* form and which from the *E* form, two methods have been used. (1) The chemical shifts of the N^2–CH_3 groups were compared to those of the N^3–CH_3 groups of 3-methyl-1,3,4-selenadiazolidine-2-selones [9] which are necessarily situated in *Z* position. (2) The solvent-induced shifts of the same signals in CCl_4, $CDCl_3$, and $C_6H_5NO_2$ were

Table 1. Preparative data for *N*-alkylsubstituted methyl diselenocarbazates and diselenocarbamates.

Compound	Formula	Method	Yield, %	M.p., °C	Analyses (C, H, N)
1 $NH_2-N(CH_3)-CSeSeCH_3$	$C_3H_8N_2Se_2$	A	79	134–135	Calc.: 15.66; 3,52; 12.18 Found: 15.44; 3.45; 12.14
2 $CH_3NH-N(CH_3)-CSeSeCH_3$	$C_4H_{10}N_2Se_2$	B	80	65.0–66.5	Calc.: 19.68; 4.13; 11.48 Found: 19.66; 4.20; 11.58
3 $(CH_3)_2N-N(CH_3)-CSeSeCH_3$	$C_5H_{12}N_2Se_2$	B	85	55–57	Calc.: 23.27; 4.69; 10.85 Found: 23.12; 4.72; 11.00
4 $(CH_3)_2N-CSeSeCH_3$	$C_4H_9NSe_2$	C	72	84–85	Calc.: 20.97; 3.96; 6.12 Found: 20.70; 3.86; 6.10
5 $(C_2H_5)(CH_3)N-CSeSeCH_3$	$C_5H_{11}NSe_2$	D	30	5.5–7.0	Calc.: 24.71; 4.56; 5.76 Found: 24.40; 4.53; 5.92
6 $(i\text{-}C_3H_7)(CH_3)N-CSeSeCH_3$	$C_6H_{13}NSe_2$	D	50	[a]	Calc.: 28.03; 5.10; 5.45 Found: 28.10; 5.16; 5.47

[a] Melted during drying at 0 °C.

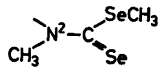
compared. The previously obtained data for 3-methyl-1,3,4-selenadiazolidine-2-selones [9] were supplemented as shown in Table 3. It is seen, that (1) the $CH_3-N-CSe$ signal of the *Z* form shows a chemical shift in $CDCl_3$ between 3.50 and 3.64 ppm, and (2) it is displaced towards lower δ-values when the compounds are recorded in CCl_4 solution, but towards higher δ-values in $C_6H_5NO_2$.

From the results listed in Table 2 it is seen, that the low-field N^2-CH_3 signals have chemical shift values in $CDCl_3$ in the range 3.48–3.88 ppm while the high-field signals are located in the range from 3.17 to 3.55 ppm. Though this indicates that it is the former signals which should be assigned to the *Z* CH_3 group there is a certain overlap of the regions and a confirmation is clearly needed for this assignment.

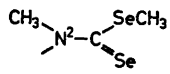
Considering next the solvent shifts, it is seen that the low-field N^2-CH_3 signals behave uniformly and differently from the high-field N^2-CH_3 signals. Thus, the low-field N^2-CH_3 signals ($CDCl_3$) are shifted to lower field in nitrobenzene and to higher field in CCl_4. The high-field N^2-CH_3 signals are found at almost identical positions in CCl_4 and $CDCl_3$, but are shifted to higher field in nitrobenzene solution. This result is only compatible with an assignment of the low-field N^2-CH_3 signals to the CH_3 group in *Z* position.

The *Z*–*E* isomer ratios for the diselenocarbamates *5* and *6* are both close to 1:1. This indicates that the steric requirements of =Se and $-SeCH_3$ are not significantly different for the compounds studied, that is provided the size of a substituent on the N^2-atom does not exceed that of an isopropyl group. In the case of the diselenocarbazates *1*–*3* there seems to be a definite tendency for predominance of the *Z* form. Walter and Reubke [7] has explained the predominance of the *E*-form in hydrazides (corresponding to the present *Z*-forms) with substituents exerting no steric influence to the tendency towards intramolecular compensation of existing dipoles. Since the diselenocarbazates *1*–*3* undoubtedly exhibit significant bond moments along the C=Se and N–N bonds, the predominance of the *Z* forms can be explained in an analogous way by the mutual repulsion of these dipoles.

Table 2. ^{1}H NMR chemical shifts (δ, ppm) at *ca.* 40 °C of *N*-alkylsubstituted methyl diselenocarbazates and diselenocarbamates. Abbreviations: s = singlet, d = doublet, q = quartet, and sep = septet. Centers of multiplets are given.



Z-isomer

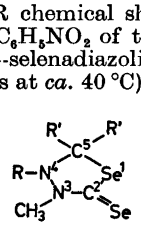


E-isomer

Compound	*Z*, %			N^2-CH_3			N^3-CH_3	$Se-CH_3$
	CCl_4	$CDCl_3$	$C_6H_5NO_2$	CCl_4	$CDCl_3$	$C_6H_5NO_2$	$CDCl_3$	$CDCl_3$
Z-isomer								
1[f]		50	80		3.88s	4.00s		2.47s
2[g]	75	75	90	3.67s	3.73s	3.83s	2.75d[a,b]	2.40s[c]
3	100	100	100	3.42s	3.48s	3.55s	2.63s	2.40s
4	(50)	(50)	(50)	3.60s	3.68s	3.62s		2.63s
5[d]	50	50	50	3.55s	3.60s	3.60s		2.65s
6[e]	45	45	45	3.41s	3.48s	3.52s		2.67s
E-isomer								
1[f]					3.55s	3.50s		2.70s
2[g]				3.42s	3.43s	3.37s	2.70d[a,b]	2.65s[c]
3								
4				3.40s	3.38s	3.27s		2.63s
5[d]				3.33s	3.32s	3.25s		2.65s
6[e]				3.16s	3.17s	3.16s		2.67s

[a] On addition of D_2O the signal collapsed to a sharp peak. [b] J_{NH-C-H} is approximately 6 Hz. [c] The signal was absent in the spectrum of the corresponding $Se-CD_3$ esters. [d] The signals from the ethyl group are: CH_3, broad triplet with center at 1.32. CH_2 (*Z*) 3.84 q, CH_2 (*E*) 4.29q. J_{CH-C-H} = *ca.* 7 Hz. [e] The signals from the isorpopyl group are: CH (*Z*) 4.79sep, CH_3 (*Z*) 1.33d, CH (*E*) 6.20sep, CH_3 (*E*) 1.27d J_{CH-C-H} = *ca.* 7 Hz. [f] NH_2 *ca.* 4.7 ($CDCl_3$), *ca.* 4.8 ($C_6H_5NO_2$). [g] Only N^3-H (*Z*) was identified with certainty: *ca.* 4.1 (CCl_4), *ca.* 4.2 ($CDCl_3$), *ca.* 4.8 ($C_6H_5NO_2$).

Table 3. ^{1}H NMR chemical shifts (δ, ppm) in CCl_4, $CDCl_3$ and $C_6H_5NO_2$ of the N^3-CH_3 group in 3-methyl-1,3,4-selenadiazolidine-2-selones. (*Ca.* 3 % solutions at *ca.* 40 °C).



Compound	CCl_4	$CDCl_3$	$C_6H_5NO_2$
R = R′ = H		3.60	3.68
R = H, R′ = CH_3[g]	3.62	3.64	3.73
R = CH_3, R′ = H	3.47	3.50	3.53
R = R′ = CH_3[g]	3.50	3.55	3.59

EXPERIMENTAL

The analyses were carried out in the microanalysis department of this laboratory. Melting points were determined in capillary tubes and were not corrected. Infrared spectra were obtained on a Perkin-Elmer model 337 grating infrared spectrophotometer and the proton magnetic resonance spectra on a Varian A-60 A instrument with tetramethylsilane as an internal standard.

The directions given below for the preparations of the methyl esters refer to the entry "Method" in Table 1.

Salts used as starting materials for the preparation of esters:

Dialkylammonium 2,2-dialkyldiselenocarbamates. One ml of 10 M aqueous sodium hydroxide was added to a suspension of the amine hydrochloride (10^{-2} mol) in pentane (100 ml) cooled in an ice bath. The mixture was shaken

vigorously and dried (KOH). Into the stirred solution of the liberated amine at 0°C a solution of carbon diselenide (5×10^{-3} mol) in pentane (40 ml) was added dropwise over a period of 2 h in a nitrogen atmosphere. The yellow solid that separated was collected on a glass filter, washed with pentane and dried *in vacuo*.

Hydrazinium diselenocarbazates. Generally, these salts were prepared analogously to the diselenocarbamates described above, starting with the free hydrazines and using dry ether as the solvent.[10]

Method A. Methyl iodide (10^{-3} mol) was added to a filtered, aqueous solution (4 ml) of the appropriate hydrazinium salt (10^{-3} mol). The reaction mixture was stirred vigorously at room temperature until it was (nearly) colourless. The crystals that precipitated were collected by centrifugation, washed with a small amount of cold water and dried *in vacuo*.

Method B. An ethanolic solution (4 ml) of methyl iodide (10^{-3} mol) was added to a filtered aqueous solution (5 ml) of the appropriate hydrazinium salt (10^{-3} mol). The reaction mixture was stirred for 5 – 10 min at room temperature and the solvent was evaporated *in vacuo* with gentle heating until a volume of ½ – 1 ml was reached. This residue was extracted with pentane and the pentane solution dried ($MgSO_4$) and taken to dryness. The residue (R) of yellow to colourless crystals was washed with a small amount of cold pentane.

Method C. An aqueous solution (5 ml) of dimethylammonium 2,2-dimethyldiselenocarbamate (2×10^{-3} mol) was added to an ethanolic solution (3 ml) of methyl iodide (2×10^{-3} mol) with stirring. More water (10 ml) was added to ensure complete precipitation. The light yellow crystalline compound was washed with water and dried *in vacuo*.

Method D. As Method B. The residue (R) consisted of an oil, which was purified by dissolution in the minimum amount of pentane and cooling to *ca.* −80 °C with scratching. The solid that separated, which in all cases melted below room temperature, was quickly collected by centrifugration, washed with a small amount of cold pentane and dried *in vacuo*.

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Fungus Pigments. XXIV*. Peniolactol Obtained from Wood Attacked by the Fungus *Peniophora sanguinea* Bres.

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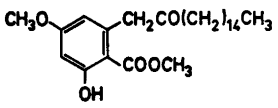
On the basis of spectral analysis, the structure 3,4-dihydro-3,6,8-trihydroxy-3-pentadecyl-1*H*-2-benzopyran-1-one (*1b*) is assigned to peniolactol.

In a paper describing the isolation of the pigment peniosanguin methyl ether from the extract of wood attacked by the fungus *Peniophora sanguinea* Bres.[1], it was noted that the pigment was accompanied by a colourless compound.

The structure *1*, which can exist in the two tautomeric forms *a* and *b*, is now proposed for this colourless compound which has been termed peniolactol. This structure proposal is based on the spectral properties of the compound and its derivatives, as outlined below.



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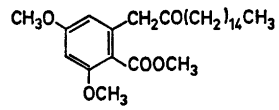

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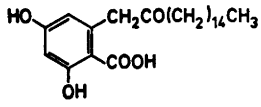
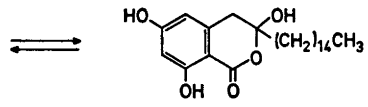
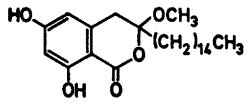


1a



1b



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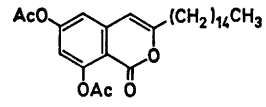

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Peniolactol has the composition $C_{24}H_{38}O_5$. In the mass spectrum only a very weak peak is found at *m/e* 406, corresponding to this molecular formula. There are instead two strong peaks at *m/e* 388 and 362, formed by loss of water and carbon dioxide, respectively. The base peak of the spectrum is at *m/e* 239, corresponding to $C_{15}H_{31}CO$.

Methylation of peniolactol with diazomethane gives a dimethyl derivative (*2*), and methylation with dimethyl sulphate and potassium carbonate of either peniolactol or its dimethyl derivative gives a trimethyl derivative (*3*).

IR-absorptions at 1654 and 1712 cm^{-1} in *2*

* Part XXIII, *Tetrahedron Lett.* (1974) 619.

and at 1712 and 1720 cm^{-1} in *3* show the presence of two carbonyl groups, one of which is an unconjugated aliphatic keto group and the other an aromatic methyl ester which is strongly chelated in *2*. Peniolactol shows only one carbonyl absorption at 1640 cm^{-1}, the aliphatic keto group being masked by lactol formation as indicated in *1b*. This requires that the aliphatic keto group is situated in a side chain *ortho* to the carboxyl group and the latter therefore lies between the aliphatic side chain and one phenolic hydroxyl. The position of the second phenolic hydroxyl in the *para*-position to the carboxyl group may be deduced from the NMR spectra of *2* and *3*, which show the presence of two *meta*-coupled aromatic protons. These NMR spectra also contain the expected two proton singlet for a methylene group between an aromatic ring and a carbonyl group.[2-4]

The structures of *2* and *3* are also supported by their UV spectra. Thus the spectrum of *2* is almost identical with that of ethyl 2-acetonyl-4-benzyloxy-6-hydroxybenzoate [2] and the mono- and diethyl esters of α-carboxy-6-hydroxy-4-methoxy-*o*-toluic acid.[5] Similarly that of *3* is almost identical with the spectrum of α-carboxy-4,6-dimethoxy-*o*-toluic acid.[6]

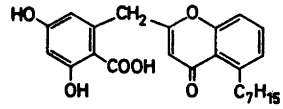
Esterification of peniolactol with methanol and sulphuric acid gives a monomethyl derivative, which must be the pseudo-ester *4*, because there is no absorption due to the aliphatic carbonyl group in its IR spectrum.

Acetylation of peniolactol gives an anhydrodiacetate (*5*). This structure is supported by the absence of the NMR signal due to the methylene group and the appearance of a one proton singlet at δ 6.09.

The NMR spectra of all the derivatives of peniolactol contain, in addition to the signals from the groups directly attached to the aromatic ring (discussed above), a very strong and somewhat broadend singlet at $\delta \sim 1.27$ which is accompanied by a three proton distorted triplet on the high field side and a two proton ill defined triplet on the low field side. This part of the spectrum is very similar to that of straight chain aliphatic acids and ketones, indicating that the group $-(CH_2)_{14}CH_3$ in the structures above is indeed the normal one.

As mentioned above, the IR spectrum indicates that, at least in the crystalline state, peniolactol exists in the lactol form *1b*. The position of this equilibrium in *o*-(2-oxoalkyl)-benzoic acids appears to depend to some extent on the substitution of the aromatic ring, although there seems also to be some confusion in the literature in this respect. Thus Buckley, Ritchie and Taylor [7] have made an extensive study of the unsubstituted 2-acetonylbenzoic acid and they conclude, on the basis of the IR spectrum in different media, that the equilibrium lies far towards the open chain form. In derivatives with a hydroxy or methoxy group in the 6-position,[2,8-10] there is no IR absorption that can be ascribed to the unconjugated carbonyl group as in peniolactol. They should therefore be regarded as existing in the lactol form, although some workers [2,10] still depict them as the open chain tautomers.

Peniolactol is evidently acetate derived and a number of compounds of analogous structure, but with a shorter aliphatic sidechain, have been isolated from fungi.[3,10,11-18] They are sometimes in a different state of oxidation or are ring closed to an isocoumarin or to a macrocyclic lactone. A number of depsides and depsidones [19] also belong to the same type of structure and a particularly interesting compound in this connection is siphulin (*6*),



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which has been isolated from the lichen *Siphula ceratites* [6] and has the same number of carbon atoms as peniolactol.

Since the extract from which peniolactol was isolated was obtained from decaying wood coloured red by the mycel of *Peniophora sanguinea* the question arises whether or not peniolactol is produced by *Peniophora sanguinea* or by some other fungus present in the decaying wood. The possibility that peniolactol is not a fungus product at all but a constituent of the wood, appears to be very improbable. In view of the very large amount of work which has been carried out on wood extracts it seems very unlikely that peniolactol, which is a nicely crystalline compound, should have been overlooked.

The presence of peniolactol in an extract can

easily be detected on TLC plates by spraying the plate with bis-diazotised benzidine. Examination of extracts obtained from different parts of the same piece of decaying wood revealed that peniolactol was never detected in those extracts obtained from non-coloured parts of the wood, whereas its presence could always be demonstrated in extracts which also contained the pigments. Furthermore, in a few fortunate cases it was possible to isolate the mycel of *Peniophora sanguinea*, seemingly free from contaminants, and in these cases the extract always contained peniolactol. It may therefore be concluded that peniolactol is indeed produced by *Peniophora sanguinea*.

EXPERIMENTAL

Melting points were determined by means of a Kofler melting point microscope and are uncorrected. Spectra were obtained using the following instruments: UV spectra on a Beckman DK-2, IR spectra on a PE 125, NMR spectra on a Varian A 60 and mass spectra on a PE 270B. Elemental analyses were carried out by Ilse Beetz, Mikroanalytisches Laboratorium, Kronach, German Federal Republic.

Isolation of peniolactol. The crude peniosanguin methyl ether [1] was dissolved in pyridine to which solution water was added until precipitation occurred. The peniosanguin methyl ether thus obtained was separated and more water was added to the mother liquor causing the precipitation of peniolactol. Further reprecipitation from pyridine gave peniolactol as slightly grey crystals. It melts at about 150 °C with decomposition. (Found: C 71.45; H 8.75, $C_{24}H_{38}O_5$ requires C 70.90; H 9.42) Mass spectrum: *m/e* 388, 362, 239, 205, 192, 150, 123. IR maxima: 3480, 2920, 2850, 1640, 1615 cm^{-1}. UV spectrum (EtOH): λ_{max} 214(4.44), 246(3.92), 264(4.02), 299(3.76); λ_{min} 240(3.82), 250(3.89), 286(3.68) nm (log ε).

Methyl 2-hydroxy-4-methoxy-6-(2-oxoheptadecyl)benzoate (2). Peniolactol (50 mg) dissolved in ether was treated with an excess of diazomethane. After standing over night the ether was evaporated and the residue purified by preparative TLC. Yield 35 mg, m.p. 96 – 97 °C (Found: C 71.54, H 9.42; $C_{26}H_{42}O_5$ requires C 71.85, H 9.74), Mass spectrum: *m/e* 434(M^+), 402, 239, 219, 206, 196, 164. IR maxima: 2920, 2850, 1712, 1654, 1620 cm^{-1}. UV spectrum (EtOH): λ_{max} 214(4.40), 263(4.13), 302(3.79); λ_{min} 239(3.60), 282 (3.55) nm (log ε). NMR spectrum ($CDCl_3$): δ 0.89 (3 H, br tr), 1,28(26 H, s), 2.41(2 H, br tr), 3.81(3 H, s), 3.82(3 H, s), 3.90(2 H, s), 6.23 (1 H, d; $J = 2.5$ Hz), 6.43 (1 H, d; $J = 2.5$ Hz), 11.62(1H, s).

Methyl 2,4-dimethoxy-6-(2-oxoheptadecyl)benzoate (3). Peniolactol (30 mg) dissolved in acetone was stirred for 8 h at room temperature with potassium carbonate (0.5 g) and dimethyl sulphate (0.2 ml). After filtration, addition of water and removal of the acetone under vacuum, a white precipitate was obtained which was purified by preparative TLC and recrystallisation from methanol. M.p. 71 – 72 °C. The same compound was also obtained when (*2*) was treated in the same way. (Found: C 72.30, H 9.22. $C_{27}H_{44}O_5$ requires C 72.28;, H 9.89). Mass spectrum: *m/e* 448(M^+), 416, 233, 220, 210, 196, 179, 178. IR maxima: 2920, 2850, 1720, 1712 1604 cm^{-1}. UV spectrum(EtOH): λ_{max} 250(3.81), 286(3.56); λ_{min} 239(3.77), 271 (3.41) nm (log ε). NMR spectrum ($CDCl_3$): δ 0.90(3 H, br tr), 1.27(26 H, s), 2.45(2 H, br tr), 3.67(2 H, s), 3.80(6 H, s), 3.82 (3H, s), 6.32(1 H, d; $J = 2$ Hz), 6.42(1 H, d; $J = 2$ Hz).

3,4-Dihydro-6,8-dihydroxy-3-methoxy-3-pentadecyl-1H-2-benzopyran-1-one (4). Peniolactol (40 mg) was refluxed for 8 h in methanol containing two drops of conc. sulphuric acid. Water was then added and the methanol removed under vacuum. Extraction with ether and recrystallisation from light petroleum gave (*4*). M.p. 92 – 93 °C (Found: C 71.35, H 9.74. $C_{25}H_{40}O_5$ requires C 71.39, H 9.59). IR maxima: 3250, 2910, 2850, 1660, 1615 cm^{-1}. UV spectrum (EtOH): λ_{max} 214(4.35), 230(infl.; 4.16), 246 (3.86), 270(4.15), 302(3.87); λ_{min} 242(3.80), 249(3.83), 291(3.84) nm (log ε). NMR spectrum ($CDCl_3$): δ 0.90(3 H, br tr), 1.28(26 H, s), 2.00(2 H, br tr), 3.08(2 H, br s), 3.38(3 H, s), 6.22(1 H, d; $J = 2.5$ Hz), 6.34(1 H, d; $J = 2,5$ Hz), 11.12(1 H, br s; exch. with D_2O).

6,8-Diacetoxy-3-pentadecyl-1H-2-benzopyran-1-one (5). Peniolactol dissolved in acetic anhydride containing a drop of pyridine was kept over night at room temperature. The anhydroacetate was precipitated with water and purified by preparative TLC. M.p. 86 – 87 °C. (Found: 70.96, H 8.38. $C_{28}H_{40}O_6$ requires C 71.16, H 8.53). Mass spectrum: *m/e* 472(M^+), 430, 388, 205, 192, 177, 163. IR maxima: 2950, 2880, 1770, 1730, 1660 cm^{-1}. UV spectrum(dioxan): λ_{max} 237(4.56), 243(infl.; 4.47), 262(3.95), 270 (4.00), 280(3.91), 323(3.67); λ_{min} 257(3.91), 264(3.94), 276(3.86), 292(3.13) nm (log ε). NMR spectrum(CCl_4): δ 0.89(3 H, br tr), 1.24(26 H, s), 2.27(3 H, s), 2.33(3 H, s), 2.20 – 2.30(2 H, m), 6.09(1 H, s), 6.77(1 H, d; $J = 3$ Hz), 6.95(1 H, d; $J = 3$ Hz).

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Studies on Intermediates Involved in the Syntheses of Pentaerythritol and Related Alcohols. VI.* Base-catalyzed Retro Condensation Reactions of Some α-Hydroxymethyl Carbonyl and Nitro Compounds

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The base-catalyzed retro aldol condensation reactions of some aldehydic intermediates in the syntheses of pentaerythritol and related alcohols have been investigated kinetically. Rate constants for these reactions, involving the splitting off of formaldehyde, have been obtained for the compounds 2,2-bis(hydroxymethyl)-3-hydroxypropanal, 3-hydroxy-2-hydroxymethyl-2-methylpropanal, 2-ethyl-3-hydroxy-2-hydroxymethylpropanal, and 2,2-dimethyl-3-hydroxypropanal. These aldehydes were all found to undergo retro aldol reaction in aqueous solution at rather low concentration of sodium hydroxide. Under the conditions used, the reactions did not proceed beyond the liberation of one molecule of formaldehyde per molecule of starting material. 2,3-Dihydroxypropanal, having one α-hydrogen, released almost no formaldehyde at 20 °C in 0.010 M NaOH. 3-Hydroxy-2-hydroxymethylpropanal, also containing one α-hydrogen, gave rise to formaldehyde formation slowly, but, as will be shown in a subsequent paper, probably in an indirect way *via* another reaction.

For comparison, the corresponding reactions of some other activated, hydroxymethyl-substituted compounds have been investigated also. The ketones 4-hydroxy-3-hydroxymethyl-3-methyl-2-butanone, 3-hydroxy-2-hydroxymethyl-2-methyl-1-phenyl-1-propanone, 2,2-bis(hydroxymethyl)-1-tetralone, 2,2-bis(hydroxymethyl)-1-indanone, and 2,3-dihydroxy-1,2-diphenyl-1-propanone also released formaldehyde, the second and the fourth of these compounds giving rise to more than one mol of formaldehyde per mol of ketone.

Of the nitro compounds examined, 2-hydroxymethyl-2-nitro-1,3-propanediol, 2-methyl-2-nitro-1,3-propanediol, 2-ethyl-2-nitro-1,3-propanediol, and 2-methyl-2-nitro-1-propanol reacted with consumption of hydroxide ion under formation of nitronate ions and one mol of formaldehyde. 2-Nitroethanol and 2-nitro-1-butanol slowly released formaldehyde, but these reactions were not investigated kinetically.

9,9-Bis(hydroxymethyl)fluorene was almost stable in 0.2 M NaOH at 55 °C.

The reactions were followed by spectrophotometric determination of free formaldehyde according to the method reported by Nash, based on the Hantzsch reaction.

This investigation is part of a series of studies undertaken with the purpose of clarifying, as to rates and mechanisms, the reaction steps involved in the syntheses of polyalcohols by condensation reactions between formaldehyde and low molecular weight aliphatic aldehydes. It is believed that a better knowledge of these reactions will help to indicate which types of side reactions are important under different reaction conditions and how these conditions should be chosen in order to improve the quality and yield of the polyalcohols produced. More specifically, if formaldehyde is used in nearly stoichiometric amounts, retro aldol condensation reactions presumably can be important during the last stages of the syntheses when the concentration of formaldehyde is low. The amounts of intermediate aldehydes still present at this stage and those of their possible

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conversion products formed in side reactions may thus be determined by retro aldol reactions.

Aldol condensation reaction are believed to be generally reversible [1] and the reversibility has been proven in many cases.[2,3] In order to examine the rates of those retro aldol condensation reactions which are possible under the conditions of synthesis of pentaerythritol and related alcohols, a suitable method was needed, by means of which the progress of these reactions could be followed. The formaldehyde determination method reported by Nash,[4] based on the Hantzsch reaction, is almost ideal for the purpose. It allows accurate and relatively rapid determinations of formaldehyde in very dilute aqueous solutions, undisturbed by the presence of most other carbonyl compounds. The procedure utilizes the reaction of formaldehyde with acetylacetone and an ammonium salt with the formation of diacetyldihydrolutidine, which is yellow and has an absorptivity of 8000 at 412 nm. The lutidine derivative is formed rapidly and quantitatively in neutral aqueous solution. Since the preferable reaction conditions are very mild, the starting materials, as was proven for all the compounds examined, neither react to form coloured products nor release additional formaldehyde during the determinations.

The fully α-hydroxymethyl-substituted aldehydes, 2,2-bis(hydroxymethyl)-3-hydroxypropanal (I, trimethylolacetaldehyde), 3-hydroxy-2-hydroxymethyl-2-methylpropanal (II, dimethylolpropionaldehyde), 2-ethyl-3-hydroxy-2-hydroxymethylpropanal (III, dimethylolbutyraldehyde), and 2,2-dimethyl-3-hydroxypropanal (IV, pentaldol), are the immediate precursors of pentaerythritol, trimethylolethane, trimethylolpropane, and neopentylglycol, respectively (Fig. 1).

For both statistical and sterical reasons, these aldehydes were expected to be most susceptible to base-catalyzed retro aldol con-

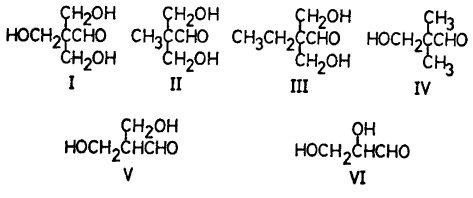


Fig. 1.

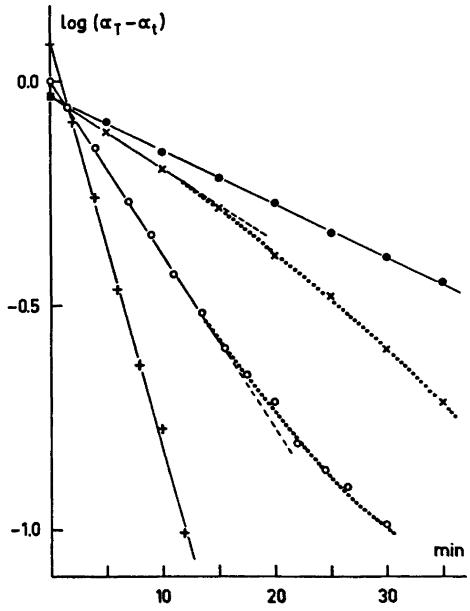


Fig. 2. Pseudo first-order plots for disappearance at 40 °C of starting materials in terms of absorbance units (α_T = theoretical maximum absorbance of the equimolar amount of formaldehyde derivative and α_t = absorbance at time t actually found for the formaldehyde derivative). +: I in 0.0020 M NaOH; ○: III in 0.0050 M NaOH; ×: IX in 0.0020 M NaOH, and ●: XI in 0.010 M NaOH.

densation with resultant loss of formaldehyde and were first examined. When treated in about 5×10^{-4} M solutions with NaOH they responded somewhat differently. Aldehyde I, within experimental errors, lost an equimolar amount of formaldehyde and gave linear pseudo first-order plots. Similar plots for compounds II and III were curved in a way which indicated that an equilibrium was reached when slightly less than an equimolar amount of formaldehyde had been formed. In Fig. 2, plots from runs with some different compounds are shown. For compound IV the pseudo first-order plots were still more curved, equilibrium being reached at an earlier stage of the reaction.

For compounds I – III rate constants could thus be evaluated graphically from pseudo first-order plots of log ($[A]_0 - [CH_2O]$) against time. Here $[A]_0$ is the initial concentration of the starting material and $[CH_2O]$ the concentration of formaldehyde at time t. Compound IV,

on the other hand, was found to obey the rate expression:

$$-\frac{d[IV]}{dt} = \frac{d[CH_2O]}{dt} = k_1'[IV] - k_{-1}'[CH_2O] \times$$
$$\times [(CH_3)_2CHCHO] = k_1'([IV]_0 - [CH_2O]) -$$
$$- k_{-1}'[CH_2O]^2$$

This leads to the integrated expression:

$$\psi = \frac{1}{\sqrt{1+4\tau[IV]_0}}$$

$$\ln \frac{1+2\tau[CH_2O]/(1+\sqrt{1+4\tau[IV]_0})}{1+2\tau[CH_2O]/(1-\sqrt{1+4\tau[IV]_0})} = k_1't$$

$$\text{where } \tau = \lim_{t\to\infty} \frac{[IV]_0 - [CH_2O]}{[CH_2O]^2}$$

The pseudo first-order rate constant k_1' was evaluated from plots of the function ψ against time (Fig. 3). The same type of expression could be used also for aldehydes II and III, but since the experimental value found for τ was rather uncertain in these cases, the use of pseudo first-order plots was considered more reliable. The difficulty in determining τ-values accurately is due to the fact that the final concentration of formaldehyde reached was not quite stable with time. On prolonged exposure to the alkaline reaction mixture it showed a tendency to decrease with a few percent, possibly due to oxidation. This was much more critical in the runs with II or III than in those with IV, since, in the former runs, the value of $([A]_0 - [CH_2O])_\infty$ was comparatively small.

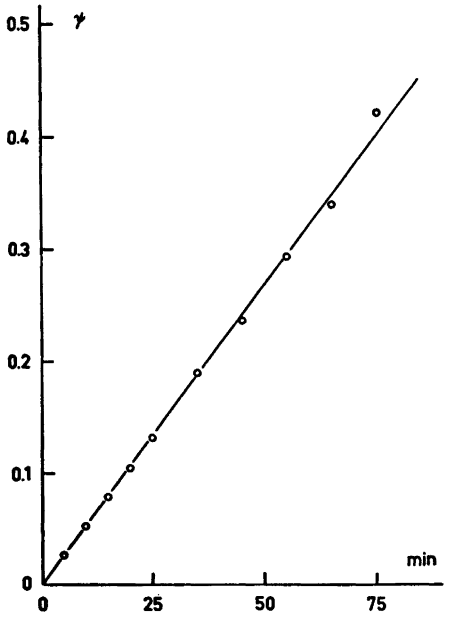


Fig. 3. Experiment with aldehyde IV at 40 °C in 0.0040 M NaOH showing function ψ against time.

That equilibria with significant amounts of starting material left were indeed established even in the rather dilute solutions examined, was proven indirectly for compound IV and its products, formaldehyde and isobutyraldehyde. The latter two aldehydes, both 2×10^{-3} M, were found to condense rather rapidly at 20 °C. For this reaction a pseudo second-order rate constant, k_{-1}'', referring to the rate expression:

$$-\frac{d[(CH_3)_2CHCHO]}{dt} = -\frac{d[CH_2O]}{dt} =$$
$$= k_{-1}''[(CH_3)_2CHCHO][OH^-] = k_{-1}''[CH_2O][OH^-]$$

was obtained from the decrease in formaldehyde concentration. The value calculated from the experimental data was $(5 \pm 2) \times 10^{-2}$ M^{-1} s^{-1}. Comparison of this value with the value 5.48×10^{-2} M^{-1} s^{-1}, obtained earlier for the same reaction in 0.1 M CH_2O by another method,[5] shows that, even at the low formaldehyde concentration of 0.002 M, the rate of formation of enolate ion from isobutyraldehyde is still probably at least partly rate determining. This was also apparent from plots of the experimental data. The reaction was neither strictly first-order nor second-order in formaldehyde concentration (equal to the concentration of isobutyraldehyde) but something in between. For this reason, the value given above was evaluated graphically from pseudo first-order plots.

If one tries to calculate a third-order rate constant from the value of k_{-1}'' given above, by dividing with the formaldehyde concentration in the run, the value obtained is 25 ± 10 M^{-2} s^{-1}. This could be used in conjunction with the value 0.00248 M^{-1} s^{-1} for the retro aldol condensation of IV (see Table 2) to calculate the equilibrium constant for the opposed reactions at 20 °C,

$$K_{IV} = \frac{[CH_2O][(CH_3)_2CHCHO]}{[HOCH_2C(CH_3)_2CHO]} = \frac{k_1'}{k_{-1}'''} = \frac{1}{\tau}$$

a value of about 10^{-4} M then being obtained. This value is not in agreement with the values of τ obtained directly from the runs with IV, as these latter values for runs at 20 °C give K_{IV}-values around 2×10^{-5} M. The reason for this discrepancy is obviously that the third-order constant calculated above is not a true third-order constant, but was obtained from runs at concentrations of formaldehyde exceeding the concentration at which the reaction is still of first-order with respect to formaldehyde. The K_{IV}-value of 10^{-4} M therefore is too high and values calculated from the τ-values, even though these are rather uncertain, probably are more correct.

From the data in Table 1 a ΔH-value of about 10 kcal mol^{-1} is obtained for the retro aldol condensation of IV. The difference in activation energies for the retro aldol reaction of IV (see Table 2) and the aldol condensation of isobutyraldehyde with formaldehyde,[5] on the other hand, amounts to only $19.2 - 15.1 = 4.1$ kcal mol^{-1}. If the large uncertainties in the τ-values and also those in the activation energies for the forward and the reverse reactions are taken into account, this discrepancy is still too large and cannot be explained as arising from experimental errors alone. Instead, it must be borne in mind that the activation energies refer to different reaction conditions with respect to formaldehyde concentration and thereby to different rate determining steps for the condensation reaction, as well as to different alkalinities, also at the same nominal concentration of NaOH.

Table 1. Equilibrium constant, K_{IV}, for the retro aldol condensation of 2,2-dimethyl-3-hydroxypropanal at different temperatures.

°C	20	30	40	50
$K_{IV} \times 10^5$ M	1.7	2.8	4.7	10

In any case, as apparent from the experimental results, the third-order rate constant for the condensation of isobutyraldehyde with formaldehyde is remarkably large when compared with the corresponding constants for the other aldehydes examined. Under comparable conditions the reaction with isobutyraldehyde goes much more nearly to completion. This confirms the conclusion drawn in an earlier paper [5] concerning the order of reactivity for isobutyraldehyde and some other aldehydes at low formaldehyde concentrations.

The observation that apparently not more than one hydroxymethyl group was split off from aldehydes I – III gave rise to the question as to whether only aldehydes with quaternary substituted α-carbons undergo retro aldol condensations of this type with ease. To examine this, the aldehydes, 3-hydroxy-2-hydroxymethylpropanal (V, dimethylolacetaldehyde) and 2,3-dihydroxypropanal (VI, D,L-glyceraldehyde), were subjected to similar treatment. Compound VI, on 5 h treatment with 0.05 M NaOH at 20 °C, lost only about 3 % of the

Table 2. Second-order rate constants and activation energies for loss of one equivalent of formaldehyde from various compounds.

Compound	k M^{-1} s^{-1} at various temperatures								E_A
	20 °C	25 °C	30 °C	35 °C	40 °C	45 °C	50 °C	55 °C	kcal mol^{-1}
I	0.302		0.740		1.71				15.8
II	0.0640		0.163		0.407				16.9
III	0.0520		0.134		0.336				17.0
IV	0.00248		0.00750		0.0208		0.0529		19.2
VIII		0.00106			0.00571			0.0248	20.4
IX		0.0827			0.313			1.02	16.3
X		0.040			0.17			0.70	18.6
XI		0.00949			0.0456			0.184	19.2
XII		0.266		0.801		2.15		5.04	19.1
XIII	7.68		19.3		42.5				15.6
XIV	2.51		6.83		17.2				17.6
XV		5.76		14.5		33.8			16.7
XVI	0.954		2.51		6.14				17.0

theoretical amount of formaldehyde. Compound V released formaldehyde slowly, about 0.5 equiv. after 2 h and about 0.8 after 24 h at 40 °C in 0.01 M NaOH. Some observations in connection with this latter reaction indicated, however, that formaldehyde here was produced as the result of a different reaction sequence. This motivates a separate discussion in a forthcoming paper. Although the number of aldehydes examined is rather small, the experimental data indicate that, of the aldehydes of the type investigated, those with a tertiary α-carbon atom lose formaldehyde much more slowly than those with a quaternary α-carbon atom.

In order to further investigate the scope of this retro aldol condensation type, some representatives of other classes of compounds were also examined. Of the ketones investigated, 4-hydroxy-3-methyl-2-butanone (VII, monomethylol methyl ethyl ketone) and 4-hydroxy-3-hydroxymethyl-3-methyl-2-butanone (VIII, dimethylol methyl ethyl ketone) are examples of simple aliphatic ketones and 3-hydroxy-2-hydroxymethyl-2-methyl-1-phenyl-1-propanone (IX, dimethylol propiophenone), 2,2-bis(hydroxymethyl)-1-indanone (X), and 2,2-bis(hydroxymethyl)-1-tetralone (XI) represent mixed aliphatic-aromatic types. 2,3-Dihydroxy-1,2-diphenyl-1-propanone (XII, hydroxymethylbenzoin) is structurally similar to aldehyde VI.

Again, the aliphatic compound with tertiary α-carbon, VII, was found to resist degradation and remained unchanged after 3 h in 0.01 M NaOH at 40 °C. The other five ketones, by contrast, all underwent retro aldol condensation. Three of them, namely compounds VIII, XI, and XII, gave linear pseudo first-order plots of log ($[A]_0 - [CH_2O]$) against time. For compounds IX and X, however, such plots were curved in a way indicating that more than one equivalent of formaldehyde was formed from the starting material. This was also evident from the total amount of formaldehyde determined after prolonged exposure to the alkaline reaction conditions. It is thus no general rule that the loss of the first and of the second of two α-hydroxymethyl groups occurs with so widely differing rates, that the reactions always can be well-distinguished kinetically (example in Fig. 2).

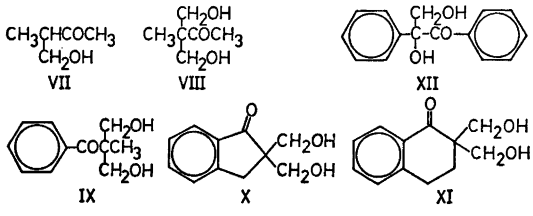


Fig. 4.

Compound XII was found to dissolve only with great difficulty in water and was therefore first dissolved in a small amount of methanol, the stock solution then being made up by dilution with water. This caused the presence of about 0.5 % of methanol as solvent in the reaction mixtures. The values obtained from these runs are therefore not strictly comparable with those from runs with the other compounds, since the methoxide ion present in equilibrium with hydroxide ion is possibly a more potent catalyst. General base catalysis should only be observed, however, if the first reaction step, that is the formation of an alkoxide ion, is rate determining, and that is probably not the case.

It is interesting to note that of the two closely similar compounds X and XI, the retro aldol condensation product from the latter seemed stable under conditions where that of the former lost more formaldehyde. Ketone IX, with a more flexible carbon side chain, also lost more than an equimolar amount of formaldehyde. It is tempting to assume that this has something to do with the spatial arrangements in compounds IX–XI. Thus, in both compounds IX and X, there is a greater possibility for the unsaturated system of the enolate ion arising by loss of formaldehyde from the initially formed alkoxide ion to be coplanar than in the corresponding ion from compound XI. One may object, that this does not hinder compound XI from first losing a molecule of formaldehyde. The rate of this reaction, however, is lower for compound XI than for the others, and the activation energy is higher. Relatively small differences may be sufficient to slow down the rate of the second retro aldol reaction in the case of XI to a level where this reaction is no longer detectable under the experimental conditions used.

For all the aldehydes and ketones for which rates of reaction were determined (see Table 2),

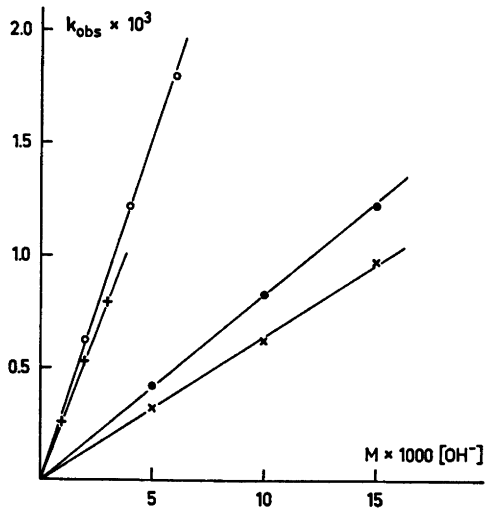


Fig. 5. Pseudo first-order rate constant observed for aldehydes I (○) and II (×) at 20 °C and ketones IX (●) and XII (+) at 25 °C.

these rates were found to be directly proportional to the hydroxide ion concentration, as illustrated in Fig. 5.

Hydroxymethyl-substituted nitro compounds, available by the Henry reaction, are also known to undergo retro aldol type reactions.[6] The rates of these reactions for the nitro analogs of compounds I–IV, 2-hydroxymethyl-2-nitro-1,3-propanediol (XIII, tris(hydroxymethyl)-nitromethane), 2-methyl-2-nitro-1,3-propanediol (XIV, bis(hydroxymethyl)nitroethane), 2-ethyl-2-nitro-1,3-propanediol (XV, bis(hydroxymethyl)nitropropane), and 2-methyl-2-nitro-1-propanol (XVI), have now been determined (compounds in Fig. 6).

The reactions proceeded rapidly to begin with, but then slowed down as seen from pseudo first-order plots. In these cases this could not be explained by equilibria being attained between starting material and product.

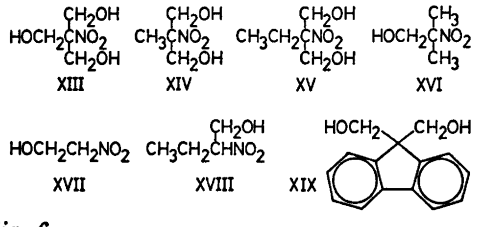


Fig. 6.

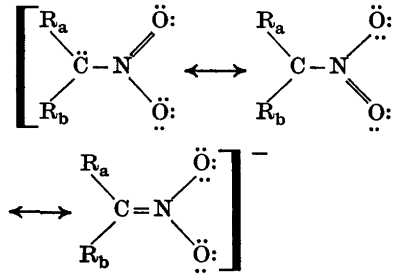
This was evident from the results of runs with varying concentrations of the catalyst, NaOH, more formaldehyde being formed if more NaOH was added. When equimolar amounts of nitro compound and NaOH were used, the reactions were found to follow the second-order rate expression (C denoting the nitro compound):

$$-\frac{d[C]}{dt} = \frac{d[CH_2O]}{dt} = k[C][OH^-] =$$
$$= k([C]_0 - [CH_2O])^2$$

These findings indicated that hydroxide ion actually was consumed during the reaction. The carbanion formed after loss of the formaldehyde molecule apparently was relatively stable under the reaction conditions, due to resonance involving the nitro group, and therefore did not react with water with regeneration of the catalyst:

$$HO^- + HOCH_2CR_aR_bNO_2 \rightleftharpoons H_2O + {}^-OCH_2CR_aR_bNO_2$$

$${}^-OCH_2CR_aR_bNO_2 \longrightarrow CH_2O +$$



Second-order plots from experimental runs with compound XV are shown in Fig. 7.

Two compounds with nitro groups on less substituted carbons, 2-nitroethanol (XVII) and 2-nitro-1-butanol (XVIII), were also examined. They lost only relatively small amounts of formaldehyde during one hour at 45 °C in 3×10^{-4} M NaOH, conditions under which the others reacted rather fast. No further determinations were made.

Finally, one hydroxymethyl-substituted hydrocarbon, 9,9-bis(hydroxymethyl)fluorene (XIX), was studied, but was found to be almost stable in 0.2 M NaOH at 55 °C for some hours.

The experimental results, in the form of rate constants and Arrhenius activation energies, are summarized in Table 2.

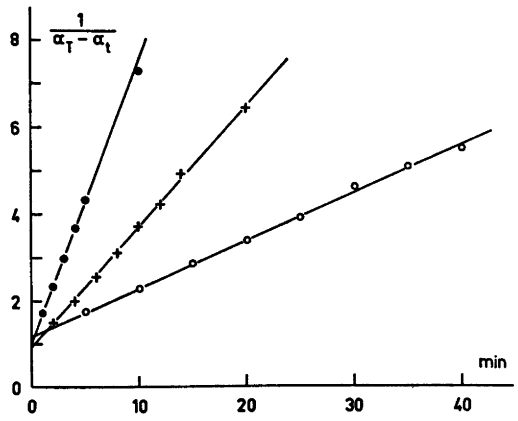


Fig. 7. Second-order plots from runs with nitro compound XV in 3×10^{-4} M NaOH at 25 °C (○), 35 °C (+), and 45 °C (●), respectively (α_T and α_t as in Fig. 2).

As seen from this table, of the compounds investigated, those activated by a nitro group react much faster than those activated by a carbonyl group. Of the latter compounds, the aliphatic aldehydes apparently react considerably faster than their ketonic analogs, as confirmed by the rate constants for the couple II and VIII. The electron attracting aromatic rings in the ketones IX and X activate these compounds to rates on a level with those of aldehydes II and III, whereas ketone XI is comparable in reactivity with aldehyde IV. As mentioned previously, the values for compound XII may not be strictly comparable with those for the other compounds, but it is evident that the second aromatic ring has a further activating effect.

The Arrhenius activation energies vary from 15.6 kcal mol^{-1} for compound XIII to 20.4 kcal mol^{-1} for compound VIII. Of the structurally related compounds II, VIII, and XIV, the aldehyde and the nitro compound have comparable activation energies, while the ketone has a distinctly higher one. The difference in rate constants between II and VIII roughly corresponds to their difference in activation energies. Nitro compound XIV, on the other hand, reacts considerably faster, which shows, that for this and the other nitro compounds, other factors contribute to facilitate their reactions.

While the limits of error for the activation energies of all the compounds appearing in Table 2, with the exception of compound X, are thought to be reliable within about ±0.5 kcal mol^{-1}, the value for this latter compound has an uncertainty of at least twice this value. Rate constants given are normally based on mean values from at least two runs and in several cases from as many as six to ten runs. They were most often reproducible within ±5 %, but for compound X they were more scattered.

EXPERIMENTAL

Materials. Aldehydes I, II, and III were prepared as described in an earlier paper.[7] A stock solution of aldehyde V was prepared by hydrolysis of its diethyl acetal on an acid ion exchanger.[8] Aldehyde IV was prepared by the method of Späth and v. Szilágyi [9] (m.p. 87–88 °C; lit.[9] 88–89 °C). Aldehyde VI (m.p. 130–132 °C; lit.[10] 131–132 °C) and nitro compounds XIII (m.p. 175 °C; lit.[11] 174 °C), XIV (m.p. 154–156 °C; lit.[12] 149–150 °C), XV (m.p. 55–57 °C; lit.[12] 56 °C), XVI (m.p. 90–91 °C; lit.[13] 90–91 °C), XVII (b.p.$_{0.5}$ 60 °C; lit.[14] b.p.$_{0.5}$ 63 °C), and XVIII (b.p.$_{10}$ 105 °C; lit.[12] b.p.$_{10}$ 105 °C) were commercial chemicals. Of these compounds, XIII–XVI were recrystallized from water to reach the melting points listed, while the others were used as received. All ketones and the substituted fluorene were kindly put to the author's disposal by Dr. B. Wesslén. They had the following physical constants: VII (b.p.$_{15}$ 93–95 °C; lit.[15] b.p.$_{11}$ 89–90 °C), VIII (m.p. 62–63 °C; lit.[16] 63 °C), IX (m.p. 95–97 °C), lit.[17] 96–97 °C), X (m.p. 92–93 °C; lit.[18] 92–93 °C), XI (m.p. 93–94 °C; lit.[18] 93–94 °C), XII (m.p. 85–86 °C; lit.[19] 85–86 °C), and XIX (m.p. 144–145 °C; lit.[20] 145.5–146.5 °C). Acetylacetone of commercial grade was redistilled before use. NaOH stock solutions were prepared from Merck Titrisol ampoules by dilution with CO_2-free, distilled water and were never used for more than a week.

Apparatus. Vis-spectra and absorption measurements were made partly with a Beckman DK 2A istrument and partly with an Unicam SP 800 instrument. Quartz cells of 1.000 cm length were used throughout the investigation.

General procedure. Normally about 0.25 mmol of the compound to be examined was weighed, introduced into a 100 ml volumetric flask, and dissolved by addition of boiled, distilled water to the mark. Compound XII was first dissolved in 5 ml of methanol before dilution to the mark with water. For each run, 10 ml of the stock solution was taken, introduced into a 100 ml volumetric flask, diluted as much as possible, allowing for later addition of the catalyst, and thermostatted in a water bath to the desired temperature ±0.05 °C. From a

NaOH stock solution, kept in the same thermostatted bath, the proper amount of catalyst was taken and the reactions were started by introduction of the latter into the reaction mixture followed by dilution to the mark with thermostatted, distilled water. At regular time intervals, often every fifth minute but sometimes as frequently as every 60 seconds, 4.0 ml samples were taken. They were immediately neutralized by introduction into 10 ml volumetric flasks containing about 0.25 to 0.5 ml of H_2SO_4 of the molarity required. To the samples, now slightly acid, were added 5 ml of the aqueous reagent described by Nash,[4] containing 150 g of NH_4OAc, 3 ml of glacial acetic acid and 2 ml of redistilled acetylacetone per l. After dilution to 10.0 ml with water, the solutions were heated 5 to 8 min at 58 °C. The absorbances of the resulting yellow solutions were then measured at 412 nm. To ensure that the absorbances were not changed due to other Vis-absorbing species in the reaction mixtures, spectra were always run between 360 and 450 nm.

Catalyst concentration ranges. With compound I: 0.002 – 0.006 M; II: 0.005 – 0.015 M; III: 0.005 – 0.015 M; IV: 0.004 – 0.040 M; V: 0.010 M; VI: 0.050 M; VII: 0.010 M; VIII: 0.050 – 0.400 M; IX: 0.001 – 0.010 M; X: 0.0025 – 0.020 M; XI: 0.003 – 0.030 M; XII: 0.0002 – 0.0030 M; XIII – XVIII: 0.0003 M; XIX: 0.20 M.

Temperature ranges used are evident from Table 2.

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Pyrylium Salts. III. Properties and Reactions of 2,6-Dicarbonyl Derivatives

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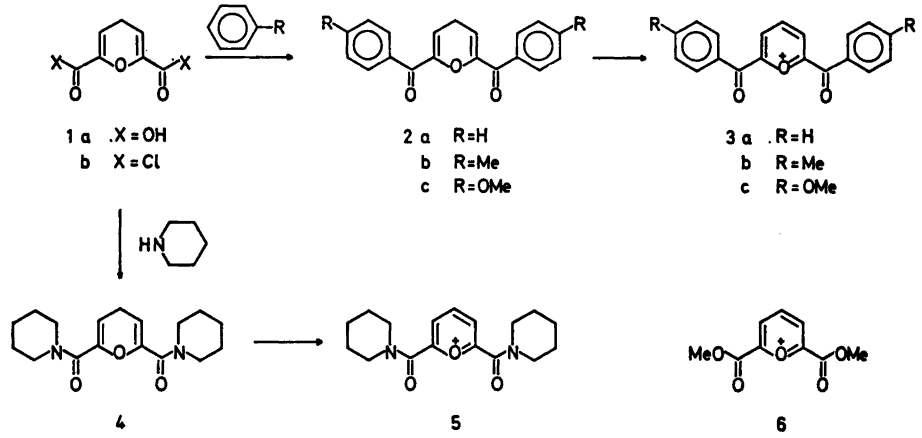
Some 2,6-dicarbonylpyrylium perchlorates have been prepared. These pyrylium ions are highly reactive and readily form 4*H*-adducts with enolisable carbonyl compounds and olefins. The adduct formation is reversible. Activated benzene derivatives are attacked. Alcohols can be dehydrogenated to the corresponding carbonyl derivatives. The equilibrium constant for pyrylium ion/4*H*-pyran pairs is related to the electron releasing properties of the substituents.

The pyrylium ion is less aromatic stabilized than the tropylium ion and without stabilising substituents the pyrylium ion is highly reactive. The initial products from reactions with nucleophiles or bases may readily be reacted further which makes pyrylium salts versatile synthetic intermediates.[2,3] We have recently reported a synthesis of the reactive 2,6-dimethoxycarbonylpyrylium perchlorate (*6*) and discussed some of its reactions.[1,4] The present work describes additional carbonyl derivatives (Scheme 1) which differ from the ester (*6*) in electronic activation. Self-condensation of the pyrylium ions is avoided by having carbonyl substituents without any activated hydrogen atom. Thus the amide (*5*) is formed from a secondary amine (*4*), and the α-carbon is part of an aromatic ring in (*3*).

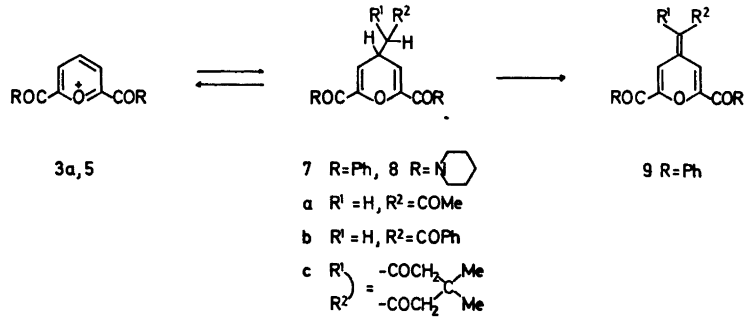


Scheme 1.

2,6-Dicarboxy-4*H*-pyran is readily available as synthetic starting material.[1,5] The amide (*4*) was prepared from its acid chloride. The latter reacted with benzene or benzene analogues under Friedel-Crafts conditions. Substituted benzenes gave exclusively (NMR) the *para*-acylated isomers (*2b,c*). The 4*H*-pyrans were then converted to the respective pyrylium salts

by reaction with triphenylmethyl perchlorate [6] in liquid SO_2. The NMR spectra in trifluoroacetic acid (TFA) have three low-field aromatic protons (AB_2) as expected for the pyrylium nucleus.[4] The shifts (Hγ 0.15 τ and Hβ 0.75 τ) for the benzoyl derivative (*3a*) are as found for the ester (*6*). The shifts are slightly affected in the expected manner by the phenyl *para*-substituents (*3b,c*). The corresponding protons in the amide (*5*) are at higher fields (0.4 and 1.25 τ), but at lower fields (1.0 and 1.5 τ), than in 2,6-diphenylpyrylium perchlorate which was prepared [2] for comparative reasons.

Variable anisotropy effects of the 2,6-substituents will affect the chemical shifts of the pyrylium protons. This may partly explain why these protons in the ester (*6*) and the benzoyl derivative (*3a*) have similar chemical shifts although the latter is the more reactive. Generally, however, the relative chemical shifts are indicative of the electron density in the pyrylium system and thereby informative about the relative reactivity as increased electron release will stabilize the pyrylium system.



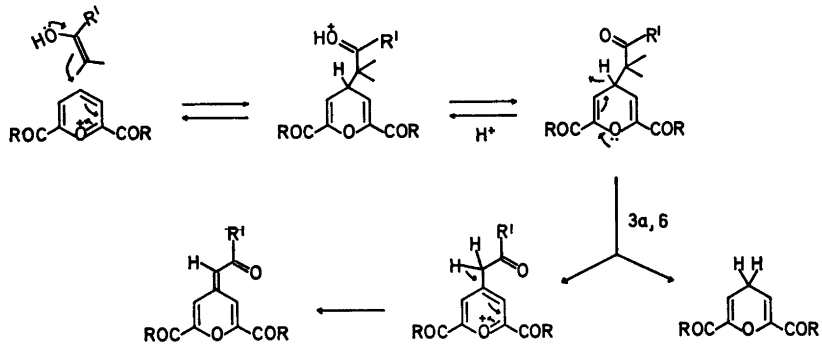
Scheme 2.

The 2,6-disubstituted pyrylium ions are attacked by a nucleophile at C-4 with formation of a 4*H*-pyran adduct. The NMR discussion above indicates that the 2,6-diphenylpyrylium ion should be the least reactive of the substances under consideration. For its reactions with activated methylene groups such as in acetonylacetone and benzoylacetone it requires the presence of a strong base.[7,8] The ester (*6*) however, attacks much less activated groups such as the methyl group in acetone or acetophenone in the cold without base addition.[1] A further comparison is available with the tropylium ion which reacts slowly with acetone on heating but readily attacks activated methylene groups in such compounds as malonic and acetoacetic ester.[9] The benzoyl derivatives (*3*) in preliminary experiments showed similar reactions and were no less reactive than the ester (*6*). Studies of the benzoyl series were therefore limited to the simplest derivative (*3a*). Both the pyrylium salts (*3a*) and (*5*) can be dissolved in acetone cooled below -10 °C without reaction. On allowing a solution of the benzoyl derivative (*3a*) in deuterioacetone (15 % concentration) to slowly warm up it was shown by NMR that a rapid reaction (1 – 2 min) ensued at about -10 °C. The amide (*5*) started to react at about -8 °C requiring about 10 min for completion of the reaction. The reaction appears autocatalytic through the perchloric acid liberated in the process. Acid catalysis was confirmed by an immediate reaction on dissolution of the pyrylium salt (*3a*) in deuterioacetone at -20 °C into which had been passed dry HCl. The function of the acid is presumably to promote enolisation of the ketonic reagent.[10]

Preparatively 4*H*-pyrans (*8a,b*) are available from the amide (*5*) by dissolution of the latter in excess acetone or acetophenone. Difficulties were experienced in preparative isolation of the benzoyl derivatives (*7a,b*) due to ease of oxidation to (*9*) and subsequent polymerisations. Such difficulties were not encountered in the reactions of the ester (*6*).[1] With equimolar quantities of the benzoyl derivative (*3a*) and the ketonic reagents in liquid SO_2 at -20 °C NMR showed only the presence of the 4-anhydro products (*9a,b*) and the parent 4*H*-pyran (*2a*). Similar hydride exchange processes have been

observed for the ester (*6*)[1] and in reactions of (*3a*) with anisole as discussed below. The carbamoyl group in (*5*), on the other hand, stabilizes the pyrylium nucleus sufficiently to prevent hydride transfer from the adducts (*8a,b*). With equimolar amounts of dimedone in SO_2 both (*3a*) and (*5*) form the 4*H*-adducts (*7c,8c*). The reduced tendency for dehydrogenation in this case may be associated with the increase in steric hindrance to the transfer of the hydride ion from C-4.

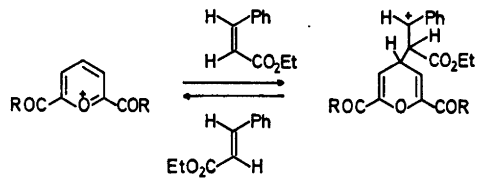
Adduct formation between the pyrylium ion



Scheme 3.

and the ketonic reagent is reversible (Scheme 3). Heating a solution of the phenacyl-4H-pyran (*8b*) in TFA at 70 °C produced some of the parent pyrylium ion (*5*). After perchloric acid addition only the pyrylium ion (*5*) was present.

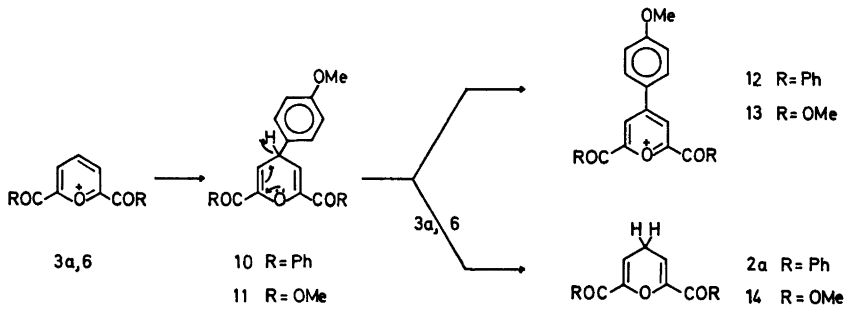
In the discussion of the reaction between the pyrylium ion and the ketonic reagents, it was postulated that the reaction proceeds *via* enolised ketone. As a consequence the pyrylium ion would also be expected to react with other suitably substituted double bonds. Thus with styrene an almost explosive, exothermic polymerisation occurred on addition of the benzoyl pyrylium salt (*3a*). The less reactive amide (*5*) required about 15 min in the cold before being consumed, while 2,6-diphenylpyrylium perchlorate had not caused any significant polymerisation after heating at 70 °C for 1 h. Ethyl cinnamate was polymerised by (*3a*) on heating. A carbonium type intermediate for this process is indicated in Scheme 4. Depending on reaction conditions the ionic intermediate can polymerise the olefin by a cationic mechanism, expel a proton to form an olefin, add the anion or any other nucleophile present, or reverse the process back to the precursor pyrylium ion. The ionic intermediate corresponds to the carbonium ion intermediate in E_1-elimination and should preferentially give the thermodynamically more stabilized olefin. Rapid and reversible formation of an ionic intermediate was strongly indicated by addition of (*3a*) or (*5*) to solutions of ethyl *cis*-cinnamate and its *p*-methoxy homologue in deuterioacetonitrile in the cold. The NMR spectra of the solutions showed only *trans*-cinnamic acid immediately after mixing the reagents.



Scheme 4.

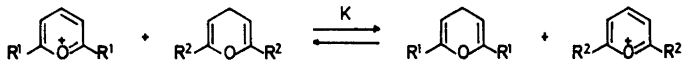
The behaviour towards aromatic nucleophiles was subsequently studied. 2,6-Diphenylpyrylium perchlorate is unreactive to simple phenyl derivatives but forms adducts at C-4 with the nucleophilic Grignard reagents.[7] Activated benzene such as phenol is attacked by the tropylium ion on heating.[11] Both the benzoyl derivative (*3a*) and the ester analogue (*6*), but not the amide (*5*), were found to react with anisole in liquid SO_2. None of the pyrylium salts would attack toluene. The 4*H*-adducts (*10*) and (*11*)[12] initially formed by *para*-substitution of anisole (NMR) reacted further to form the correspond-

ing 4-anisylpyrylium salts (*12,13*). The hydride abstraction by unsubstituted pyrylium ion from the 4*H*-adduct is a rapid process compared to anisole addition since the anisylpyrylium salts (*12,13*) and the respective 4*H*-pyrans are formed in almost equimolar amounts. Presumably the driving force for the redox process is the great increase in resonance stabilisation of the pyrylium nucleus brought about by the anisole radical.



Scheme 5.

A guide to relative redox potentials for the various cations investigated is available by equilibration studies. The triphenylmethyl cation is used both to generate the tropylium ion from cycloheptatriene and the pyrylium ions from the respective 4*H*-pyrans. Mixing equimolar amounts of cycloheptatriene and 2,6-dimethoxycarbonylpyrylium perchlorate in acetonitrile results in complete (NMR) hydride transfer from cycloheptatriene to the pyrylium nucleus.[1] Both the benzoyl derivative (*3a*) and the amide (*5*) were found to react with cycloheptatriene in the same manner. Within the pyrylium series equilibration studies between redox pairs were attempted in several solvents (15 % concentration) at 20 °C by the use of NMR. The hydride exchange reactions (Scheme 6) under these conditions required days (5 – 7) to reach equilibrium and reproducible quantitative measurements were difficult to achieve because of competing side reactions. The best results were obtainable in TFA.



Scheme 6.

By these crude measurements the equilibrium constants were of the order: $K_{3a/6} = 0.9$; $K_{5/3a} = 1.3$ and $K_{5/6} = 1.2$. The values show that the pyrylium ion with the more electron releasing substituents is thermodynamically the more stable. It is also evident that increase in the electron releasing properties of the substituents stabilizes the pyrylium nucleus to a greater extent than it stabilizes the 4*H*-pyran. As a consequence pyrylium ions without electron releasing substituents are most effective for hydride abstractions and can be used for dehydrogenation of other kinds of molecules. Thus the pyrylium salts (*3a,5,6*) on heating with molar amounts of diphenylcarbinol in nitromethane for half an hour fully converted the carbinol (chromatography) to benzophenone.

EXPERIMENTAL

All NMR spectra were determined on a 60 MHz Varian A-60A spectrometer.

Synthesis of 2,6-dibenzoyl-4H-pyrans (2). Anhydrous aluminium chloride (0.048 mol) was added gradually (15 – 30 min) at 10 – 25 °C to 2,6-dichlorocarbonyl-4*H*-pyran (0.024 mol) in benzene or its analogues (0.144 mol). Reaction temperature and time were varied individually as follows: (*2a*)(50 °C, 4 h); (*2b*)(25 °C, 6 h); (*2c*)(10 °C, 4 h). The reaction mixture was next

poured onto ice (100 g) and conc. HCl (5 ml), and the precipitated ketone collected by filtration.

2,6-Dibenzoyl-4H-pyran (2a). Yield 82 %, m.p. 189–190 °C (benzene). (Found: C 78.42; H 4.90. Calc. for $C_{19}H_{14}O_3$: C 78.61; H 4.86), τ (TFA) 3.85 (2 H, t, $H^{3,5}$), 6.65 (2 H, t, H^4, $J_{3,4}$ 4 Hz), 2.0–2.7 (10 H, m, 2 × Ph), ν_{max} (KBr) 1645 cm^{-1} (CO).

2,6-Di-p-methylbenzoyl-4H-pyran (2b). Yield 69 %, m.p. 162 °C (EtOH). (Found: C 78.97; H 5.69. Calc. for $C_{21}H_{18}O_3$: C 79.19; H 5.70), τ (TFA) 3.85 (2 H, t, $H^{3,5}$), 6.65 (2 H, t, H^4 $J_{3,4}$ 4 Hz), 2.1–2.9 (8 H, A_2B_2, 2 × Ph), 7.5 (6 H, 2 × Me) ν_{max} (KBr) 1645 cm^{-1} (CO).

2,6-Di-p-methoxybenzoyl-4H-pyran (2c). Yield 60 %, m.p. 160 °C (EtOH). (Found: C 71.88; H 5.26. Calc. for $C_{21}H_{18}O_5$: C 71.99; H 5.18), τ (TFA) 3.85 (2 H, t, $H^{3,5}$), 6.65 (2 H, t, H^4, $J_{3,4}$ 4 Hz), 1.8–2.9 (8 H, A_2B_2, 2 × Ph), 5.95 (6 H, 2 × OMe), ν_{max} (KBr) 1640, 1650 cm^{-1} (CO).

Synthesis of 2,6-di-N-piperidylcarbonyl-4H-pyran (4). A solution of piperidine (13.30 g, 0.14 mol) in dioxane (40 ml) was added over 1 h to a solution of 2,6-dichlorocarbonyl-4*H*-pyran (4.94 g, 0.024 mol) in dioxane (150 ml) and benzene (20 ml) in the cold. The solution was poured into water (200 ml) after 3 h, the solution acidified with HCl and the amide isolated by ether extraction. The combined ether extracts were evaporated and the residual yellow material crystallised from ethanol; m.p. 146–148 °C, yield 3.80 g (52 %). (Found: C 67.30; H 8.14. Calc. for $C_{17}H_{24}N_2O_3$: C 67.08; H 7.95). τ (TFA) 4.25 (2 H, t, $H^{3,5}$), 6.85 (2 H, t, H^4, $J_{3,4}$ 4 Hz), 5.9–6.4, 8.0–8.4, (20 H, m, 2 × piperidine ν_{max} (KBr) 1630 cm^{-1} (CO).

Synthesis of 2,6-dibenzoylpyrylium perchlorate (3). The 2,6-dibenzoyl-4*H*-pyran (0.015 mol) was added to liquid SO_2 (100 ml) at −30 °C and the mixture stirred while triphenylmethyl perchlorate (0.015 mol) was added gradually under anhydrous conditions. The reaction mixture was left at room temperature after completion of the addition until all the SO_2 had evaporated. The residue was treated with anhydrous ether (150 ml) and stirred vigorously for 10 min. If the oily residue after SO_2 evaporation did not crystallise, it was solidified at about −40 °C and the solid pulverised under ether. The treatment with anhydrous ether was generally repeated three times. The insoluble pyrylium salt thus obtained was pure enough for elemental analysis. It is highly hygroscopic and must be stored in dry atmosphere in an air-tight flask.

2,6-Dibenzoylpyrylium perchlorate (3a). Yield 87 %, m.p. 164–166 °C (decomp). (Found: C 58.74; H 3.60. Calc. for $C_{19}H_{13}O_3.ClO_4$: C 58.70; H 3.37). τ (TFA) 0.75 (2 H, AB_2, $H^{3,5}$), 0.15 (H, H^4, $J_{3,4}$ 8 Hz), 1.8–2.8 (10 H, 2 × Ph).

2,6-Di-p-methylbenzoylpyrylium perchlorate (3b). Yield 82 %, m.p. 150–152 °C (decomp.). (Found: C 60.25; H 4.35. Calc. for $C_{21}H_{17}O_3.ClO_4$: C 60.51; H 4.11). τ (TFA) 0.95 (2H, AB_2, $H^{3,5}$), 0.25 (H, H^4, $J_{3,4}$ 8 Hz), 1.8–2.8 (8 H, A_2B_2, 2 × Ph), 7.5 (6 H, 2 × Me).

2,6-Di-p-methoxybenzoylpyrylium perchlorate (3c). Yield 79 %, m.p. 156–158 °C (decomp.). (Found: C 55.98; H 4.01. Calc. for $C_{21}H_{17}O_5.ClO_4$: C 56.20; H 3.82). τ (TFA) 0.85 (2 H, AB_2, $H^{3,5}$), 0.20 (H, H^4, $J_{3,4}$ 8Hz), 1.6–3.0 (8 H, A_2B_2, 2 × Ph), 5.95 (6 H, 2 × OMe).

2,6-Di-N-piperidylcarbonylpyrylium perchlorate (5). The pyrylium salt was prepared from 2,6-di-*N*-piperidylcarbonyl-4*H*-pyran in the same way as the ketones above. The yield was 85 %, m.p. 150–152 °C (decomp.). (Found: C 50.43; H 5.74. Calc. for $C_{17}H_{23}N_2O_3.ClO_4$: C 50.69; H 5.76) τ (TFA) 1.25 (2 H, AB_2, $H^{3,5}$), 0.4 (H, H^4, $J_{3,4}$ 8 Hz), 5.6–6.5, 7.9–8.4 (20 H, 2 × piperidine).

4-Acetylmethyl-2,6-di-N-piperidylcarbonyl-4H-pyran (8a). 2,6-Di-*N*-piperidylcarbonylpyrylium perchlorate (2.01 g, 0.005 mol) was added to acetone (25 ml) with stirring and the solution left in the cold for 1 h. Evaporation left an oily material which on trituration with ether gave a crystalline solid, m.p. 100–102 °C; yield 1.30 g (72 %). (Found: C 66.49; H 7.80. Calc. for $C_{20}H_{28}N_2O_4$: C 66.64; H 7.83) τ ($CDCl_3$) 4.45 (2 H, d, $H^{3,5}$, $J_{3,4}$ 3.5 Hz), 7.1 (2 H, d, CH_2CO, $J_{CH_2,4}$ 6 Hz), 7.85 (3 H, CH_3CO), 6.0–6.6, 8.0–8.5 (20 H, 2 × piperidine); H^4 is superimposed on the piperidine protons in the 6.0–6.6 region; ν_{max} (KBr) 1630 (CO–N), 1710 cm^{-1} (–CO–C).

4-Benzoylmethyl-2,6-di-N-piperidylcarbonyl-4H-pyran (8b) was prepared as above from acetophenone in 78 % yield, m.p. 154–156 °C. (Found: C 70.80; H 7.02. Calc. for $C_{25}H_{30}N_2O_4$: C 71.07; H 7.16), τ (TFA) 4.5 (2 H, d, $H^{3,5}$, *J* 3.5 Hz), 6.85 (2 H, d, CH_2CO, $J_{CH_2,4}$ 6 Hz), 2.2–3.0 (5 H, Ph), 6.2–6.8, 8.3–8.6 (20 H, 2 × piperidine); H^4 is superimposed on the piperidine protons in the 6.2–6.8 region; ν_{max} (KBr) 1630 (CO–N), 1680 cm^{-1} (CO–C).

4-(5,5-Dimethyl-1,3-dione-2-cyclohexyl)-2,6-di-N-piperidylcarbonyl-4H-pyran (8c). 2,6-Di-*N*-piperidylcarbonylpyrylium perchlorate (2.01 g, 0.005 mol) was dissolved in liquid SO_2 (25 ml) at −30 °C and dimedone (0.70 g, 0.005 mol) added with stirring. The SO_2 was then allowed to evaporate and the residual oily material crystallised by trituration with ether; yield 1.57 g (71 %), m.p. 140–142 °C. (Found: C 67.92; H 7.92. Calc. for $C_{35}H_{34}N_2O_5$: C 67.85; H 7.74), τ (CD_3CN) 4.75 (2 H, d, $H^{3,5}$, $J_{3,4}$ 4 Hz), 5.5 (H, t, H^4), 7.4–7.6 (4 H, CH_2CO), 8.9–9.0 (6 H, 2 × Me), 6.2–6.8, 8.2–8.6 (20 H, 2 × piperidine). These values are for the enolic tautomer (75 %); non-enolised molecules differ in that the H^4 proton and the COCHCO proton seem to be in the 6.2–6.6 region; ν_{max} (KBr) 1630 (CO–N), 1600 cm^{-1} (CO–C=).

2,6-Dibenzoyl-4-(5,5-dimethyl-1,3-dione-2-cyclohexyl)-4H-pyran (7c) was prepared as above from 2,6-dibenzoylpyrylium perchlorate and

dimedone in 78 % yield, m.p. 122–124 °C. (Found: C 75.51; H 5.42. Calc. for $C_{27}H_{24}O_5$: C 75.68; H 5.65), τ (CD_3CN) 4.2 (2 H, d, $H^{3,5}$, $J_{3,4}$ 4 Hz), 5.2 (H, t, H^4), 7.2–7.4 (4 H, 2 CH_2CO), 8.8–9.0 (6 H, 2 Me), 2.0–2.7 (10 H, 2 × Ph); these values are for the enolic tautomer (85 %); ν_{max} (KBr) 1645 (CO-aryl), 1590 cm^{-1} (CO–C=).

*2,6-Dibenzoyl-4-*p*-methoxyphenylpyrylium perchlorate (12).* 2,6-Dibenzoylpyrylium perchlorate (1.94 g, 0.005 mol) was dissolved in liquid SO_2 (25 ml) at −30 °C and anisole (0.66 g, 0.006 mol) added gradually with stirring under anhydrous conditions. The solution was then left at room temperature until all the SO_2 had evaporated. The residual oily material was solidified on treatment with anhydrous ether and the ether decanted. The ether treatment was repeated three times before the residue was extracted twice with anhydrous dioxane to remove 2,6-dibenzoyl-4*H*-pyran. The remaining pyrylium salt (1.05 g) corresponds to 82 % of theoretical yield of the pyrylium salt; m.p. 155–156 °C (decomp.). (Found: C 62.93; H 4.01. Calc. for $C_{26}H_{19}O_4.ClO_4$: C 63.10; H 3.87), τ (TFA) 0.95 (2 H, $H^{3,5}$), 5.8 (3 H, OMe), 1.3–2.8 (4 H, A_2B_2, subst. Ph), 1.3–2.8 (10 H, 2 × Ph).

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N-Quaternary Compounds. XXXVI.[1] Nitro- and Diazonium-group Activation in Reactions of Pyridinium Derivatives

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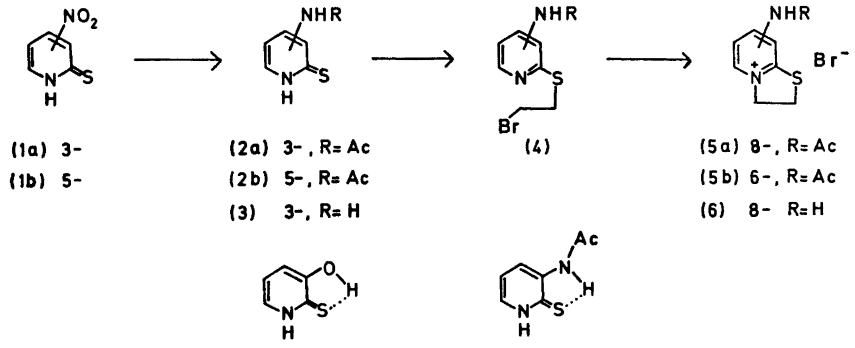
6-(8)-Nitrodihydrothiazolo[3,2-*a*]pyridinium salts as pseudo-bases are readily brominated. Diazotisation of the 8-amino derivative is followed by re-arrangement of the diazonium salt into dihydrothiazolo[2,3-*e*]*v*-triazoles.

Simple pyridinium derivatives are electron deficient and therefore not reactive towards electrophiles. The pyridinium ring in dihydro thiazolo[3,2-*a*]pyridinium-8-oxide, however, is readily substituted by electrophiles because of the activation from the oxide function.[2] In the present work we have investigated effects of other substituents on the reactivity of the pyridinium ring. Syntheses of amino derivatives are shown in Scheme 1. The acetamides (*2a*) and (*2b*) required for the syntheses were prepared by dithionite reduction [3] of the corresponding nitropyrid-2-thiones [4] and the amides isolated after acetylation. The 3-amine (*3*) was prepared from the amide (*2a*) by acid hydrolysis.

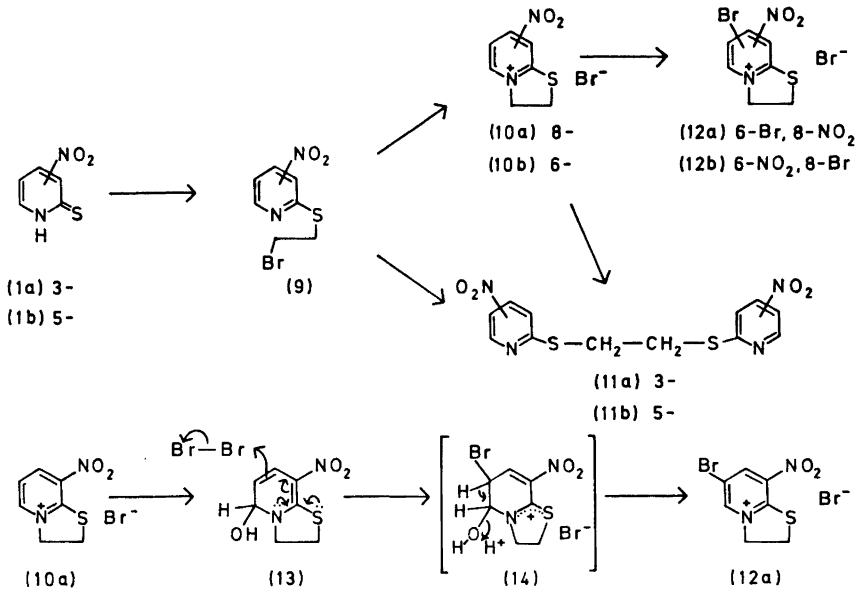
Pyrid-2-thiones are initially attacked on the sulphur atom by electrophilic carbon.[5,6] A 3-ethoxy group increases the reaction rate by virtue of its electron releasing properties while a 3-hydroxy group has the opposite effect. The latter has been attributed to intramolecular hydrogen bonding (*7*).[6] In the present work it was noticeable that the 3-acetamide was less reactive than the 5-isomer as expected on the basis of steric interference and hydrogen bonding (*8*).

Generally, in aminopyridines it is the pyridine nitrogen atom which is the more basic and nucleophilic. Accordingly, cyclisation of the *S*-alkylated intermediate (*4*) should occur over the pyridine nitrogen atom to the dihydrothiazolo[3,2-*a*]pyridinium derivatives (*5a-6*) even in the case of the 3-amine (*3*). This was confirmed by acid hydrolysis of the bicyclic product from (*2a*) which gave the condensation product from (*3*). The latter product contains a diazotisable amino group as discussed below.

S-Alkylation is the overall rate determining step in the reaction sequence with very rapid cyclisation of the *S*-alkylated intermediate



Scheme 1. (7) (8)

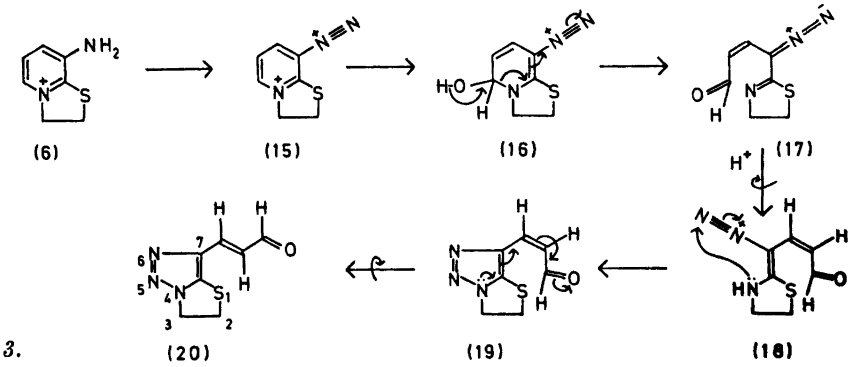


Scheme 2.

since the latter was not observed in the reaction. In the reaction of the nitro analogues (*1*), however, a dipyridyl thioether (*11*) was isolated. The nitro group serves to decrease the nucleophilicities of both the sulphur and the pyridine nitrogen atoms. The reduced cyclisation rate could account for formation of (*11*). The bicyclic product (*10*), however, is reactive towards nucleophiles and (*11*) could equally well have been formed by a nucleophilic ring-opening of (*10*). The latter path was demonstrated by addition of the parent thione (*1*) as sodium salt to the pyridinium salt (*10*) which resulted in formation of (*11*).

Dihydrothiazolo[3,2-*a*]pyridinium derivatives with a hydroxy group in 8-position are very readily substituted by electrophiles and even undergo diazo-coupling reactions.[2] The electron releasing properties of the amino group and the acetamido group, however, were not sufficient to effect direct bromination in (*5*) and (*6*) under mild conditions.[2] Contrary to deductive expectations, however, the nitro-substituted pyridinium derivatives (*10*) are readily brominated in aqueous methanol. This observation is understandable in terms of pseudo-base formation as illustrated in Scheme 2 for the 8-nitro derivative (*13*). As distinct from the amino salts (*5,6*) the electron withdrawing effect by the nitro group increases the electron deficiency of the pyridinium ring to the extent that hydroxyl addition occurs. Pseudo-base formation in pyridinium systems has been found to occur preferentially in an α-position to the heteroatom.[7] The pseudo-base thus formed can be regarded both as an *N*-vinyl and *S*-vinyl derivative activated for electrophilic substitution in 6- or 8-position, respectively (Scheme 2). The mechanistically postulated substitution pattern is further supported by the NMR spectra which show two pyridinium protons with chemical shifts less than 1 τ and coupling constants $J = 1.5 - 2.0$ Hz as expected for 1,3-coupling.

In order to prove the dihydrothiazolo[3,2-*a*]-pyridinium structure with a primary amino group, after the reaction of 3-amino-pyrid-2-thione (*3*), the condensation product was diazotised using isoamyl nitrite in aqueous acetic acid. The diazonium group is interesting since it is by far the most strongly electron-attracting group known.[8] It is an electrophilic reagent which can attack a vicinal thioether group with 1,2,3-thiadiazole formation.[9] Any appreciable product formation with opening of the dihydrothiazole ring, however, was not



Scheme 3.

observed for (*6*). Nor was the diazotised pyridinium molecule sufficiently stabilised from the thiother group for heterolytic reactions with water in which N_2 is the leaving group. Instead the highly electron deficient pyridinium system adds a hydroxyl ion similar to the observation for the nitro derivatives (*10*) discussed above. The pseudo-base then re-arranges to two main products identified as the v-thiazoles (*19*) and (*20*). The *trans*-acraldehyde (*20*) is the more stable and is readily formed from the *cis*-isomer by isomerisation about the double bond. The formyl group in IR absorbs at 1670 cm^{-1}. Both isomers have UV absorption maxima at 314 and 239 nm. High resolution mass spectrometry shows molecular ion at *m/e* 181 with the presented elemental compositions. The fragmentation is characterised by $[M-N_2]$ being the major intermediate for lower mass fragments. The coupling constant between the α-(3.8 τ) and the β-proton (2.2 τ) is $J=16$ Hz for the *trans*-isomer. Between the α-proton and the formyl proton (0.4 τ) the coupling constant is $J=8$ Hz. The vicinal vinyl coupling between the α- (4.0 τ) and the β-proton (2.7 τ) in the *cis*-isomer is $J=12$ Hz, the coupling between the α-proton and the formyl proton being as in the *trans*-isomer. The chemical shift for the formyl proton in the *cis*-isomer, however, has been moved downfield to -0.7 τ. A similar observation for *cis*-3-(v-triazolo[1,5-*a*]pyridyl)acraldehydes relative to their *trans*-isomers has been attributed to the vicinal nitrogen lone-pair in the triazole.[10] The isomer shifts in the present case are of the same order which could mean that the lone pairs on the sp^3-hybridised sulphur atom have little influence in this respect.

X-Ray analysis of the *trans*-isomer (*20*) shows that the acraldehyde side-chain has a conformational preference.[11] It lies with its substituents in the ring-plane and has the α-hydrogen atom close to the sulphur atom (*20*). The triazole ring is planar. The dihydrothiazole ring has the envelope conformation with the 1- and 3-atoms nearly coplanar with the triazole ring and the C-2 atom 0.4 Å out of plane.[11] The envelope conformation corresponds to that found in dihydrothiazolo-pyridines.[12]

The crude product after the diazotisation reaction is an almost equimolar mixture of the *cis*- and *trans*-isomers. In weak acid solution the *cis*-isomer is converted to the *trans*-isomer. Warming a solution of the 1:1 isomer mixture in deuterium oxide also led to the *trans*-isomer. No deuterium was incorporated in the molecule. Isomerisation therefore occurs without any exchange of vinyl protons.

The re-arrangement leading to the *v*-triazoles is explained by initial hydroxyl addition to the carbon next to the hetero-atom followed by re-arrangement as indicated in Scheme 3. The reaction appears general in that simple 1-alkyl- and 1-aryl-3-aminopyridinium salts are convertible into 3-(1-alkyl or aryl-4-v-triazolyl)-acraldehydes.[13,14] Similar re-arrangements to v-triazoles have also been observed from a number of 1-aminoquinolizinium salts in aqueous nitrous acid.[10]

No attempts have been made to study the intermediate diazonium salts (Scheme 3). In simple 3-aminopyridinium salts, however, a transient and quickly disappearing diazo-coupling ability was observed supporting the formulation of an initial diazonium salt.[13,14]

The cyclisation corresponds to the well established formation of benzotriazoles by diazotisation of *o*-phenylenediamine.[15]

The exocyclic double bond in the initially formed triazole would be expected to have retained the *cis*-configuration. The presence in the reaction product of almost equal amounts of the *cis*- and *trans*-isomers is presumably due to the ease of isomerisation of the *cis*-isomer under the experimental conditions used.

EXPERIMENTAL

All NMR spectra were determined on a 60 MHz Varian A-60A spectrometer.

3-Acetamidopyrid-2-thione (2a) was prepared by sodium dithionite reduction of 3-nitropyrid-2-thione [4] followed by acetylation as described for 5-acetamidopyrid-2-thione (*2b*).[3] Yield 38 %, m.p. 196–197 °C (H_2O). (Found: N 16.85; S 18.94. Calc. for $C_7H_8N_2OS$: N 16.65; S 19.06).

8-Acetamidodihydrothiazolo[3,2-a]pyridinium bromide (5a). 3-Acetamidopyrid-2-thione (0.85 g, 0.005 mol), potassium carbonate (0.35 g, 0.0025 mol) and 1,2-dibromoethane (0.94 g, 0.005 mol) were stirred together in anhydrous dimethylformamide (30 ml) in the cold. Chromatography showed the reaction to be over after 3 h. The reaction mixture was then evaporated at reduced pressure and the residue dissolved by heating in dilute methanol. The title compound was precipitated on cooling; yield 38 %, m.p. 269–270 °C (decomp.) (dilute MeOH). (Found: C 38.85; H 3.98; N 10.36. Calc. for $C_9H_{11}BrN_2OS$: C 39.28; H 4.03; N 10.18), τ (TFA) 6.0 (2 H-2), 4.6 (2 H-3), 1.3 (H-5), 2.3 (H-6), 1.3 (H-7), 7.5 ($COCH_3$); $J_{5,6}$ 6.0, $J_{6,7}$ 8.0, $J_{5,7}$ 2.0 Hz.

6-Acetamidodihydrothiazolo[3,2-a]pyridinium bromide (5b) was prepared as (*5a*) in 60 % yield, m.p. 320–323 °C (decomp.) (dil. MeOH). Reaction time 30 min. (Found: S 11.33; Br 29.24. Calc. for $C_9H_{11}BrN_2OS$: S 11.65; Br 29.04). τ (TFA) 6.1 (2 H-2), 4.7 (2 H-3), 0.5 (H-5), 1.6 (H-7), 2.2 (H-8), 7.6 ($COCH_3$); $J_{7,8}$ 9.0, $J_{5,7}$ 2.0 Hz.

8-Nitrodihydrothiazolo[3,2-a]pyridinium bromide (10a) was synthesised as (*5a*). The reaction time was 1 day. The solid residue after evaporation of the reaction mixture was extracted with hot dilute methanol (4:1) and the yellow, insoluble material crystallised from acetone. The product thus obtained in 15 % yield, m.p. 148–149 °C, has been identified as *1,2-di-(3-nitro-2-pyridylthio)ethane* (*11a*); molecular ion composition by high resolution MS: $C_{12}H_{10}N_4O_4S$; τ (TFA) 6.1 (4 H-2 CH_2), 0.8 (H-4), 2.0 (H-5), 0.8 (H-6).

The title compound was precipitated from the dilute ethanol solution on cooling; yield 38 %, m.p. 300 °C (decomp.). (Found: C 32.26; H 2.68; S 11.95. Calc. for $C_7H_7BrN_2O_2S$: C 31.95; H 2.68, S 12.19), τ (TFA) 6.1 (2 H-2), 4.5 (2 H-3), 0.8 (H-5), 2.1 (H-6), 0.8 (H-7); $J_{5,6}$ 6.0, $J_{6,7}$ 8.0, $J_{5,7} < 1$ Hz.

6-Nitrodihydrothiazolo[3,2-a]pyridinium bromide (10b) was prepared as (*10a*). The reaction time was 4 h. The yield of co-formed *1,2-di-(5-nitro-2-pyridylthio)ethane* (*11b*) was 18 %, m.p. 153–156 °C (acetone); molecular ion composition by high resolution MS: $C_{12}H_{10}N_4O_4S_2$; τ (TFA) 6.4 (4 H-2 CH_2), 1.9 (H-3), 1.0 (H-4), 0.5 (H-6).

The yield of the title compound (*10b*) was 60 %; no melting point due to gradual decomposition on heating (dil. MeOH). (Found: C 31.93; H 2.66; S 12.17. Calc. for $C_7H_7BrN_2O_2S$: C 31.95; H 2.68; S 12.19), τ (TFA) 5.8 (2 H-2), 4.4 (2 H-3), 0.3 (H-5), 1.0 (H-7), 1.9 (H-8); $J_{7,8}$ 9.0, $J_{5,7}$ 2.0 Hz.

8-Aminodihydrothiazolo[3,2-a]pyridinium bromide (6). (a) 3-Acetamidopyrid-2-thione (4.8 g, 0.04 mol) was dissolved in a solution of conc. HBr (5 ml) and ethanol (50 ml) and the resultant solution heated under reflux for 4 h. The precipitated 3-aminopyrid-2-thione (*3*) as hydrobromide was filtered off from the cold reaction mixture and reacted further with 1,2-dibromoethane in DMF as described for (*2a*) using an additional equivalent of potassium carbonate for neutralisation of the hydrobromide. The reaction time was 15 min; yield 59 %, m.p. 155–157 °C (dil. EtOH). (Found: C 33.48; H 4.43; N 11.20. Calc. for $C_7H_9BrN_2S.H_2O$: C 33.48; H 4.41; N 11.15), τ (TFA) 5.9 (2 H-2), 4.6 (2 H-3), 1.4 (H-5), 2.3 (H-6), 1.6 (H-7); $J_{5,6}$ 6.0, $J_{6,7}$ 8.0, $J_{5,7} < 1$ Hz.

(b) 8-Acetamidodihydrothiazolo[3,2-*a*]pyridinium bromide (*5a*) (9.0 g, 0.033 mol) was dissolved in water (100 ml) and Amberlite IRA-400 (OH^-) ion exchanger added. The ion exchanger was filtered off after stirring for 3 h, conc. HCl (100 ml) added to the filtrate and the solution heated under reflux for 1 h. Evaporation gave the amine (*6*) as chloride in 91 % yield (5.6 g).

6-Bromo-8-nitrodihydrothiazolo[3,2-a]pyridinium bromide (12a). Bromine (0.32 g, 0.002 mol) in methanol (2 ml) was added dropwise to an ice-cold solution of (*10a*) (0.52 g, 0.002 mol) in MeOH:H_2O (4:1) (10 ml). The bromine was decolourised at once. The precipitated bromo compound was recrystallised from methanol; yield 0.45 g (66 %), m.p. 303 °C (decomp.). (Found: C 24.61; H 2.06. Calc. for $C_7H_6Br_2N_2O_2S$: C 24.58; H 1.77), τ (TFA) 6.0 (2 H-2), 4.4 (2 H-3), 0.8 (H-5), 0.9 (H-7); $J_{5,7}$ 2.0 Hz.

8-Bromo-6-nitrodihydrothiazolo[3,2-a]pyridinium bromide (12b) was prepared as (*12b*) in 80 % yield, m.p. 300 °C (decomp.) (MeOH). (Found: C 24.72; H 1.85; N 8.31; Calc. for $C_7H_6Br_2N_2O_2S$: C 24.58; H 1.77, N 8.19), τ (TFA) 5.8 (2 H-2), 4.3 (2 H-3), 0.3 (H-5), 0.9 (H-7); $J_{5,7}$ 1.5 Hz.

trans-*3-(7-Dihydrothiazolo[2,3-*e]v-*triazolyl)-acraldehyde. (20).* 8-Aminodihydrothiazolo[3,2-*a*]pyridinium chloride (5.60 g, 0.03 mol) in water solution (125 ml) was stirred with Amberlite IRA-400 (OH^-) ion exchanger for 2 h. The ion exchanger was then removed by filtration and acetic acid (125 ml) added to the filtrate. Isoamyl nitrite (4.00 g, 0.034 mol) dissolved in acetic acid (20 ml) was added dropwise to the ice-cold solution over 3 h. At the end of this period the solution was evaporated almost to dryness at reduced pressure, a little water added, the pH adjusted to 7 with sodium carbonate and the solution extracted several times with ethyl acetate. The dried ethyl acetate extracts were evaporated and the residual material crystallised from water or chloroform; yield 1.4 g (25 %), m.p. 200 °C (Found: C 46.40; H 4.12; N 23.40. Calc. for $C_7H_7N_3SO$: C 46.41; H 3.90; N 23.20), τ (DMSO-d_6) 5.8 (2 H-2), 5.3 (2 H-3), 3.8 (H-2, quartet, $J_{2,3}$ 16 Hz, $J_{2,CHO}$ 8 Hz), 2.2 (H-3, doublet), 0.4 (H-CHO, doublet), λ_{max} (MeOH) 239, 314 nm (log *E* 4.03 and 4.11); *m/e* 181 (M^+) 58 %, [M−N_2] 22, [M−N_2H] 18, [M−N_2CO] 10, [M−$N_2C_2H_4$] 23, [M−N_2CHO] 20, and *m/e* 60 [C_2H_4S] 100.

The data refer to the *trans*-isomer. NMR of the crude reaction product showed this to contain the *cis-(19)* and *trans-(20)* isomers in the ratio 1:1. Warming the crude product for a short time in acetic acid led to complete isomerisation to the *trans*-form *(20)*.

NMR of the *cis*-isomer *(19)*, as it appears in the crude reaction mixture, shows the methylene protons as in *(20)* with the following shifts for the side-chain protons: τ (DMSO−d_6) 4.0 (H-2, quartet, $J_{2,3}$ 12 Hz, $J_{2,CHO}$ 8 Hz), 2.7 (H-3, doublet), −0.7 (H−CHO).

In an isomerisation experiment the crude mixture of the isomers (1:1) (45 mg) was heated in deuterium oxide (10 ml) for 2 ½ h. The NMR spectrum of the quantitatively recovered product showed only the *trans*-isomer present with no deuterium incorporation.

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Tobacco Chemistry. 23. Structures and Syntheses of Four New Norisoprenoid Furans from Greek *Nicotiana tabacum* L.

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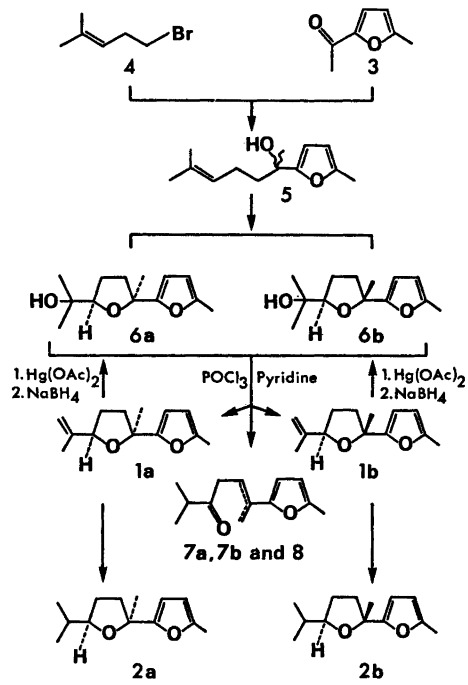
The structures of *cis* and *trans* (±)-2-methyl-5-(2-methyl-5-isopropenyltetrahydro-2-furyl)-furan, *1a* and *1b*, and their dihydro derivatives, (±)-2-methyl-5-(2-methyl-5-isopropyltetrahydro-2-furyl)furan, *2a* and *2b*, isolated from Greek tobacco were elucidated from spectral data, and subsequently confirmed by total syntheses. The *cis-trans* assignments were based upon the observation of an $Eu(DPM)_3$ induced upfield shift of the furan methyl signal in the NMR-spectrum of the *cis*-isomer of the synthetic intermediate (±)-2-[5-methyl-5-(5-methyl-2-furyl)tetrahydro-2-furyl]-2-propanol, *6a*.

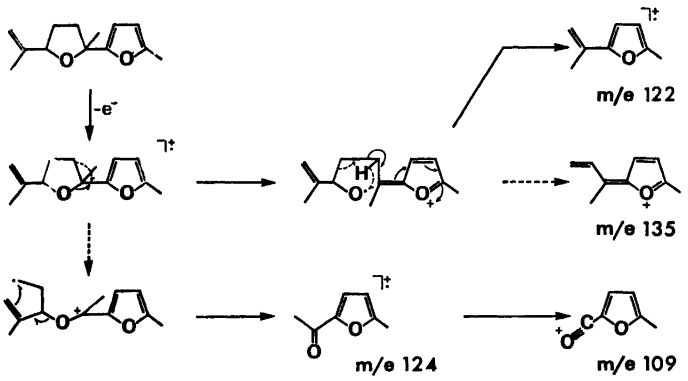
Recent research into tobacco flavour has suggested that a large number of the tobacco constituents are degradation products of terpenoid compounds.[1-3] The present communication describes the structural elucidation and syntheses of further new tobacco constituents possessing carbon skeletons derivable from those of aliphatic terpenoids.

The mass spectrum of the first isolate (from fraction C9) [4] displayed a molecular ion at *m/e* 206 corresponding to the elemental composition $C_{13}H_{18}O_2$ or $C_{14}H_{22}O$. The former alternative was favoured since signals in the NMR spectrum indicated the presence of two oxygen atoms incorporated in a furan ring and a cyclic ether. A 2-methylfur-5-yl moiety was apparent from NMR signals at δ 5.84 (1 H, doublet of quartets, J 1.0 and 3.0 Hz), δ 6.06 (1 H, doublet of quartets, J 0.4 and 3.0 Hz) and δ 2.24 (3 H, doublet of doublets, J 0.4 and 1.0 Hz).[5] The long-range spin-spin couplings between the methyl group and the furyl protons were established by spin decoupling experiments. Absence of further couplings to the proton in position 4 suggested that a fully substituted



Scheme 1.

carbon atom was attached to position 5 of the methylfuryl moiety. The second oxygen atom represented part of a cyclic ether grouping in which the two oxygenated carbon atoms carried a methyl group (δ 1.57, singlet) and an allylic proton (δ 4.45, triplet with further splittings) respectively. Double resonance experiments demonstrated that two olefinic protons (δ 4.78, 5.02) and a vinylic methyl group (δ 1.72) were weakly, mutually coupled (J *ca.* 1 Hz) and could



Scheme 2.

be ascribed to an isopropenyl group, $CH_2=C-(CH_3)-$. The remaining atoms, two methylene groups, were responsible for multiplets in the δ 1.2 – 1.8 region and were readily accomodated in the tetrahydrofuran ring of the postulated structure that could be advanced: 2-methyl-5-(2-methyl-5-isopropenyltetrahydro-2-furyl)furan. Methyl resonances of low intensity at δ 1.55 and δ 1.68 indicated that the isolate was a mixture of diastereomers (ratio 3:7). Thus both the *cis* and the *trans* tetrahydrofuran compounds (*1a* and *1b*) were present in the tobacco.

Subsequently the isomer *1a*, free of the less polar isomer (*1b*) was isolated from a more polar fraction of the tobacco by preparative gas chromatography. Its elemental composition was confirmed by accurate mass determination.

The mass spectra of the two isomers revealed prominent ions at *m/e* 109, 122, 124 and 135 for which fragments, the genesis shown in Scheme 2 may be invoked. High resolution mass spectrometry manifested the elemental compositions of the structures suggested in Scheme 2.

The mass spectrum of the second isolate, obtained by preparative gas chromatography from the same fraction [4] as *1a* and *1b*, exhibited a molecular ion at *m/e* 208, corresponding to $C_{13}H_{20}O_2$, and was similar to the mass spectra of *1a* and *1b* (abundant ions at *m/e* 109, 122, 124, and 135) indicating a structural relationship. The 2-methylfur-5-yl moiety was apparent from IR absorption at 782 cm^{-1} and signals in the NMR spectrum [5] at δ 2.26 (3 H, broad singlet), δ 5.82 (1 H, doublet with further splittings, J 3.0 Hz) and δ 6.02 (1 H, doublet, J 3.0 Hz). The methyl substituted tetrahydrofuran ring was indicated by a three-proton singlet at δ 1.54 and a one-proton multiplet at δ *ca.* 3.74. The chemical shift and multiplicity of the latter signal suggested that the proton on the oxygenated carbon was non-allylic, and that this isolate was also a diastereomeric mixture. The observed lack of clear resolution of the long-range spin couplings might be explained by small differences in chemical shifts of these protons. The diastereomeric nature (ratio 2:3) of this isolate was further supported by the presence of a seven line multiplet in the δ 0.8 – 1.0 region (6 H) which could be ascribed to two isopropyl groups. The two diastereomers could be assigned the structures *cis* and *trans* 2-methyl-5-(2-methyl-5-isopropyltetrahydro-2-furyl) furan (*2a* and *2b*).

The proposed structures of *1a*, *1b*, *2a*, and *2b* were confirmed by synthesis as outlined in Scheme 1, which also provided a means of determining their relative stereochemistry. Thus the alcohol *5*, conveniently prepared by reacting 4-methyl-3-penten-1-ylmagnesium bromide with 2-methyl-5-acetylfuran (*3*) in a Grignard reaction, was converted to a 1:1 mixture of the diastereomeric tetrahydrofuran derivatives *6a* and *6b* in one step by epoxidation followed by spontaneous cyclization.[7] Dehydration was effected by phosphorus oxychloride in pyridine to yield, after chromatography on silica, the desired pure olefins *1a* and *1b*, which exhibited spectral data and gas chromatographic behaviour (co-injection on a capillary column) indistinguishable from those of the natural mixture.

The last step also yielded three by-products evidently arising *via* rearrangement of an enol ether, namely the isomeric unsaturated ketones 2-methyl-6-(5-methyl-2-furyl)-6-hepten-3-one, (*8*), and 5*E* and 5*Z* isomers of 2-methyl-6-(5-methyl-2-furyl)-5-hepten-3-one, (*7a* and *7b*).

The pure dihydro derivatives *2a* and *2b* were prepared from *1a* and *1b*, respectively, by catalytic hydrogenation of the isopropenyl groups. Synthetic *2a* and *2b* exhibited spectral data identical to those of the natural products. The seven line multiplet in the NMR-spectrum, ascribed to the isopropyl signals, could be reconstructed by superposition of the spectra of synthetic *2a* and *2b*. Furthermore, synthetic *2a* and *2b* had different GC retention times but when coinjected separately with the natural diastereomeric mixture each, in turn, enhanced the signal for the respective natural isomer.

The relative stereochemistry of the tetrahydrofuran compounds was determined by measuring the rel. lanthanide induced chemical shifts [2,14] for the alcohols *6a* and *6b*. Attempts to separate the synthetic intermediate mixture of *6a* and *6b* were only moderately successful, even by preparative gas chromatography, and these compounds were therefore prepared by hydration [8] of the respective olefins *1a* and *1b*, which could be separated easily by liquid chromatography.

Addition of the shift reagent $Eu(DPM)_3$ to the alcohol *6* obtained from the more polar olefin *1a*, produced an upfield shift of the furan methyl signal, while for the other isomer, this signal showed the usual downfield shift. Upfield shifts are predicted for O–Eu–H angles between 54.7 and 125.3° and have been observed in a number of cases.[9] Only when the hydroxy-isopropyl group and furan ring have a *cis* relationship (*6a*) can the angle subtended by the europium atom, the hydroxyl oxygen and the furan methyl group be such as to give rise to an upfield lanthanide induced shift. Also, in a competition experiment on a 1:1 mixture, the *trans* isomer showed much stronger co-ordination to the Eu-complex.

The skeletons of these four new tobacco compounds *1a*, *1b*, *2a*, and *2b* suggest they are of terpenoid origin and pseudoionone, a known tobacco constituent,[10] could be a possible precursor. The co-occurrence of an unsaturated isoprenoid and its saturated counterpart (*e.g. 1a* and *2a*) is relatively common in tobacco isolates.[10] *1a* isolated from tobacco showed no optical activity, and it is assumed that *1a* and the related tobacco constituents described here are racemates.

EXPERIMENTAL

NMR, IR, UV, rotations, and mass spectra were recorded on Varian A60-A; HA-100D and XL-100, Digilab FTS-14, Beckmann DK-2A, Perkin-Elmer 141, and LKB 9000 (70 eV) instruments, respectively. Accurate mass determinations were carried out at the Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm. Analytical and preparative gas chromatography was performed on a Varian 1700 instrument using steel capillary columns (50 m × 0.25 mm) coated [11] with Ucon Oil HB 2000, and a 3 m × 3.2 mm glass column packed with 5 % Carbowax 20 M on Chromosorb G, respectively. The extraction of 295 kg sun-cured Greek tobacco, *Nicotiana tabacum* L., and the fractionation of the extract has been described in previous communications.[4,6] The natural compounds were isolated by preparative gas chromatography.

2-Methyl-5-(2-methyl-5-isopropenyltetrahydro-2-furyl)furan (1a+1b). Isolated as a mixture of *1a* and *1b* in ratio 3:7 (7.2 mg) from fraction C9.[4] For spectral data, see synthetic *1a* and *1b*.

(±)-cis-2-Methyl-5-(2-methyl-5-isopropenyltetrahydro-2-furyl)furan (1a): Isolated from subfraction No. 5 of fraction B2 [6] (8 mg). Spectral data are given under synthetic *1a*. Zero optical rotation was observed at λ 589, 578, 546, 436, and 365 nm.

2-Methyl-5-(2-methyl-5-isopropyltetrahydro-2-furyl)furan (2a and 2b). Isolated from fraction C9,[4] isomeric ratio 2:3. Spectral data are given under synthetic *2a* and *2b*.

Synthetic products

2-Methyl-5-acetylfuran (3) was prepared as described by Farrar and Levine.[12]

4-Methyl-3-penten-1-yl bromide (4) was obtained 95 % pure from dimethylcyclopropyl carbinol as outlined by Medina and Manjarrez [13] except for omitting the $NaHCO_3$-treatment and subsequent distillation.

(±)-6-Methyl-2-(5-methyl-2-furyl)-5-hepten-2-ol (5). 4-Methyl-3-penten-1-yl bromide (*4*, 2.6 g) in anhydrous ether (10 ml) was added dropwise to a suspension of Mg (340 mg) and ether (40 ml). Stirring was prolonged until the Mg was dissolved and the ketone (*3*, 600 mg) in ether (20 ml) was added dropwise to the cooled (0 °C) solution of the Grignard reagent. The reaction mixture was stirred for 1 h after the addition of the ketone and the temperature maintained at 0 °C. Crushed ice was added and the mixture stirred for 10 min, followed by

extraction with ether. Removal of the solvent and subsequent chromatography on silica gel furnished the furan derivative (*5*, 580 mg, 57 %) as a colourless oil. MS: *m/e* 208 (M^+, 11), 125 (100), 147 (46), 43 (33), 122 (31), 126 (18), 190 (15), 69 (15), 41 (15); $\nu_{max}^{(film)}$ 3400 (broad), 2978 (s), 2926 (s), 2860 (m), 1450 (m), 1385 (m), 1378 (m), 1221 (m), 1117 (m), 1100 (m), 1022 (s), 944 (m), 786 (s); δ ($CDCl_3$): 1.47 (3 H, s), 1.55 (3 H, m), 1.67 (3 H, m), 1.82 (2 H, m), 2.23 (3 H, d, *J* 0.8 Hz), 2.9 (OH), 5.09 (1 H, m), 5.83 (1 H, dq, *J ca.* 0.8 and 3.0 Hz), 6.03 (1 H, d, *J* 3.0 Hz).

cis *and* trans *(±)-2-[5-Methyl-5-(5-methyl-2-furyl)tetrahydro-2-furyl]-2-propanol* (6a + 6b). To a cooled (−5 °C) solution of the alcohol (*5*, 500 mg) in ether (50 ml) was added 3-chloroperbenzoic acid (600 mg) and the reaction mixture was kept at −5 °C overnight. The ether solution was extracted with diluted NaOH, washed with water, concentrated, and chromatographed on silica gel furnishing the tetrahydrofuran derivative as a colourless oil (216 mg, 40 %). The two diastereomers being present in equal amounts could only be separated by capillary GC.

cis *Isomer (6a)*. A solution of $Hg(OAc)_2.H_2O$ (171 mg) in water (0.9 ml) was diluted with tetrahydrofuran (0.9 ml) and the pure olefin *1a* (*vide infra*, the more polar of the isomers, 90 mg) added to the yellow suspension. The solution was stirred for 55 min before adding 2 M NaOH (1.5 ml) and $NaBH_4$ (15 mg). The reaction mixture was diluted with ether, washed with brine and dried (Na_2SO_4). The pale yellow residue after evaporation was chromatographed on silica (25 % ether in pentane) to yield *6a*, (39 mg). MS: *m/e* 224 (M^+, 9), 122 (100), 165 (53), 123 (43), 43 (42), 109 (21), 59 (16), 147 (13), 137 (12). IR: ν_{max}(film): 3560 (m), 3460 (broad), 3110 (w), 2980 (s), 1385 (m), 1375 (m), 1224 (m), 1092 (s), 1086 (s), 1052 (m), 1024 (s), 784 (s). NMR: The *τ* values given refer to relative induced shifts,[14] on addition of $Eu(DPM)_3$, δ ($CDCl_3$): 1.11 (3 H, s, *τ* 1.0), 1.23 (3 H, s, *τ* 0.98), 1.55 (3 H, s, *τ* 0.21), 1.7−2.3 (4 H, m), 2.25 (3 H, broad s, *τ* −0.015), 3.90 (1 H, m, *τ* 0.88), 5.85 (1 H, dq, *J* 3.0 and 1.0 Hz, *τ* 0.009), 6.04 (1 H, d, *J* 3.0 Hz, *τ* 0.13).

trans *Isomer (6b)*. It was prepared as described for the *cis* isomer but from *1b* (30 mg) to yield 11 mg. MS: *m/e* 224 (M^+, 5), 122 (100), 43 (34), 123 (28), 165 (27), 109 (16), 59 (14), 135 (9), 127 (9). IR: ν_{max}(film): 3470 (broad), 3110 (w), 2980 (s), 1386 (m), 1375 (m), 1224 (m), 1118 (s), 1056 (m), 1052 (m), 1026 (s), 1022 (s), 785 (s). NMR: For notations, see *cis* isomer. δ ($CDCl_3$): 1.15 (3 H, s, *τ* 1.0), 1.23 (3 H, s, *τ* 0.97). 1.55 (3 H, s, *τ* 0.17), 1.7−2.3 (4 H, m), 2.26 (3 H, broad s, *τ* 0.048), 3.89 (1 H, t, *J* 7.0 Hz, *τ* 0.93), 5.85 (1 H, dq, *J* 3.0 and 1.0 Hz, *τ* 0.048), 6.05 (1 H, d, *J* 3.0 Hz, *τ* 0.12).

(±)-2-Methyl-5-(2-methyl-5-isopropenyltetrahydro-2-furyl)furan (1a and 1b). To a cooled (−10 °C) solution of the diastereomeric alcohols *6a* and *6b* (500 mg) in dry pyridine (50 ml) was added $POCl_3$ (1.5 ml) dissolved in pyridine (5 ml). The mixture was kept at −10 °C for 24 h, diluted with water and extracted with ether. The extract was washed with diluted H_2SO_4, water, $NaHCO_3$, water, concentrated and chromatographed on silica gel. The two diastereomers *1a* and *1b* were separated and collected in fractions 2 and 1, respectively. Three slightly more polar products (double-spot on TLC) were found in fraction 3 (160 mg). ν_{max}(film): 1714 cm^{-1}; NMR of fraction 3 revealed the presence of the following three compounds (ratio *ca.* 5:1:4) :2-methyl-6-(5-methyl-2-furyl-5*E*-hepten-3-one, (*7a*), δ 1.91, (3 H, m), δ 3.34 (2 H, d, *J* 7 Hz); irradiation at δ 6.18, and δ 1.91 respectively simplified the multiplet at δ 1.91, and increased (6 %) the intensity of the doublet at δ 3.34, 2-methyl-6-(5-methyl-2-furyl)-5*Z*-hepten-3-one, (*7b*, δ 2.02 (*ca.* 3 H, m), δ 3.66 (*ca.* 2 H, d, *J* 7 Hz), and 2-methyl-6-(5-methyl-2-furyl)-6-hepten-3-one (*8*), δ 4.89 (1 H, m), δ 5.45 (1 H, m). Signals also occurred at δ 1.12 (6 H, dd), δ 2.30 (3 H, m), δ *ca.* 2.66 (*ca.* 4 H, m), δ 5.95 (1 H, m), δ *ca.* 6.18 (*ca.* 2 H, m). Less of the desired olefins and more of these side-products were produced when higher reaction temperatures were employed.

trans *Isomer (1b)* (70 mg, yield: 31 %, based on the weight of one of the diastereomers of *6*): MS: *m/e* 206 (M^+, 6), 109 (100), 43 (55), 124 (48), 67 (37), 122 (27), 121 (21), 125 (20), 41 (16), 82 (14); ν_{max}(film): 1373 (m), 1301 (w), 1262 (m), 1221 (m), 1181 (w), 1149 (w), 1100 (s), 1044 (m), 1020 (s), 958 (w), 943 (w), 900 (s), 873 (w,) 784 (s); δ ($CDCl_3$): 1.58 (3 H, s), 1.73 (3 H, m), 2.24 (3 H, dd, *J* 1.0 and 0.4 Hz), 4.45. (1 H, t, *J ca.* 6.5 Hz), 4.79 (1 H, broad s), 5.02 (1 H, broad s), 5.83 (1 H, dq, *J* 1.0 and 3.0 Hz), 6.05 (1 H, d, *J* 3.0 and 0.4 Hz). Irradiation at δ 1.73 simplified the peaks at δ 4.79 and 5.02, while irradiation at δ 2.24 simplified the signals at δ 5.83 and 6.05 to doublets. No separation from the natural isomer could be observed when co-injected with fraction B1 on a capillary GC column.

cis *Isomer (1a)* (75 mg, yield 33 %). MS: *m/e* 206 (M^+, 17), 109 (100), 43 (78), 67 (30), 122 (26), 148 (25), 135 (25), 41 (23), 124 (23), 121 (20).

Accurate mass determinations (measured on natural *1a*)

Composition:	$C_{13}H_{18}O_2$	$C_9H_{11}O$	$C_7H_8O_2$	$C_8H_{12}O$
found =	206.1311	135.0807	124.0524	124.0888
calc. =	206.1307	135.0810	124.0524	124.0888

Composition:	$C_8H_{10}O$	$C_6H_5O_2$	C_7H_9O	C_8H_{13}
found =	122.0739	109.0286	109.0657	109.1022
calc. =	122.0732	109.0290	109.0653	109.1017

The tree ions at *m/e* 109 are given in the order of decreasing abundance. ν_{max}(film): 1373 (m), 1309 (w), 1289 (w), 1269 (w), 1222 (m), 1206 (w),

1181 (w), 1153 (m), 1100 (s), 1022 (s), 982 (w), 960 (w), 946 (w), 928 (w), 901 (s), 870 (w), 848 (w), 785 (s); δ ($CDCl_3$): 1.56 (3 H, s), 1.69 (3 H, m), 2.23 (3 H, dd, J 0.4 and 1.0 Hz), 4.46 (1 H, m), 4.78 (1 H, broad s), 5.0 (1 H, broad s), 5.81 (1 H, dq, J 1.0 and 3.0 Hz), 6.05 (1 H, dq, J 0.4 and 3.0 Hz).

The synthetic product was indistinguishable from the natural compound isolated from fraction B2 when coinjected on a capillary GC column.

trans (±)-*2-Methyl-5-(2-methyl-5-isopropyltetrahydro-2-furyl)furan (2b). 1b* (22 mg) in ethanol (2 ml) was hydrogenated at room temperature and atmospheric pressure using platinum oxide (*ca.* 5 mg) as catalyst. The hydrogen uptake was completed in 10 min and the reaction mixture was diluted with water and extracted with pentane. Removal of the solvent furnished the dihydro derivative (19 mg) essentially pure. MS: *m/e* 208 (M^+, 5), 43 (100), 41 (40), 109 (32), 69 (19), 55 (19), 193 (16), 165 (15), 123 (11), 122 (10), 121 (8), 125 (8), 124 (6); ν_{max}(film): 1388 (m), 1371 (m), 1278 (w), 1222 (m), 1178 (w), 1105 (m), 1047 (s), 1022 (s), 958 (w), 944 (w), 902 (w) 869 (w), 785 (s); δ ($CDCl_3$): 0.88 (3 H, d, J 6.8 Hz), 0.95 (3 H, d, J 6.0 Hz), 1.53 (3 H, s), 2.24 (3 H, d, J 1.0 Hz), 3.78 (1 H, q, J *ca.* 6.5 Hz), 5.83 (1 H, dq, J 1.0 and 3.0 Hz), 6.02 (1 H, d, J 3.0 Hz).

cis(±)-*2-Methyl-5-(2-methyl-5-isopropyltetrahydro-2-furyl)furan (2a). 1a* (28 mg) was hydrogenated as described above yielding the pure dihydro derivative (27 mg). MS: *m/e* 208 (M^+, 9), 43 (100), 109 (73), 69 (40), 193 (35), 165 (32), 41 (30), 123 (28), 125 (25), 124 (20), 55 (20), 121 (15), 122 (13); ν_{max}(film): 1386 (m), 1370 (m), 1290 (w), 1279 (w), 1221 (m), 1108 (s), 1041 (m), 1025 (s), 958 (w), 945 (w), 918 (w), 901 (w), 845 (w), 785 (s); δ ($CDCl_3$): 0.84 (3 H, d, J 6.8 Hz), 0.98 (3 H, d, J 6.5 Hz), 1.51 (3 H, s), 2.21 (3 H, dd, J 0.4 and 1.0 Hz), 3.72 (1 H, q, J *ca.* 7 Hz), 5.80 (1 H, dq, J 1.0 and 3.0 Hz), 6.02 (1 H, dq, J 0.4 and 3.0 Hz). The two dihydro derivatives could only be separated on capillary GC columns and were found indistinguishable from the natural diastereomeric mixture when co-injected.

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Organic Hydroxylamine Derivatives. X.* Structural Analogues of γ-Aminobutyric Acid (GABA) of the Isoxazole Enol-betaine Type. Synthesis of 5,6,7,8-Tetrahydro-4*H*-isoxazolo[4,5-*d*]azepin-3-ol Zwitterion and 4,5,6,7-Tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol Zwitterion

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The syntheses of the isoxazole enol-betaines 5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*d*]azepin-3-ol zwitterion (VIII) and 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol zwitterion (IX), both of which are new heterocyclic compounds, are described. The syntheses of (VIII) and (IX) are based on an acid catalyzed hydrolysis and subsequent cyclization of the ethylene ketals of the appropriate β-oxohydroxamic acids. The constitutions of (VIII) and (IX) are established by spectroscopic methods. The pK_A values of (VIII) and (IX) have been determined to be 4.84 ± 0.05 and 9.20 ± 0.02, and 4.33 ± 0.05 and 9.06 ± 0.04, respectively. On the basis of Dreiding stereomodels the most probable conformers of (VIII) and (IX) are postulated.

Structure-activity correlations of conformationally restricted analogues of γ-aminobutyric acid (GABA) play a decisive part in the elucidations of the structural characteristics of the GABA receptors and of the active sites of the GABA transaminase enzymes.[1-5] As part of our investigations of the active conformations of GABA, syntheses and X-ray structure determinations of GABA analogues of the isoxazole enol-betaine type have been performed.[6-9] At present we are synthesizing a series of model compounds, which are conformationally restricted bicyclic isoxazole enol-betaines in which the *intra*-molecular distance between the zwitterionic centers is systematically changed. This paper presents the syntheses of two members of this series, namely 5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*d*]azepin-3-ol zwitterion (VIII) and 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol zwitterion (IX), which are structural analogues of GABA and β-alanine, respectively. Studies of Dreiding stereomodels seem to indicate that two conformers of (VIII) (A and B in Fig. 1) are almost equally favourable, whereas (IX) can adopt only one almost frozen conformation. The approximate *intra*-molecular distances between the charged oxygen atoms and the charged nitrogen atoms in the respective conformers of (VIII) and (IX) are shown in Fig. 1.

An X-ray diffraction analysis of (VIII) is in progress, and investigations of the biological properties of (VIII) and (IX) will be initiated in the near future.

The syntheses of the 3-hydroxyisoxazoles (VIa – c) were accomplished utilizing a reaction sequence analogous to that described by Jacquier *et al.*[10] for the preparation of 3-hydroxyisoxazoles using β-oxoesters as starting materials. The cyclic β-oxoesters (IIIb,c) were synthesized by reaction of compound (I) with ethyl and methyl chloroformate, respectively. The compounds (II) and (IIIb,c) were converted into the dioxolanes (IVa – c), which in turn were transformed into the corresponding hydroxamic acids (Va – c). Attempts to accomplish acid catalyzed hydrolyses and subsequent cyclizations of the

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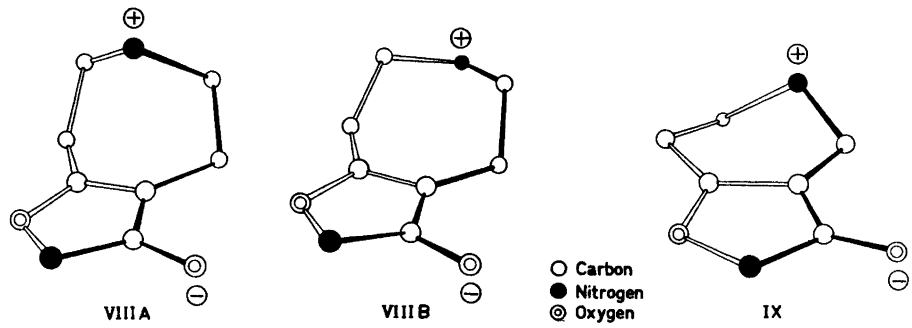
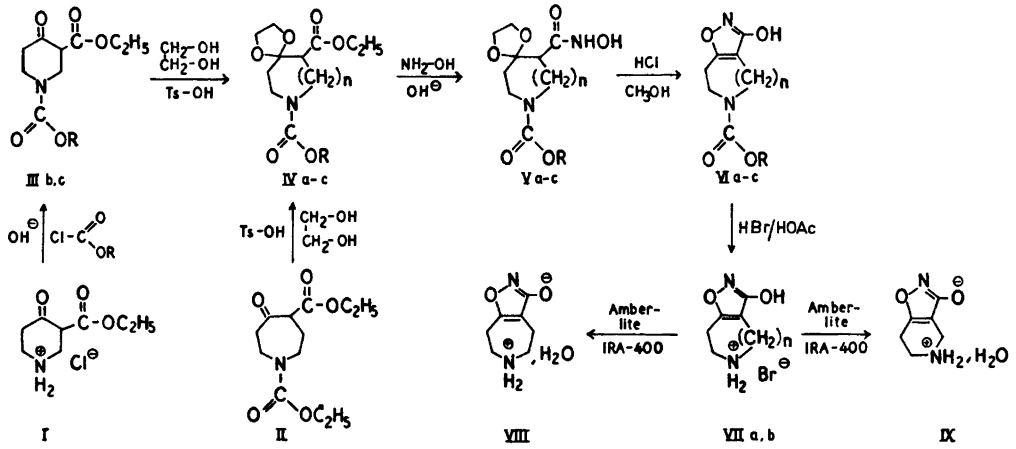


Fig. 1. Some structural characteristics of 5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*d*]azepin-3-ol (VIII) and 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol (IX). The structural similarities of VIII and IX to GABA and β-alanine, respectively, are indicated by heavy black lines. The N^+ to O^- distances were estimated by measurements on Dreiding stereomodels: 4.5 Å (VIIIA), 5.3 Å (VIIIB), 4.4 Å (IX). pK_A-Values (H_2O): 4.84 ± 0.05, 9.20 ± 0.02 (17 °C; VIII); 4.33 ± 0.05, 9.06 ± 0.04 (20 °C; IX).

crude reaction products of the dioxolane hydroxamic acids (Va – c) to give the corresponding 3-hydroxyisoxazoles (VIa – c) according to the method described by Jacquier *et al.*[10] were rather unsuccessful. Thus in all cases complex reaction mixtures were obtained, from which no products were isolated. Isolation of the hydroxamic acids (Va – c) in a pure state using chromatography on silica gel columns and subsequent treatment of (Va – c) with hydrochloric acid, however, gave the 3-hydroxyisoxazoles (VIa – c) in moderate to good yields.

Cleavage of (VIa – c) to the corresponding hydrobromides (VIIa,b) was accomplished by treatment with 43 % solutions of hydrogen bromide in glacial acetic acid. The drastic reaction conditions including prolonged heating requisite for the cleavage of the ethoxycarbonyl group of (VIa,b) caused some decomposition of the compounds, especially of (VIb). The methoxycarbonyl group of (VIc), however, was easily cleaved by the above mentioned reagent to give (VIIb) in a pure state and in a high yield. The enol-betaines (VIII) and (IX) were



Scheme 1.

isolated from (VIIa,b), respectively, using a strongly basic ion exchange resin, and both compounds crystallized as monohydrates. The pK_A values of (VIII) and (IX) are 4.84 ± 0.05 and 9.20 ± 0.02, and 4.33 ± 0.05 and 9.06 ± 0.04, respectively.

The structure determinations of (IIIb,c), (IVa–c), and (Va–c) are based on IR and ^{1}H NMR spectroscopy and are supported by elemental analyses. Carbonyl absorption bands in the IR spectra of (IIIb,c) at 1660 cm^{-1} supported by resonance signals in the ^{1}H NMR spectra in tetrachloromethane solutions at δ *ca.* 12 and of intensities corresponding to 0.6 H and 0.8 H, respectively, reveal that the β-ketoesters (IIIb,c) are largely in the *intra*-molecular hydrogen bonded enol form. Structure elucidations of the 3-hydroxyisoxazoles (VIa–c) and (VIIa,b) were performed by IR, UV, and ^{1}H NMR spectroscopy and were supported by elemental analyses. The spectroscopic data originating in the 3-hydroxyisoxazole moieties of (VIa–c) and (VIIa,b) are in accordance with the general findings described by Jacquier *et al.*[10]

The aromatic character of the isoxazole nucleus of (VIII) and (IX) is evident from the IR absorption bands in the 1520–1460, 1630–1600, and 1680–1650 cm^{-1} regions.[11] Absorptions over the range 3600–1900 cm^{-1} and at 2140 and 2250 cm^{-1}, respectively, suggest ammonium salt character of (VIII) and (IX). The UV maxima of (VIII) and (IX) at 216 and 215 nm, respectively, are in agreement with those observed (210 and 211 nm, respectively) for 3-hydroxy-5-(2-aminoethyl)isoxazole,[8] and 3-hydroxy-5-(3-aminopropyl)isoxazole,[12] the structures of which have been established by X-ray diffraction analyses.[8,12] The above mentioned

Table 1. Some IR and UV data of the compounds (III)-(IX).

	IR data[a] (cm^{-1})	UV data[b] λ_{max}(nm)	$\varepsilon \times 10^{-3}$
IIIb[c]	3600–3100(w), 1760(m), 1700(s), 1600(s), 1620(m)		
IIIc[c]	3600–3100(w), 1730(m), 1710(s), 1660(s), 1620(m)		
IVa[c]	1730(s), 1690(s)		
IVb[c]	1730(s), 1700(s)		
IVc[c]	1730(s), 1710(s)		
Va[d]	3700–3000(m), 1685(s), 1675(s)		
Vb[d]	3600–3000(m), 1690(s), 1675(s)		
Vc[d]	3600–3000(m), 1695(s), 1690(s)		
VIa	3600–2100(s), 1695(s), 1650(m), 1540(s)	213	5.86
VIb	3600–2100(s), 1705(s), 1675(m), 1550(m), 1535(m)	211	6.55
VIc	3600–2100(s), 1705(s), 1675(m), 1550(m), 1535(m)	212	6.23
VIIa	3700–3300(m), 3300–2300(m), 1665(m), 1590(m), 1530(s)	211	6.23
VIIb	3700–3300(s), 3300–2300(s), 1670(s), 1630(m), 1610(m), 1540(s)	210	5.77
VIII	3700–1800(s), 2140(w), 1660(s), 1630(m), 1500–1420(s)	216	4.65
IX	3700–1800(s), 2250(m), 1675(s), 1615(m), 1510–1430(s)	215	5.31

[a] Unless otherwise stated the IR spectra were recorded in the solid state (KBr). [b] The UV spectra were recorded in ethanol solutions. [c] The IR spectra were recorded using the film technique. [d] The IR spectra were recorded in chloroform solutions.

observations combined provide conclusive evidence of the proposed constitutions of (VIII) and (IX).

Some spectroscopic data of the new compounds (IIIb,c), (IVa–c), (Va–c), (VIa–c), (VIIa,b), (VIII), and (IX) are listed in Table 1.

EXPERIMENTAL

Unless otherwise stated the determination of melting points, the recording of IR, UV, and ^{1}H NMR spectra, the designation of the patterns of the ^{1}H NMR spectra, and the performance of microanalyses were accomplished as described in a previous paper.[13] pH values were measured on a Radiometer pH meter 26, and the pK_A values were determined according to the method of Albert and Serjeant [14] as described in a previous paper.[8] Thin layer and column chromatographic procedures were accomplished using silica gel GF_{254} plates (Merck) and silica gel, 0.05–0.20 mm (Merck), respectively.

Diethyl 4-oxoperhydroazepine-1,5-dicarboxylate ethylene ketal (IVa). (IVa) was synthesized according to the general reaction for the preparation of β-oxoester ethylene ketals described by Jacquier *et al.*[10] using 11.7 g (46 mmol) of (II),[15] 14.0 g (230 mmol) of ethylene glycol, 0.6 g of 4-toluenesulfonic acid, and 400 ml of benzene. After reaction for 76 h the reaction mixture was worked up to give 12.6 g of crude product, which was subjected to column chromatography (silica gel: 200 g; eluent: methylene chloride to which increasing amounts of ethyl acetate were added). Obtained was 8.62 g of product, which after distillation gave 7.56 g (55 %) of (IVa) as a colourless oil, b.p. 152–154 °C/0.2 mmHg. (Found: C 55.75; H 7.72; N 4.59. Calc. for $C_{14}H_{23}NO_6$: C 55.80; H 7.69; N 4.65). ^{1}H NMR data (CCl_4): δ 4.05 [two coincident q (J = 7 Hz), 4 H, 2 × O–C*H*$_2$–CH$_3$]; 3.87 (s, 4 H, O–C*H*$_2$–C*H*$_2$–O); 3.8–3.1 (m, 4 H, CH$_2$–C*H*$_2$–N–C*H*$_2$–CH$_2$); 2.8–2.5 (perturbed t, 1 H, C–C*H*–CH$_2$); 2.5–1.5 (m, 4 H, CH$_2$–C*H*$_2$–C and CH$_2$–C*H*$_2$–CH); 1.25 [two coincident t (J = 7 Hz), 6 H, 2 × O–CH$_2$–C*H*$_3$].

1-Ethoxycarbonyl-4-oxoperhydroazepine-5-carbohydroxamic acid ethylene ketal (Va). To a stirred solution of 4.5 g (*ca.* 65 mmol) of potassium hydroxide in methanol (45 ml) was added 3.6 g (52 mmol) of hydroxylamine hydrochloride. The mixture was stirred for further 90 min and after cooling in an ice bath a solution of 4.02 g (13 mmol) of (IVa) in methanol (12 ml) was added, and the mixture was allowed to stand at 8 °C for 18 d. Upon addition of 5 ml of glacial acetic acid the mixture was filtered and the filtrate was evaporated *in vacuo* to give a thick mass which was worked up utilizing column chromatography [silica gel: 130 g; eluent: ethyl acetate-methanol-formic acid (90:10:1)]. Obtained was 1.76 g (46 %) of (Va) as a reddish, glassy compound. (Found: C 49.78; H 7.11; N 9.65. Calc. for $C_{12}H_{20}N_2O_6$: C 49.99; H 6.99; N 9.72). ^{1}H NMR data ($CDCl_3$): δ 9.4–7.7 (broad signal, 2 H, CO–N*H*–O*H*); 4.08 [q (J = 7 Hz), 2 H, O–C*H*$_2$–CH$_3$]; 3.92 (s, 4 H, O–C*H*$_2$–C*H*$_2$–O); 3.8–3.1 (m, 4 H, CH$_2$–C*H*$_2$–N–C*H*$_2$–CH$_2$); 2.9–2.4 (m, 1 H, C–C*H*–CH$_2$); 2.4–1.6 (m, 4 H, CH$_2$–C*H*$_2$–C and CH$_2$–C*H*$_2$–CH); 1.23 [t (J = 7 Hz), 3 H, O–CH$_2$–C*H*$_3$].

Ethyl 3-hydroxy-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepine-6-carboxylate (VIa). To a solution of 1.84 g (6.4 mmol) of (Va) in methanol (5 ml) was added 3 ml of concentrated hydrochloric acid. After heating to 70 °C for 5 min the solution was evaporated *in vacuo* to give an oil which was subjected to column chromatography [silica gel: 120 g; eluent: methylene chloride-ethyl acetate-formic acid (85:15:1) to which increasing amounts of ethyl acetate were added]. Obtained was 1.36 g (94 %) of (VIa) in a crystalline state. An analytical sample was recrystallized (benzene-cyclohexane) to give colourless crystals, m.p. 95.5–97.5 °C. (Found: C 53.15; H 6.25; N 12.35. Calc. for $C_{10}H_{14}N_2O_4$: C 53.09; H 6.24; N 12.38). ^{1}H NMR data (CCl_4): δ 9.5–8.8 (broad signal, 1 H, O*H*); 4.13 [q (J = 7 Hz), 2 H, O–C*H*$_2$–CH$_3$]; 3.8–3.4 (m, 4 H, CH$_2$–C*H*$_2$–N–C*H*$_2$–CH$_2$); 3.05–2.65 [t, 2 H, CH$_2$–C*H*$_2$–C(O)=C]; 2.65–2.35 [t, 2 H, CH$_2$–C*H*$_2$–C(C)=C]; 1.25 [t (J = 7 Hz), 3 H, O–CH$_2$–C*H*$_3$].

3-Hydroxy-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-d]azepinium bromide (VIIa). A solution of 1.40 g (6.2 mmol) of (VIa) in 8 ml of glacial acetic acid containing 43 % of hydrogen bromide was refluxed for a total of 2 h (bath temperature: 100 °C). After reflux for 30 and 60 min, respectively, further amounts of 8 ml of glacial acetic acid containing 43 % of hydrogen bromide were added. After cooling to 25 °C the mixture was evaporated to dryness *in vacuo*. The crystalline residue was recrystallized (methanol-ether) to give 1.13 g (78 %) of (VIIa) as colourless crystals, m.p. 253 °C (decomp.). (Found: C 35.73; H 4.68; N 11.97; Br 34.07. Calc. for $C_7H_{11}BrN_2O_2$: C 35.75; H 4.73; N 11.91; Br 34.01). ^{1}H NMR data [D_2O (sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard)]: δ 4.74 (s. *ca.* 4 H, DO*H*); 3.7–3.3 (m, 4 H, CH$_2$–C*H*$_2$–NH$_2^+$–C*H*$_2$–CH$_2$); 3.3–3.0 [m, 2 H, CH$_2$–C*H*$_2$–C(O)=C]; 3.0–2.6 [m, 2 H, CH$_2$–C*H*$_2$–C(C)=C].

5,6,7,8-Tetrahydro-4H-isoxazolo[4,5-d]azepin-3-ol zwitterion (VIII). A solution of 792 mg (3.4 mmol) of (VIIa) in water (10 ml) was passed through a column containing an ion exchange resin [Amberlite IRA 400, (OH), 100 ml] using acetic acid (1 M) as an eluent. Crude product (605 mg) was obtained. Recrystallization (water-ethanol) gave 334 mg (58 %) of (VIII) as colourless crystals, m.p. 211 °C (decomp.). (Found: C 48.75; H 7.01; N 16.37. Calc. for $C_7H_{10}N_2O_2.1H_2O$: C 48.83; H 7.03; N 16.27). [Found after drying of (VIII) over P_2O_5 (48 h;

125 °C; 0.05 mmHg): C 54.40; H 6.69; N 18.22. Calc. for $C_7H_{10}N_2O_3$: C 54.53; H 6.54; N 18.17]. ^{1}H NMR data [D_2O-CF_3COOD (19:1) (sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard)]: δ 4.95 (s, *ca.* 5 H, DO*H*); 3.6–3.3 (m, 4 H, $CH_2-CH_2-NH_2^+-CH_2-CH_2$); 3.3–2.6 (m, 4 H, $CH_2-CH_2-C=C-CH_2-CH_2$).

Diethyl 4-oxopiperidine-1,5-dicarboxylate (IIIb). To an ice-cooled solution of 12.3 g (59 mmol) of (I) (Fluka) in water (40 ml), an ice-cooled solution of 3.9 g (*ca.* 55 mmol) of potassium hydroxide and 11.0 g (80 mmol) of potassium carbonate in water (75 ml) was added with stirring, and immediately thereafter 12.9 g (118 mmol) of ethyl chloroformate was added over a period of 15 s. Stirring was continued at 0 °C for 1 h and finally at 25 °C for further 1 h. The reaction mixture was extracted with four 75 ml portions of ether and the combined ether phases were dried and evaporated to give 11.4 g of a red oil. Distillation gave 9.9 g (69 %) of (IIIb) as a colourless oil, b.p. 133–138 °C/0.7 mmHg. (Found: C 54.50; H 6.94; N 5.77. Calc. for $C_{11}H_{17}NO_5$: C 54.31; H 7.04; N 5.76). ^{1}H NMR data (CCl_4): δ 12.0 (s, 0.6 H, enol O*H*); 4.4–3.2 (m, consisting of several partially overlapping patterns of resonance signals, total 8.3 H); 2.7–2.2 [m, 2 H, $CH_2-CH_2-C=O$(oxo form) and $CH_2-CH_2-C=C$(enol form)]; 1.5–1.1 (m, consisting of several overlapping t, total 6 H, $O-CH_2-CH_3$).

Ethyl 1-methoxycarbonyl-4-oxopiperidine-5-carboxylate (IIIc). (IIIc) was prepared as described above for (IIIb) using 12.3 g (59 mmol) of (I) and 11.2 g (118 mmol) of methyl chloroformate, which gave 10.2 g of crude product as a red oil. Distillation gave 9.20 g (68 %) of (IIIc) as a colourless oil, b.p. 131–133 °C/0.3 mmHg. (Found: C 52.45; H 6.58; N 6.09. Calc. for $C_{10}H_{15}NO_5$: C 52.39; H 6.60; N 6.11). ^{1}H NMR data (CCl_4): δ 12.1 (s, 0.8 H, enol O*H*); 4.4–4.0 (two overlapping q, $O-CH_2-CH_3$) and 4.1–3.9 [m, $N-CH_2-CH$ (oxo form) and $N-CH_2-C=C$ (enol form)], total 4 H; 3.9–3.3 [m, $N-CH_2-CH_2$ (oxo and enol form)], 3.67 (s, $O-CH_3$), and 3.65 (s, $O-CH_3$), total 5 H; 2.6–2.1 [m, 2 H, $CH_2-CH_2-C=O$ (oxo form) and $CH_2-CH_2-C=C$ (enol form)]; 1.5–1.1 (two overlapping t, 3 H, $O-CH_2-CH_3$).

Diethyl 4-oxopiperidine-1,5-dicarboxylate ethylene ketal (IVb). (IVb) was synthesized as described above for (IVa) using 11.4 g (47 mmol) of (IIIb) and 14.9 g (240 mmol) of ethylene glycol. After reaction for 81 h the reaction mixture was worked up to give 12.2 g of crude product as an oil, which without previous purification by column chromatography was distilled to give 9.20 g (68 %) of (IVb) as a colourless oil, b.p. 152–156 °C/0.2 mmHg. (Found: C 54.20; H 7.21; N 4.75. Calc. for $C_{13}H_{21}NO_6$: C 54.34; H 7.37; N 4.88). ^{1}H NMR data (CCl_4): δ 4.04 [q (J=7 Hz), $O-CH_2-CH_3$], 4.00 [q (J=7 Hz), $O-CH_2-CH_3$], and 3.88 (s, $O-CH_2-CH_2-O$), total 8 H; 3.8–3.1 (m, 4 H, $CH_2-CH_2-N-CH_2-CH$); 2.6–2.3 (perturbed t, 1 H, $C-CH-CH_2$); 2.3–1.3 (m, 2 H, CH_2-CH_2-C); 1.24 [t (J=7 Hz), $O-CH_2-CH_3$] and 1.21 [t (J=7 Hz), $O-CH_2-CH_3$], total 6 H.

Ethyl 1-methoxycarbonyl-4-oxopiperidine-5-carboxylate ethylene ketal (IVc). (IVc) was synthesized as described above for (IVa) using 9.21 g (40 mmol) of (IIIc) and 12.4 g (200 mmol) of ethylene glycol. After reaction for 69 h the reaction mixture was worked up to give 9.8 g of crude product as an oil, which without previous purification by column chromatography was distilled to give 6.60 g (60 %) of (IVc) as a colourless oil, b.p. 160–162 °C/0.4 mmHg. (Found: C 52.80; H 7.08; N 5.23. Calc. for $C_{12}H_{19}NO_6$: C 52.74; H 7.01; N 5.13). ^{1}H NMR data (CCl_4): δ 4.05 [q (J=7 Hz), $O-CH_2-CH_3$] and 3.91 (s, $O-CH_2-CH_2-O$), total 6 H; 3.8–3.3 (m, $CH_2-CH_2-N-CH_2-CH$) and 3.60 (s, $O-CH_3$), total 7 H; 2.6–2.4 (perturbed t, 1 H, $C-CH-CH_2$); 2.3–1.3 (m, 2 H, CH_2-CH_2-C); 1.25 [t (J=7 Hz), 3 H, $O-CH_2-CH_3$].

1-Ethoxycarbonyl-4-oxopiperidine-5-carbohydroxamic acid ethylene ketal (Vb). (Vb) was synthesized as described above for (Va) using 4.00 (14 mmol) of (IVb), 4.9 g (*ca.* 70 mmol) of potassium hydroxide, and 3.9 g (56 mmol) of hydroxylamine hydrochloride. After standing at 8 °C for 6 d the reaction mixture was worked up as described above for (Va) to give 2.42 g (63 %) of (Vb) in a crystalline state. An analytical sample was recrystallized (ethanol-benzene) to give (Vb) as colourless crystals, m.p. 134.5–136.5 °C. (Found: C 48.40; H 6.72; N 10.32. Calc. for $C_{11}H_{18}N_2O_6$: C 48.17; H 6.62; N 10.21). ^{1}H NMR data [CCl_4-$CDCl_3$ (2:1)]: δ 9.2–8.4 (broad signal, 2 H, $CO-NH-OH$); 4.09 [q (J=7 Hz), $O-CH_2-CH_3$], 3.98 (s, $O-CH_2-CH_2-O$), and 4.4–3.0 (m, $CH_2-CH_2-N-CH_2-CH$), total 10 H; 2.8–2.3 (m, 1 H, $C-CH-CH_2$); 2.3–1.3 (m, 2 H, CH_2-CH_2-C); 1.30 [t (J=7 Hz), 3 H, $O-CH_2-CH_3$].

1-Methoxycarbonyl-4-oxopiperidine-5-carbohydroxamic acid ethylene ketal (Vc). (Vc) was synthesized as described above for (Va) using 7.72 g (28 mmol) of (IVc), 9.8 g (*ca.* 140 mmol) of potassium hydroxide, 7.8 g (112 mmol) of hydroxylamine hydrochloride, and 90 ml of methanol. After standing at 8 °C for 6 d the reaction mixture was worked up as described above for (Va) using 10 ml of glacial acetic acid and 160 g of silica gel to give 3.50 g (48 %) of (Vc) in a crystalline state. An analytical sample was recrystallized (ethanol-benzene) to give (Vc) as colourless crystals, m.p. 162–165 °C (decomp.). (Found: C 46.45; H 6.16; N 10.63. Calc. for $C_{10}H_{16}N_2O_6$: C 46.15; H 6.20; N 10.77). ^{1}H NMR data ($CDCl_3$): δ 9.5–8.5 (broad signal, 2 H, $CO-NH-OH$); 3.97 (s, $O-CH_2-CH_2-O$), 3.66 (s, $O-CH_3$), and 4.2–2.9 (m, $CH_2-CH_2-N-CH_2-CH$), total 11 H; 2.8–2.3 (m, 1 H, $C-CH-CH_2$); 2.1–1.1 (m, 3 H, CH_2-CH_2-C).

Ethyl 3-hydroxy-4,5,6,7-tetrahydroisoxazolo-[4,5-c]pyridine-5-carboxylate (VIb). (VIb) was

synthesized and isolated from the reaction mixture as described above for (VIa) using 2.30 g (8.4 mmol) of (Vb). (VIb) (1.33 g, 75 %) was obtained in a crystalline state. An analytical sample was recrystallized (benzene-cyclohexane) to give colourless crystals, m.p. 118.0 – 120.5 °C. (Found: C 50.85; H 5.67; N 13.13. Calc. for $C_9H_{12}N_2O_4$: C 50.94; H 5.70; N 13.20). ^{1}H NMR data ($CDCl_3$): δ 10.60 (s, 1 H, O*H*); 4.4 – 4.1 (m, N – C*H*$_2$ – C = C) and 4.12 [q (J = 7 Hz), O – C*H*$_2$ – CH$_3$], total 4 H; 3.9 – 3.5 (t, 2 H, N – C*H*$_2$ – CH$_2$); 2.8 – 2.5 (t, 2 H, CH$_2$ – C*H*$_2$ – C = C); 1.27 [t (J = 7 Hz), 3 H, O – CH$_2$ – C*H*$_3$].

Methyl 3-hydroxy-4,5,6,7-tetrahydroisoxazolo-[4,5-c]pyridine-5-carboxylate (VIc). (VIc) was synthesized as described above for (VIa) using 3.20 g (12.3 mmol) of (Vc), 15 ml of methanol, and 8 ml of concentrated hydrochloric acid. The reaction mixture was evaporated *in vacuo* to give an oil, which was dissolved in chloroform (30 ml). The chloroform solution was washed with a saturated solution of sodium chloride, dried, and evaporated *in vacuo* to give 2.28 g of crude (VIc). Recrystallization (benzene-cyclohexane) gave 1.16 g (48 %) of (VIc) as colourless crystals, m.p. 155.0 – 156.5 °C. (Found: C 48.50; H 5.07; N 14.08. Calc. for $C_8H_{10}N_2O_4$: C 48.48; H 5.09; N 14.14). ^{1}H NMR data ($CDCl_3$): δ 10.50 (s, 1 H, O*H*); 4.4 – 4.2 (m, 2 H, N – C*H*$_2$ – C = C); 3.9 – 3.6 (t, N – C*H*$_2$ – CH$_2$) and 3.69 (s, O – C*H*$_3$), total 5 H; 2.9 – 2.5 (t, 2 H, CH$_2$ – C*H*$_2$ – C = C).

3-Hydroxy-4,5,6,7-tetrahydroisoxazolo[4,5-c]-pyridinium bromide (VIIb). Method a. (VIIb) was synthesized as described above for (VIIa) using 1.15 g (5.4 mmol) of (VIb). The crude product was recrystallized (methanol-ether) to give 781 mg (65 %) of (VIIb) as colourless crystals, m.p. 168 – 170 °C (decomp.). (Found: C 32.46; H 4.18; N 12.67; Br 36.28. Calc. for $C_6H_9BrN_2O_2$: C 32.60; H 4.10; N 12.67; Br 36.14). ^{1}H NMR data [D_2O (sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard)]: δ 4.68 (s, *ca.* 5 H, DO*H*); 4.2 – 4.0 (m, 2 H, NH_2^+ – C*H*$_2$ – C = C); 3.7 – 3.4 (t, 2 H, NH_2^+ – C*H*$_2$ – CH$_2$); 3.2 – 2.8 (t, 2 H, CH$_2$ – C*H*$_2$ – C = C).

Method b. A solution of 1.15 g (5.8 mmol) of (VIc) in 10 ml of glacial acetic acid containing 43 % of hydrogen bromide was heated for 25 min (bath temperature: 100 °C). Evaporation of the reaction mixture to dryness *in vacuo* and recrystallization (methanol-ether) of the residue afforded 1.13 g (88 %) of (VIIb) as colourless crystals, m.p. 169 – 171 °C (decomp.). The IR spectrum was identical with that of the product of method a.

4,5,6,7-Tetrahydroisoxazolo[4,5-c]pyridin-3-ol zwitterion (IX). (IX) was prepared as described above for (VIII) using 581 mg (2.6 mmol) of (VIIb) and 65 ml of ion exchange resin. (IX) (205 mg, 49 %) was obtained as colourless crystals, m.p. 254 – 256 °C (decomp.) (water-ethanol). (Found: C 45.50; H 6.38; N 17.82. Calc. for $C_6H_8N_2O_2.1H_2O$: C 45.56; H 6.37; N 17.71). [Found after drying of (IX) over P_2O_5 (48 h; 125 °C; 0.05 mmHg): C 51.65; H 5.88; N 20.03. Calc. for $C_6H_8N_2O_2$: C 51.42; H 5.75; N 19.99]. ^{1}H NMR data [D_2O-CF_3COOD (19:1) (sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard)]: δ 4.88 (s, *ca.* 6 H, DO*H*); 4.2 – 4.0 (m, 2 H, NH_2^+ – C*H*$_2$ – C = C); 3.7 – 3.4 (t, 2 H, NH_2^+ – C*H*$_2$ – CH$_2$); 3.2 – 2.8 (t, 2 H, CH$_2$ – C*H*$_2$ – C = C).

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The Chemistry of Dibenzo[*d*,*f*][1,3]diazepines. 3.[1] The Electrolytic Preparation of 2,2′-Dihydroxylaminobiphenyl and its Reaction with Aldehydes and Ketones

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Preparative scale electrolytic reduction of 2,2′-dinitrobiphenyl gives 2,2′-dihydroxylaminobiphenyl which, although not isolated, combines either *in situ* or after electrolysis with various aliphatic aldehydes and ketones to give the corresponding 5,7-dihydroxy-6,7-dihydrodibenzo[*d*,*f*][1,3]diazepine derivatives. A few activated heterocyclic aldehydes and cycloalkanones underwent this reaction, but generally aromatic aldehydes and ketones and some aliphatic ketones did not react under similar conditions.

The structures of the products were established according to spectroscopic and chemical evidence. A comparison of the results of controlled potential electrolyses and *in situ* catalytic hydrogenations suggests that the former method is usually the preferred method.

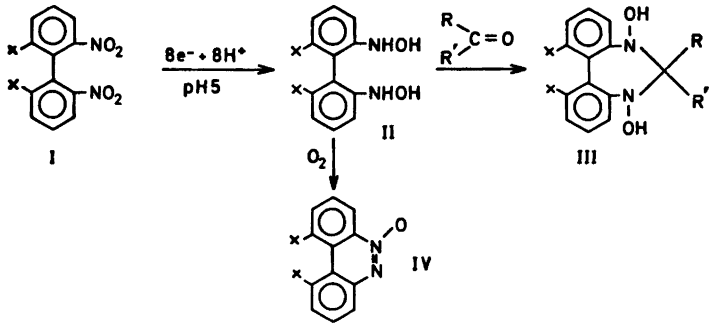
Recently Laviron and Lewandowska [2] have shown that 2,2′-dihydroxylaminobiphenyl (II) is formed by cathodic reduction of 2,2′-dinitrobiphenyl (I). Isolation of the bishydroxylamine (II) was not recorded but compelling evidence for its formation comes from the use of electroanalytical techniques and air (or anodic) oxidation to benzo[*c*]cinnoline-*N*-oxide (IV). The investigations have since been extended to 2,2′,6,6′-tetranitrobiphenyl [3,4] with similar results. The reductive cyclization of 2,2′-dinitrodiphenyl with a few aliphatic aldehydes to give 6-alkyl-5,7-dihydroxy-6,7-dihydrodibenzo[*d*,*f*][1,3]diazepines (III) has been described by Becher [5] and their mass spectra have been discussed.[1] The catalytic hydrogenation method gives low yields [5] and accordingly an electrochemical approach was adopted with the aim of exploring the scope of the cyclization reaction by using a variety of carbonyl compounds.

The trapping of hydroxylamines from electrolytic reduction of nitroalcohols by *in situ* reaction with aldehydes and ketones has been reported by Petrov and Barkhash.[6] In this case, however, the primary condensation products were not isolated but the nitrones postulated as intermediates were further reduced to the corresponding amines at the amply negative cathode potential employed. The present work thus provides new examples of a general reaction type in cathodic electrolysis, *i.e.* the reaction of an electrophile (the carbonyl compound) with an electrochemically generated nucleophile [the bishydroxylamine (II)]. The same principle has been used by Lund [7] to effect many electrochemical cyclizations leading to heterocyclic systems. In such cases the reducible group (which should be transformed into a nucleophile by cathodic reduction) and the unsaturated electrophilic function were present within the same molecule.

A related condensation with ketones was found by Volodarsky and Kutikova who prepared the 1-hydroxy-3-imidazoline-3-oxide derivatives by reaction of α-hydroxylamino oximes with acetone.[8]

RESULTS AND DISCUSSION

Electrochemical preparation. From investigations of the cathodic reduction of aromatic



nitro compounds [9] it has been concluded that weakly acidic media will favour hydroxylamine formation. Similar optimum conditions are required [10] for the reaction between carbonyl compounds and N-containing nucleophiles (which lose their nucleophilic activity by protonation). It proved convenient to conduct preparative scale electrolyses in an acetate buffer (*ca.* pH 5) and add varying amounts of organic polar solvents which by increasing the solubility of the depolarizer allowed the passage of a larger cell current with a consequent shortening of the electrolysis time.

The reductions were performed at room temperature using a mercury cathode and a conventional H-type 3-electrode cell of 250 ml volume. Potentials less cathodic than -0.8 V *vs.* Ag/AgCl were used with 1 – 2 g of the 2,2′-dinitrobiphenyl (I) and in most cases a large excess (5 ml or 5 g) of the carbonyl compound. For the more reactive carbonyl compounds the excess could be smaller. A few experiments using a 500 ml cell with 10 – 12 g of I and acetaldehyde, acetone, or cyclopentanone, gave similar results.

For aldehydes and ketones which were not reduced at ≥ -0.8 V *in situ* techniques could be used. However, an electrochemically prepared solution of II was found to be stable in the absence of oxygen and similar yields of the dihydrodibenzodiazepines (III) were obtained by adding the carbonyl compound to a freshly electrolysed solution of (I). This procedure was necessary for condensations involving the easily reducible heteroaromatic aldehydes. If oxygen is not excluded, oxidation to IV predominates. The *in situ* method is quicker and therefore preferable when the reduction potential of the carbonyl component will allow it. The products are reducible but at more negative potentials than used in this work. The further electroreduction has not yet been investigated. However, for these reasons careful control of the cathode potential is essential. For both procedures the consumption of electricity was generally very close to the theoretical value of 8 F/mol, which thus confirms the findings of Laviron.[9]

Following electrolysis the catholyte was kept under nitrogen at room temperature for 1 – 7 days to allow complete condensation. The product was isolated by filtration and/or extraction with ether after partial evaporation, and purified by recrystallization from a suitable solvent. The yields were estimated from the 60 MHz ^{1}H NMR spectra (DMSO-d_6) of the crude materials and are given in Table 1 together with the yields of recrystallized, pure substances; results from elemental analysis and melting points are to be found in Table 3 in the experimental section. The pH-conditions, methods of isolation and purification for every product were not optimised.

In the original experiments ethanol [9] was used as a co-solvent which was suitable for condensations involving the lower aliphatic aldehydes and acetone. With less reactive carbonyl reagents such as ethyl methyl ketone or cyclohexanone, NMR-analysis of the crude product revealed the presence of IIIb and IV in addition to the expected products. Compound IV was detected in all cases in amounts ranging from traces to its being the main product. It was presumably formed from unreacted II by air oxidation during the work-up, and could be isolated in high yield after purging the electrolysed solution of I with oxygen. The mechanism of formation of IIIb is not yet clear, but it is suggested that an Oppenauer-type of

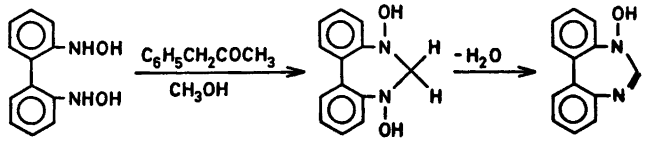
Table 1. Yields of dibenzo[*d,f*][1,3]diazepine derivatives.

Compound	R	R'	X	% Crude	% Recryst.	Solvent
IIIa	H	H	H	(45)	–	B
IIIb	CH_3	H	H	80	66	A
IIIc	CH_2CH_3	H	H	38	22	B
IIId	$(CH_2)_2CH_3$	H	H	85	30	A
IIIe	$(CH_2)_3CH_3$	H	H	45	25	A
IIIf	CH_3	CH_3	H	79	61	A
IIIg	CH_2CH_3	CH_3	H	56	45	B
IIIh	$(CH_2)_2CH_3$	CH_3	H	52	28	B
IIIi	CD_3	CD_3	H	76	48	A
IIIj	$(CH_2)_4$		H	90	72	B
IIIk	$(CH_2)_5$		H	47	25	C
IIIl	4-pyridyl	H	H	64	32	C
IIIm	4-methyl-2-thiazolyl	H	H	55	36	C
IIIn	CH_3	H	CH_3	73	20	A
IIIo	CH_3	CH_3	CH_3	77	42	A

oxidation of the ethanol takes place with the added carbonyl reagent as an oxidant in the presence of II. The acetaldehyde formed would then react rapidly with II to give IIIb. The alcohol corresponding to the carbonyl reagent has not yet been detected but support for the suggestion comes from an experiment using benzyl methyl ketone and methanol as co-solvent which resulted in the main product (V) being derived from formaldehyde. Compound V is the dehydrated derivative 5-hydroxydibenzo[*d,f*][1,3]diazepine, a hitherto unknown and simple representative of the parent ring system (eqn. 2).

In view of this complication other solvents which would not form carbonyl compounds by oxidation were tried. Acetone-water could of course only be used for the derivative IIIf and did work satisfactorily. Acetonitrile-water mixtures were suitable solvents for the aromatic dinitro derivatives but could not be buffered satisfactorily because of the low solubility of sodium acetate. *t*-Butyl alcohol-water was also tried but proved to be too poor a solvent which led to inconveniently long electrolysis times. The most convenient mixture was 1:1 DMF-water which even so made work-up difficult in some cases because of its relatively low volatility and good dissolving power for the products. The present results show clearly that care must be exercised when alcohols and acetone (which are common co-solvents for polarographic work [2,3] in aqueous media) are considered for use in preparative electrolysis water-organic solvent mixtures.

Because of its thermal instability the formaldehyde derivative (IIIa) could not be isolated with a sufficient purity for elemental analysis. Evaporation of an ethereal solution at below 10 °C gave a solid material from which a satisfactory ^1H NMR spectrum was obtained. It was stored for several weeks at below −20 °C but deteriorated rapidly to a brownish tar at room temperature.



IIa IIIa V

From Table 1 it is seen that the condensation goes well with the lower aliphatic aldehydes, acetone, and cyclopentanone. Higher straight-chain methyl ketones, cyclohexanone and some heterocyclic aldehydes were also found to react, a longer reaction time being necessary with the ketones.

Surprisingly benzyl methyl ketone did not condense, even after 10 days, and the same was true for diethyl ketone and cycloheptanone. The sterically hindered ketone methyl isopropyl ketone did not react nor did two aromatic ketones (acetophenone and α,α,α-trifluoroacetophenone). Aromatic aldehydes, exemplified by benzaldehyde and *p*-nitrobenzaldehyde failed to condense (benzaldehyde was even tried at 70 – 80 °C for two days with negative result). It is, however, known that 2,3-dihydroxylamino-2,3-dimethylbutane needs more severe conditions to condense with aromatic aldehydes to give a 5-membered ring.[12] Despite the fact that 4-methyl-2-thiazolealdehyde and 4-pyridinealdehyde did react, no condensed product could be isolated from 2-pyridinealdehyde, *N*-benzyl-2-imidazolealdehyde and 5-nitro-2-furanaldehyde. The deuterated derivative IIIi and the dimethyl derivatives IIIn and IIIo were prepared for use in a MS-investigation,[13] but the corresponding cyclopentanone derivative could not be obtained. Cyclobutanone did react with IIa but TLC analysis showed the presence of several products and the characteristic hydroxyl proton signal did not feature in the ^{1}H NMR spectrum of the product mixture. The nature of the products has not yet been investigated. Negative results were also found with a number of α,β-unsaturated carbonyl compounds.

Catalytic reduction. A few condensations initiated by hydrogenation were performed to allow comparison with the electrochemical procedure. It has previously been found [5] that the yields of IIIb and IIIc were in the range 26 – 37 %, and that the use of larger aliphatic aldehydes to condense with the intermediate II gave poor results. However, when the hydrogenation of I was carried out in a mixture of acetone and acetate buffer, IIIf could be isolated in 76 % yield. This is similar to that obtained with much less acetone by the electrochemical method. A lower yield of 61 % was found with cyclopentanone under similar conditions, but the corresponding electrochemical yield of 90 % of IIIj is superior. These results suggested that a more general investigation of the hydrogenation method would be unrewarding.

It may well be that a catalytic hydrogenation procedure can be devised for the more reactive carbonyl compounds in which case a medium of pH 5 – 6 is probably preferable to the neutral conditions originally used.[5] For the less reactive ketones it would be necessary to use a catalyst which does not promote the further reduction of the bishydroxylamine II to the corresponding 2,2′-diaminophenyl. Apart from the simple experimental procedure the advantage of the controlled potential electrolysis lies in the fact that a stable solution of the intermediate II can be prepared thus allowing the use of carbonyl reagents with other reducible functions in the moecule.

Properties of products. The tentative assignment [5] of the *N*,*N*′-dihydroxydiazepine structure III for the condensation products is supported by the interpretation of the fragmentation pattern evident in the mass spectra of members of this family of compounds.[1,13] Further spectroscopic and chemical evidence is presented below which supports the 7-ring structure, and so far all of the information collected is consistent with this conclusion. However, the direct proof which would follow from an X-ray crystal structure determination has yet to be obtained. The symmetric acetone derivative IIIf has been selected for this purpose, and a structure determination has been undertaken in collaboration with the Inorganic Department of the University of Aarhus. Suitable single crystals could be grown from isopropanol solution, and preliminary findings show that IIIg crystallizes in the monoclinic space group $P2_1/c$ with 2 asymmetric units and the following cell constants: $a = 18.78$ Å, $b = 15.53$ Å, $c = 10.04$ Å, $\cos\alpha = \cos\gamma = 0$, $\cos\beta = -0.412$.

[13] *C NMR analysis.* The best evidence to date comes from the proton decoupled Fourier Transform (FT) ^{13}C NMR spectrum of IIIg (Fig. 1) in which the numbering of the biphenyl system has been retained for simplicity. The compound has a total of 15 C-atoms and the presence of only 8 lines clearly points to a symmetric structure. The intensity ratio for seven pairs and one single carbon would in

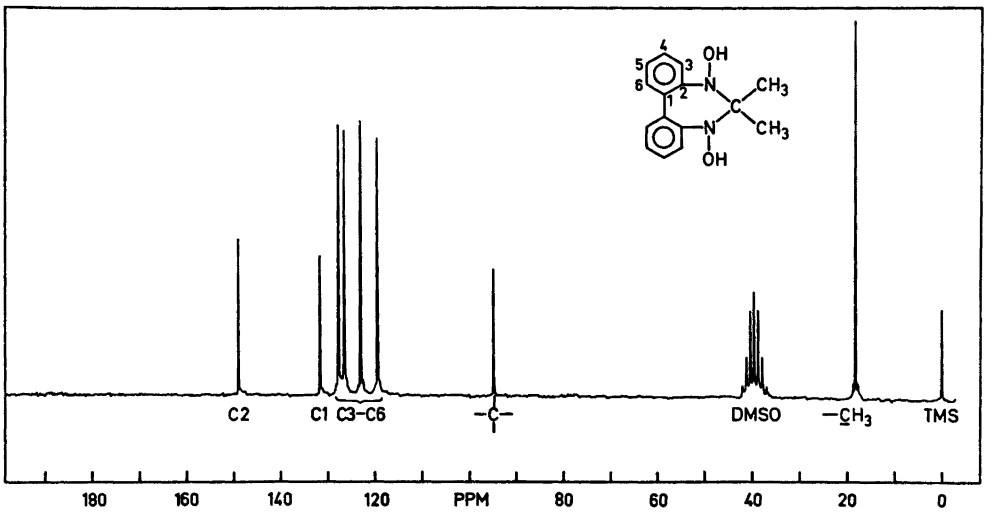


Fig. 1. ^{13}C NMR spectrum of 6,6-dimethyl-5,7-dihydroxy-6,7-dihydrodibenzo[*d,f*][1,3]diazepine IIIf in DMSO-d_6.

practice differ from 2:2:2:2:2:2:1:2 because of differences in the nuclear Overhauser effect which tend to give lines from nuclei bound to hydrogen an enhanced intensity.[14]

By considering likely substituent effects on chemical shifts[15] the interpretation of the spectrum is straightforward. The line at lowest field occurs in the region observed for C1 in aniline and is attributed to C2 bearing the hydroxylamine function. The next line upfield (C1) is due to the quaternary biphenyl carbon, and the group of 4 lines (C4-C6, C5 and C3, respectively) correspond to the remaining biphenyl carbons attached to protons and these carbon signals show an increased intensity relative to C1 and C2. The next upfield signal originates from the quaternary aliphatic carbon, showing more than half of the intensity of C1, and the last and most intense line corresponds to the methyl carbons.

Open structures and a 6-ring (with oxygen in the ring) are not symmetric and thus could not give a simple 8 line spectrum. Symmetry arguments, however, could not distinguish between a 7- and a 9-membered ring, but the 9-ring is highly improbable both because of the presence of absorption lines attributed to the NOH function in the ^1H NMR and IR spectra (see below), and because the nitrogen atom in hydroxylamines is known to be the nucleophilic centre in similar additions to carbonyl compounds. Furthermore IIIf is easily acetylated at the NOH groups (see below and experimental).

IR, ^1H NMR and UV-spectra. Cyclic and acyclic *N,N'*-dihydroxyamines have been studied by Zinner and Kliegel[16] and their spectroscopic data are similar to those found for the derivatives under study here thus confirming the presence of the NOH functions. The IR-spectra (Table 2) of nearly all the *N,N'*-dihydroxydiazepines IIId–IIIo show a strong absorption due to OH in the range 3230–3280 cm^{-1} in all cases with nearly the same intensity. An absorption with exactly the same shape and intensity was found at 3240 cm^{-1} for the known 1,3-dihydroxy-2-phenyl-4,4,5,5-tetramethylimidazoline.[17]

The ^1H NMR spectra (Table 2) of the diazepines IIId–IIIo showed the same pattern as found previously.[5] However, for the dimethylated derivative (IIIn) two NOH signals were observed at room temperature which coalesced on heating above *ca.* 80 °C.

All of the diazepines show UV absorption at 231–239 nm (Table 2) which is characteristic of benzene rings. For the aldehyde derivatives with unsubstituted benzene rings (IIId, IIIe, and IIIl and IIIm) the long wavelength absorption falls within the range 291–296 nm.

Table 2. Spectral properties of dibenzo[*d*,*f*][1,3]diazepine derivatives.

Compound	IR absorption spectra in KBr NOH band in cm^{-1}	UV absorption spectra in abs. ethanol λ_{max} nm	(log ε)	NMR spectral assignments chemical shifts in τ ppm[b] J in cps, recorded in DMSO-d_6, internal ref. TMS.
IIId	3260	295	(3.45)	τ 9.50–8.17 (m, 7 H, alkyl H), τ 5.40
		232	(4.34)	(t, 1 H, CH), τ 3.08–2.42 (m, 8 H, aryl H), τ 1.63 (s, 2 H, NOH)
IIIe	3280	296	(3.42)	τ 9.55–8.00 (m, 9 H, alkyl H), τ 5.45
		231	(4.35)	(m, 1 H, CH), τ 3.10–2.45 (m, 8 H, aryl H), τ 1.29 (s, 2 H, NOH)
IIIf	3260	285	(3.56)	τ 8.48 (s, 6 H, CH_3), τ 3.02–2.45 (m, 8 H,
		233	(4.54)	aryl H), τ 1.48 (s, 2 H, NOH)
IIIg	3260	289 sh[a]	(3.26)	τ 9.06 (t, J=7.5, 3 H, CH_3), τ 8.62 (s, 3
		234	(4.24)	H, CH_3), τ 7.96 (q, J=7.5, 2 H, CH_2), τ 3.05–2.40 (m, 8 H, aryl H), τ 1.55 (s, 2 H, NOH)
IIIh	3250	290 sh	(3.56)	τ 9.20 (t, 3 H, CH_3), τ 8.61 (s, 3 H, CH_3), τ
		235	(4.43)	7.90–8.75 (m, 4 H, CH_2), τ 3.06–2.41 (m, 8 H, aryl H), τ 1.55 (s, 2 H, NOH)
IIIi	3270	285	(3.59)	τ 3.05–2.40 (m, 8 H, aryl H), τ 1.46
		233	(4.56)	(s, 2 H, NOH)
IIIj	3250	286	(3.24)	τ 8.21 (m, 4 H, CH_2), τ 7.94 (m, 4 H, CH_2),
		233	(4.22)	τ 2.93–2.34 (m, 8 H, aryl H), τ 1.42 (s, 2 H, NOH)
IIIk	3240	307	(3.48)	τ 8.70–7.85 (m, 10 H, CH_2), τ 2.93–2.50
		239	(4.44)	(m, 8 H, aryl H), τ 1.78 (s, 2 H, NOH)
IIIl	3340(sharp)	297 sh	(2.91)	τ 4.46 (s, 1 H, CH), τ 2.97–2.52 (m, 10 H,
		252 sh	(3.42)	aryl H), τ 1.55 (d, d, J=1.5, 4.5, 2 H,
		231	(3.81)	H_2 and H_6 τ 1.15 (s, 2 H, NOH)
IIIm	3540(sharp)	295 sh	(2.51)	τ 7.62 (d, J=1, 3 H, CH_3) τ 4.15 (d, J=1,
	3150	258 sh	(3.05)	1 H, alkyl CH), τ 2.93–2.50 (m, 9 H,
		237	(3.45)	aryl H), τ=1.48 (s, 2 H, NOH)
IIIn	3260	279 sh	(3.79)	τ 8.80 (d, J=5.2, 3 H, CH_3), τ 7.94 (s, 3 H,
		234	(4.29)	aryl CH_3), τ 7.90 (s, 3 H, aryl CH_3), τ 5.72 (q, J=5.2, 1 H, CH), τ 3.11–2.53 (m, 6 H, aryl H), τ 1.63 and τ 1.58 (s, s, 2 H, NOH)
IIIo	3240	279 sh	(3.44)	τ 8.79 (s, 6 H, alkyl CH_3), τ 7.93 (s, 6 H,
		235	(4.34)	aryl CH_3), τ 3.13–2.65 (m, 6 H, aryl H), τ 1.45 (s, 2 H, NOH)
V	3200	305	(3.72)	τ 5.37 (s, 1 H, CH), τ 2.90–2.35 (m, 4 H,
		249	(4.39)	aryl H), τ 2.04–1.81 (m, 2 H, aryl H), τ
		243	(4.40)	1.35–1.00 (m, 2 H, aryl H), τ 0.93 (s)
		231	(4.42)	1 H, NOH)

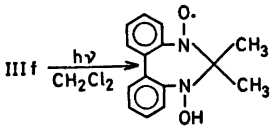
[a] sh, shoulder. [b] s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet.

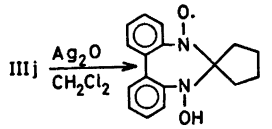
For the ketone derivatives (IIIf–IIIi) this absorption is in the range 285–290 nm, the cyclohexanone derivative being an exception. When the benzene rings are substituted (IIIn and IIIo) a hypsochromic shift to 279 nm is observed.

The spectral observations for V (Table 2) are also in good accord with the proposed structure, which is further supported by its mass spectrum (M^+: m/e 210) in which a large peak at m/e = 193 due to the $M^+ - OH$ ion is present together with the benzo[*c*]cinnoline ion (m/e 180) and the expected [1] peaks at m/e 166 and 167.

ESR-spectroscopy. It was originally reported [5] that oxidation of the bishydroxylamines IIIb and IIIc gave a mixture of relatively stable radicals. When IIIf was irradiated at room temperature with UV light in methylene chloride solution in the cavity of an ESR-spectrometer, strong signals appeared com-

prising a triplet with $a_N = 14.2$ G which compares well with $a_N = 14.0$ for the *t*-butyl-*o*-tolyl nitroxide radical.[18] The radical was quite stable and the ESR spectrum contained no hyperfine splitting, facts which are consistent with the postulation of the nitroxide:





Similarly oxidation of the spiro cyclopentane derivative IIIj either with lead dioxide in benzene on with silver oxide in methylene chloride yielded the corresponding nitroxide with a characteristic triplet in the ESR spectrum ($a_N = 13.7$ G). The use of other reagents such as alkaline DMSO [19] for oxidation of the bishydroxylamines III gave in most cases radicals or mixtures of radicals showing rather complex (often time-dependent) unsymmetrical ESR-signals. The spectra have not yet been assigned fully but the line pattern did not vary with the 6-substituent. The possibility of stabilizing the radicals by appropriate substitution in the biphenyl rings is currently under investigation.

Chemical reactions. The compounds have been formulated as cyclic secondary bishydroxylamines and their thermal and chemical stability has been found to depend strongly on the nature of substituents in the 6-position. Thus a pure sample of the unsubstituted derivative IIIa has not been obtained and the aliphatic aldehyde derivatives are relatively unstable and difficult to handle especially when impure.

The heteroaromatic aldehyde products and the ketone derivatives are relatively stable and a preliminary exploration of their chemistry has been attempted. From the ESR-work described above it is known that all of the derivatives can be easily oxidized chemically, but the products have not yet been isolated and identified. The compounds are also electroreducible (see above) although the electrode reaction has not been investigated.

The aldehyde derivatives would be expected to undergo dehydration under suitable conditions, but so far our attempts have been unsuccessful, and the parent compound V has been obtained indirectly in a side reaction as mentioned above.

Acylation of the acetone product IIIf by treatment with an excess of acetic anhydride gave the bis-acetylated derivative VI in 80 % yield. Product VI was also obtained by reaction with one equivalent of acetic anhydride, and contained none of the monoacetyl derivative. This correlates well with the findings of Zinner and Wilwing [20] who recently acylated a series of 1,3-dihydroxy-4,4,5,5-tetramethylimidazolines. Attempted acetylation of the aliphatic aldehyde products under the same conditions failed, and only black tars were obtained.

EXPERIMENTAL

Apparatus. The electrochemical and MS euipment has been described.[21,1] Microanalyses were carried out at the Microanalytical Laboratory of the University of Copenhagen by Mr. P. Hansen and by Dr. A. Bernhardt, Mülheim/Ruhr, BRD. Melting points (uncorrected) were determined on a Büchi melting point apparatus. IR-spectra were recorded using a Perkin-Elmer Model 137 or Model 457 spectrophotometer and UV-spectra were recorded using a Beckman Acta III spectrophotometer. ^{1}H NMR spectra were recorded using a JEOL C-60 HL or a Varian A-60 NMR spectrometer. The FT ^{13}C NMR spectrum of IIIg was measured at Varian Ass., Zug, Switzerland, using a Varian XL-100 NMR spectrometer. ESR-spectra were recorded using a Varian E-3 EPR spectrometer. Unit cell constants for IIIf were determined using a Picker four-circle diffractometer.

Materials. 2,2′-Dinitrobiphenyl (Ia) was obtained from Aldrich Chemical Company, Inc. and used without further purification and 6,6′-dimethyl-2,2′-dinitrobiphenyl (Ib) was prepared by Lothop's method.[22]

The aldehydes and ketones were obtained commercially and were mostly used without further purification. The preparation of the heteroaromatic aldehydes has been described [11,23] and 5-nitro-2-furanaldehyde was prepared from the diacetate.[24]

Preparative scale electro-reduction; general procedure. A conventional H-type cell of about 175 ml catholyte volume was used immersed

in a water bath and the cathode potential was controlled using a Juul Electronic potentiostat. Usually a suspension of 1.0 or 2.0 g of Ia (or Ib) in an acetate buffer mixture (A, B, or C, see below) was reduced at room temperature at potentials more positive than −0.8 V *vs.* Ag/AgCl with consumption of approximately 8 F/mol. Nitrogen was bubbled slowly through the catholyte, the middle compartment of the cell was filled with saturated aqueous potassium chloride, and the anolyte was 1:1 (approx.) ethanol (resp. methanol or DMF) – water saturated with sodium chloride. An excess (usually 5 ml or 5 g) of the carbonyl compound was added either before or after the electrolysis which was normally of 1 – 2 days duration. The catholyte was kept under nitrogen for further 1 – 7 days to allow completion of the cyclization reaction. Three different aqueous solvent mixtures were used:

A: 60 % EtOH, 0.2 M acetic acid, 0.4 M potassium acetate
B: 80 % MeOH } 15 g sodium acetate +
C: 50 % DMF } 5 ml acetic acid/200 ml

For electrolyses involving greater amounts of starting material additional acetic acid was added during the reduction. The products from the aliphatic aldehydes were usually soluble in the reaction mixture whereas those from the ketone and heteroaromatic aldehydes were partially precipitated. The catholyte was diluted with 1 – 2 volumes of water (except in the formaldehyde case) and cooled to −15 °C (except for C which was cooled to 0 °C). The precipitate was removed by filtration, washed with water, and dried under reduced pressure over silica gel. The filtrate was worked up by partial evaporation, neutralisation, and shaking with ether which led to the extraction of a small crop of usually impure product contaminated with IV. The extraction procedure (without prior dilution with water) was used in some of the early experiments but purer products (free from IV) are obtained by the precipitation method even though some material is lost with the filtrate. For the formaldehyde derivative IIIa, however, the extraction procedure is unavoidable, and in the final evaporation of the ethereal solution the temperature must be kept below 10 °C to avoid decomposition of the product which should then be kept in a refrigerator. Table 3 summarises microanalytical results, melting points and recrystallisation solvent.

Benzo[c]*cinnoline-N-oxide (IV).* Ia (1.0 g) was reduced as described above (solvent B), and after the electrolysis oxygen was bubbled through the catholyte (4 h). The methanol was

Table 3. Melting points and analytical results for the dibenzo[*d*,*f*][1,3]diazepine derivatives.

Compound	Melting point °C	Solvent of recrystallization	Formula	Analyses Calculated	Found
IIId	120 – 122 d	methylcyclohexane	$C_{16}H_{18}N_2O_2$	C 71.09, H 6.71, N 10.36	C 70.91, H 6.85, N 10.17
IIIe	110 – 112 d	ether-pentane	$C_{17}H_{20}N_2O_2$	C 71.80, H 7.09, N 9.85	C 72.00, H 7.28, N 9.67
IIIf	231 – 232 d	benzene	$C_{15}H_{16}N_2O_2$	C 70.29, H 6.29, N 10.93	C 70.46, H 6.39, N 11.00
IIIg	188 – 189 d	benzene	$C_{16}H_{18}N_2O_2$	see above	C 70.89, H 6.56, N 10.22
IIIh	202 – 203 d	benzene	$C_{17}H_{20}N_2O_2$	see above	C 71.60, H 7.10, N 9.95
IIIi	227 – 230 d	benzene	$C_{15}H_{10}D_6N_2O_2$	C 68.70, D+H 6.15, N 10.67	C 68.54, D+H 6.34, N 10.79
IIIj	202 – 204 d	ethanol	$C_{17}H_{18}N_2O_2$	C 72.32, H 6.43, N 9.92	C 72.09, H 6.34, N 9.99
IIIk	175 – 178 d	benzene	$C_{18}H_{20}N_2O_2$	C 72.95, H 6.80, N 9.45	C 72.81, H 6.66, N 9.60
IIIl	215 – 217 d	methanol	$C_{16}H_{16}N_3O_2$	C 70.80, H 4.95, N 13.76	C 70.60, H 4.96, N 13.87
IIIm	185 – 186 d	ethanol-water	$C_{17}H_{15}N_3O_2S$	C 62.75, H 4.65, N 12.91, S 9.85	C 62.70, H 4.65, N 12.83, S 9.72
IIIn	145 – 147 d	methylcyclohexane-benzene	$C_{16}H_{18}N_2O_2$	see above	C 70.96, H 6.79, N 10.17
IIIo	187 – 189 d	benzene	$C_{17}H_{20}N_2O_2$	see above	C 72.00, H 6.92, N 10.03
V	186 – 188 d	benzene	$C_{13}H_{10}N_2O$	C 74.27, H 4.79, N 13.33	C 74.07, H 4.92, N 13.53

evaporated *in vacuo*, the precipitate filtered off, washed with water, and dried over silica gel giving 0.8 g of light yellow material m.p. 117–119 °C, identified as nearly pure IV by comparing its IR and ^{1}HNMR spectrum with those of an authentic specimen.

5-Hydroxydibenzo[d,f][*1,3*]*diazepine (V).* Ia (1.0 g) was reduced in the presence of benzyl methyl ketone (3.0 ml, solvent B) as described above and kept at room temperature under nitrogen for totally 4 days. The clear yellowish catholyte was partially evaporated *in vacuo*, neutralized by addition of solid sodium hydrogencarbonate, and extracted twice by ether (100 ml + 50 ml). Removal of the solvent after drying over magnesium sulphate left a semi-solid residue (1.4 g) which by recrystallisation (chloroform – petrol ether) gave 0.4 g of crude product, m.p. 153 – 5°d. TLC showed some IV to be present, and an analytically pure sample (0.12 g, bright yellow needles, m.p. 186 – 8°d) was obtained by recrystallizing 0.32 g of the crude product twice from benzene.

Catalytic reductions

6,6-Dimethyl-5,7-dihydroxy-6,7-dihydrodibenzo[d,f][*1,3*]*diazepine (IIIf).* A mixture of 2,2′-dinitrobiphenyl (Ia), (5.0 g), 50 ml of acetone, 100 ml of aqueous acetate buffer (0.5 M acetic acid, 1.0 M potassium acetate) and 0.5 g of 10 % palladium on barium sulfate were shaken for 20 h with hydrogen (1.05 atm) at room temperature. Subsequent addition of 100 ml of water to this suspension and filtration gave 4.5 g of a mixture of IIIf and the catalyst, *i.e.* the yield of IIIf was 4.0 g (76 %). Recrystallization from benzene (1.5 l) gave white needles (3.64 g), m.p. 231 – 232 °C, d.

5,7-Dihydroxy-6,7-dihydrodibenzo[d,f][*1,3*]*diazepine-6-spirocyclopentane (IIIj).* Cyclopentanone (10 ml) and ethanol (50 ml) were hydrogenated as above. Similar work-up gave grey crystals (4.05 g) corresponding to 3.55 g (61 %) of crude IIIj. Recrystallization of this material from benzene (1.4 l) yielded white crystals (0.91 g, 16 %), m.p. 178 – 180 °C, d. Concentration of the mother liquor yielded additional impure material (0.6 g). Recrystallization of the first crop gave analytically pure IIIj m.p. 202 – 204 °C, d.

Acetylation

6,6-Dimethyl-5,7-diacetoxydibenzo[d,f][*1,3*]*diazepine (VI).* IIIf (0.15 g) was stirred for 1 h at 50° with a mixture of acetic anhydride (15 ml) and one drop of pyridine. Concentration under reduced pressure and washing with pentane yielded pale grey crystals of VI (0.16 g, 80 %) m.p. 128 – 130 °C. Recrystallization of a sample from methanol gave VI as white crystals, m.p. 140 – 141 °C, d. IR (KBr): Strong absorption at 1775 cm^{-1} (ester C=O). UV (abs. ethanol): λ 288 (sh, log ε 2.26), λ 254 (sh, log ε 3.94) and λ_{max} 231 (log ε 4.37). NMR (DMSO-d_6): τ 8.51 (s, 6 H, CH_3), τ 8,00 (s, 6 H, $OCOCH_3$) and τ 2.70 – 2.45 (m, 8 H, aryl H).

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Diels-Alder Reactions of 2,4-Cyclohexadienones.* II.** Stereoselectivity in the Dimerisation of *o*-Quinols

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Chemical evidence is presented for the steric arrangement at carbon atoms 5 and 9 of *o*-quinol dimers.

Formation of the phenolic compounds *9*—*11* on treatment of the diacetates of dimers *1*—*3* with ethanolic potassium hydroxide is interpreted as being due to an E2 elimination of acetic acid requiring *trans*-position of the acetoxy group at C-5 with regard to the hydrogen atom at C-4a.

Furthermore, it is shown that the C-9, C-10 ketol bridge in dimers *1*—*3* is cleaved by periodate, whereas dimers *4* and *5* are stable towards the same oxidant. Considering the accepted mechanism of ketol cleavage by periodate, these results indicate that the hydroxyl group at C-9 is oriented as shown in formulae *1*—*5*.

The structural interpretations given in this paper are in harmony with the structures of dimers *1*, *2* and *4* earlier established by X-ray crystallography.

Periodate oxidation of 2,6-,[4] 2,5-,[2] and 2,4-dimethylphenol [3] as well as of 2,4,6-trimethylphenol [4] results in the formation of the corresponding 6-hydroxy-6-methyl-2,4-cyclohexadienones ("*o*-quinols"), which rapidly dimerise by Diels-Alder reaction to give compounds *2*—*5*. The parent compound (*1*) [5] of these methyl homologous dimers has been obtained by acid hydrolysis of 6-acetoxy-6-methyl-2,4-cyclohexadienone which, in turn, was prepared by treatment of *o*-cresol with lead tetraacetate. In each case, only one of several possible isomers with different structural and steric orientation has been detected, indicating that these Diels-Alder dimerisations are regiospecific and stereospecific or at least highly selective in both respects.

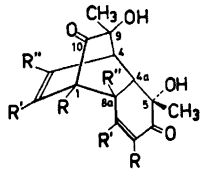
As reported in the preceding paper,[2] the structure of the parent dimer (*1*) was established by the finding that it is reduced by Raney nickel to a product which is also obtained from a dibromo derivative (*1*, CH_2Br instead of CH_3),[6] the structure of which had been determined by X-ray analysis.[7] After completion of the work described in the present paper (*cf.* also preliminary communication [1]), X-ray analyses of dimers *2* and *4* were reported; they showed these dimers to be analogous to *1* with regard to both structural orientation and the steric arrangement of the *tert.* carbinol groups (C-5 and C-9).[8]

Although it seemed rather likely that the *o*-quinol dimers obtained from 2,5-dimethylphenol and 2,4,6-trimethylphenol, *i.e.* dimers *3* and *5*, would have the same structural and steric characteristics as dimers *1*, *2* and *4*, experimental confirmation was desirable. The structural orientation of the diene and dienophile moieties in dimer *5* has been clarified earlier from its NMR spectrum.[1,2] Furthermore, this orientation as well as *endo* configuration of *5* has been established by photochemically induced intramolecular cycloaddition.[9] In the present paper, chemical evidence regarding the stereochemistry of C-atoms 5 and 9 in the *o*-quinol dimers will be presented.

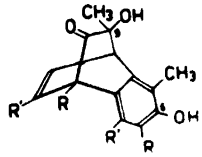
Steric arrangement at C-5. — In previous work [4] it was shown that the diacetates *6* and *7* of *o*-quinol dimers *1* and *2* on treatment with ethanolic potassium hydroxide give the phenolic

* Part XII in the series "Periodate Oxidation of Phenols". Preliminary communication, see Part X.[1]

** Part I, see Ref. 2.



1 R=R′=R″=H
2 R=CH_3; R′=R″=H
3 R′=CH_3; R=R″=H
4 R″=CH_3; R=R′=H
5 R=R″=CH_3; R′=H
6=*1*, OAc instead of OH
7=*2*, OAc instead of OH
8=*3*, OAc instead of OH



9 R=R′=H
10 R=CH_3; R′=H
11 R′=CH_3; R=H
11a=*11*, OAc instead of 6-OH
11b=*11*, OAc instead of 6-OH and 9-OH

compounds *9* and *10* in addition to the non-acetylated dimers. Similarly, phenol *11* has now been obtained from the diacetate *8* [2] of dimer *3*.

Kende and MacGregor [10] found that the fluorine-containing analogue of dimer *2* (F instead of OH), when treated with KOH-C_2H_5OH, is converted into the fluorine analogue of phenol *10* (F instead of the *tert.* OH). Assuming this reaction to be due to E2 elimination of hydrogen fluoride from the unbridged ring, these authors concluded that the fluorine atom at C-5 is in a position *trans* to the hydrogen atom at C-4a, the favourable *anti* coplanar orientation of the leaving groups then being easily attained.

By analogy, the formation of phenols *9*–*11* from diacetates *6*–*8* suggested that OAc at C-5 is in a position *trans* to H at C-4a, favouring E2 elimination of HOAc * with concomitant aromatisation of the unbridged ring. For the *endo* forms of the corresponding non-acetylated dimers *1*–*3* the steric arrangement at C-5 then could be assumed to be as shown in the formulae given above. As mentioned earlier, this steric orientation as well as *endo* configuration has been finally established for dimers *1* [2,6,7] and *2* [8] by X-ray diffraction.

The base-catalysed elimination of acetic from the unbridged ring of diacetates *6*–*8* may be assumed to be preceded by proton abstraction from C-8a, aromatisation of the resulting enol constituting a driving force for the elimination (*cf.* also Ref. 10). The latter reaction then competes successfully with the hydrolysis of the C-5 acetoxy group.

In the diacetates of dimers *4* and *5*, however, enolisation and aromatisation of the unbridged ring are prevented by the methyl substituent at C-8a. Treatment of these diacetates with KOH-C_2H_5OH simply produces the corresponding dialcohols, no elimination of acetic acid being observed.[3,4]

Steric arrangement at C-9. In the periodate treatment of 2,6- [4] and 2,4-dimethylphenol [3] as well as of 2,4,6-trimethylphenol [4] both the formation of the *o*-quinols and their Diels-Alder dimerisation are fast reactions, consumption of the starting phenols and formation of dimers *2*, *4*, and *5* being complete after reaction times of a few minutes. These dimers contain two ketol groupings and therefore could be expected to consume further periodate undergoing ketol cleavage. In fact, prolonged treatment of 2,6-dimethylphenol with excess sodium metaperiodate or periodic acid produced a monocarboxylic acid (*13*).

Oxidation of 2,5-dimethylphenol with sodium periodate proceeds more slowly than that of the above-mentioned phenols, unchanged starting material still being detectable after reaction times of 1–2 h. From the reaction mixtures, dimer *3* as well as its cleavage product (*14*) was isolated. The latter was also obtained on oxidation of 2,5-dimethylphenol with periodic acid.

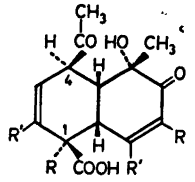
Acids *13* and *14* also formed when the isolated dimers *2* and *3*, respectively, were oxidised with periodate. Similar oxidation of dimer *1* [5] provided acid *12*.

Periodate as well as periodic acid thus cleaved only one of the two ketol groupings

* This view is supported by results discussed in a recent monograph according to which the *anti* mechanism is greatly preferred to the *syn* mechanism for E2 reactions in six-membered rings (Saunders, Jr., W. H. and Cockerill, A. F. *Mechanism of Elimination Reactions*, Wiley, New York 1973).

present in dimers *1–3*. The resulting monocarboxylic acids remained unchanged even on several hours' treatment with these oxidants.

Assignment of structures *12–14* is based on spectroscopic evidence. The UV spectra



12 R=R'=H
13 R=CH_3; R'=H
14 R'=CH_3; R=H

of *o*-quinol dimers *1–5* in ethanol exhibit a high intensity band with λ_{max} around 210 nm (ε about 10 000),[4] which can be ascribed to charge transfer within the β,γ-unsaturated carbonyl system (2,3-ethylenic bond and C-10 carbonyl group) in the photoexcited state[11] ("photodesmotic band"[12]); *cf.* also Ref. 6. They further show a comparatively strong absorption band around 310 nm (ε=150–330) involving the $n \rightarrow \pi^*$ absorption of the α,β-unsaturated carbonyl system as well as the characteristically intensified $n \rightarrow \pi^*$ absorption of the β,γ-unsaturated carbonyl system. In the spectra of acids *12–14*, however, the photodesmotic band is lacking, and the $n \rightarrow \pi^*$ bands (λ_{max} 328 nm) due to the α,β-unsaturated carbonyl grouping are of normal intensity (ε=83, 83, and 100 for *12*, *13*, and *14*, respectively). The $\pi \rightarrow \pi^*$ absorptions of the latter chromophores in acids *12–14* are very similar to those reported[4] for the corresponding dimers *1–3*, their locations being in harmony with the predicted[13] ones. Finally, the $n \rightarrow \pi^*$ absorption of the CO group at C-9 in *12–14* is partially overlapped by the $\pi \rightarrow \pi^*$ and the $n \rightarrow \pi^*$ bands of the conjugated carbonyl system and gives rise to shoulders at λ_{max} 280, 285, and 295 nm for *12*, *13*, and *14*, respectively.

The UV spectra of the methyl esters prepared from acids *12–14* were closely similar to those of the free acids.

Thus, UV spectroscopy clearly indicated that of the two ketol groupings present in dimers *1–3* the 9,10-ketol bridge was cleaved specifically. This finding is corroborated by the following IR spectroscopic data (Table 1).

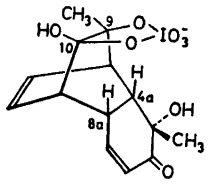
Cleavage of the 9,10-ketol bridge in dimers *1–3* gives rise to a non-conjugated carboxylic acid containing an unconjugated keto group as well as the conjugated carbonyl group initially present. Cleavage of the 5,6-hydroxyketone grouping, however, would produce an α,β-unsaturated carboxylic acid containing two non-conjugated keto groups. The locations of the lowest frequency bands of the acids and their methyl esters (Table 1) agree with those expected for α,β-unsaturated keto groups (*cf.* the corresponding bands for dimers *1–3*) but are below the range typical of α,β-unsaturated COOH (1690–1715 cm^{-1})[14] and far below the range accepted for α,β-unsaturated esters (1717–1730 cm^{-1}),[14] respectively.

Contrary to dimers *1–3*, dimers *4* and *5* proved to be stable towards periodate. The presence of a methyl substituent at C-8a in

Table 1. Infrared absorption bands (cm^{-1}, KBr) of *o*-quinol dimers *1–3*, carboxylic acids *12–14* and methyl esters *12a–14a*.

Substance	CO (C-10)	CO (at C-4)	COOH	$COOCH_3$	α,β-unsaturated CO
Dimer *1*	1723				1679
Acid *12*		1733	1700		1682
Ester *12a*		1720		1742	1679
Dimer *2*	1723				1680
Acid *13*		1720	1695		1672
Ester *13a*		1716		1729	1670
Dimer *3*	1724				1662
Acid *14*		1728	1710		1640
Ester *14a*		1720		1730	1663

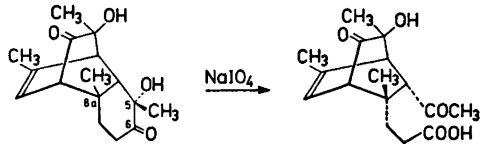
the two latter dimers suggests the following explanation for this striking difference in the behaviour of the two groups of dimers.



15

According to Bunton and Shiner,[15] periodate cleavage of α-hydroxy ketones is initiated by nucleophilic attack of periodate oxygen upon the carbonyl group, probably accompanied by co-ordination of the α-hydroxyl group to the periodate, resulting in the formation of a cyclic periodate ester intermediate.[16] Since the stereochemistry of the C-9 carbinol group is established for dimers *1* [2] and *2* [3] (see formulae), and formation of the cyclic intermediate requires approach from the side of the C-9 hydroxyl group, the structure of this intermediate will be as illustrated by formula *15*.

The resistance to periodate of dimers *4* and *5* then suggested that these dimers have the same steric arrangement at C-9 as dimer *1*, the C-9 hydroxyl group being directed towards the C-4a, C-8a linkage.[1] With this arrangement it is easily understood that the methyl group at C-8a in *4* and *5* by steric hindrance prevents the periodate to approach the ketol bridge and to form intermediates of type *15*.* As mentioned earlier, this configuration of C-9 has been confirmed in the case of dimer *4* by X-ray analysis.[8]



16 *17*

* If the configuration of C-9 would be opposite to that given in formulae *1*–*5*, periodate cleavage would be possible for all dimers. Inspection of Dreiding models indicates that, in this case, the C-10 hydroxyl group of the cyclic intermediate corresponding to *15* would not sterically interfere with the C-8a methyl group.

The fact that neither the α,β-conjugated ketol group of the *o*-quinol dimers *1*–*5* nor that present in acids *12*–*14* is cleaved by periodate may be explained by the assumption that in the conjugated system the carbonyl C-atom (C-6) is not sufficiently electropositive to be susceptible to attack by periodate oxygen. Steric hindrance could also be considered at least in the case of the rather rigid dimers but seems less probable in the acids *12*–*14*. The importance of the α,β-conjugation is strongly supported by the finding that the 7,8-dihydro derivative *16* [3] of dimer *4* on treatment with periodate suffers ketol cleavage between C-5 and C-6 to yield monocarboxylic acid *17*. Again, the methyl group at C-8a prevented the 9,10 ketol bridge from being affected.

Conclusions

The results presented above provide chemical evidence for the steric arrangement at carbon atom 5 of *o*-quinol dimers *1*–*3* as well as at carbon atom 9 of *o*-quinol dimers *4* and *5*. These assignments are in agreement with the completely established structures of dimers *1*, *2* and *4*.[2,8] It then seems justified to assume that the steric arrangements at C-9 and C-5 of dimers *3* and *5*, respectively, for which there is no direct experimental proof, are analogous to those found in dimers *1*, *2*, and *4*.

In all cases, therefore, the stereochemical orientation at C-5 and C-9 is the same and is certainly due to steric approach control in the dimerisation of the *o*-quinols, the bulky methyl groups being directed away from the reaction center. The same principle holds true for the formation of dimers from other 2,4-cyclohexadienones containing an asymmetric C-atom in the 6-position. Thus, in the Diels-Alder dimers of spiro(oxirane-2,4-cyclohexadienones)[6,3] the CH_2-O groupings of the oxirane rings at C-5 and C-9 have been shown to be oriented analogously to the CH_3,OH substituents at the same C-atoms of the *o*-quinol dimers. Furthermore, dimerisation of the parent *o*-quinol acetate, 6-acetoxy-6-methyl-2,4-cyclohexadienone, and that of its 2-methyl homologue are known to give the diacetates (*6* and *7*) of compounds *1* and *2*, the latter compounds being the dimerisation products of the corresponding non-acetylated *o*-quinols.[5,4] Finally, in the halogen containing dimers described by Kende and MacGregor [10] (*2*, F instead of OH) and by Lindgren and Ericsson [17] (*2*, Cl instead

of OH) at least the arrangement at C-5 has been shown to be analogous to that now established for the *o*-quinol dimers.

From the results discussed in the preceding [2] as well as in the present paper it can be concluded that the Diels-Alder dimerisation of the 2,4-cyclohexadienones hitherto examined proceeds in a uniform fashion with regard to both regiospecificity and stereochemical specificity.

EXPERIMENTAL

UV spectra were recorded on a Cary Model 14 Spectrophotometer; IR and NMR spectra were obtained using Beckman 9A and Varian A-60 instruments, respectively. Chemical shifts are given in δ (ppm) units, TMS being used as internal standard.

1,4-Dihydro-6,9-dihydroxy-2,5,8,9-tetramethyl-[1,4]ethanonaphthalen-10-one (11).* The diacetate *8* [2] of *o*-quinol dimer *3* [2] (765 mg) was added to a 10 % solution of KOH in ethanol (25 ml), and the mixture was kept under nitrogen for 16 h. Ethanol was removed under vacuum, water being repeatedly added during the distillation. The alkaline aqueous mixture was extracted three times with dichloromethane; the extract gave dimer *3* [2] (13 %). When the aqueous phase was neutralised with CO_2, a crystalline product deposited, m.p. 201–202° after recrystallisation from ethanol; yield, 69 %. (Found: C 74.55; H 7.08. Calc. for $C_{16}H_{18}O_3$: C 74.40; H 7.02). UV spectrum (ethanol): λ_{max}, nm (log ε) 208 (4.37) (charge transfer band of β,γ-unsaturated CO system), 280 (3.25), 288 (3.25) (aromatic ring), 314 (2.91) (intensified $n \rightarrow \pi^*$ absorption of β,γ-unsaturated CO system). IR (KBr): ν_{max}, cm^{-1} 1513, 1600, 1616 (aromatic ring), 1715 (CO), 3425, 3356 (OH).

Monoacetate 11a* was obtained with acetic anhydride-pyridine. M.p. 153–154° (ethanol-water). (Found: C 71.84; H 6.65; CH_3CO 13.96. Calc. for $C_{18}H_{20}O_4$: C 71.98; H 6.71; CH_3CO 14.33).

Diacetate 11b* was prepared using Ac_2O-$HClO_4$ in ethyl acetate.[18] M.p. 134–135° (ethanol-water). (Found: C 70.02; H 6.38. Calc. for $C_{20}H_{22}O_5$: C 69.98; H 6.39).

4-Acetyl-1,4,4a,5,6,8a-hexahydro-5-hydroxy-5-methyl-6-oxo-1-naphthalenecarboxylic acid (12).** The solution of *o*-quinol dimer *1* [5] (200 mg) in acetic acid (25 ml) was mixed with a solution of sodium metaperiodate (0.75 g) in 60 % aqueous acetic acid (25 ml). After 2 h (room temp.) a few drops of ethylene glycol were added to reduce excess periodate, the solvent was removed under vacuum and the residue extracted with dichloromethane. From the extract acid *12*, m.p. 174–175° after recrystallisation from chloroform-hexane, was obtained. (Found: C 63.95; H 6.19. Calc. for $C_{14}H_{16}O_5$: C 63.63; H 6.10). UV (ethanol): λ_{max}, nm (log ε) 224 (4.01), sh 280 (2.08), 328 (1.92). IR (KBr): ν_{max}, cm^{-1}, see Table 1, and 2400–3300 (COOH), 3510 (OH). NMR (DMSO-d_6): δ 1.30 (s, 3 H, CH_3), 2.10 (s, 3 H, CH_3CO), 2.88 (2 H, 2 CH), 3.41 (2 H, 2 CH), 5.21 (broad s, 1 H, OH), 5.51, 5.92, 5.94 and 6.73 (doublets, 1 H each, 4 olefinic H).

Methyl ester 12a. Prepared by adding an ethereal solution of diazomethane to a methanolic solution of *12*. M.p. 142–143° (benzene-hexane). (Found: C 65.0; H 6.7. Calc. for $C_{15}H_{18}O_5$: C 64.7; H 6.5). UV (ethanol): λ_{max}, nm (log ε) 224 (3.99), sh 280 (2.05), 326 (1.92). IR (KBr): ν_{max}, cm^{-1}, see Table 1, and 1622 (C=C), 3480 (OH). NMR spectrum ($CDCl_3$) resembles that of acid *12*, but shows an additional signal at δ 3.79 (s, 3 H, ester-CH_3).

4-Acetyl-1,4,4a,5,6,8a-hexahydro-5-hydroxy-1,5,7-trimethyl-6-oxo-1-naphthalenecarboxylic acid (13). An aqueous solution (100 ml) of periodic acid (H_5IO_6, 41 g, 180 mmol) was added to a solution of 2,6-dimethylphenol (9.15 g, 75 mmol) in water (2250 ml). After 2 h (25°) ethylene glycol (7 g) was added and the solution extracted with six 250 ml portions of chloroform. The combined organic phases were washed with aqueous sodium thiosulphate, dried over anhydrous sodium sulphate and evaporated to dryness. The remaining oil on addition of ethyl ether (10 ml) gave colourless crystals (yield, 54 %, m.p. 180–182°) which after recrystallisation from ethanol melted at 183–184°. (Found: C 65.51, H 6.97; equiv. wt. 294. Calc. for $C_{16}H_{20}O_5$: C 65.74; H 6.90; equiv. wt. 292). UV (ethanol): λ_{max}, nm (log ε) 240 (3.87), sh 285 (2.11), 328 (1.92). IR (KBr): ν_{max}, cm^{-1}, see Table 1, and 1630 (C=C), 2300–3300 (COOH), 3410 and 3500 (OH). NMR (DMSO-d_6): δ 1.30 and 1.46 (singlets, 3 H each, 2 CH_3), 1.76 (t, 3 H, olefinic CH_3), 2.10 (s, 3 H, CH_3CO), 2.86 (broad s, 2 H, 2 CH), 3.30 (broad s, 1 H, CH), 5.12 (s, 1 H, OH), 5.50 and 5.74 (doublets, 1 H each, 2 olefinic H), 6.73 (broad s, 1 H, olefinic CH).

Methyl ester 13a. From *13* and diazomethane. After recrystallisation from benzene-hexane, m.p. 88–89°. (Found: C 66.68; H 7.29. Calc. for $C_{17}H_{22}O_5$: C 66.64; H 7.23.) UV (ethanol): λ_{max}, nm (log ε) 238 (3.87), sh 285 (2.07), 327 (1.93). IR (KBr): ν_{max}, cm^{-1}, see Table 1, and 1630 (C=C), 3462 (OH). NMR spectrum ($CDCl_3$) resembles that of acid *13*, but shows an additional signal at δ 3.75 (s, 3 H, ester-CH_3).

4-Acetyl-1,4,4a,5,6,8a-hexahydro-5-hydroxy-2,5,8-trimethyl-6-oxo-1-naphthalenecarboxylic acid (14). The acid was obtained from 2,5-dimethylphenol and periodic acid, using the procedure described above for acid *13*. Yield, 32 %, m.p. 205–206°. (Found: C 65.65; H 7.01. Equiv. wt. 294. Calc. for $C_{16}H_{20}O_5$: C 65.74; H 6.90. Equiv. wt. 292). UV (ethanol): λ_{max},

* Experiments carried out by tekn. lic. Ingrid Jansson.

** Experiment carried out by Dr. Britt Berggren.

nm (log ε) 237 (3.94), sh 295 (2.11), 328 (2.00). IR (KBr): ν_{max}, cm^{-1}, see Table 1, and 1610 (C=C), 2400–3300 (COOH), 3485 (OH). NMR (DMSO-d_6): δ 1.27 (s, 3 H, CH_3), 1.89 (s, 6 H, 2 olefinic CH_3), 2.09 (s, 3 H, CH_3CO), 2.92 and 3.37 (singlets, 2 H each, 4 CH), 5.06 (s, 1 H, OH), 5.35 and 5.84 (broad singlets, 1 H each, 2 olefinic H).

Methyl ester 14a. From *14* and diazomethane, m.p. 135–136° (benzene-hexane). (Found: C 66.44; H 7.25. Calc. for $C_{17}H_{22}O_5$: C 66.64; H 7.23). UV (ethanol): λ_{max}, nm (log ε) 236 (4.01), sh 287 (2.12), 320 (200). IR (KBr): ν_{max}, cm^{-1}, see Table 1, and 1620 (C=C), 3440 (OH). NMR spectrum ($CDCl_3$) resembles that of acid *14*, but shows an additional signal at δ 3.78 (s, 3 H, ester-CH_3).

Periodate oxidation of dimers 2 *and* 3. The dimers, dissolved in ethanol-water (2:3), were treated with a 2.5-fold excess of $NaIO_4$ for 16 h at room temperature. Chloroform extraction gave the almost pure acids *13* and *14* in yields of 66 and 62 %, respectively, identified by m.p., mixed m.p. and IR spectra.

3-Acetyl-8-hydroxy-2,5-dimethyl-7-oxo-bicyclo-[2.2.2]oct-5-ene-2-propanoic acid (17). A solution of $NaIO_4$ (1.28 g, 6 mmol) in 60 % aqueous acetic acid (35 ml) was added to a solution of dihydrodimer *16*[3] (0.83 g, 3 mmol) in the same solvent (150 ml). Extraction with dichloromethane (four 100 ml portions) after a reaction time of 18 h (25°) provided a crystalline solid, which was recrystallised from ethyl acetate to give 75 % of *17*, m.p. 193.5–194.5°. (Found: C 65.23; H 7.58. Calc. for $C_{16}H_{22}O_5$: C 65.29; H 7.53). UV (ethanol): λ_{max}, nm (log ε) sh 210 (3.58) (β,γ-unsat. CO), 308 (2.39) (CO and β,γ-unsat. CO). IR (KBr): ν_{max}, cm^{-1} 1713 (CO and COOH), 2400–3300 (COOH), 3400 (OH). NMR (DMSO-d_6): δ 1.12 and 1.22 (singlets, 3 H each, 2 CH_3), 1.94 (d, 3 H, olefinic CH_3), 2.15 (s, 3 H, CH_3CO), 1.85–3.50 (7 H, 2 CH_2 and 3 CH), 5.47 (s, 1 H, OH), 5.64 (d, further split by allylic coupling, 1 H, olefinic H).

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Preparation and Purification of Fructose-1,6-diphosphate

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Some investigations have been made concerning the accumulation of fructose-1,6-diphosphate (FDP) by yeasts. The concentration of glucose-6-phosphate and fructose-6-phosphate during fermentation has been determined. Optimum conditions for the precipitation of FDP have been determined and some of the impurities of Ca_2FDP have been analyzed. A new simple purification method is introduced.

Fructose-1,6-diphosphate (FDP) is a well-known intermediate of sugar metabolism. It is accumulated in a fermentation mixture of yeast, sugar, inorganic phosphate and toluene.[1,2] FDP is recovered from the solution usually as a Ca- or Ba-salt. Complete precipitation occurs when ethanol is added to the solution.[3] Crude salts contain many impurities derived from yeast and starting materials. FDP has been purified by acid and charcoal treatment [3,4] or by ion exchange.[5] The purity achieved by these methods has been 70 – 80 %. To obtain pure FDP it is usually converted to salts with organic bases.[6,7] The main aim of this work was to find optimum conditions for the precipitation of FDP and to develop a simple purification method.

MATERIAL AND METHODS

FDP was prepared by fermentation using fresh brewer's yeast, sucrose, NaH_2PO_4 and toluene. Optimum conditions for FDP accumulation were determined earlier [8] and were as follows: pH 6.6, temperature 30 °C, the amount of yeast (25 % dry matter) 500 g in 1000 g of reaction mixture, sucrose concentration 7 – 8 % (w/w), and concentration of $NaH_2PO_4.2H_2O$ 5 – 6 % (w/w). About 45 ml toluene in 1000 g of reaction mixture were needed for maximum FDP accumulation.

After fermentation the proteins were precipitated with 100 ml of 80 % trichloroacetic acid per 1000 g of reaction mixture. The mixture was centrifuged, neutralized and filtered. The concentration of FDP in the clear solution was 50 – 55 g/l. FDP was precipitated as a Ca-salt using 50 % (w/w) $CaCl_2$ solution. Final precipitation occurred when ethanol was added to the mixture. Ca_2FDP was filtered off from the mixture and washed with 70 % ethanol and subsequently with 94 % ethanol. The product was freeze-dried.

FDP was determined enzymatically according to the method of Bergmeyer.[9] Commercial $Na_3FDP.8H_2O$ (Boehringer Mannheim, GmbH) was used as standard. Inorganic and total phosphorus was determined by Allen's [10] method. Nitrogen content of the precipitated Ca_2FDP was determined by the Kjeldahl method with an ammonia specific electrode. A thermoanalyzer was used to determine the water content of Ca_2FDP. Fermentable sugars [11] and sugar phosphates [12] were separated by ion exchange and determined by an autoanalyzer [13] using the anthrone method. The flow sheet of the reverse-osmosis system used in the purification tests is presented in Fig. 1. Cellulose acetate membranes prepared according to van Oss [14] were used. The heat treatment of the membranes was accomplished by incubating the membranes in hot water for 4 min.

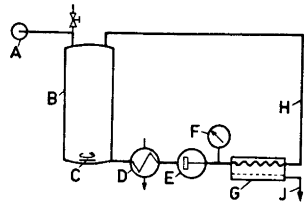

Fig. 1. The reverse osmosis system. A compressed nitrogen, B stirred tank, C magnetic stirrer, D heat exchanger, E pump, F manometer, G reverse osmosis unit, H concentrate and J permeate.

RESULTS

Only yeast that had been used in beer fermentation was able to accumulate FDP. Fresh unused brewer's yeast, fresh baker's yeast, *Rhodotorula glutinis*, and *Candida utilis* did not cause accumulation of FDP. The ability of the yeast to accumulate FDP depended on the length of time it had been used in beer fermentation. During ten days' fermentation the yeast's ability to produce FDP increased and reached its maximum level during the last few days. The conversion of sucrose to FDP was about 15 % when using yeast from the suspension in the fermentation tank and about 40 % with the yeast settled at the bottom of the tank. The yeast used in this work had passed 3 – 5 beer fermentations. When FDP was produced the end point of the fermentation was determined by measuring the concentration of inorganic phosphate in the reaction mixture (Fig. 2). The FDP-concentration of the mixture reached maximum with the lowest concentration of inorganic phosphate.

Glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) were also found in the fermentation mixture. Shortly before the end point of the fermentation their concentrations were quite high. During fermentation samples were taken from the reaction mixture as shown in Fig. 2 (black dots) for the determination of monophosphates (Fig. 3).

The precipitated Ca_2FDP was purest in the experiments with the highest initial sugar concentration (Table 1) and with an amount of

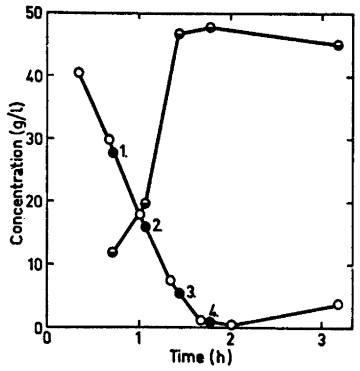

Fig. 2. The concentration of FDP (◒) and inorganic phosphate (calculated as $NaH_2PO_4 \cdot 2H_2O$) (○) in the reaction mixture during fermentation.

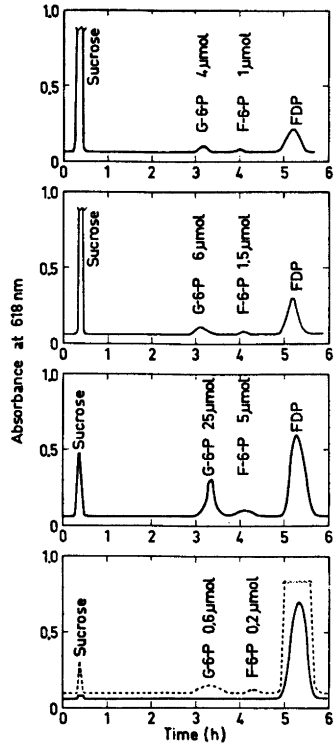

Fig. 3. The amount of G-6-P and F-6-P in the mixture during fermentation. The pictures from top to bottom refer to the sampling times 1, 2, 3, and 4 given in Fig. 2, in this order. Dilutions were 1:10 (—) and 1:1 (---).

Table 1. Dependence of purity of the precipitated Ca_2FDP on the initial sugar concentration.

Initial sugar conc. g/l	Conc. of FDP g/l	Purity of Ca_2FDP %	P_{inorg} %	N %
50	29	69	2.2	0.47
81	52	79	0.9	0.34

inorganic phosphate somewhat below the optimum. Inorganic phosphate precipitated as an impurity when FDP was recovered. Therefore it is advisable to stop the reaction when the concentration of inorganic phosphate is lowest.

FDP was recovered as a Ca-salt. Final precipitation occurred when ethanol was added. When the volume of ethanol used was equal to the volume of fermentation solution most of the

Table 2. Precipitation of FDP from 50 ml of fermentation solution with 5 ml of 50 % (w/w) $CaCl_2$ and ethanol. The concentration of FDP was 42.5 g/l.

Ethanol	Yield	Purity of Ca_2FDP	P_{inorg}	N
ml	g	%	%	%
–	2.2	65	1.19	0.41
40	4.9	58	0.41	0.45
50	4.8	63	0.48	0.46
70	4.9	64	0.60	0.44
100	4.4	64	0.78	0.47

Ca_2FDP precipitated (Table 2). Ca_2FDP precipitated instantly and could be filtered off immediately. The purity of Ca_2FDP, precipitated as mentioned above, was about 70 %. The water content of the product was 5 – 7 %, $Ca_3(PO_4)_2$ content 1 – 2 %, the amount of nitrogenous compounds (6.25N) 2 – 5 %, and the amount of monophosphates less than 1 %. The rest of the impurities were organic compounds.

The purification tests were aimed at finding a simple, preliminary method to purify the fermentation solution. Charcoal treatment, ion exchange, and ultrafiltration were tested. Best results were obtained when the solution was concentrated by reverse osmosis (Fig. 1). Because of the technical limitations of the system used the solutions were diluted with water before concentration. The results obtained with membranes heated at different temperatures are shown in Fig. 4.

The retention of FDP was at least 99 % with all these membranes. By diluting the fermentation solution before concentration a purer product was obtained. The purity was increased by washing the concentrate with water. Table 3 shows the effect of dilution of the fermentation solution and of washing of the concentrate on the purity of the precipitated Ca_2FDP.

By the above method 13 – 15 % of the impurities were removed. The purification did not affect inorganic phosphate or nitrogen content of Ca_2FDP. Most of the nitrogenous compounds and the colour of the product could be removed by treating the concentrate with charcoal. After this, there were still at least 5 % of unknown impurities in the product. Some of these could be removed by ion exchange. The composition of the purified product was as follows:

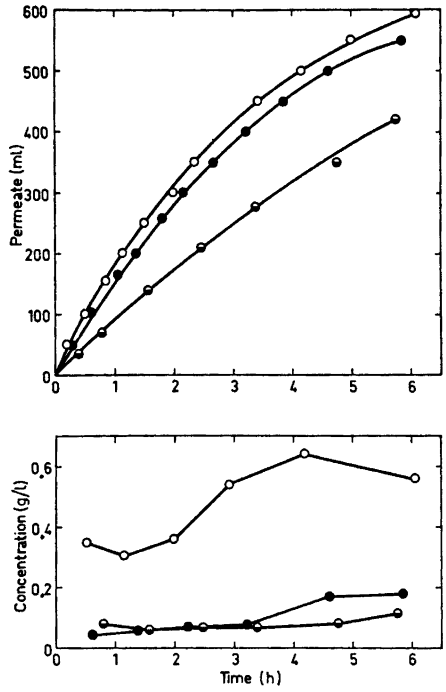

Fig. 4. Concentration of fermentation solution by reverse osmosis. Initial FDP-concentration and heat treatment of the membranes were 23 g/l, 66 °C (○); 18 g/l, 70 °C (●), and 23 g/l, 77 °C. (◒). The volume and FDP-concentration of the permeate is presented as a function of time.

Ca_2FDP	83 – 85 %
H_2O	6 – 7 %
$Ca_3(PO_4)_2$	1 – 2 %
6.25N	0.2 – 0.5 %
Monophosphates	1 %
	92 – 95 %

DISCUSSION

Obviously the membrane of the yeast has to be modified or partly destroyed to make the accumulation of FDP possible. The permeation characteristics of the membrane are changed by drying [15] or by plasmolytic agents, *e.g.* toluene. Addition of toluene is not always necessary when using dry yeast.[2] Some fresh baker's yeasts fail to cause any accumulation of FDP whilst others produce considerable amounts of this compound in the same procedure.[16] The membrane of the yeasts causing accumulation of FDP may be more sensitive to toluene or

Table 3. Purification of the fermentation solution with a cellulose acetate membrane (heat treatment 72 °C). The pressure was 2.9 MPa (30 kp/cm²) and the temperature 22 °C. Initial volume was 700 ml and final volume of the concentrate 200 ml.

Initial conc. g/l	Washing ml H_2O	Purity of Ca_2FDP %	P_{inorg} %	N %
50[a]	–	71	0.24	0.46
25	–	74	0.24	0.49
12	–	78	0.22	0.45
11	2 × 500	84	0.40	0.59

[a] Reference sample, not concentrated.

other plasmolytic agents. According to our experience even results obtained with a single yeast strain depend very much on the history of the yeast, *e.g.* its previous use in the brewing process. Many years ago Meyerhof [17] proved that the slow fermentation of FDP was caused by the destruction of the sensitive and structurally bound adenylpyrophosphatase, since adenosine triphosphate can be split only by means of this enzyme in the absence of a phosphate acceptor. However, this does not explain the considerable stimulation of the accumulation of FDP by an addition of a phosphate acceptor, *e.g.* adenosine monophosphate.[5] A similar stimulation has been found to occur with several purine compounds. Thus the detailed reasons for an efficient accumulation of FDP still seem to be somewhat unclear.

When optimum amounts of starting materials are used in the reaction mixture and the reaction is stopped at the right moment a purer product is obtained (75 %) than with earlier preparation methods. The use of maximum sugar concentration and somewhat less than an optimum amount of inorganic phosphate is important. The reaction should be stopped at the highest FDP concentration, since the amount of inorganic phosphate is lowest at this point and thus only a small amount of $Ca_3(PO_4)_2$ precipitates as an impurity with FDP. At the same time the concentration of monophosphates is also low. On the other hand, a slightly shorter reaction time might also offer a new possibility for preparation of G-6-P and F-6-P.

The purity of the product increases considerably when the fermentation solution is diluted and then concentrated by reverse osmosis before the precipitation of FDP. Washing of the concentrate with water increases the purity. Most of the unknown impurities are removed by this method. However, in some cases a considerable amount of FDP (0 – 50 %) disappeared during the concentration. The reason for this is not known; no FDP was found in the permeate.

Most of the unknown impurities of the purified Ca_2FDP were organic compounds. In X-ray diffraction analysis only phosphorus and calcium could be detected. Only traces of other elements ($\ll 1$ %) were present.

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Reactions of Some *N*-Phenylcarbamates of Glycosides under Hydrolytic Conditions

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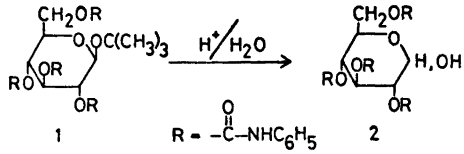
Syntheses and acid hydrolysis studies of *N*-phenylcarbamates of some glycosides are reported. *tert*-Butyl 2,3,4,6-tetra-*O*-(*N*-phenylcarbamoyl)-β-D-glucopyranoside was easily hydrolysed, yielding 2,3,4,6-tetra-*O*-(*N*-phenylcarbamoyl)-D-glucose. The 2-*O*-(*N*-phenylcarbamoyl) derivatives of methyl-α-D-glucopyranoside and methyl β-D-mannopyranoside on hydrolysis reacted further, giving bicyclic oxazolidin-2-one derivatives. The glucose derivative yielded an α-furanosidic oxazolidin-2-one, the mannose derivative a mixture of β-furanosidic and β-pyranosidic oxazolidin-2-ones.

DISCUSSION

The condensation of *N*-phenylcarbamates of sugars to give oligo- and polysaccharides seemed, from the results reported by Husemann and Müller,[1] to be a promising method. Recent results by Eby and Schuerch,[2,3] however, indicate that the value of this approach is limited. We have obtained similar results and arrived at the same conclusion. We now report the syntheses of *N*-phenylcarbamates of some glycosides and their reactions under hydrolytic conditions.

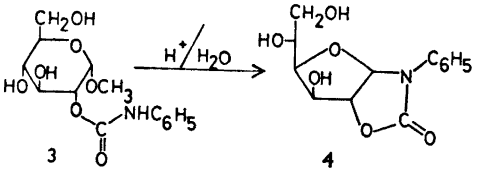
Introduction of the slightly basic *N*-phenylcarbamate group into glycosides renders them more resistant to acid hydrolysis. Thus the methyl 2,3,6- and 2,3,4-tri-*O*-(*N*-phenylcarbamoyl)-α-D-glucopyranosides could not be hydrolysed.[3] Eby and Schuerch [2] prepared 2,3,4-tri-*O*-(*N*-phenylcarbamoyl)-D-glucose by hydrolysis of the corresponding 1,6-anhydro-D-glucopyranose derivative. We have now prepared 2,3,4,6-tetra-*O*-(*N*-phenylcarbamoyl)-D-glucose (*2*) by acid hydrolysis of the fully carbanilated *tert*-butyl β-D-glucopyranoside (*1*).



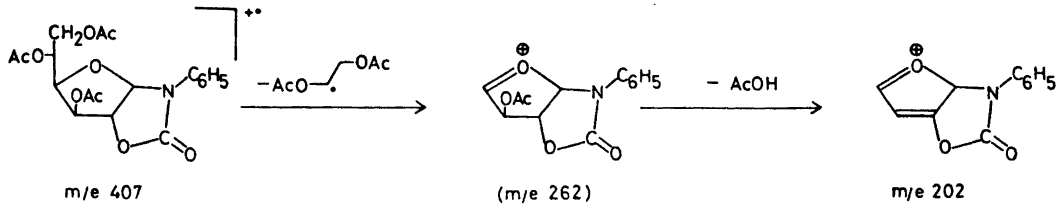
1 2

Methanolysis of *2* yielded a mixture of fully carbanilated methyl α- and β-D-glucopyranosides in the approximate proportion 2:3. The β-anomer predominated but the stereoselectivity of the reaction was not very high.

Although methyl tri- and tetra-*O*-(*N*-phenylcarbamoyl)-glucopyranosides are resistant to acid hydrolysis, the corresponding monoesterified products should be more labile. Methyl 2-*O*-(*N*-phenylcarbamoyl)-α-D-glucopyranoside (*3*) was therefore prepared by carbanilation of methyl 3-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside,[4] followed by mild acid hydrolysis. Under stronger hydrolytic conditions, the glucoside was hydrolysed but the product reacted further, yielding the bicyclic α-furanosidic oxazolidin-2-one derivative (*4*). A first order analysis of the NMR spectra of the acetate of *4*, using spin decoupling in order to identify the individual protons, was in agreement with the postulated structure (Table 1). The mass spectrum of the acetate also agreed with this structure. The



3 4



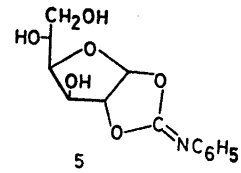
Scheme 1.

Table 1. NMR spectra of the acetylated oxazolidin-2-one derivatives.

	Compound 4	7	8
Chemical shifts (δ-values)			
H-1	6.05	5.87	5.61
H-2	4.88	5.12	4.84
H-3	5.60	5.47	5.29
H-4	4.37	4.29	5.29
H-5	5.30	5.33	3.83
H-6	4.15	4.10	4.09
H-6′	4.55	4.51	4.28
-OAc	1.97	2.01	2.00
	2.03	2.04	2.04
	2.11	2.10	2.09
Coupling constants (Hz)			
$J_{1,2}$	5.5	6.2	3.7
$J_{2,3}$	0	5.7	3.5
$J_{3,4}$	3.0	4.0	–
$J_{4,5}$	9.1	9.0	6.0
$J_{5,6}$	5.0	5.0	3.0
$J_{5,6'}$	2.5	2.5	5.0
$J_{6,6'}$	12.0	12.1	12.3

origin of the main fragment is indicated in Scheme 1.

The results of these analyses do not preclude the cyclic imidocarbonate (*5*) structure. Imidocarbonates are, however, easily hydrolysed whilst *4* is resistant to acid hydrolysis. The observed IR absorption at 1755 cm^{-1} also favours the oxazolidin-2-one structure. Substance *4* was recovered unchanged after treatment with methanolic hydrogen chloride.

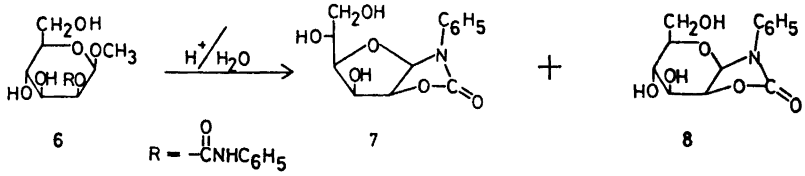


5

Methyl 2-*O*-(*N*-phenylcarbamoyl)-β-D-mannopyranoside (*6*) was prepared by carbanilation of methyl 3-*O*-benzyl-4,6-*O*-benzylidene-β-D-mannopyranoside,[5] followed by catalytic hydrogenation. Acid hydrolysis of *6* yielded two products, identified by NMR, MS, and optical rotation of their acetates as the β-furanosidic (*7*) and β-pyranosidic (*8*) oxazolidin-2-one derivatives. The results of the NMR analyses are given in Table 1 and the pathways leading to the main fragments in the mass spectrum of *8* are indicated in Scheme 2. The mass spectrum of *7* was similar to that of *4*. From the spectrochemical analyses cyclic imidocarbonate structures cannot be precluded but are considered most unlikely.

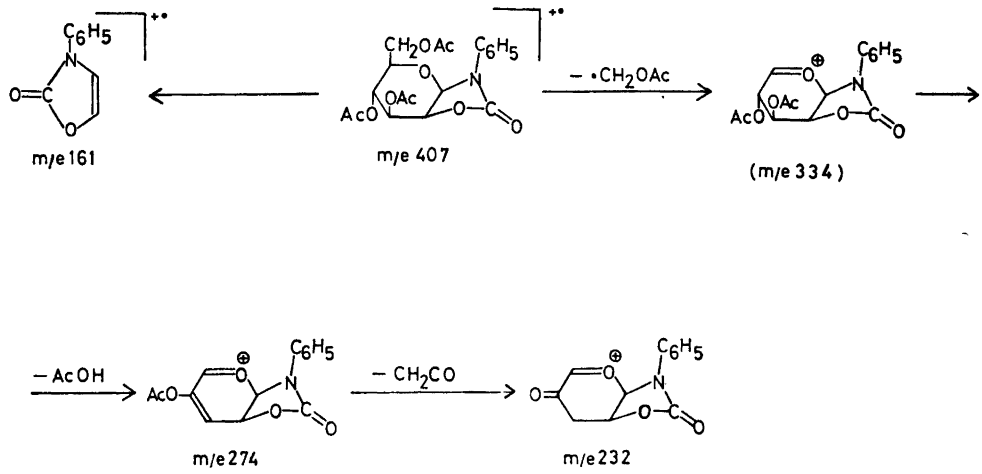
On treatment of either *7* or *8* with strong acid in water or methanol, the equilibrium between them was reestablished but no other products were formed. At equilibrium, the ratio between furanoside and pyranoside was approximately 1:3.

The α-D-glucofuranosidic oxazolidin-2-one derivative (*4*) should be considerably more stable than the corresponding α-D-glucopyranosidic derivative, in agreement with the findings. In the mannose series, however, the



6 7 8

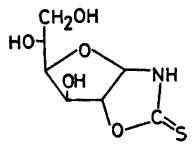
R = $-\overset{O}{\overset{\|}{C}}NHC_6H_5$



Scheme 2.

steric compression from the substituents in the β-furanoside (*7*) is much greater than that in the β-pyranoside (*8*), which may explain the somewhat greater relative stability of the latter.

A number of bicyclic oxazolidin-2-ones, imidazolidin-2-ones, and corresponding 2-thione derivatives of carbohydrates have been prepared (compare Ref. 6). For several of these, in which the anomeric carbon atom is part of both rings, the ring structure (pyranoside or furanoside) has not been convincingly established. The furanosidic nature of the oxazolidin-2-thione (*9*) which is similar to the D-glucose derivative (*4*) above, has been demonstrated by Schwarz.[7] It seems probable, at least in the glucose series, that several of these derivatives are furanosidic.



9

EXPERIMENTAL

Concentrations were performed at reduced pressure at bath temperatures not exceeding 40°. Melting points are corrected. TLC was performed on Silica Gel F_{254} (Merck). Optical rotations were determined with a Perkin-Elmer 141 polarimeter. NMR spectra were recorded with a Varian XL-100 spectrometer, using tetramethylsilane as internal reference Chemical shifts (δ) are given as ppm downfield from tetramethylsilane. NMR spectra were determined for all new substances and were in agreement with the postulated structures. For GLC-MS the compounds were injected into a glass column (190 × 0.15 cm) containing 3 % UCW-98 on Gas Chrom Q fitted in a Perkin-Elmer 270 gas chromatograph-mass spectrometer.

*tert-Butyl 2,3,4,6-tetra-*O-(N-*phenylcarbamoyl*)-β-D-*glucopyranoside* (*1*). Phenyl isocyanate (1.6 ml) was added to a solution of *tert*-butyl β-D-glucopyranoside [8] (0.8 g) in anhydrous pyridine (4.0 ml). The mixture was heated 1 h at 100°, cooled, diluted with methanol (1.5 ml) to destroy excess phenyl isocyanate, and heated for another 10 min. The reaction mixture was concentrated, dissolved in acetone (20 ml) and applied to a Sephadex LH-20 column (5 × 40 cm) which was irrigated with acetone. The first fraction to be eluted (*tert*-butyl 2,3,4,6-tetra-*O*-(*N*-phenylcarbamoyl)-β-D-glucopyranoside) was concentrated, yielding crystals (2.10 g), m.p. 214–217°, $[\alpha]_D$ +2° (*c* 1.01, acetone). (Found: C 63.9; H 5.60; N 8.03. $C_{38}H_{40}N_4O_{10}$ requires: C 64.0; H 5.66; N 7.86).

*2,3,4,6-Tetra-*O-*(*N-*phenylcarbamoyl)-*D-*glucose* (*2*). *tert*-Butyl 2,3,4,6-tetra-*O*-(*N*-phenylcarbamoyl)-β-D-glucopyranoside (250 mg) was hydrolysed with 5.0 M sulphuric acid (2.0 ml) in dioxane (8.0 ml) for 2 h at 100°. The reaction mixture was neutralised with barium carbonate, filtered, and concentrated to dryness. The crude product was purified by preparative TLC (acetone-chloroform, 1:4) yielding crystals (190 mg), m.p. 244–247° (decomp.), $[\alpha]_D$ +30° (*c* 0.60, acetone). (Found: C 62.1; H 4.84;

N 8.45. $C_{34}H_{32}N_4O_{10}$ requires: C 62.2; H 4.91; N 8.53).

*Methanolysis of 2,3,4,6-tetra-*O*-(N-phenylcarbamoyl)-*D*-glucose.* 2,3,4,6-Tetra-*O*-(*N*-phenylcarbamoyl)-D-glucopyranose (20 mg) was refluxed in 3 % (w/v) methanolic hydrogen chloride (5 ml) for 16 h. The reaction mixture was neutralised with silver carbonate, filtered and concentrated to dryness. TLC of the products (acetone-chloroform, 1:4) showed the presence of two compounds in the approximate proportions 2:3, with the same mobility as fully carbanilated methyl α- and β-D-glucopyranoside,[9] respectively.

*Methyl 3-*O*-acetyl-4,6-*O*-benzylidene-2-*O*-(N-phenylcarbamoyl)-α-D-glucopyranoside.* Phenyl isocyanate (1.2 ml) was added to a solution of methyl 3-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside[4] (2.2 g) in anhydrous pyridine (3.0 ml). The mixture was heated for 1 h at 100°, cooled, diluted with methanol (1.5 ml) and heated for another 10 min. The reaction mixture was concentrated, and the product crystallised from methanol (2.4 g), m.p. 198–199°, $[\alpha]_D$ +69° (*c* 0.63, chloroform). (Found: C 62.0; H 5.60; N 3.00. $C_{23}H_{25}O_8N$ requires: C 62.3; H 5.69; N 3.15).

*Methyl 2-*O*-(N-phenylcarbamoyl)-α-D-glucopyranoside (3).* Methyl 3-*O*-acetyl-4,6-*O*-benzylidene-2-*O*-(*N*-phenylcarbamoyl)-α-D-glucopyranoside (2.3 g) was treated with 2 % hydrogen chloride in methanol (60 ml) for 36 h. The reaction mixture was neutralised with silver carbonate, filtered, and concentrated to dryness. The crude product was purified by preparative TLC (ethyl acetate) giving a chromatographically pure syrup (1.3 g), $[\alpha]_D$ +103° (*c* 0.95, ethanol).

*Acid hydrolysis of methyl 2-*O*-(N-phenylcarbamoyl)-α-D-glucopyranoside. 3* (1.0 g) was hydrolysed with 1.0 M sulphuric acid (20 ml) for 14 h at 100°. The reaction mixture was neutralised with barium carbonate, filtered, and concentrated to dryness. The product was acetylated with acetic anhydride (2.5 ml) in pyridine (2.5 ml) for 20 min at 100°. The reaction product (*4*) (1.0 g) gave one spot on TLC (ethyl acetate–light petroleum, 1:2) and crystallised from ethyl ether, m.p. 129–130°, $[\alpha]_D$ +138° (*c* 0.55, chloroform). (Found: C 56.2; H 5.20; N 3.49. $C_{19}H_{21}O_9N$ requires: C 56.0; H 5.21; N 3.44). The NMR spectrum is given in Table 1. The mass spectrum showed, *inter alia*, the following peaks (relative intensities in brackets): 43(100), 77(16), 91(6), 104(13), 119(12), 130(8), 147(12), 161(7), 162(16), 174(9), 201(10), 202(29), 203(5), 217(5), 245(3), 305(1), 347(1), and 407(14).

Methanolysis of acetylated 4. Acetylated *4* (10 mg) was refluxed in 15 % (w/v) methanolic hydrogen chloride (2.5 ml) for 16 h. The reaction mixture was neutralised with silver carbonate, filtered, and concentrated to dryness. The crude product was acetylated with acetic anhydride (1.0 ml) in pyridine (1.0 ml) for 20 min at 100°. GLC-MS gave one product identical with the starting material.

*Methyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-*O*-(N-phenylcarbamoyl)-β-D-mannopyranoside.* Phenyl isocyanate (1.8 ml) was added to a solution of methyl 3-*O*-benzyl-4,6-*O*-benzylidene-β-D-mannopyranoside[5] (3.0 g) in anhydrous pyridine (5.0 ml). The mixture was heated for 1 h at 100°, cooled, diluted with methanol (2.5 ml) and heated for another 10 min. The reaction mixture was concentrated, and the product was purified on a silicic acid column (40 × 4 cm; ethyl acetate–light petroleum, 1:2), yielding a syrup (3.2 g), $[\alpha]_D$ −75° (*c* 0.96, chloroform).

*Methyl 2-*O*-(N-phenylcarbamoyl)-β-D-mannopyranoside (6).* Methyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-*O*-(*N*-phenylcarbamoyl)-β-D-mannopyranoside (2.9 g) in ethanol (40 ml) was hydrogenated over palladium on carbon (10 %, 0.5 g) at room temperature and atmospheric pressure. When the hydrogen consumption had ceased, the catalyst was filtered off. Concentration yielded a syrup (1.8 g), $[\alpha]_D$ −76° (*c* 0.87, ethanol).

*Acid hydrolysis of methyl 2-*O*-(N-phenylcarbamoyl)-β-D-mannopyranoside (6).* Substance *6* (1.0 g) was hydrolysed with 1.0 M sulphuric acid (20 ml) for 14 h at 100°. The reaction mixture was neutralised with barium carbonate, filtered, and concentrated to dryness. The crude product was acetylated with acetic anhydride (2.5 ml) in pyridine (2.5 ml) for 20 min at 100°. The reaction mixture gave two spots on TLC. The mixture was separated on a silicic acid column (40 × 3 cm, acetone–chloroform, 1:19). The separation was followed by polarimetry and TLC. The first fraction to be eluted (acetylated *8*) on concentration yielded a syrup (0.82 g), $[\alpha]_D$ −117° (*c* 0.88, chloroform). The next fraction (acetylated *7*) yielded a syrup which crystallised from ethanol–light petroleum (1:2, v/v) (0.29 g), m.p. 168–170°, $[\alpha]_D$ −161° (*c* 0.56, chloroform). (Found: C 56.0; H 5.22; N 3.22. $C_{19}H_{21}O_9N$ requires: C 56.0; H 5.21; N 3.44). The NMR spectra are given in Table 1. The mass spectrum of acetylated *8* showed, *inter alia*, peaks at *m/e* 43(100), 77(12), 104(13), 119(9), 161(17), 162(6), 190(5), 203(5), 204(6), 232(27), 245(2), 305(1), 347(1) and 407(16). The mass spectrum of acetylated *7* showed peaks at *m/e* 43(100), 77(9), 104(7), 119(7), 130(11), 147(6), 162(12), 190(6), 202(41), 203(6), 217(4), 245(2), 305(1), 347(1) and 407(12).

Methanolysis and acid hydrolysis of acetylated 7 *and* 8. Methanolysis of acetylated *7* and *8* was performed in 3 % (w/v) methanolic hydrogen chloride as described above. Acid hydrolysis of acetylated *7* and *8* was performed with 1.0 M sulphuric acid as described above. The reaction mixtures were acetylated with acetic anhydride in pyridine and analysed on GLC-MS. All reactions produced the same mixture (1:3) of *7* and *8*.

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The Crystal and Molecular Structure of *N*-Acetyl-5-methoxy-tryptamine (Melatonin)

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The structure of melatonin has been determined by X-ray methods using 2543 observed reflections collected by counter methods. The crystals are monoclinic, space group $P2_1/c$, with unit cell dimensions $a = 7.71_4$ Å; $b = 9.27_6$ Å; $c = 17.11_8$ Å and $\beta = 96.9_1°$. The refinements yielded a conventional R-factor of 5 %; standard deviations in bond lengths and in angles not involving hydrogen atoms are 0.002 Å and 0.1°, respectively. The molecular geometry and the hydrogen bond system in the crystal is discussed. The molecule has also been studied by X-ray methods by Quarles [1] and by Wakahara, Fujiwara and Tomita.[2]

As a part of a series of structure investigations of tyrosine and tryptophan derivatives the crystal and molecular structure of *N*-acetyl-5-methoxytryptamine (melatonin) has been determined. The compound occurs naturally in mammals and its biological activity is believed to involve changes in certain neurones in the central nervous system. Bioactive monoamines, which includes melatonin, have in the recent years received increased interest and much work has been devoted to reach information useful for an explanation of the relation between structure and biological activity. We have studied the melatonin molecule in order to obtain data relevant in this discussion, for the study of the conformation of such compounds in the crystalline phase and for the comparison with several theoretical calculations made on analogous molecules. During the refinement of the structure model we became aware of studies of the same molecule by Quarles [1] and also by Wakahara *et al.*[2] However, even if the present determination yields the same general structural information it is based on more than twice of their number of data, and less than half of their standard deviations in bond lengths were obtained.

EXPERIMENTAL

Large prismatic crystals of melatonin were formed by slow evaporation of an ethanolic solution at room temperature. A preliminary study showed the crystals to be monoclinic, space group $P2_1/c$.

The intensity data were recorded on an automatic Picker diffractometer using graphite crystal monochromated MoK-radiation. The specimen was cut from a large crystal to the dimensions $0.3 \times 0.3 \times 0.3$ mm^3 and was mounted on a glass fibre with the crystallographic a-axis along the ϕ-axis. The $\omega - 2\theta$ scanning mode was employed with a 2θ scan speed of 2° min^{-1} through a scan range from 0.5° below $2\theta(\alpha_1)$ to 0.5° above $2\theta(\alpha_2)$. Background counts were taken for 10 s at each of the scan range limits. The intensities of three standard reflections measured for every 150 reflections of the data set showed slow changes of up to 10 % and the intensity data were accordingly adjusted. The estimate of the standard deviations of the measurements was based on counting statistics with an additional term of 2 % of the intensity. Out of 3306 unique reflections with $\sin\theta/\lambda$ less than 0.7, 2543 reflections were found to have intensities larger than twice their standard deviation and were regarded as observed. The remaining reflections were excluded from the refinement procedure.

The intensity data were corrected for Lorentz and polarization effects. Atomic scattering factors used during the calculations (for computer programs *cf.* Ref. 3) were those of Hansen *et al.*[4] for oxygen, nitrogen, and carbon, and of Stewart *et al.*[5] for hydrogen. Unit cell dimensions were calculated from diffractometer measurements on 17 general reflections.

Table 1. Observed and calculated structure factors. The columns are h, k, l, $10F_o$, and $10|F_c|$.

0	0	2	147 - 166	0	6	5	139 - 149	1	11	2	21 - 21	1	7	3	107 - 102	1	4	-4	146 - 144	1	1	1	241 - 241
0	0	4	250 - 231	0	6	6	151 - 145	1	11	3	48 - 37	1	7	4	241 - 236	1	4	-5	245 - 247	1	1	2	593 - 558
0	0	6	317 - 286	0	6	7	61 - 71	1	11	5	64 - 61	1	7	5	159 - 162	1	4	-7	67 - 57	1	1	4	172 - 178
0	0	8	283 - 259	0	6	8	93 - 107	1	11	6	29 - 20	1	7	6	284 - 289	1	4	-8	145 - 142	1	1	6	161 - 159
0	0	10	167 - 158	0	6	9	91 - 92	1	11	7	25 - 26	1	7	7	67 - 68	1	4	-10	134 - 129	1	1	8	109 - 92
0	0	12	291 - 288	0	6	10	199 - 196	1	11	9	44 - 44	1	7	8	229 - 239	1	4	-11	24 - 25	1	1	10	196 - 178
0	0	14	97 - 92	0	6	11	85 - 85	1	11	10	19 - 0	1	7	9	27 - 27	1	4	-12	51 - 49	1	1	12	137 - 125
0	0	16	287 - 299	0	6	12	75 - 79	1	11	11	31 - 26	1	7	10	46 - 46	1	4	-13	47 - 42	1	1	14	396 - 368
0	0	20	32 - 21	0	6	13	57 - 58	1	11	13	31 - 34	1	7	11	41 - 40	1	4	-14	132 - 135	1	1	9	24 - 22
0	1	13	40 - 41	0	6	14	93 - 94	1	10	10	46 - 48	1	7	12	25 - 27	1	4	-15	45 - 44	1	1	10	129 - 126
0	1	17	23 - 26	0	6	15	44 - 44	1	10	8	73 - 66	1	7	13	27 - 24	1	4	-16	32 - 36	1	1	11	18 - 18
0	1	16	57 - 64	0	6	16	19 - 17	1	10	7	16 - 17	1	7	14	28 - 28	1	4	-17	43 - 40	1	1	12	118 - 110
0	1	15	171 - 173	0	6	17	21 - 29	1	10	6	27 - 26	1	7	15	26 - 28	1	4	-18	46 - 38	1	1	13	263 - 265
0	1	14	86 - 87	0	6	18	18 - 18	1	10	5	103 - 109	1	7	16	23 - 21	1	4	-19	25 - 23	1	1	14	130 - 139
0	1	13	36 - 37	0	6	19	57 - 56	1	10	4	43 - 50	1	7	19	113 - 104	1	4	-20	32 - 30	1	1	15	35 - 39
0	1	12	53 - 54	0	6	20	20 - 19	1	10	2	42 - 35	1	7	19	19 - 12	1	3	-18	29 - 22	1	1	16	53 - 57
0	1	11	131 - 122	0	7	19	35 - 27	1	10	1	94 - 97	1	6	19	23 - 26	1	3	-17	37 - 33	1	1	17	53 - 56
0	1	10	93 - 97	0	7	17	43 - 43	1	10	0	63 - 58	1	6	19	15 - 5	1	3	-16	89 - 89	1	1	18	14 - 18
0	1	9	111 - 109	0	7	16	20 - 11	1	10	-1	100 - 106	1	6	16	93 - 91	1	3	-14	71 - 70	1	1	19	18 - 3
0	1	8	423 - 426	0	7	15	37 - 37	1	10	-2	21 - 24	1	6	15	19 - 21	1	3	-13	140 - 137	1	0	20	29 - 32
0	1	7	171 - 160	0	7	14	22 - 21	1	10	-4	44 - 41	1	6	14	16 - 17	1	3	-12	14 - 18	1	1	13	58 - 56
0	1	6	69 - 61	0	7	13	66 - 66	1	10	-5	57 - 57	1	6	13	45 - 48	1	3	-11	115 - 109	1	0	16	55 - 50
0	1	5	99 - 90	0	7	12	60 - 60	1	10	-6	24 - 20	1	6	11	97 - 92	1	3	-9	61 - 57	1	0	14	268 - 278
0	1	4	224 - 214	0	7	11	41 - 41	1	10	-9	32 - 34	1	6	10	154 - 155	1	3	-8	153 - 136	1	0	12	297 - 304
0	1	3	261 - 260	0	7	10	167 - 170	1	10	-10	15 - 23	1	6	9	93 - 89	1	3	-7	429 - 417	1	0	10	370 - 364
0	1	2	272 - 274	0	7	9	56 - 64	1	10	-11	17 - 8	1	6	8	131 - 134	1	3	-6	140 - 162	1	0	8	94 - 89
0	1	1	457 - 458	0	7	8	76 - 83	1	9	-15	81 - 78	1	6	7	235 - 230	1	3	-5	41 - 34	1	0	6	504 - 493
0	2	0	174 - 174	0	7	7	19 - 14	1	9	-15	22 - 21	1	6	6	107 - 102	1	3	-4	15 - 11	1	0	4	67 - 51
0	2	1	423 - 429	0	7	6	277 - 277	1	9	-14	13 - 14	1	6	4	102 - 101	1	3	-3	219 - 201	1	0	2	1103 - 1234
0	2	2	337 - 332	0	7	5	113 - 114	1	9	-13	78 - 73	1	6	3	23 - 24	1	3	-2	149 - 130	1	0	0	356 - 323
0	2	3	191 - 192	0	7	4	178 - 181	1	9	-12	74 - 68	1	6	2	54 - 52	1	3	-1	9 - 14	1	0	-2	561 - 532
0	2	4	91 - 101	0	7	3	118 - 113	1	9	-11	29 - 37	1	6	1	54 - 48	1	3	0	91 - 82	1	0	-4	742 - 739
0	2	5	111 - 111	0	7	2	89 - 86	1	9	-9	65 - 67	1	6	0	261 - 266	1	3	1	257 - 251	1	0	-6	101 - 97
0	2	6	329 - 334	0	7	1	49 - 55	1	9	-8	62 - 60	1	6	-1	204 - 204	1	3	2	91 - 76	1	0	-8	231 - 212
0	2	7	3 - 26	0	8	0	70 - 64	1	9	-7	93 - 96	1	6	-2	81 - 76	1	3	3	530 - 474	1	0	-10	339 - 296
0	2	8	325 - 294	0	8	1	42 - 43	1	9	-6	129 - 132	1	6	-3	175 - 174	1	3	4	188 - 169	1	0	-12	77 - 79
0	2	9	121 - 114	0	8	2	142 - 143	1	9	-5	56 - 55	1	6	-4	50 - 57	1	3	5	331 - 334	1	0	-14	706 - 705
0	2	10	103 - 102	0	8	3	97 - 97	1	9	-4	48 - 47	1	6	-5	41 - 41	1	3	6	204 - 191	1	0	-16	24 - 27
0	2	11	136 - 106	0	8	4	107 - 110	1	9	-3	141 - 143	1	6	-6	116 - 115	1	3	7	57 - 49	1	0	-18	32 - 32
0	2	12	135 - 130	0	8	5	40 - 41	1	9	-2	77 - 74	1	6	-8	229 - 242	1	3	8	308 - 279	2	0	-18	101 - 111
0	2	13	18 - 17	0	8	6	99 - 97	1	9	-1	119 - 118	1	6	-9	221 - 223	1	3	9	153 - 139	2	0	-16	151 - 151
0	2	14	87 - 82	0	8	7	159 - 160	1	9	0	159 - 158	1	6	-10	121 - 124	1	3	10	14 - 12	2	0	-14	144 - 146
0	2	15	72 - 75	0	8	8	46 - 46	1	9	1	35 - 33	1	6	-14	54 - 55	1	3	11	141 - 143	2	0	-12	70 - 71
0	2	17	57 - 52	0	8	9	163 - 160	1	9	2	43 - 50	1	6	-15	44 - 51	1	3	14	44 - 44	2	0	-10	261 - 254
0	2	20	52 - 50	0	9	10	24 - 20	1	9	3	91 - 49	1	6	-16	13 - 19	1	3	15	121 - 130	2	0	-8	121 - 111
0	2	21	24 - 23	0	9	11	61 - 65	1	9	5	81 - 80	1	6	-19	21 - 23	1	3	16	30 - 26	2	0	-6	17 - 28
0	2	19	64 - 57	0	9	12	67 - 66	1	9	7	51 - 49	1	6	-21	61 - 59	1	3	17	29 - 27	2	0	-4	253 - 242
0	2	22	47 - 44	0	9	14	34 - 29	1	9	8	73 - 74	1	5	-18	24 - 20	1	3	18	22 - 18	2	0	-2	1429 -1619
0	2	23	82 - 86	0	9	15	27 - 19	1	9	10	39 - 37	1	5	-16	77 - 80	1	3	20	44 - 38	2	0	0	241 - 257
0	3	15	78 - 76	0	9	16	51 - 45	1	9	12	33 - 34	1	5	-15	59 - 61	1	2	19	29 - 25	2	0	2	442 - 462
0	3	14	95 - 87	0	9	17	47 - 44	1	9	14	71 - 65	1	5	-13	53 - 57	1	2	18	27 - 16	2	0	4	99 - 92
0	3	13	307 - 309	0	9	18	34 - 26	1	9	16	66 - 57	1	5	-12	57 - 58	1	2	17	16 - 20	2	0	6	332 - 300
0	3	12	21 - 22	0	9	9	47 - 45	1	9	19	19 - 19	1	5	-11	157 - 167	1	2	16	42 - 32	2	0	8	10 - 7
0	3	11	127 - 126	0	9	7	42 - 45	1	9	17	17 - 17	1	5	-10	71 - 70	1	2	14	131 - 132	2	0	10	127 - 129
0	3	10	61 - 62	0	9	6	43 - 51	1	9	15	65 - 67	1	5	-9	23 - 28	1	2	13	164 - 163	2	0	12	57 - 50
0	3	9	151 - 148	0	9	5	25 - 28	1	9	14	61 - 56	1	5	-8	17 - 16	1	2	12	134 - 128	2	0	14	217 - 218
0	3	8	173 - 164	0	9	4	55 - 55	1	9	13	69 - 67	1	5	-7	59 - 58	1	2	11	115 - 107	2	0	16	70 - 77
0	3	7	51 - 55	0	9	3	93 - 93	1	8	12	47 - 38	1	5	-6	246 - 245	1	2	10	11 - 6	2	0	18	44 - 44
0	3	6	124 - 116	0	9	2	129 - 138	1	8	11	23 - 25	1	5	-5	111 - 116	1	2	9	149 - 143	2	1	0	64 - 61
0	3	5	114 - 105	0	9	1	72 - 77	1	8	10	27 - 26	1	5	-4	107 - 104	1	2	8	405 - 395	2	1	19	97 - 82
0	3	4	97 - 85	0	10	3	56 - 56	1	8	9	33 - 28	1	5	-3	22 - 21	1	2	7	461 - 427	2	1	18	22 - 12
0	3	3	157 - 150	0	10	2	67 - 65	1	8	8	17 - 13	1	5	-2	166 - 159	1	2	6	509 - 474	2	1	17	16 - 12
0	3	2	101 - 90	0	10	1	13 - 19	1	8	7	31 - 32	1	5	-1	237 - 223	1	2	5	154 - 140	2	1	16	20 - 24
0	3	1	154 - 144	0	10	3	44 - 52	1	8	6	49 - 48	1	5	0	373 - 347	1	2	4	46 - 64	2	1	15	114 - 120
0	4	1	291 - 289	0	10	4	31 - 31	1	8	5	21 - 22	1	5	1	351 - 371	1	2	3	50 - 41	2	1	14	190 - 201
0	4	2	264 - 277	0	10	5	88 - 88	1	8	4	220 - 223	1	5	2	73 - 72	1	2	2	17 - 31	2	1	13	64 - 69
0	4	3	1 - 7	0	10	6	41 - 39	1	8	3	193 - 192	1	5	3	236 - 198	1	2	1	265 - 254	2	1	12	91 - 98
0	4	4	137 - 142	0	10	7	19 - 34	1	8	2	115 - 119	1	5	4	182 - 169	1	2	0	403 - 384	2	1	11	31 - 32
0	4	5	151 - 144	0	10	8	61 - 56	1	8	1	57 - 61	1	5	5	37 - 26	1	2	-1	139 - 127	2	1	10	157 - 151
0	4	7	134 - 133	0	10	9	39 - 25	1	8	0	38 - 41	1	5	6	150 - 143	1	2	-2	167 - 173	2	1	9	112 - 112
0	4	8	37 - 86	0	11	10	17 - 17	1	8	-1	76 - 79	1	5	7	26 - 23	1	2	-3	503 - 495	2	1	8	75 - 69
0	4	9	126 - 127	0	11	11	18 - 8	1	8	-2	89 - 96	1	5	8	76 - 70	1	2	-4	183 - 169	2	1	7	11 - 4
0	4	10	117 - 108	0	11	12	23 - 17	1	8	-4	170 - 145	1	5	9	41 - 41	1	2	-5	335 - 288	2	1	6	27 - 25
0	4	11	45 - 44	0	11	13	32 - 36	1	8	-5	101 - 102	1	5	10	16 - 16	1	2	-6	184 - 168	2	1	5	332 - 312
0	4	12	77 - 77	0	11	14	71 - 71	1	8	-6	45 - 46	1	5	11	53 - 59	1	2	-8	135 - 136	2	1	4	146 - 178
0	4	13	17 - 11	0	11	11	57 - 45	1	8	-7	121 - 126	1	5	12	122 - 123	1	2	-9	194 - 181	2	1	3	325 - 297
0	4	14	32 - 29	0	11	10	54 - 57	1	8	-8	117 - 117	1	5	13	14 - 22	1	2	-10	437 - 349	2	1	2	224 - 208
0	4	15	74 - 39	0	11	7	19 - 18	1	8	-9	14 - 17	1	5	14	43 - 50	1	2	-11	69 - 67	2	1	1	572 - 518
0	4	16	25 - 27	0	11	6	25 - 26	1	8	-10	64 - 72	1	5	15	41 - 47	1	2	-13	69 - 69	2	1	0	564 - 558
0	4	17	33 - 34	0	11	5	28 - 34	1	8	-11	16 - 20	1	5	17	27 - 19	1	2	-14	14 - 15	2	1	-1	1174 - 1155
0	4	18	16 - 19	0	11	2	16 - 10	1	8	-12	29 - 29	1	5	18	37 - 35	1	2	-15	160 - 167	2	1	-2	1695 - 1719
0	4	19	24 - 20	0	12	3	24 - 29	1	8	-13	14 - 12	1	5	19	56 - 50	1	2	-16	53 - 62	2	1	-3	107 - 94
0	5	17	53 - 50	0	12	4	24 - 16	1	8	-14	22 - 29	1	4	20	33 - 37	1	2	-17	104 - 109	2	1	-4	457 - 476
0	5	16	14 - 16	0	12	5	44 - 44	1	8	-15	16 - 16	1	4	17	79 - 77	1	2	-18	16 - 12	2	1	-5	304 - 281
0	5	15	51 - 57	0	12	7	29 - 24	1	8	-16	73 - 72	1	4	16	16 - 2	1	2	-20	24 - 28	2	1	-6	177 - 169
0	5	14	125 - 121	0	12	8	17 - 11	1	8	-17	41 - 38	1	4	15	97 - 98	1	1	-19	29 - 26	2	1	-7	206 - 193
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3	1	-2	231	-216
3	1	-1	193	181
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3	1	16	45	40
3	1	20	54	51
3	0	20	23	-28
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3	0	16	43	-46
3	0	14	83	-79
3	0	12	335	345
3	0	10	71	-61
3	0	8	75	74
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3	0	0	76	72
3	0	-2	304	282
3	0	-4	427	394
3	0	-6	202	-199
3	0	-8	304	300
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3	0	-20	31	-28
4	0	-14	165	165
4	0	-12	175	-176
4	0	-10	132	-127
4	0	-8	79	-79
4	0	-6	297	[illegible]
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4	0	2	292	271
4	0	4	100	-91
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4	0	8	16	-21
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4	1	14	27	-26
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4	1	2	63	57
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Table 1. Continued.

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4 1 -11	170 170	4 5 6	13 16	4 9 3	76 - 74	5 7 3	42 - 46	5 3 0	92 - 86	6 1 0	15 14
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4 3 0	74 - 74	4 7 10	34 31	5 9 -17	20 21	5 5 9	33 29	5 1 4	97 92	6 3 1	60 62
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4 3 -2	134 - 97	4 7 8	63 - 69	5 9 -11	69 - 66	5 5 11	26 28	5 1 6	29 - 21	6 3 -2	22 - 25
4 3 -3	244 237	4 7 7	71 74	5 9 -10	34 - 29	5 5 12	25 28	5 1 7	12 8	6 3 -4	207 206
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4 3 -6	34 - 41	4 7 4	91 - 92	5 9 -6	29 28	5 5 16	59 50	5 1 10	117 119	6 3 -11	55 54
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4 3 -8	57 - 51	4 7 2	27 - 20	5 9 -4	24 - 32	5 4 17	27 24	5 1 12	16 - 10	6 3 -15	34 36
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4 3 -10	124 128	4 7 0	57 - 55	5 9 -1	57 - 56	5 4 14	59 - 59	5 1 14	40 - 38	6 3 -19	32 28
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4 3 -16	77 - 69	4 7 -16	47 - 40	5 8 13	39 - 34	5 4 2	104 104	5 0 8	109 - 112	6 4 -7	23 - 21
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4 3 -14	29 31	4 7 -18	31 - 26	5 8 11	43 - 41	5 4 0	56 - 59	5 0 -4	48 51	6 4 -5	117 107
4 3 -12	15 - 17	4 8 -16	24 - 25	5 8 9	21 20	5 4 -1	96 85	5 0 -6	131 - 122	6 4 -4	53 52
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4 3 -11	64 - 65	4 8 -12	34 32	5 8 5	57 48	5 4 -4	72 71	5 0 -10	139 - 138	6 4 -2	34 - 36
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4 3 0	64 67	4 8 -4	35 38	5 8 -6	27 29	5 4 -12	75 - 77	6 0 -12	116 119	6 4 6	47 - 47
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4 3 5	86 - 94	4 8 1	28 30	5 8 -12	61 60	5 4 -17	27 32	6 0 -4	66 63	6 4 13	17 20
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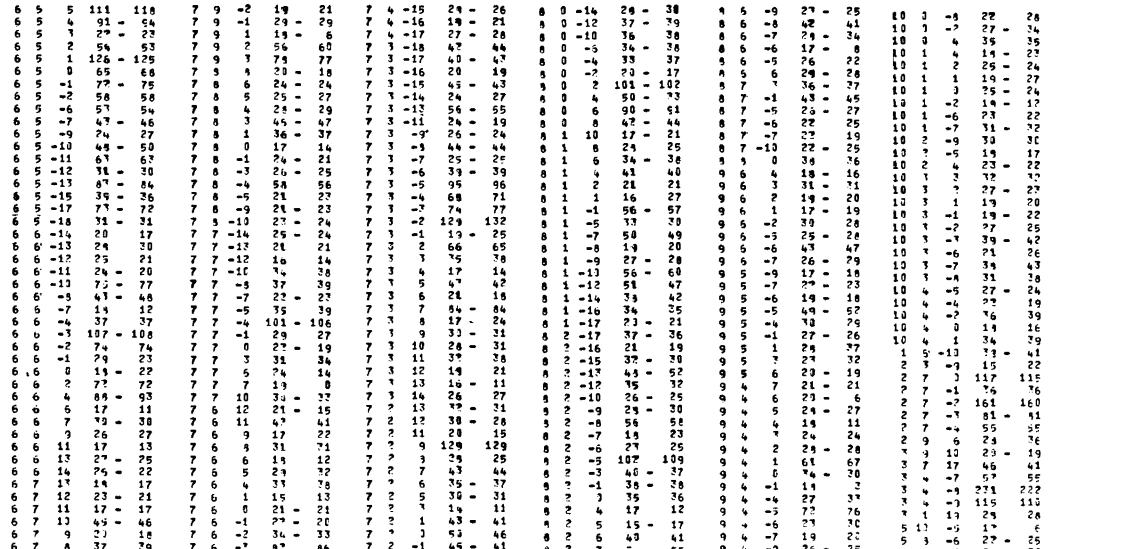
Table 1. Continued.



CRYSTAL DATA

Melatonin (*N*-acetyl-5-methoxytryptamin) $C_{13}H_{16}N_2O_2$, monoclinic. $a = 7.714(0.002)$ Å; $b = 9.276(0.002)$ Å; $c = 17.118$ (0.004) Å; $\beta = 96.91$ (0.03). $V = 1214$ Å^3; $M = 232.28$; $F(000) = 496$; $Z = 4$; $D_{calc} = 1.27$ gcm^{-3}. Absent reflections: $(h0l)$ for l odd, $(0k0)$ for k odd; space group $P2_1/c$.

STRUCTURE DETERMINATION

The structure was determined by the application of the symbolic addition procedure and

Table 2. Fractional atomic coordinates and thermal parameters with standard deviations ($\times 10^5$) for non-hydrogen atoms.

	x	y	z	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
O5	6647(16)	−25333(11)	37530(7)	2729(27)	1008(13)	460(5)	−879(30)	−29(19)	−283(13)
O13	61358(14)	48624(10)	73724(5)	2794(25)	701(10)	316(4)	−462(26)	312(16)	−26(11)
N1	27247(16)	31323(12)	37572(6)	2030(25)	909(14)	296(5)	−31(30)	148(17)	−264(13)
N12	50897(16)	26613(11)	70553(6)	2206(26)	630(12)	240(4)	−183(28)	118(16)	33(11)
C2	33649(19)	33006(14)	45301(8)	1766(27)	784(15)	317(5)	−61(33)	210(19)	41(15)
C3	31897(17)	20627(13)	49447(7)	1455(24)	753(15)	255(5)	30(32)	127(17)	23(14)
C4	18833(18)	−4044(14)	44452(7)	1530(25)	838(15)	255(5)	30(32)	127(17)	23(14)
C5	11844(18)	−11065(15)	37747(8)	1562(26)	916(16)	340(6)	−86(34)	123(19)	−174(15)
C6	9623(20)	−4022(17)	30442(8)	1790(29)	1299(21)	288(5)	51(40)	−80(20)	−278(17)
C7	14300(19)	10096(17)	29723(8)	1783(28)	1364(21)	225(5)	363(40)	46(19)	134(16)
C8	21407(17)	17329(14)	36436(7)	1378(23)	890(15)	262(5)	264(31)	185(16)	107(14)
C9	23855(16)	10404(13)	43808(7)	1271(22)	812(14)	232(5)	138(29)	184(16)	−11(13)
C10	37056(18)	17623(14)	58001(7)	1692(26)	778(15)	253(5)	−106(33)	139(17)	−2(14)
C11	43736(21)	30646(14)	62666(7)	2366(32)	759(15)	240(5)	−107(36)	96(20)	27(14)
C13	59679(18)	35796(13)	75510(7)	1703(26)	727(14)	253(5)	−45(32)	343(18)	−62(13)
C14	67334(22)	30028(16)	83307(8)	2214(32)	1015(18)	289(5)	−297(39)	8(21)	33(16)
C15	8773(24)	−33064(17)	44714(11)	2300(35)	866(18)	577(9)	−177(42)	279(28)	70(20)

Sayre's equation and refined by Fourier- and full-matrix least-squares methods. Final refinements included individual isotropic thermal parameters for hydrogen atoms and anisotropic temperature factors expressed by $\exp-(B_{11}h^2+B_{22}k^2+B_{33}l^2+B_{12}hk+B_{13}hl+B_{23}kl)$ for the other atoms in addition to all positional parameters. The final conventional R-factor is 0.052.

The observed and calculated structure factors are listed in Table 1; positional and thermal parameters of non-hydrogen atoms are listed in Table 2 and those of hydrogen atoms in Table 3. Bond lengths and angles are tabulated in Table 4; estimated standard deviations were calculated from the correlation matrix. An analysis of the rigid body vibrations of the indol nucleus was carried out and the bond lengths corrected for thermal librations are given in Table 4.

Table 3. Fractional atomic coordinates ($\times 10^3$) with standard deviations and thermal parameters for hydrogen atoms.

	x	y	z	B
HN1	282(2)	378(2)	338(1)	4.1 (0.3)
HC2	376(2)	429(2)	472(1)	3.6 (0.3)
HC4	204(2)	−84(2)	494(1)	4.1 (0.3)
HC6	45(2)	−92(2)	259(1)	4.3 (0.4)
HC7	124(2)	145(2)	249(1)	3.2 (0.3)
H110	269(2)	137(2)	605(1)	3.4 (0.3)
H210	461(2)	103(2)	587(1)	3.8 (0.3)
H111	348(2)	379(2)	629(1)	4.4 (0.4)
H211	532(2)	349(2)	601(1)	4.2 (0.3)
H115	18(3)	−285(2)	489(1)	6.5 (0.5)
H215	46(3)	−425(2)	432(1)	6.8 (0.5)
H315	207(2)	−333(2)	471(1)	4.7 (0.4)
HN12	502(2)	178(2)	720(1)	3.0 (0.3)
H114	680(3)	200(2)	835(1)	6.2 (0.5)
H214	613(2)	340(2)	873(1)	5.3 (0.4)
H314	788(3)	338(3)	845(1)	7.4 (0.5)

DESCRIPTION OF THE STRUCTURE

The structure of the melatonine molecule as found in the present determination is shown in Fig. 1. The bond lengths and angles in the indole-ethylamine part of the molecule is in very good agreement with those given by Falkenberg [6] as the averaged structure based on several precise structure analyses of compounds containing this group. In the indole nucleus there are consistent and systematic variations in the bond lengths and angles indicating a reduced aromatic character; the same variations are also found in the theoretically calculated structure.[7] The C11−N12 bond length of 1.445 Å may be considered normal for a C(sp^3)−N(sp^2) distance and the N-acetyl group shows dimensions typical for a peptide.

The methoxy group is close to the plane of the indole moiety and the conformation about the O5−C15 bond is strictly staggered. It is

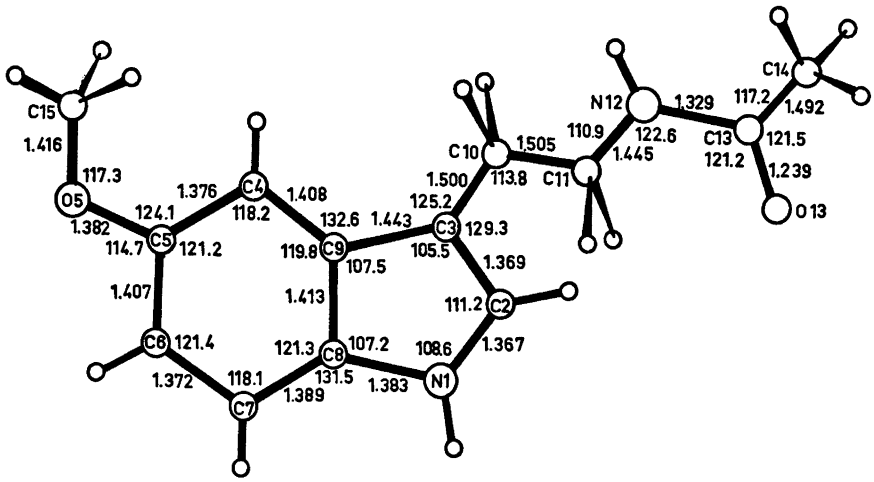


Fig. 1. Conformation of the melatonin molecule. Corrected bond lengths (Å) and angles (°) are indicated.

interesting to note that the external angles at C5 differ in a way (C4 – C5 – O5 = 124.1°, ∠C6 – C5 – O5 = 114.7) corresponding to that found at the OH group in phenols in which the H-atom is situated in the plane of the aromatic ring. The opening of the angles C15 – O5 – C5, O5 – C5 – C4 and also of C5 – C4 – HC4 relative to their standard values indicate a steric strain; nevertheless the arrangement of the atoms involved is planar, the dihedral angle C4 – C5 – O5 – C15 is 0° within the accuracy of determination.

The six- and five-membered rings are each strictly planar, the deviations from the corre-

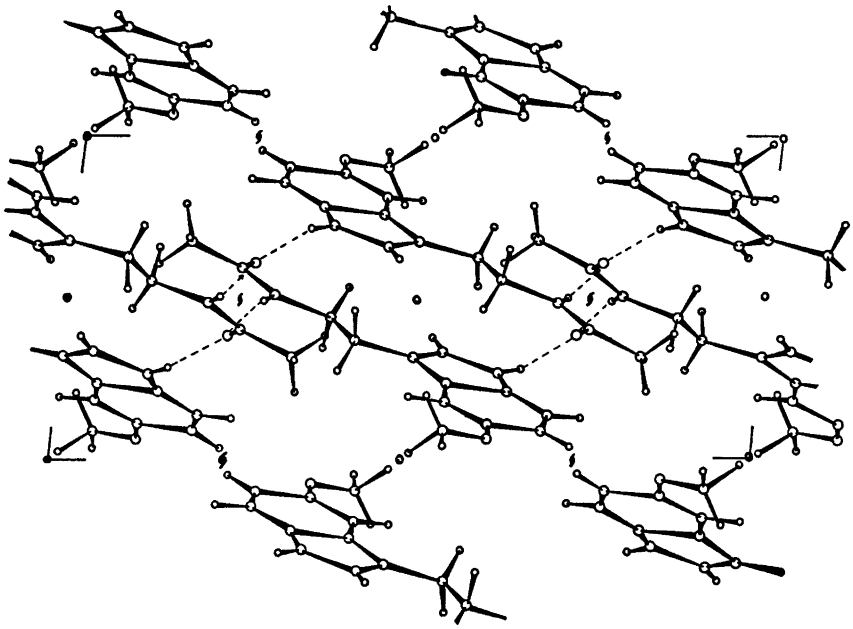


Fig. 2. The crystal structure of melatonin as viewed along the *b* axis.

Table 4. Interatomic distances (Å) and bond angles (°) with estimated standard deviations. For the indole part bond lengths corrected for rigid-body librations are given in parentheses. Standard deviations are 0.002 Å in bonds and 0.1° in angles between heavy atoms, 0.02 Å and 1.0°, respectively, if one hydrogen atom is involved.

Distances

N1–C2	1.365	(1.367)	C15–H115	1.03
C2–C3	1.365	(1.369)	C15–H215	0.95
C3–C9	1.440	(1.443)	C15–H315	0.96
C9–C4	1.403	(1.408)	C2–HC2	1.01
C4–C5	1.373	(1.376)	C4–HC4	0.96
C5–C6	1.403	(1.407)	C6–HC6	0.96
C6–C7	1.368	(1.372)	C7–HC7	0.91
C7–C8	1.386	(1.389)	C10–H110	1.01
C8–C9	1.408	(1.413)	C10–H210	0.97
C8–N1	1.380	(1.383)	C11–H111	0.97
C3–C10	1.497	(1.500)	C11–H211	0.98
C5–O5	1.382		C14–H114	0.93
O5–C15	1.416		C14–H214	0.94
C10–C11	1.505		C14–H314	0.95
C11–N12	1.445		N1–HN1	0.90
N12–C13	1.329		N12–HN12	0.85
C13–O13	1.239			
C13–C14	1.492			

N1 ···O13	$(1-x,1-y,1-z)$	2.895	N12–O13	$(1-x,y-\frac{1}{2},3/2-z)$ 2.969
HN1···O13	$(1-x,1-y,1-z)$	2.03	HN12–O13	$(1-x,y-\frac{1}{2},3/2-z)$ 2.19

Angles

N1–C2–C3	111.2	C5–C4–HC4	123
C2–C3–C9	105.5	C6–C7–HC7	120
C3–C9–C8	107.5	C7–C6–HC6	119
C9–C8–N1	107.2	C8–C7–HC7	122
C8–N1–C2	108.6	C9–C4–HC4	117
C3–C9–C4	132.6	C8–N1–HN1	126
C9–C4–C5	118.2	C2–N1–HN1	125
C4–C5–C6	121.2	N1–C2–HC2	118
C5–C6–C7	121.3	C3–C2–HC2	130
C6–C7–C8	118.1	C3–C10–H110	111
C7–C8–C9	121.3	C3–C10–H210	111
C8–C9–C4	119.8	H110–C110–H210	106
C7–C8–N1	131.5	C11–C10–H110	108
C4–C5–O5	124.1	C11–C10–H210	108
C6–C5–O5	114.7	C10–C11–H111	112
C5–O5–C15	117.3	C10–C11–H211	108
C2–C3–C10	129.3	H111–C11–H211	108
C9–C3–C10	125.2	N12–CH–H111	110
C3–C10–C11	113.8	N12–C11–H211	108
C10–C11–N12	110.9	C11–N12–HN12	119
C11–N12–C13	122.6	C13–N12–HN12	119
N12–C13–O13	121.3	C13–C14–H114	113
N12–C13–C14	117.2	C13–C14–H214	110
C14–C13–O13	121.5	C13–C14–H314	111
		H114–C14–H314	109
O5–C15–H115	120	H114–C14–H214	113
O5–C15–H215	103	H214–C14–H314	103
O5–C15–H315	112		
H115–C15–H215	112		
H115–C15–H315	113		
C5–C6–HC6	119		

sponding least-squares planes being less than 0.01 Å; the angle between the two planes is 1.4°. The non-hydrogen atoms of the longer side-chain are also roughly co-planar; a plane through these atoms does not quite coincide with the pyrrole-plane, however, the dihedral angle C2 – C3 – C10 – C11 being 5.5°.

The molecular arrangement in the crystal and the hydrogen bond system is illustrated in Fig. 2. Two hydrogen donors, N1 and N12, each with one hydrogen atom are available for hydrogen bond formation. There are also two potential acceptor atoms, but O13 is the acceptor for two hydrogen bonds and O5 for none. Each molecule is hydrogen bonded to three neighbouring molecules. The structure may be described as centrosymmetric hydrogen bonded dimers which are again hydrogen bonded to four neighbouring dimers forming helices along a set of screw axes. In this way the crystal consists of layers of hydrogen bonded molecules parallel to the $b-c$ plane. Between these layers there are only weak van der Waals interactions.

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Conformational Analysis of 2,5-Substituted 1,3-Dioxa-2-silacyclohexanes

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Results of the NMR analysis indicate that the free-energy difference between chair and boat conformations of the *cis*-2-*tert*-butyl-2,5-dimethyl-1,3-dioxa-2-silacyclohexane ring system is unusually low. The free energy difference between axial and equatorial substituents in position 5 of the 1,3-dioxa-2-silacyclohexanes is larger as compared to the pertinent cyclohexanes. Isomers with an axial substituent in the ring position 5 have not been detected.

Conformational studies on 1,3-dioxa-2-silacyclohexanes are scanty. Krieble and Buckhard [1] published the synthesis of 2,2-dimethyl-1,3-dioxa-2-silacyclohexane and later several substituted compounds have been prepared.[2–9] NMR studies on substituted 1,3-dioxa-2-silacyclohexanes have been reported twice.[6,8] Hellier [8] reported the NMR spectra of 2,2,5,5-tetra-substituted compounds, but only chemical shift data have been reported for the 5,5-dimethyl substituted compound.

This work was undertaken, partly because no relevant data on silicon analogues to 1,3-dioxanes or trimethylene sulfites were available in literature, and partly to obtain information on the conformational dependence of elongated bonds in cyclic sixmembered ring systems. Moreover, it was of interest to compare the effect of substituents in sixmembered ring systems containing silicon in the ring position 2, with analogous compounds containing sulphur or phosphorus. Only compounds with substituents in positions 2 and 5 have been studied by NMR.

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EXPERIMENTAL

The 1,3-dioxa-2-silacyclohexanes were synthesized from the appropriate diols by the following procedures:

Method A. The 1,3-diol together with two mol of *N,N*-dimethyl amine were dissolved in a suitable solvent (ether, chloroform or benzene). An equimolar amount of the appropriate dichlorosilane was added dropwise and the mixture was refluxed for 4 h, filtered and the pure compound was obtained, after removal of solvent by distillation.

Method B. The 1,3-diol and a small amount of sodium methanolate were dissolved in benzene or mesitylene. The equivalent amount of the appropriate dimethoxysilane was added dropwise and the methanol formed was removed by azeotropic distillation with the solvent. The pure compound was obtained by distillation.

All non-commercial available 1,3-diols were synthesized from the appropriate malonates [10] by reduction with $LiAlH_4$.

2,2-Dimethyl-1,3-dioxa-2-silacyclohexane (I).[7] $B.p._{760}$ 122 °C ($b.p._{22}$ 35 °C), n_D^{20} 1.4132, GC purity 99 %. Yield method A, 74 %, and method B, 85 %.

2,2,5-Trimethyl-1,3-dioxa-2-silacyclohexane (II). $B.p._8$ 29 °C, n_D^{20} 1.458, GC purity 98 %. Yield method A, 55 %, and method B, 72 %.

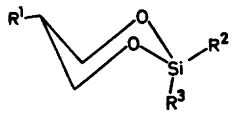
5-tert-*Butyl-2,2-dimethyl-1,3-dioxa-2-silacyclohexane (III).* Method A: $B.p._{9.5}$ 69 – 71 °C, n_D^{20} 1.4373, GC purity 98 %, yield 68 %. Method B: $B.p._7$ 67 – 68 °C, n_D^{20} 1.434, GC purity 98 %, yield 60 %.

2-tert-*Butyl-2-methyl-1,3-dioxa-2-silacyclohexane (IV).* $B.p._{30}$ 38 °C, GC purity 98 %.

2-tert-*Butyl-2,5-dimethyl-1,3-dioxa-2-silacyclohexane* [cis (V) and trans (VI)]. $B.p._{16}$ 60 – 61 °C, azeotropic distillation with mesitylene. (The mesitylene was used as solvent during the preparation of the chlorosilane.)

GC specifications: The compounds were analysed and purified on a preparative gas chromatographic column, 2.1 m × 6 mm, containing either 15 % Apiezon L on Chromosorb W HMDS

Table 1. Coupling constants (Hz) and chemical shifts (ppm from TMS) at 27 °C.



Compound	R^1	R^2	R^3	$^2J_{4(6)}$	$^2J_5$	$^3J_{4a5a}$	$^3J_{4e5a}$	$^4J_{4a6a}$	$^4J_{4a6e}$	$^4J_{4e6e}$	$^3J_{CH_3,H}$	$\nu_{4a(6a)}$	$\nu_{4e(6e)}$	ν_{5a}	ν_{R^1}	ν_{R^2}	ν_{R^3}	RMS
I	H	CH_3	CH_3	[a]	[a]	7.10	3.46	[a]	[a]	[a]		3.93	3.93	1.72	1.72[b]	0.8	0.08	0.10
II	CH_3	CH_3	CH_3	−10.89		9.49	3.97	0.47	−0.45	1.85	6.85	3.57	3.88		0.70	0.05	0.05	0.06
II[b]	CH_3	CH_3	CH_3	−10.96		9.39	3.97	0.25	−0.31	1.78	6.82	3.56	3.89		0.69	0.05	0.06	0.05
III	*t*-Bu	CH_3	CH_3	−11.06		10.55	4.06	−0.27	−0.59	1.88		3.80	4.05	1.74	0.82	0.08	0.08	0.12
IV	H	*t*-Bu	CH_3	[a]	−13.0	4.25	6.50	[a]	[a]	[a]		3.93	3.93	1.63	1.75[c]	0.08	0.97	[d]
V	CH_3	*t*-Bu	CH_3	−11.0		9.5	4.0	−0.1	−0.3	1.9	6.9	3.59	3.93		0.67	0.08	0.95	[d]
VI	CH_3	CH_3	*t*-Bu	−11.0		10.5	4.0	−0.1	−0.3	1.9	6.9	3.51	3.87		0.57	0.95	0.08	[d]

[a] Parameters cannot be calculated from spectra. [b] Temperature 58 °C. [c] R^1=H. [d] Non-iterative calculations (see the text); errors in the coupling constants are *ca.* 0.3 Hz.

or 10 % Apiezon L on Fluoropack HMDS.

NMR specifications: The materials were introduced into 5 mm o.d. NMR sample tubes, directly from the gaschromatograph. A suitable solvent as well as a small amount of TMS were added to serve as locking and reference substance. The samples were degassed by the freeze-pump-thaw technique. The spectra were recorded on a 60 MHz JEOL-C-60-H instrument. All spectra were recorded in internal lock mode with frequency sweep at *ca.* 50 Hz sweep width and calibrated every 5 Hz using a frequency counter. The counter was accurate to 0.1 Hz and the line positions were taken as the average of several spectra. The computations were carried out on an IBM 360/50 computer and the graphical output was obtained using a Calcomp plotter.

SPECTRAL ANALYSIS

The spectrum of 2,2-dimethyl-1,3-dioxa-2-silacyclohexane (I) can be divided into three regions; a triplet at δ 3.9, a multiplet at δ 1.7, and a singlet at δ 0.1. The triplet at high frequency is assigned to the protons in positions 4 and 6, and the multiplet to protons at carbon 5. The singlet is due to the methyl groups attached to the silicon. The spectrum was analysed as an $[ABC]_2$ spin-system (Table 1). The RMS value obtained was 0.1 when 67 theoretical lines were correlated to observed transitions and 14 parameters were allowed to vary.

The spectrum of 2,2,5-trimethyl-1,3-dioxa-2-silacyclohexane (II) contained four bands at δ 0.05, δ 0.7, δ 2.0, and δ 4.0 – 3.7. The two low frequency bands are assigned to the methyl-groups. The high frequency band is assigned to protons at carbond 4 and 6 and the band at δ 2.0 to the protons in the ring position 5. The spectrum was analysed as an $[AB]_2CD_3$ spin-system. Spectra obtained at 27 °C and 50 °C were analysed (Table 1). The RMS value obtained in both cases was 0.06 when 18 parameters were allowed to vary and 185 lines were correlated to observed transitions.

The spectrum of 5-*tert*-butyl-2,2-dimethyl-1,3-dioxa-2-silacyclohexane (III) consists of four bands at δ 0.08, δ 0.82, δ 1.74, and δ 4.1 – 3.7. The two low frequency bands are due to the two methyl groups and the *tert*-butyl group. The bands at δ 4.1 – 3.7 and δ 1.7, assigned to protons at $C_4(C_6)$ and C_5 (Fig. 1), respectively, were analysed as an $[AB]_2C$ spin system. The

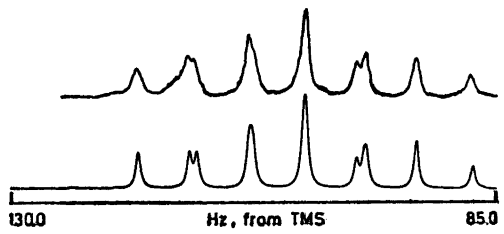


Fig. 1. The observed spectrum (upper) and the calculated spectrum of proton 5 of 5-*tert*-butyl-2,2-dimethyl-1,3-dioxa-2-silacyclohexane (III).

RMS value obtained was 0.12 when 40 theoretical lines were correlated to observed transitions and 15 parameters were allowed to vary.

The NMR spectrum of 2-*tert*-butyl-2-methyl-1,3-dioxa-2-silacyclohexane(IV) appeared to be deceptively simple, with a triplet at δ 3.93, a complex band at δ 1.63 and singlets at δ 0.97 and δ 0.08 integrating to 4:2:9:3, respectively. The spectrum could be reproduced by assuming an $[A]_4CD$ spin system, but iterative calculations were not feasible, as the experimental spectrum had too little fine structure. Parameters used in the non-iterative calculation are listed in Table 1. The errors in the parameters are *ca.* 0.3 Hz. The GC analysis of 2-*tert*-butyl-2,5-dimethyl-1,3-dioxa-2-silacyclohexane (V and VI) dissolved in mesitylene showed only one peak for this compound. The NMR spectrum, however, gave a complex pattern with singlets at δ 0.95 and δ 0.08, two doublets at δ 0.67 and δ 0.57, a broad unresolved band centered at *ca.* δ 2.0, and a region at δ 4.0 – 3.4 containing eleven complex peaks (Fig. 2). The signals at δ 4.0 – 3.4, assigned to the protons at carbons 4 and 6, were analysed as two overlapping $[AB]_2$ parts of $[AB]_2CD_3$ spin systems. Only non-iterative calculations were performed. The calculated spectrum of parameters of Table 1 is shown in Fig. 2. The errors in the coupling constants are *ca.* 0.3 Hz.

RESULTS AND DISCUSSIONS

2,2-Dimethyl-1,3-dioxa-2-silacyclohexane (I). The observed chemical shifts (Table 1) are close to those reported by Hellier [3] and Voronkow *et al.*[4] The reported [3] vicinal coupling constant 5.4 Hz is, however, the mean value

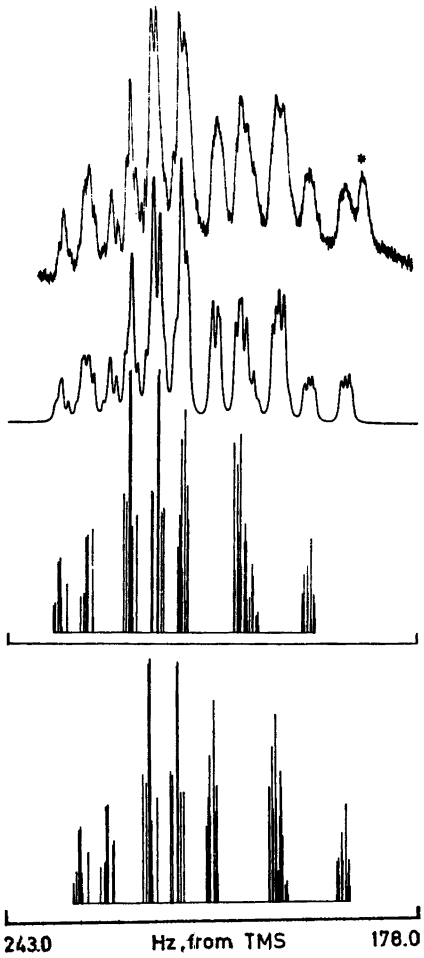


Fig. 2. The observed spectrum (upper) of protons at carbons 4 and 6 of *cis-* and *trans*-2-*tert*-butyl-2,5-dimethyl-1,3-dioxa-2-silacyclohexane (V and VI). The theoretical spectrum is shown below. The stickplots show the individual spectra of the *cis-* (upper) and *trans-* (lower) isomer (see the text). * = impurity.

of the coupling constant obtained by us, 7.09 Hz and 3.46 Hz (Table 1). The chemical shift data and the lack of any observable geminal coupling constants suggest a rapid equilibrium between two equivalent chair conformations. The 1,3-dioxane has been reported [11,12] to exist in a 1:1 equilibrium between two chair conformations. The observed vicinal couplings of compound I are of the same magnitude as observed in dioxanes. Moreover, considering the symmetry of I, it is reasonable to assume a 1:1 equilibrium between two chair conformations for compound I too. The NMR spectrum of compound I in CS_2 was examined in the temperature range +27 °C to −88 °C,* but the coalescence temperature was not reached and no noticeable changes of the spectra were observed. A coalescence temperature below −88 °C indicates a lower inversion barrier as compared to 1,3-dioxane. This is a reasonable assumption because coupling constants and chemical shift differences of the siloxanes examined (Table 1) are close to pertinent values obtained for dioxanes.[13] Regardless of the transition states, inversion between chairforms involves rotations about single bonds. To the authors knowledge, no values of the rotation barrier about Si−O bonds have been reported. The Si−O bond length, 1.63 Å,[14] as compared to C−O, 1.43 Å, causes a "flattening" of O−Si−O ring moiety as compared to the O−C−O part of the dioxane ring. The effect of longer bonds on the rotation barrier can be seen from CH_3-SiH_3, where the barrier is estimated to *ca.* 1.9 kcal/mol [14] as compared to the ethane barrier of *ca.* 3.0 kcal/mol. Introduction of a single bond, into a cyclic system, with lower rotation barrier should lower the inversion barrier of the ring-system. Geminal methyl groups at carbon 2 in dioxane lowers the barrier from 9 kcal/mol to about 7.8 kcal/mol,[15] and the methyl groups attached to the silicon atom might cause a similar lowering of the barrier in compound I.

Assuming an equilibrium between two equally populated chair forms, the two observed vicinal coupling constants can be expressed by ${}^3J_{obs} = \frac{1}{2}(J_{aa} + J_{ee})$ and ${}^3J_{obs} = \frac{1}{2}J_{ae} + J_{ea})$. This enables us to calculate dihedral angles for the chair conformation by the *R*-value method,[16] giving $R = 2.05$, corresponding to a dihedral angle about 57° of the $-CH_2-CH_2-CH_2-$ moiety. The *R*-value is essentially the same as those obtained from NMR measurements on various six-membered ring compounds,[16-18] which are shown to exist in chair conformations.

2,2,5-Trimethyl-1,3-dioxa-2-silacyclohexane (II). The NMR data obtained for this compound clearly indicates that it exists in a chair

* The temperature ranges for compounds I and II (*vide infra*) are decided from solubility restrictions.

conformation or an equilibrium between two chair forms. The silicon methyl groups appear to be a broad but split signal, indicating two different methyl groups. The couplings 9.5 Hz and 3.97 Hz (Table 1), assigned to $^3J_{4a5a}$ and $^3J_{4e5a}$, indicate that the methyl group at carbon 5 is predominantly in the equatorial position. The coupling constants suggest that compound II exists almost entirely in a conformation with an equatorial methyl group at carbon 5. The NMR data obtained at 58 °C are not sufficiently different from those obtained at 27 °C, to make any calculations of conformational energies feasible. There was, however, no significant changes in the spectrum between −70 and +58 °C. The latter observation might indicate a chair-twist rather than a chair-chair equilibrium where an equatorial methyl group at carbon atom 5 is expected to be at least 0.8 kcal/mol [19] more stable as compared to the axial position. The large value of $^4J_{ee}$, 1.85 Hz, suggests a chair-twist equilibrium or that II exists in a rigid chair form.

5-tert-*Butyl-2,2-dimethyl-1,3-dioxa-2-silacyclohexane (III).* The coupling constants obtained are close to values of compound II, except for $^3J_{aa}$, which is substantially larger, 10.6 Hz. The coupling constants (Table 1) indicate that compound III exists predominantly in a chair conformation with the tertiary butyl group in the equatorial position. No significant changes of the spectrum was observed between −107 and +197 °C, and it is therefore reasonable to assume that III constitutes an anancomeric system.

2-tert-*Butyl-2-methyl-1,3-dioxa-2-silacyclohexane (IV).* The spectrum of compound IV appeared to be deceptively simple. The protons at C_5 are, however, not equivalent with chemical shift difference about 0.1 ppm. The coupling constants obtained, 6.5 Hz and 4.3 Hz, are substantially different from the values of I and this suggests an equilibrium different from that assumed for compound I. An equilibrium between two twist conformations can explain a chemical equivalence of protons at carbons 4 and 6 and a non-equivalence of protons at carbon 5. An estimate of the vicinal coupling constants of an equally populated twist-twist equilibrium, *ca.* 6.5 Hz and *ca.* 4.3 Hz, can be calculated from coupling values suggested by Wücherpfennig.[20] Recently Pihlaja *et al.*[21] reported the existance of twist forms in 1,3-dioxanes. The longer Si−O bond as compared to the C−O bond could reduce the equatorial preference of a 2-*tert*-butyl group, but it might also make twist conformations more probable.

cis- *and* trans-*2*-tert-*Butyl-2,5-dimethyl-1,3-dioxa-2-silacyclohexane (V and VI).* The NMR spectrum of this sample clearly indicated, in spite the appearence of only one peak in GC, the existance of more than one compound. The complexity of the spectrum made it feasible only to analyse the region δ 3.4−4.0 (Fig. 2), assigned to protons in ring-positions 4 and 6. The region assigned to the proton at C_5 did not show any resolved fine-structure, and hence it was not possible to obtain any information from this band.

The band at $\delta \sim 3.5$ can only be reproduced upon the following assumptions (see Fig. 2): (a) The spectrum consists of two superimposed spectra due to closely related spin-systems and (b) The methylgroup at carbon 5 occupies the equatorial position in both compounds. The two superimposed spectra are both of the $[AB]_2CD_3$ type. From the magnitude of relevant coupling constants and chemical shifts (Table 1) and the symmetry of the spin system, it is reasonable to assume that both isomers exist in a chair conformation. Entry VI is tentatively assigned to the *trans* isomer with the methyl group at carbon 5 and the *tert*-butyl group in the equatorial position. The existence of a large coupling constant in both isomers indicates equatorial methyl groups. This implies that the *tert*-butyl group in the *cis* isomerV is situated axial or V exists in a boat conformation or in a chair-boat equilibrium. The Si−O bond (1.63 Å) is considerably longer compared to the C−O (1.43 Å) and the P−O bond (1.54 Å). The equatorial preference of a *tert*-butyl group on silicon might be less as compared to pertinent carbon and phosphorus analogues. The large coupling $^3J_{aa}$ excludes any equilibrium between twist conformations.

Boat forms of six-membered saturated ring compounds are normally 5−6 kcal/mol higher in free energy (ΔG°_{25}) than the corresponding chair conformations. ΔH° (chair-boat) for cyclohexane and 1,3-dioxane are roughly those estimated from the rotational barriers in propane [27] and dimethyl ether.[23] It is expected that for other heterocycles the ΔG°_{25} (chair-

boat) may be less than 6 kcal/mol depending primarily on rotational barriers, as yet unknown. It is assumed that the rotational barrier of $CH_3-CH_2-Si-CH_3$[24] is less than the value of the carbon analogue. The longer Si–X bond as compared to the C–X bond may be the reason for this. A lower rotational barrier about the Si–X bond,[24] as compared to the C–X bond, together with a more favourable equatorial *tert*-butyl group suggest the existence of a boat conformation or a chair-boat equilibrium for compound V. The existence of a boat conformation for *cis*-2,5-di-*tert*-butyl-1,3,2-dioxaphosphorinan-2-one [25] is shown, based on the magnitude of the coupling between the phosphorus atom and the protons at carbons 4 and 6. Certain 1,3-dioxanes, substituted at carbons 2 and 5, have been reported to exist in boat conformations.[26–29]

CONCLUSION

The barrier to ring inversion in siloxanes is probably less than the barrier observed for analogous 1,3-dioxanes. Twist and boat conformations must be considered when discussing conformational equilibria of 1,3-siloxanes. Contribution from non-chair forms is assumed to be due to a lower rotational barrier together with a substantial longer Si–X bond as compared to carbon analogues. There is no evidence for the existence of isomers with an axial substituent at carbon 5. The free energy difference between axial and equatorial substituents in position 5 is apparently larger for 1,3-siloxanes as compared to cyclohexanes and 1,3-dioxanes.

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Short Communications

An Anodic Synthesis of 2,5-Dimethyl-2,5-diphenyltetrahydrofuran

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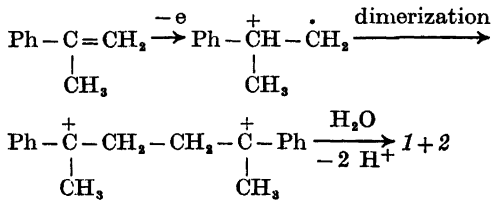
The anodic oxidation of aromatic hydrocarbons with at least one benzylic hydrogen partly results in the formation of the corresponding olefin *via* proton loss from an intermediate benzylic cation. Since the olefin formed is oxidized at a lower potential than the starting material, further oxidation takes place – possibly in the adsorbed state – with formation of the two-electron oxidation product from the olefin, as has been shown in the case of indan.[1] If cumene is oxidized according to the same mechanism it should under suitable conditions give α-methylstyrene as the primary product. This might then be oxidized further to phenylacetone by analogy with the known behaviour of α-methylstyrene upon lead tetraacetate oxidation.[2,3] Hence, it might be possible to realize a direct route from cumene to this important intermediate.

The oxidation of cumene in acetonitrile/water (volume ratio 98:2) with sodium perchlorate or tetrabutylammonium tetrafluoroborate as supporting electrolyte gives, however, 2-phenylpropanol-2 as the main product. To see if it was possible to obtain phenylacetone from α-methylstyrene itself, this compound was oxidized under identical conditions, but no trace of phenylacetone was detectable. GLC analysis of the reaction product showed, besides starting material, only two well-resolved peaks (retention times 5.1 and 5.8 min on a 5 % neopentylglycol succinate column (2 m × 0.3 mm) at 170 °C, ratio 52:48), with identical mass spectra. After distillation, the product partly crystallized on standing. Recrystallization from ethanol gave crystals which on GLC analysis were found to correspond to one of the peaks in the gas chromatogram (with retention time 5.8 min). From the mother liquor, the second compound could be isolated as an oil. NMR and elemental analysis led to the identification of the two compounds as the *cis* (*1*) and *trans* (*2*) isomers of 2,5-dimethyl-2,5-diphenyltetrahydrofuran. The formation of these compounds is in accordance with earlier mechanistic work on the additive dimerization of arylolefins.[4]



The NMR spectra of *1* and *2* have been reported [5] and agree well with those given in the experimental section.

Experimental The concentric capillary gap cell used has been described in a recent paper.[6] Analytical procedures were as described earlier.[7]

Oxidation of cumene. Cumene (120 g, 1 mol), $NaClO_4.H_2O$ (2.8 g, 0.01 mol) and water (18 ml, 1 mol) were dissolved in acetonitrile (1000 ml). At the beginning of the electrolysis the applied voltage was 13 V. The current was kept at 25 A throughout the experiment. The voltage increased slowly and as soon as it reached 30 V, 4.5 ml of a solution of $NaClO_4$. H_2O (2.8 g) in water (36 ml) was added. After passing a charge of 2.70 F the electrolysis was interrupted and the solvent removed by evaporation *in vacuo*. Ether was added to the residue and the solution washed with water and dried over $MgSO_4$. After removing the ether, the residue (57.7 g) was distilled to give the following fractions: 1, b.p. 87 °C/11 mmHg (11.0 g); 2, b.p. 87 – 92 °C/11 mmHg (28.1 g); 3, residue (13.4 g). Fraction 2 was found to be pure 2-phenylpropanol-1 by comparison with an authentic sample.

Oxidation of α-methylstyrene. α-Methylstyrene (118 g, 1 mol), $NaClO_4.H_2O$ (2.8 g, 0.02 mol) and water (18 ml, 1 mol) were dissolved in acetonitrile (1000 ml). The electrolysis was run at 50 A until 1 F had passed. The applied voltage rose from 12 to 26 V during the run. The reaction solution was worked up as above. The residue after evaporation of the ether (100.5 g) was distilled to give the following fractions: 1, 103 °C/0.4 mmHg (8.7 g); 2, 103 – 128 °C/0.4 mmHg (4.8 g); 3, 128 – 143 °C (30.6 g); 4, residue (43.3 g). GLC of fraction 3 showed two well-resolved peaks with an integral ratio of 52:48. On standing this fraction partly crystallized. A sample of the solid compound was recrystallized from ethanol giving colourless

crystals, m.p. 74 – 75 °C (lit.[5] 75 °C). By suction of fraction 3 the oily isomer was obtained contaminated with 20 % of the solid isomer. NMR of the crystalline isomer:[5] δ 1.53 ppm (s, 6, methyl protons), 2.15 (an AA'BB' spectrum centered at 2.15 ppm, 4, methylene protons), 7.12 – 7.63 (m, 10, aromatic protons). NMR of the liquid isomer:[5] δ 1.60 ppm (s, 6, methyl protons), 2.20 (s, 4, methylene protons), 7.00 – 7.58 ppm (m, 10, aromatic protons). The two isomers showed identical mass spectra, *m/e* (% of base peak): 238(15), 237(100), 219(7), 117(21), 105(95), 91(11), 77(16), 43(15). (Found: C 85.3; H 7.97; O 6.49. Calc. for $C_{18}H_{20}O$: C 85.7; H 8.00; O 6.34).

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4-Chlorobuten-3-yne: a Spectrochemical Characterization

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True acetylenic hydrogens are generally replaced by halogen by action of alkaline hypohalites. This reaction has been applied to vinylacetylene with different halogen atoms by Carothers and Jacobson.[1] We prepared 4-chlorobuten-3-yne by adding monovinylacetylene to a potassium hypochlorite solution.

The substance was isolated and purified with gas liquid chromatography at a temperature of 100 °C. The column was packed with diethylhexyl sebacate (15 %) absorbed on Chromosorb.

The identification is primarily based on the mass spectrum obtained by using a combined GLC – MS. After correction for background, the largest peaks are: *m/e*(rel. intensity), 49(19.5), 50(46.5), 51(100.0), 52(4.5), 60(10.5), 62(3.5), 84(10.5), 85(12.0), 86(98.5), 87(8.5), 88(30.5) and 89(1.5).

The occurrence of chlorine is demonstrated by the intensity relation 3:1 of the mass numbers 86:88 corresponding to the molecular ions with ^{35}Cl and ^{37}Cl and the mass numbers 60:62 probably corresponding to the ions $CHC^{35}Cl^+$ and $CHC^{37}Cl^+$. The molecular weight is consequently 86.5. The relation $^{13}C:^{12}C$ in the substance appears most evident from the intensity relation 4.5:100.0 between the mass numbers 52:51, corresponding to the ions $^{13}CC_3H_3^+$ and $C_4H_3^+$. Hence the assumption of four carbon atoms is confirmed.

A UV-spectrum of the substance was run between 340 and 200 nm. An absorption at 277 – 278 nm was observed.

For monovinylacetylene, absorption at 221 nm and 227.5 nm has been reported by Georgieff *et al.*[2]

The IR-spectrum of gaseous 4-chlorobuten-3-yne is given in Fig. 1. A theoretical calculation of the frequencies, from a normal coordinate analysis using a simple diagonal valence force field, shows good agreement with the obtained spectrum for some of the peaks.[3,4]

At 3120 cm^{-1} we observed the asymmetric hydrogen stretch for the vinyl group. The peak at 3035 cm^{-1} belongs either to the symmetric hydrogen stretch for the vinyl group or a stretch of the remaining hydrogen. At 2220 cm^{-1} the characteristic stretching of $-C\equiv C-$ was observed and at 1600 cm^{-1} the $-C=C-$ stretch. There is no strong absorption around 3300 cm^{-1} which shows the purity of the substance and eliminates the isomeric chlorobutenyne structures.[5,6]

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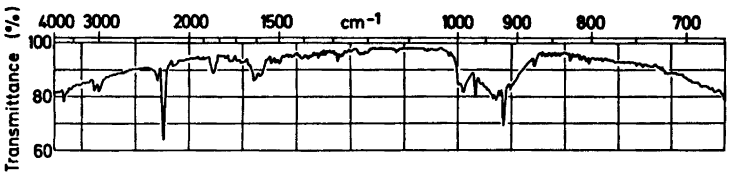

Fig. 1. IR spectrum of 4-chlorobuten-3-yne. Vapour mixed with He, 1 m cell, KBr optics. The curved background line depends on multiple reflections in the 1 m cell; no reference cell could be used.

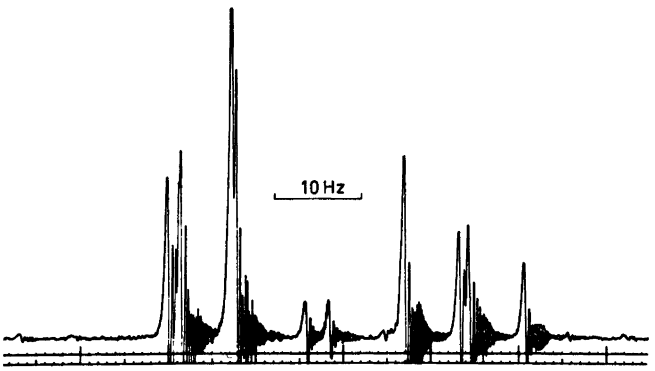

Fig. 2. High resolution NMR spectrum of 4-chlorobuten-3-yne at 98 MHz with C_6D_6 as solvent.

The NMR-spectra of 4-chlorobuten-3-yne were recorded at 60 MHz with carbon tetrachloride as solvent and at 98 MHz with deuteriochloroform and deuteriobenzene as solvents. The spectra run with CCl_4 and $CDCl_3$ as solvents showed a surprising resemblance to an A_2B spin system spectrum. With C_6D_6 as solvent a spectrum was obtained with quite a different appearance (see Fig. 2) as expected from the molecular structure. The observed thirteen lines were used in a least squares fitting procedure, LAOCN 3 [7] and the following chemical shifts and spin-spin coupling constants were obtained.

The three vinyl protons are labelled C *trans* to A and *cis* to B; $\nu_A = 534.6$ cps [$\delta_A = 5.45(5)$ ppm], $\nu_B = 507.3$ cps [$\delta_B = 5.18$ ppm], $\nu_C = 535.0$ cps [$\delta_C = 5.45(9)$ ppm], $J_{AB} = 0.9$ cps, $J_{AC} = 17.7$ cps, and $J_{BC} = 11.9$ cps.

The values of the chemical shifts are measured from TMS used as internal standard. The obtained values of the coupling constants are in good agreement with those obtained from other vinyl compounds.[8] The chemical shift values are somewhat low, probably due to the solvent, C_6D_6, but their relative positions are in agreement with other vinyl compounds.[8,9]

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Indolizine Derivatives. I. A Novel One-step Synthesis of Pyrrolo[2,1,5-*cd*]-indolizines. The Mechanism of the Acylative Cyclization of 2-Pyridine-carbaldehyde and Unsaturated Carbonyl Compounds

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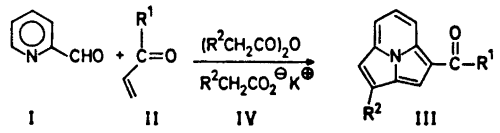
Some fifteen years ago practical syntheses of many pyrrolo[2,1,5-*cd*]indolizine derivatives starting from indolizines [1] were developed. Later, the preparation of pyrrolo[2,1,5-*cd*]-indolizines from pyrrolizines,[2] alkylpyridines,[3] and pyridineacrylates [4] have been reported. The overall yields based on commercial starting materials were seldom better than moderate.

As shown in this paper, the acylative cyclization between the readily available 2-pyridine-carbaldehyde (I) and an α,β-unsaturated carbonyl compounds such as II seems to provide a new, convenient route to 1-acyl-substituted pyrrolo[2,1,5-*cd*]indolizines (III).



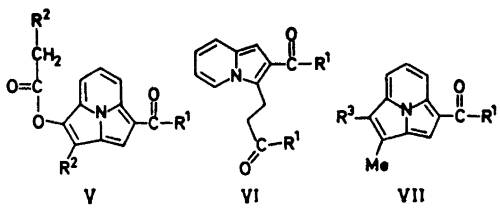
I II IV III

R^1 = Me, Ph, MeO
R^2 = H, Me

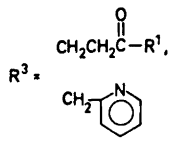
In a typical procedure I and IIa, Ac_2O and KOAc (rather arbitrary mol ratios 2:3:20:10, respectively) are refluxed for 15 min. After a standard work-up* the product IIIa is freed of coloured by-products, best by benzene elution over alumina or silica. The yield is 60 – 70 %.

In order to study the scope and mechanism of this peculiar cyclization reaction, other unsaturated carbonyl compounds were used in place of IIa. Similar cyclizations in a propionate system were examined. The results are shown in Table 1.

The structures of the cyclization products are in accord with analyses and spectral data. The nature and sites of the substituents in pyrrolo[2,1,5-*cd*]indolizines are easily determined by NMR-spectroscopy.[4] The substitution



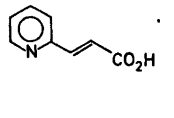
V VI VII



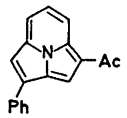
pattern in the resulting pyrrolo[2,1,5-*cd*]indolizines, that is, the acyl group at C-1 and the methyl group (when R^2 = Me) at C-3, verifies that C-1 and C-2 must originate from the α- and β-carbons of II, respectively, and C-2a and C-3 from the 1- and 2-carbons of IV, respectively.

The reaction may be visualized (Scheme 1) as beginning with a Perkin reaction to give the bicyclic intermediate VIII, followed by nucleophilic addition of the anion IX to furnish the intermediate X, which then rearranges into the tricyclic intermediate XI. The aromatization completes the reaction. In the propionate case, besides III, V is also formed, probably owing to steric factors. The propionyloxy group at C-4 originates from the aldol intermediate VIII'. A molecule of hydrogen is then lost from the subsequent intermediate XI'.

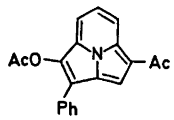
The mechanism is supported by the following findings: Pyridineacrylic acid XII, prepared by the Doebner reaction, reacts as well, yielding with IIa only IIIa in any acylate system. The



XII



XIII



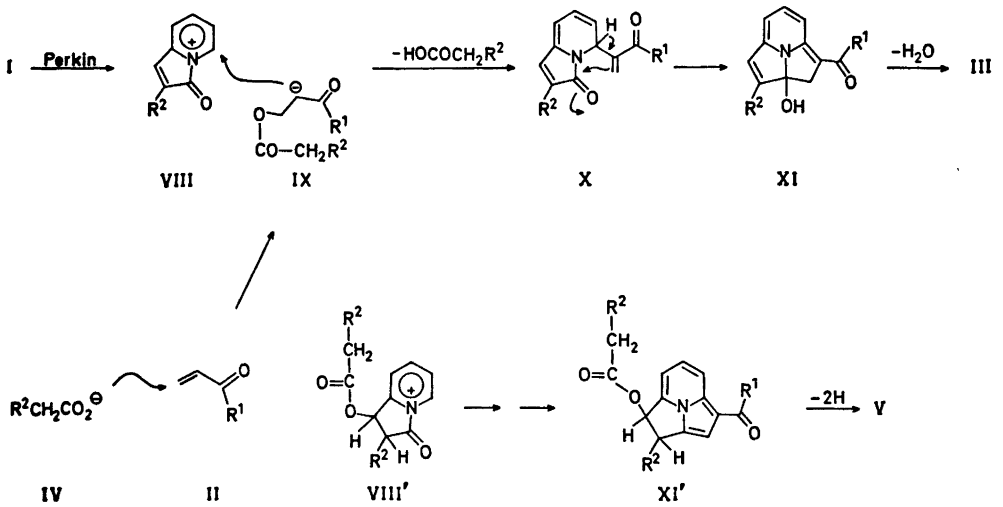
XIV

reaction between I, IIa, and phenylacetic acid gives the compounds XIII and XIV (the phenyl group at C-3). If $KOCOCH_2R^2$ is omitted, neither II nor XII gives rise to pyrrolo[2,1,5-*cd*]indolizines. In mere $(R^2CH_2CO)_2O$ I and the

* The cooled acylating mixture is stirred with water untill acetic anhydride has disappeared. The product is extracted into ether, washed and dried.

Table 1.

	R^1	R^2	Products	(%) of III	Ref.
a	Me	H	IIIa	60–70	(1)
b	Me	Me	IIIb, Vb VIIb	~50	
c	Ph	H	IIIc, VIc	~40	
d	Ph	Me	IIId, Vd, VId, VIId	~20	
e	OMe	H	IIIe	~15	(1)
f	Benzalacetone		None		



Scheme 1.

unsaturated ketones II yield the indolizine VI as the sole product, whereas I and the unsaturated ester IIe do not react at all. Irrespective of the amount of added $KOCOCH_2R^2$, I and IIc always form some indolizine VIc, presumably because the enolate anion IXc is able to compete with an anhydride anion in the first condensation step. Owing to electronic effects, benzalacetone (IIf) does not give sufficiently of the corresponding anion IXf, hence no Xf, which could lead to IIIf.

This mechanism explains the decreasing cyclization tendency IIa > IIc > IIe > IIf; the more II is inclined to form IX, the higher is the reactivity, and makes allowance for the need of a salt catalyst also after the Perkin step.

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Improved *O*-Methylation of Carotenoids

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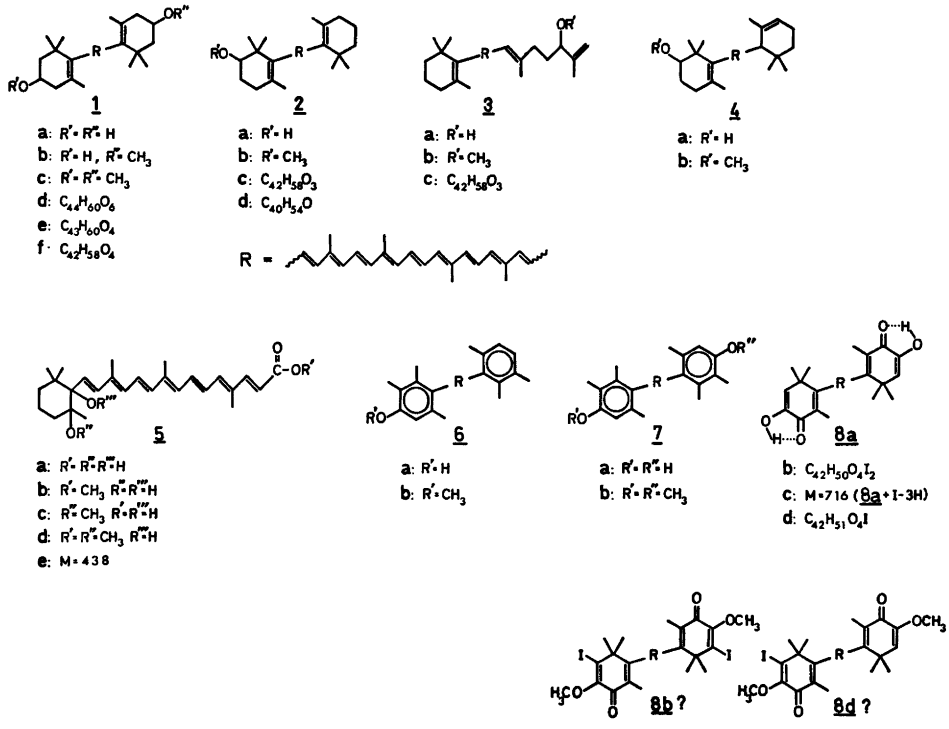
Methods available for *O*-methylation of carotenoids have recently been reviewed.[1] These include selective methylation of allylic, secondary hydroxy groups by hydrogen chloride in methanol [2–4] and methylation of non-allylic secondary hydroxy groups with methyl iodide and potassium *t*-amyloxide [5] or better by the Kuhn procedure [6–10] with methyl iodide and silver oxide or barium oxide in dimethylformamide or dimethylformamide/dimethyl sulfoxide. By the latter procedure tertiary hydroxy group may also be methylated.[11] Methylation of enolic and phenolic hydroxy groups in the carotenoid series is not achieved with diazomethane.[12–13]

In the present work, the original Kuhn procedure with silver oxide [6] was shown to result in abnormal products for zeaxanthin (*1a*) and *β,β*-caroten-2-ol (*2a*), Scheme 1. Zeaxanthin (*1a*) gave three products *1d, e, f* more polar than zeaxanthin dimethyl ether (*1c*) with unchanged electronic spectrum and molecular ion *m/e* 684.4392 (684.4390 calc. for $C_{44}H_{60}O_6$), 640 and 626 respectively, and with fragment ions M − 14, M − 30, M − 44, and M − 60 in addition to M − 92 and M − 106 ascribed to eliminations from the polyene chain.[14] Product *1d* has formally added $2 \times C_2H_2O_2$, and the molecular ions of *1e* and *1f* are compatible with the addition of ($C_2H_2O_2 + CH_2$) and $C_2H_2O_2$, respectively. *β,β*-Caroten-2-ol (*2a*) gave a product *2c* (20 % of recovered carotenoid; 45 % total recovery), more polar than the methyl ether *2b*, with molecular ion *m/e* 610, again compatible with a formal addition of $C_2H_2O_2$. The same type of abnormal product has recently been observed for aleuriaxanthin (*3a*).[15]

The BaO/DMF modification [7] gave for *β,β*-caroten-2-ol (*2a*) 49 % pigment recovery, 25 % thereof was a didehydro-*2a* = *2d* (4 nm hypsochromic shift in methanol, *m/e* 550.4170 = M = $C_{40}H_{54}O$).

The BaO-DMF/DMSO procedure,[8] tested for zeaxanthin (*1a*), *β,β*-caroten-2-ol (*2a*),[10] and *β,ε*-caroten-2-ol (*4a*) gave the desired methyl ethers. Thus zeaxanthin (*1a*) gave the dimethyl ether (*1c*, 30 % of recovered carotenoid), the mono-



Scheme 1.

methyl ether (*1b*, 20 %), and unreacted *1a* (50 %); total pigment recovery 50 % after 40 h. β,ε-Caroten-2-ol (*4a*) gave the methyl ether *4b* (35 % of recovered carotenoid) and unreacted *4a*; total pigment recovery 48 %.

These results demonstrate that the Kuhn procedures[6,7] may give abnormal products in the carotenoid series. Yields of methylated products are variable, *cf.* Refs. 9 and 4, and particularly unsatisfactory for secondary, sterically hindered alcohols (*2a*[10] and *4a*) and tertiary alcohols.[11]

Recently Stoochnoff and Benoiton[16] reported that methyl iodide and sodium hydride in tetrahydrofuran effect methylation of weakly acidic or sterically hindered hydroxy groups. The potentiality of this method in the carotenoid series has now been studied:

Secondary, allylic hydroxyl. Aleuriaxanthin (*3a*) gave the methyl ether *3b* (60 % of recovered carotenoid) besides unreacted *3a*; total pigment recovery 66 %.

The methyl ether *3b* had *m/e* 566 (M), 550.4176 (M−CH_4; calc. for $C_{40}H_{54}O$ 550.4175), M−28, M−79, M−92, M−106, M−158.

Secondary, non-allylic hydroxyl. Zeaxanthin (*1a*) gave the monomethyl ether *1b* (15 % of recovered carotenoid) and the dimethyl ether *1c* (85 %); total recovery 92 %. Both methyl ethers exhibited the predicted molecular ions and M−31, M−92, M−106, and M−158 fragment ions; *1b* also an M−18 ion.

Sterically, hindered hydroxyl. β,ε-Caroten-2-ol (*4a*) gave the methyl ether *4b* (60 % of recovered carotenoid) and unreacted *4a*; total recovery 72 %.

Azafrin (*5a*) gave (24 h, room temperature) the methyl ester *5b* (50 % of recovered pigment), *5e* (20 %) and unreacted *5a*; total recovery 85 %. Modified conditions (45 °C, 5 h, 20 °C, 12 h) gave small amounts of the presumed monomethyl ether *5c* and the presumed methyl ester monomethyl ether *5d*; total recovery 75 %.

The methyl ester *5b* had electronic spectrum like azafrin (*5a*), *m/e* 440 (M), M−18, M−31 *etc.*; inseparable from authentic *5b* on cochromatography. Silylation afforded the mono(trimethylsilyl) ether, *m/e* 512 (M); *cf.* Ref. 17.

Phenolic hydroxyl. 3-Hydroxyisorenieratene (*6a*) gave the methyl ether *6b* (100 % of recovered carotenoids) and unreacted *6a*; pigment recovery 80 %. 3,3′-Dihydroxyisorenieratene (*7a*) provided the dimethyl ether *7b* (100 % of recovered carotenoid); pigment recovery 30 %.

Enolic hydroxyl. Astacene (*8a*), see Scheme 1, gave products *8b* (60 % of recovered carotenoid), *8c* (15 %) and *8d* (25 %); pigment recovery 25 %. All products had electronic spectra (round) like canthaxanthin (β,β-caroten-4,4′-dione).

Product *8b* had *m/e* 872.1762 (calc. 872.1799 for $C_{42}H_{50}O_4I_2$), M−78, M−92, M−106, and M−158 ascribed to elimination from the polyene chain,[14] M−126 (?) and double peaks at both *m/e* 127 and *m/e* 128 partly ascribed to I^+ and HI^+. No phenazine derivative[1] was obtained with *o*-phenylenediamine under conditions where astacene (*8a*) gave such derivatives.

Product *8c* had *m/e* 716 (M), M−15, M−92, M−106, M−126 and double peaks at both *m/e* 127 and *m/e* 128.

Product *8d* had *m/e* 746 (M, compatible with $C_{42}H_{51}O_4I$), M−92, M−126 (?) and double peaks at both *m/e* 127 and *m/e* 128.

Data for products *8b* and *8c* are consistent with the structures suggested in Scheme 1. The assumed mechanism for their formation involves abstraction of a proton by the hydride to a resonance stabilized enolate/carbanion, followed by *O*-methylation by methyl iodide and a synchronous or subsequent Michaeltype addition of iodide to C-2. That hydride, rather than iodide, serves as leaving group on reforming the keto group in 4-position, is unexpected. Alternative *C*-methylation should lead to products capable of forming a phenazine derivative.

Only one other iodine derivative of carotenoids is previously described, namely "isocarotintetraiodid",[18] now considered to be a π-complex.[19]

Conclusion. *O*-Methylation with methyl iodide and sodium hydride seems to represent a rather universal method in the carotenoid series.

Thus methylation of phenolic carotenoids was achieved for the first time. Enolic hydroxy groups like in astacene (*8a*) also appear to undergo *O*-methylation, but complications in form of iodinated products arose.

The results obtained indicate that the CH_3I/NaH procedure is superior to previous methods for methylation of non-allylic and allylic (to one double bond) secondary hydroxy groups, including sterically hindered ones.

Judged by the results for azafrin (*5a*) tertiary hydroxy groups appear to be rather unreactive and carboxylic acids provide methyl esters.

Experimental details are reported elsewhere.[20] General conditions used for *O*-methylation with CH_3I/NaH were: To the carotenoid (0.1−1.0 mg) in dry tetrahydrofuran (2 ml) was added freshly distilled CH_3I (1 ml) and NaH (40 mg). The reaction mixture was kept at room temperature in darkness in inert atmosphere for 16−24 h, and worked up in the usual manner. The products were purified by TLC. R_F-values on kieselguhr paper (Schleicher and Schüll No. 287) of the compounds studied are given below with the percentage acetone in petroleum ether used for development given in parenthesis: *1a* 0.40 (10), *1b* 0.39 (2), *1c* 0.89 (2), *1d* 0.58 (2), *1e* 0.65 (2), *1f* 0.31 (2), *2a* 0.85 (5), *2b* 0.55 (0), *2c* 0.93 (2), *2d* 0.40 (0), *3a* 0.45 (2), *3b* 0.54 (0), *4a* 0.54 (1), *4b* 0.86 (1), *5a* 0.0 (100), *5b* 0.72 (5), *6a* 0.52 (5), *6b* 0.68 (2), *7a* 0.27 (10), *7b* 0.85 (5), *8a* 0.40 (10), *8b* 0.72 (10), *8c* 0.69 (10) and *8d* 0.42 (10 %).

Acknowledgement. G.N. was supported by a research grant from Hoffmann-La Roche, Basel, to SLJ.

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The Reaction between Phosphonium Ylides and Isocyanates, a Convenient Route to Ketenimines

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Reactions of phosphonium ylides with isocyanates have been reported in a few papers. In 1919 Staudinger and Meyer [1] found that triphenyl ketenimine (II) could be prepared from phenyl isocyanate by reaction with diphenylmethylene triphenylphosphorane (I; $R^1 = R^2 =$ Ph)

$$Ph_3P{=}CR^1R^2 + Ph{-}N{=}C{=}O \rightarrow$$

(I)

$$Ph{-}N{=}C{=}C\begin{matrix}R^2\\ \diagup\\ \\ \diagdown\\ R^1\end{matrix} + Ph_3P{=}O$$

(II)

Although this was the first preparation of a ketenimine, no yield was reported and no attempt was made to extend the synthesis to other ketenimines. Later, however, Trippett and Walker [2] studied the reactions of phenyl isocyanate with a series of ylides, but no ketenimine was formed during these reactions. It was reported that the reaction between the "non-stabilized" ylide dimethylmethylene triphenylphosphorane (I; $R^1 = R^2 = CH_3$) and phenyl isocyanate stops at the betaine stage. In other cases, when the ylide under investigation contained an α-hydrogen atom, migration of that hydrogen occurred, a new ylide being formed:

$$Ph_3P{=}CHR + Ph{-}N{=}C{=}O \rightarrow$$

$$Ph_3\overset{+}{P}{-}CHR \quad \overset{-}{O}{-}\overset{|}{C}{=}NPh \rightarrow Ph_3P{=}CR{-}C(O)NHPh$$

Finally, Ratts and Partos [3] attempted the following reaction:

$$Ph_3P{=}C{=}C{=}CPh_2 + CH_3N{=}C{=}O \rightarrow$$

$$Ph_2C{=}C{=}C{=}C{=}NCH_3$$

(III)

and were able to isolate the amide of the cumulene imine (III). As far as the present author knows, there have been no other attempt to make ketenimines along this route. Thus, no ordinary ketenimine, except the one synthesized by Staudinger and Meyer has been prepared by

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Table 1. Ketenimines from the reaction $Ph_3P=CR^1R^2 + RN=C=O \rightarrow RN=C=CR^1R^2 + Ph_3P=O$

No.	R−N=C=CR¹R²	M.p., °C (B.p. °C/mmHg)	Method of isolation	Yield %	% C Calc.	% C Found	% H Calc.	% H Found	N=C=C stretching, cm^{-1}
1	R=Ph R_1=Me, R_2=COOEt	(96/0.3)	Dist.	95	70.96	70.81	6.45	6.47	2020
2	R=*p*-FC_6H_4 R_1=Me, R_2=COOEt	(120/0.03)	Dist.	80	65.15	64.70	5.47	5.45	2040
3	R=α-$C_{10}H_7$ R_1=Me, R_2=COOEt	(160/0.03)	Dist.	90	75.87	75.50	5.97	5.77	2040
4	R=Et R_1=Me, R_2=COOEt	(38/0.03)	Dist.	90	61.95	61.94	8.45	8.31	2058
5	R=*t*-Bu R_1=Me, R_2=COOEt	(40/0.05)	Dist.	80	65.54	64.10	9.35	9.04	2045
6	R=Ph R_1=Me, R_2=COPh		TLC	90	81.68	81.35	5.57	5.49	2025
7	R=Et R_1=Me, R_2=COPh		TLC	90					2045
8	R=Et R_1=R_2=Ph	(116/0.01)	Dist.	90	86.84	86.94	6.83	6.86	2020
9	R=cyclohexyl R_1=R_2=Ph	(156/0.01)	Dist.	90	87.24	86.11	7.68	7.19	2015
10	R=R_1=R_2=Ph	55	Cryst.	85	89.18	89.12	5.61	5.47	2000
11	R=α-$C_{10}H_7$ R_1=R_2=Ph	132	Cryst.	80	90.25	90.41	4.39	4.24	2000
12	R=Ph R_1=R_2=Me	(96/10)	Dist.	35	82.78	82.77	7.64	7.53	2020
13	R=α-$C_{10}H_7$ R_1=R_2=Me	(115/0.02)	Dist.	40	86.12	86.04	6.71	6.81	2022
14	R=Ph R_1=CN, R_2=COOEt			0					

the ylide-isocyanate reaction. The lack of interest in the Staudinger-Meyer synthesis of ketenimines is remarkable, particularly in view of the cumbersome methods currently used for the preparation of these compounds. No single reaction for the preparation of ketenimines has the character of a general reaction. The method most widely used is perhaps the dehydration of *N*-monosubstituted amides,[4] but this preparation gives good yields only in the case of triarylsubstituted compounds.

In order to test the synthetic potential of the ylide-isocyanate method, the present author has undertaken a study of the formation of ketenimines from the reaction of isocyanates with a series of phosphonium ylides $Ph_3P=CR^1R^2$, representing both non-stabilized ylides, *i.e.* pK of the carbon acid $CH_2R^1R^2 > 37$, and stabilized ylides *i.e.* pK of $CH_2R^1R^2 < 35$. The ylide used in the reactions 8, 9, 10, and 11 (Table 1) is of borderline stability (pK of $CH_3Ph_2 \simeq 35$ on the McEwen scale.) The expected ketenimine was isolated in all cases except one. A summary of the results is given in Table 1. The reaction appears in most cases to proceed quantitatively as judged by the amount of triphenylphosphine oxide isolated and by the characteristic infrared absorption at approximately 2000 cm^{-1}. In several cases, however, some material is lost by polymerization during the work-up.

As can be seen from Table 1, the reaction works equally well with aliphatic and aromatic isocyanates, although the latter react under milder conditions. Non-stabilized ylides do undergo the reaction, but not as readily as moderately stabilized ylides. Thus in the case of 12 and 13 (Table 1) comparatively stable intermediates were formed and the reactions could not be completed at 80 °C (boiling benzene). When the ylide was present in a pure state the second step of the reaction could be performed at 130 – 150 °C in mesitylene. However, non-stabilized ylides are most conveniently prepared *in situ* from the corresponding phosphonium salt and alkyl or aryl lithium. When this procedure is applied, lithium halides will be present in the reaction mixture and seriously affect the reactivity of the ylides. In the case of 13 an attempt was made to perform the reaction along this route. The first step of the reaction went smoothly, but the subsequent decomposition of the intermediate to triphenylphosphine oxide and *N*-α-naphthyl dimethylketenimine could not be performed under the abovementioned conditions. Heating for one hour at 170 °C (boiling mesitylene) gave only a trace of the expected products. This result was not unexpected, however, since it is well known from studies of the Wittig reaction that Lewis acids, particularly lithium salts, co-ordinate with the ylides and with the forming intermediates, and the olefin forming step may only be performed under forcing conditions.

In the case of (14), $R^1=CN$, $R^2=COOC_2H_5$) no reaction was observed in boiling benzene. This negative result may be attributed to the extraordinary strong electron-withdrawing groups linked to the carbanion portion of the ylide. The interaction between the ylide carbon and the cyano and ester groups is reflected in a considerable decrease in the nucleophilicity of the ylide, and thereby in its reactivity towards isocyanates. Disubstituted phosphonium ylides were applied in all cases because monosubstituted ylides ($Ph_3P=CHR$) undergo a prototropic shift at the intermediate stage of the reaction forming a new ylide.[2]

Experimental. Materials. The phosphonium ylides were prepared by the "salt method" on treatment of a solution of the appropriate phosphonium salt with a suitable base. The stabilized ylides were generated with sodium ethoxide in anhydrous ethanol and recrystallized from benzene. Dimethyl- and diphenylmethylene triphenylphosphorane were prepared in liquid ammonia, using sodium amide as a base.

Synthesis of ketenimines (Table 1). In a dry 150 ml, two necked, roundbottomed flask equipped with a magnetic stirrer, a nitrogen inlet, and a reflux condenser with drying tube was placed 50 ml of dry benzene, and 0.01 mol of ylide. Thereafter 0.01 mol of isocyanate was slowly added. The reaction mixture was vigorously stirred during the addition. In the case of 4, 5, 9, 10, and 11 (Table 1) the reaction mixture was refluxed for several hours in a slow current of dry nitrogen. 8 was heated at 100 °C in an autoclave for 24 h. In the case of 12 and 13 the reactions were completed after about 5 min at 140 °C in mesitylene. In the other cases, however, the reaction went to completion in a few minutes at ambient temperature. The ketenimine formation was always recognized on the strong infrared absorption at approximately 2000 cm^{-1}. The product was worked up without further heating if no sign of the infrared isocyanate band at 2150 cm^{-1} was observed. The benzene was evaporated *in vacuo* whereafter the ketenimine was extracted with petroleum ether. After evaporation of this solvent, the ketenimines were purified by distillation or by recrystallization. In two cases (6 and 7, Table 1) the product was unstable and could not be purified by distillation.

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An Inulin-like Fructan Produced by *Streptococcus mutans*, Strain JC2

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In studies of polysaccharides elaborated by bacteria, isolated from human dental plaque, *Streptococcus mutans*, strain JC2,[1] was one of the organisms investigated. An enzyme preparation with hexosyltransferase activity was isolated from the supernatant fluid of a glucose broth culture by chromatography on hydroxylapatite.[2] When the enzyme preparation was incubated with sucrose, alcohol-precipitable polysaccharide material was formed. This, on acid hydrolysis, yielded comparable amounts of D-glucose and D-fructose. Judging from previous results,[3,4] the mixture most probably contained a glucan and a fructan. Indeed, a fructan, $[\alpha]_D^{22} -33°$ (*c* 0.3, 0.1 M NaOH), was isolated by precipitation with barium hydroxide as was a glucan, $[\alpha]_D +192°$ (*c* 0.3, 0.1 M NaOH) by precipitation with ethanol. Gel filtration chromatography on Sepharose 2B, as devised by Arturson and Granath,[5] showed that both polysaccharides had high molecular weights, $\overline{M}_w \simeq 2 \times 10^7$.

The two polysaccharides were subjected to methylation analyses as previously described for dextrans [6] and fructans [7] and the methylated sugars obtained were analysed by GLC-MS.[8] The results, given in Table 1, show that the glucan is of the type generally elaborated by *Streptococcus* species, *e.g.* an α-glucan with 3-, 6-, and 3,6-linked residues. The fructan, however, was not of the levan type with (2→6)-linkages, known to be produced by several bacteria, but of the inulin type, with (2→1)-linked β-fructofuranose residues. Some branching through the 6-position was observed. The sugars obtained in the methylation analysis were reduced with sodium borodeuteride. The deuterium labelling observed on MS showed that the tri-*O*-methyl-hexitol came from 3,4,6-tri-*O*-methyl-D-fructose and not from 1,3,4-tri-*O*-methyl-D-fructose. The alditol acetate derivatives of these sugars cannot be separated by GLC; however, the corresponding acetylated sugars are readily separated,[7] and not even traces of the 1,3,4-tri-*O*-methyl derivative were observed.

Until recently, only fructans of the levan type were known to be formed by bacterial fructosyltransferases. After the completion of the present work, another report [9] on a bacterial (2→1)-linked fructan, from *Streptococcus mutans*, strain Ingbritt, has come to our knowledge.

Table 1. Methyl ethers from the hydrolysate of the methylated fructan (A) and the methylated glucan (B).

Sugars	T[a]	Mol %	
		A	B
1,3,4,6-Tetra-*O*-methyl-D-fructose	0.82	5	
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00		22
3,4,6-Tri-*O*-methyl-D-fructose	1.82	89	
2,4,6-Tri-*O*-methyl-D-glucose	1.82		10
2,3,4-Tri-*O*-methyl-D-glucose	2.22		46
2,4-Di-*O*-methyl-D-glucose	4.21		22
3,4-Di-*O*-methyl-D-fructose	4.31	6	

[a] Retention times of the corresponding alditol acetates on the OV-225 column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

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Trimethyloxosulphonium Chloride from the Iodide through Ion Pair Extraction

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Trimethyloxosulphonium halides are important reagents as precursors of dimethyloxosulphonium methylid.[1] Whereas the synthetic procedure yields the iodide,[2] the trimethyloxosulphonium chloride has the advantage of being more soluble in tetrahydrofuran. The preparation of the ylid is thus made possible in this solvent.

The conversion of the iodide to the chloride has been carried out with elemental chlorine.[1] The same change of anion can be more simply and rapidly achieved using ion pair extraction. If trimethyloxosulphonium iodide and an equimolar amount of a suitable quaternary ammonium chloride, *e.g.*, the benzyltributylammonium salt, are distributed between dichloromethane and water, the latter will contain trimethyloxosulphonium chloride, whereas the organic phase will contain benzyltributylammonium iodide. Since iodides are extracted into dichloromethane and similar solvents a thousandfold better than the corresponding chlorides,[3] the ion exchange is practically complete.

The reason for choosing benzyltributylammonium chloride in the present work was that this salt could be simply prepared from inexpensive starting materials, tributylamine and benzyl chloride, in acetonitrile. Kantor and Hauser have reported [4] that this quaternization fails in refluxing benzene or in the absence of solvent. An early report [5] of a successful preparation of the salt in benzene solution could not be reproduced by us and is probably erroneous. The melting point, 185 °C, given in Ref. 5 disagrees with the one found in the present work, 162 – 164 °C. To obtain a good yield in acetonitrile, one week's refluxing was necessary.

Experimental. Benzyltributylammonium chloride. A mixture of 185.4 g (1 mol) of freshly distilled tributylamine, 139.0 g (1.1 mol) of benzyl chloride, and 300 ml of acetonitrile, purified according to O'Donnell and his coworkers,[6] was kept at gentle reflux for one week. Most of the acetonitrile was removed at aspirator vacuum, and 500 ml of dry ether was added, which caused the quaternary salt to precipitate. This was filtered off and washed with dry ether until colourless (*ca.* 500 ml). Drying *in vacuo* yielded 268.2 g (86 %) of crude product. Recrystallization from ethyl acetate containing some ethanol gave an odourless product, m.p. 162 – 164 °C (unchanged on further recrystallization). The recovery was only 60 %. Argentometric titration showed the purified salt to contain 11.3 % of chloride; calc. for $C_{19}H_{34}NCl$, 11.4 %.

Trimethyloxosulphonium chloride. A solution of 15.6 g (0.05 mol) of benzyltributylammonium chloride in 100 ml of water was shaken with 10.2 g (0.05 mol) of trimethyloxosulphonium iodide [2] and 75 ml of dichloromethane until a clean two-phase liquid system was obtained. The aqueous phase was washed with 50 ml of dichloromethane and evaporated, first at aspirator vacuum and then for several hours at an oil pump until constant weight. A quantitative yield of trimethyloxosulphonium chloride was obtained (5.6 g), m.p. 222 – 223 °C, lit.[1] 220 – 222 °C. Iodide was absent as indicated by a spot test with 30 % hydrogen peroxide. From the 60 MHz ^{1}H NMR spectrum, recorded in deuterium oxide solution, it could be concluded that a trace (less than 0.2 mol %) of benzyltributylammonium ion was present which can be disregarded in synthetic use.

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Intramolecular Transfer of Chirality from Sulfur to Carbon: Dehydrocyclization of Optically Active *o*-Benzylsulfinylbenzoic Acid with Dicyclohexylcarbodiimide

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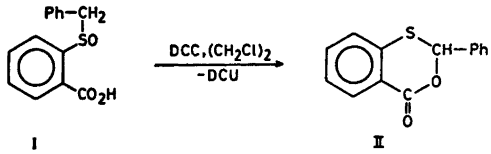
We have found that *o*-benzylsulfinylbenzoic acid (I) is readily converted to 2-phenyl-3,1-benzoxathian-4-one (II) by dicyclohexylcarbodiimide (DCC) in over 90 % yield. The reaction product is obviously the result of a Pummerer-type of rearrangement.[1] It was also found, as expected, that the transformation I→II could be performed using the classical Pummerer reagent acetic anhydride. The latter reaction was reported recently by Numata and Oae [2] after the initiation of our investigation. The lactone II was first described in 1961 as the product from a condensation of thiosalicylic acid with benzaldehyde.[3]



I II

Starting with optically active I we found that product II, after repeated chromatography on silica gel, possesses optical activity. A remarkable difference in the stereochemical course of the reaction, depending upon the nature of the Pummerer reagent and the solvent used, was observed, however. While the DCC-method gave II with an opposite sign of rotation to I, the situation with the use of acetic anhydride (AA) was found to be more complex. While a small excess of AA in benzene similarly gave II with opposite sign of rotation, the reverse was found with AA as both reagent and solvent. The results are given in Table 1.

These data imply that the DCC-reaction in 1,2-dichloroethane proceeds with the highest degree of stereoselectivity, while the reaction in acetic anhydride is associated with much more of racemization. This is not surprising, however, in view of the higher temperature needed in the latter case and the known.[4,5] ability of this medium to cause racemization of sulfoxides due to an acetoxy-interchange at the sulfur atom. Unfortunately, the optical yield of the DCC-reaction remains unestimated because the rotation of optically pure II is not yet known.

To the best of our knowledge, the only stereoselective Pummerer rearrangement so far reported is the reaction of 2,2-dialkyl-1,3-oxathiolan-5-one *S*-oxides with acetic anhydride in dichloromethane to give 4-acetoxy-2,2-dialkyl-1,3-oxathiolan-5-ones, where the 85–90 % stereoselectivity observed was interpreted in terms of an intramolecular migration of the acetoxy-group on one side of the ring.[6]

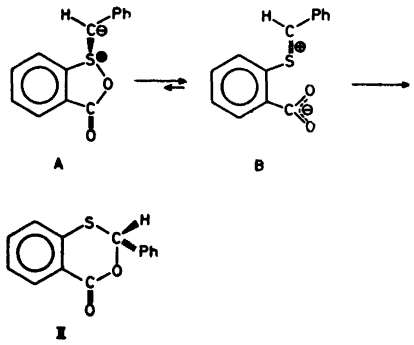
However, the presently described conversion of I to II appears to be the only reported Pummerer type of reaction in which an intramolecular transfer of chirality has occurred with the formation of an optically active product. Although a more detailed investigation of the mechanism is needed, some remarks and conclusions may nevertheless be made.

From Moffatt's investigations on the reactions of DMSO-DCC with carboxylic acids,[7] it was established that the necessary intermediate for the formation of an α-acyloxysulfide in this reaction is an acyloxysulfonium ylide. We feel that the key step responsible for the chirality-transfer is the opening of the cyclic ylide (A) and recombination to the ring-expanded product II, most probably *via* an ion pair (B) with restricted rotation around the partially double C–S-bond.

Stereoselectivity is thus induced with the formation of A as a consequence of the different reactivities of the diastereotopic protons [8] at the prochiral benzylic carbon atom. The ylide A should be a common intermediate in the DCC as well as the AA reaction, although with loss

Table 1.

I, $[\alpha]_D^{25}$	Pummerer reagent	Solvent	Temp. °C	II, $[\alpha]_D^{25}$	Yield %	Reaction time h
+451	DCC	$(CH_2Cl)_2$	25	−46.3	91	15
+451	DCC/H_3PO_4	THF	25	−7.9	89	2
+451	AA	C_6H_6	80	−30.2	91	5
+451	AA	AA	100	+17.3	95	2
+451	AA	AA NaOAc added	100	+8.2	98	2



of stereochemical integrity in more acidic media. The steps preceding A should follow the normal Pummerer mechanism, the difference from the normal route being due to internal nucleophilic participation by the carboxyl group present in the molecule.

Experimental. o-*Benzylthiobenzoic acid*, prepared by benzylation of thiosalicylic acid, was obtained in 85 % yield. M.p. 183 – 185 °C (lit.[9] m.p. 189 °C).

o-*Benzylsulfinylbenzoic acid (I).* o-Benzylthiobenzoic acid (15 g, 0.0615 mol) was dissolved in 600 ml of acetone-acetic acid (2:1) and oxidized with 5 % excess of a 15 % solution of peracetic acid in acetic acid. The reaction mixture was kept at 5 °C for 5 h and then at 25 °C for 20 h. After evaporation of the solvent, the product was recrystallized from acetone-petroleum ether. The yield was 13.2 g (82 %) of pure I, m.p. 164 – 165 °C (dec.).

Resolution of I. Racemic I (8.5 g, 0.0327 mol) was dissolved, together with brucine (dihydrate; 14.1 g, 0.0327 mol), in 350 ml of boiling acetone-ethyl acetate (6:1). The solution was left at 25 °C for 24 h, and the salt (11.1 g), which had crystallized, was isolated. The acid, liberated from a small amount of this salt, showed $[\alpha]_D^{25} = -373°$ (EtOH). The salt was treated with 300 ml of boiling acetone and, after cooling to room temperature, filtered by suction and dried. An amount of 8.6 g was obtained. A small crop of liberated acid showed an increased negative rotation, $[\alpha]_D^{25} = -444°$ (EtOH). This procedure was repeated, using 100 ml of acetone, which yielded 8.4 g of salt from which the acid, $[\alpha]_D^{25} = -443°$ (EtOH), was liberated. One recrystallization of the acid from acetone-ethanol gave 2.5 g of optically pure (–)-I, $[\alpha]_D^{25} = -449°$ (EtOH, $c = 0.5$). M.p. 165 – 166 °C (dec.).

The other enantiomer was liberated from the mother liquor from the first crystallization, $[\alpha]_D^{25} = +332°$ (EtOH). The optical purity was increased by two recrystallizations of the acid from acetone-ethanol. In this way (+)-I (2.5 g, m.p. 165 – 166 °C (dec.) with maximum rotation, $[\alpha]_D^{25} = +451°$, was obtained.

2-Phenyl-3,1-benzoxathian-4-one (II). A. I + DCC. DCC (0.103 g, 0.5 mmol) was dissolved in 5 ml of dry 1,2-dichloroethane, the solution cooled to 0 °C and finely powdered (racemic) I (0.130 g, 0.5 mmol) was added. The reaction mixture was kept for 1 h at 0 °C and for 14 h at 25 °C. Acetic acid (0.075 g, 1.25 mmol) was added and after stirring for 30 min the precipitated *N,N'*-dicyclohexylurea (DCU) was removed by filtration and the remaining filtrate evaporated to dryness. The product was then chromatographed on silica gel 60 (120 – 230 mesh) with chloroform as the eluent. Pure (racemic) II (0.110 g, 91 %) of m.p. 85.5 – 87.5 °C, (lit.[3] 83 – 84 °C, lit.[2] 90 – 91 °C) was obtained.

Starting with (+)-I this procedure yielded (–)-II, $[\alpha]_D^{25} = -46.3°$ (EtOH, $c = 1$), m.p. 88 – 90 °C, of unknown optical purity. The optical activity remained unchanged when (–)-I was rechromatographed with benzene as the eluent.

DCC (0.309 g, 1.5 mmol) in THF (3 ml) was added at 0 °C to a stirred solution of (+)-I (0.130 g, 0.5 mmol) and anhydrous orthophosphoric acid (0.025 g, 0.25 mmol) in THF (4 ml). Almost immediately crystalline DCU separated. After 30 min at 0 °C the mixture was kept at room temperature for 1.5 h. Oxalic acid (0.19 g, 1.5 mmol) was added and after 30 min the DCU was removed by filtration and the filtrate was evaporated. The residue was dissolved in benzene (10 ml), filtered to remove a small amount of DCU, and evaporated. The product was chromatographed as described above with chloroform as the eluent. 0.108 g (89 %) of II with m.p. 87 – 89 °C and $[\alpha]_D^{25} = -7.9°$ (EtOH, $c = 1$) was obtained.

B. I + AA. Treatment of I (0.130 g, 0.5 mmol) with an excess of acetic anhydride[2] (5 ml), evaporation and chromatographic purification of the product as described above, gave II in more than 90 % yields. By this method, however, (+)-I was converted to a product with $[\alpha]_D^{25} = +17.3°$ (EtOH, $c = 1$), m.p. 89 – 90 °C. The optical rotation was retained when the product was rechromatographed with benzene or ether as the eluents.

Addition of sodium acetate (0.1 g, 1.22 mmol) gave II with $[\alpha]_D^{25} = +8.2°$ (EtOH, $c = 1$), m.p. 89 – 90 °C.

When the reaction was performed in benzene (10 ml) with a 3:1 molar ratio of acetic anhydride (0.153 g, 1.5 mmol), the rotatory power of the chromatographed product was $[\alpha]_D^{25} = -30.2°$ (EtOH, $c = 1$) and m.p. 88 – 89.5 °C.

The optical rotations were determined at 589 nm with a Perkin-Elmer model 141 photoelectric polarimeter with the use of 1 ml microcells of 10 cm length.

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Electrolytic Generation of Nucleophiles. III.[1] Reductive Acetylation of Nitro and Nitroso Compunds

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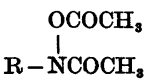
During the current investigations [2] of the reaction between electrophiles and electrolytically generated nucleophiles reductive acetylation of *N*-heterocyclic compounds was found useful.[3] Below is reported the analogous electrolysis of nitro and nitroso compounds in the presence of acetic anhydride to *N*,*O*-diacetyl-*N*-substituted hydroxylamines (I).



I

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Electrolytic reduction at a suitable potential of nitro or nitroso compounds in an aprotic medium, such as acetonitrile, containing an excess of acetic anhydride gives the desired compounds in one step in fair to good yield (47 – 87 % isolated yield). In Table 1 are given the yields from some electrolytic reductions and some of the properties of the isolated products.

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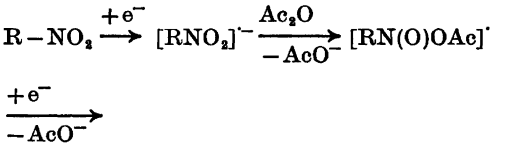

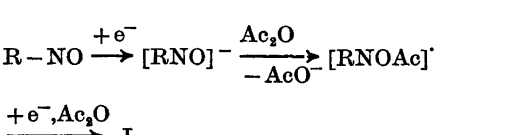

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Electrolytic Generation of Nucleophiles. III.[1] Reductive Acetylation of Nitro and Nitroso Compunds

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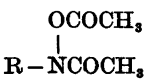
During the current investigations [2] of the reaction between electrophiles and electrolytically generated nucleophiles reductive acetylation of *N*-heterocyclic compounds was found useful.[3] Below is reported the analogous electrolysis of nitro and nitroso compounds in the presence of acetic anhydride to *N*,*O*-diacetyl-*N*-substituted hydroxylamines (I).



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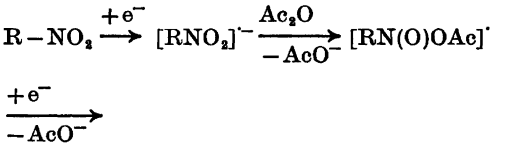

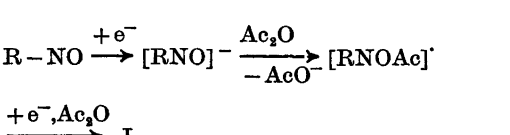

The same experimental procedure, which here has been applied to nitro and nitroso compounds and previously to heteroaromatic compounds,[3] has also been applied to the reductive acetyla-

Table 1. Yields and properties of *N,O*-diacetylhydroxylamines from electroreductive acetylations of some nitro and nitroso compounds.

Substrate used	Product I obtained % Yield crude	isolated	Physical properties (isolated sample)	Properties of authentic sample[a] of I
$MeNO_2$	68[b]	54	b.p. 90–100 °C (13 mmHg) n_D^{26} 1.4305	b.p. 87–88 °C (12 mmHg) n_D^{26} 1.4270; Lit.[15]
t-$BuNO_2$	63[b] 59[c]	47	b.p. 90–100 °C (13 mmHg) n_D^{26} 1.4295	b.p. 95–98 °C (18 mmHg) n_D^{26} 1.4320; lit.[16]
t-BuNO	73[b] 75[c]	57[d]	n_D^{26} 1.4320	see above
$PhNO_2$	70[b]	55	m.p. 39–42 °C (43.5–44.5)[e]	lit.[17] m.p. 43 °C
PhNO	83[b]	71	see above	see above
p-$MeC_6H_4NO_2$	–	77	b.p. 118–121 °C (0.5 mmHg)	see Experimental
m-$C_6H_4(NO_2)_2$	88[b]	87	m.p. 113 °C (119 °C)[e]	see Experimental

[a] Unless otherwise indicated, data are for a sample prepared by acetylation of the corresponding hydroxylamine. [b] From NMR analysis. [c] From VPC analysis. [d] By preparative VPC. [e] Analytically pure sample.

tion of anthraquinone, benzoquinone, and 2,3,5,6-tetramethoxybenzoquinone in yields of 70–85 %. The simplicity in controlling the degree of the reduction probably makes the procedure the method of choice where a reductive acylation to a partly reduced substrate is sought.

Experimental. Apparatus. An H-type 3-electrode cell of conventional design[13] has been used in combination with a Juul Electronic 100V/3A or 100V/10A potentiostat. Spectra were recorded on a Varian A60 NMR-spectrometer and a Perkin-Elmer Infracord IR-spectrophotometer. Analytical VPC was performed on a FM810 gas chromatograph and preparative VPC on a Perkin-Elmer F21. Melting and boiling points are uncorrected.

Materials. The nitro and nitroso compounds were either commercially available or prepared according to published procedures.[12,14]

General procedure. Electroreductions were mostly performed at room temperature (water bath surrounding the cell) at a stirred mercury cathode maintained at controlled potential, usually −0.9 to −1.3 V *vs* an Ag/AgI or Ag/Ag *p*-toluenesulfonate reference electrode. The solvent/electrolyte was anhydrous acetonitrile (dried over type A4 molecular sieves) containing 0.8 M sodium perchlorate and 10 % (by volume) of redistilled acetic anhydride throughout the cell. The anode was made of platinum gauze, because a graphite rod rapidly breaks down when used as an anode in aprotic perchlorate medium. The substrate (usually 1–2.3 g) was dissolved in the catholyte (180 ml) and current was passed through at the chosen potential until it ceased, usually after uptake of 5–10 % more than the theoretical consumption. Especially for the aliphatic compounds it was found advantageous to work up immediately and use short electrolysis times to minimize the formation of by-products. During the electrolysis a white precipitate was formed and the catholyte turned more or less yellowish. The precipitate could be filtered off, washed with acetonitrile, and identified as sodium acetate by its IR-spectrum; it was formed in an amount closely corresponding to the amount of electricity consumed.

Typically the resulting catholyte was concentrated *in vacuo*, diluted with methylene chloride or chloroform, filtered to remove the inorganic salts and evaporated *in vacuo*. The residue was analyzed by NMR or VPC (2 m 15 % SE30 on Chromosorb W, isothermal at 50 °C for 5 min, then programmed to 200 °C with 10 °C/min, biphenyl as internal standard). The products were isolated and purified by means of distillation, recrystallization, preparative VPC (2.7 m 15 % SE30, conditions similar to the analytical VPC) or column chromatography (chloroformsilica gel), and identifiel by means of IR (*N*-Ac, 1670–1690; *O*-Ac, 1780–1800 cm^{-1}), NMR and comparison with authentic samples and/or literature data (Table 1).

N,O-Diacetyl-*p*-tolylhydroxylamine, amber

liquid; IR-spectrum (film, cm^{-1}): 1780(s), 1670(s) 1170(br, s), 850(br, m), 820(br, m). NMR-spectrum (CCl_4): δ 1.94 (s, 3 H); δ 2.10 (s, 3 H); δ 2.36 (s, 3 H); δ 7.0 – 7.5 (multiplet, 4 H). The IR- and NMR-spectra correspond to those previously published.[6]

N,N′,O,O′-*Tetraacetyl*-m-*dihydroxylaminobenzene* (IV). *m*-Dinitrobenzene (1.0 g) was reduced at 5 °C in acetonitrile containing $NaClO_4$ in the presence of 10 ml acetic anhydride at -0.2 V *vs* Ag/AgI-electrode, $n = 8$. The solvent was evaporated and the residue extracted with methylene chloride. Evaporation of CH_2Cl_2 left 5.55 g of a residue; from NMR-analysis of the residue a yield of 88 % of tetraacetyl-1,3-dihydroxylaminobenzene was found. The residue was dissolved in 5 ml CH_2Cl_2 and 5 ml ether and then petroleum ether (b.p. < 50 °C) were added to beginning turbidity. A small amount of coloured material was filtered off and the filtrate cooled to -20 °C. The crystals, 1.60 g, m.p. 113 °C was recrystallized from methanol yielding a compound m.p. 119 °C (1 °C/min). NMR δ 2.11 (s, 6 H); δ 2.22 (s, 6 H); δ 7.3 – 7.7 (multiplet, 4 H). (Found: C 54.76; H 5.44; N 9.10. Calc. for $C_{14}H_{16}N_2O_6$: C 54.54; H 5.23; N 9.09).

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Palladium(II) Catalyzed Aromatic Acetoxylation. IV. Nuclear Acetoxylation in the Gas Phase: Reversal of the Usual Isomer Distribution Pattern in Aromatic Substitution

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Division of Organic Chemistry 1, Chemical Center, University of Lund, P.O. Box 740, S-220 07 Lund 7, Sweden

The acetoxylation of aromatic compounds in the gas phase using different palladium based catalysts has been studied. Low yields (of the order of 1 % per pass) of acetoxylated products were obtained on catalysts in which promotors, such as bismuth or silver-bismuth, had been added to the palladium. For monofunctional benzenes the isomer distributions observed were close to the statistically expected ones. However, when a cooxidant (*e.g.* potassium dichromate) was incorporated in the catalyst, the *meta* isomer became predominant, as has earlier been observed in homogeneous palladium acetate catalyzed acetoxylation reactions. ESCA studies on two of the catalysts indicated that the catalyst favoring *meta* orientation has a relatively high surface concentration of Pd(II) species.

Previous work from this laboratory[1-4] has shown that the homogeneous palladium(II) acetate catalyzed acetoxylation of monofunctional benzene derivatives that takes place in acetic acid in the presence of oxygen (eqn. 1) shows a reversal of the normal substituent effect, *i.e.*,

$$2Ar-H + 2HOAc + 1/2O_2 \xrightarrow[HOAc]{Pd(II)} 2Ar-OAc + H_2O \quad (1)$$

ortho,para-directing substituents give predominantly *meta* acetoxylation and *meta*-directing ones *ortho,para* acetoxylation. Similar findings have been reported by other investigators.[5,6]

Under the conditions referred to above, the reaction is, however, very slow, the degree of conversion being typically of the order of 100 % per 24 h, based on the amount of palladium used. We have therefore initiated a study of the factors influencing the rate and selectivity of this acetoxylation process with the goal of attaining practically useful reaction rates while retaining the selectivity for *meta* substitution. Since it was known that aromatic acetoxylation is also feasible in the gas phase over solid palladium based catalyst,[7-9] we decided to investigate this type of reaction first. This paper shows that it is indeed possible to achieve a modest degree of *meta* selectivity in the gas phase process too, and that possibly the presence of Pd(II) species on the catalyst surface is necessary for the *meta* selectivity.

RESULTS AND DISCUSSION

Product studies. A number of palladium catalysts for the gas phase acetoxylation of benzene have already been described by Arpe and Hörnig.[9] It was shown that gold is an efficient promotor for the formation of phenyl acetate, and that impregnation with activators like potassium and cadmium acetate—alone or in combination—was necessary to suppress the formation of phenol and concomitant resinification of the catalyst. The catalyst carrier in all these cases was silica (Davison, grade 62).

We have repeated these experiments with benzene as the substrate using the apparatus and conditions described in the experimental section with essentially the same results as those

* To whom inquiries should be addressed.

Table 1. Catalysts used for gas phase acetoxylation of aromatic compounds; palladium content = 1.0 %; impregnated with KOAc and $Cd(OAc)_2$[a].

Designation	Promotor(s) atom-% of Pd	Carrier[b]
A	Bi 30	Alumina pellets
B	Bi 30	Alumina granules
C	Bi 30	Glass wool
D	Au 55, Bi 20	Alumina pellets
E	Ag 40, Bi 10	Alumina pellets
F	Ag 20, Bi 10, $K_2Cr_2O_7$ 36[c]	Alumina pellets
G	Bi 30, $K_2Cr_2O_7$ 36[c]	Alumina pellets

[a] Without these activators the yield of acetoxy compounds was considerably lower. [b] With activated carbon or silica as carrier, no acetoxy compounds were formed. [c] Before reduction.

Table 2. Gas phase acetoxylation of benzene at 185 ± 5 °C over different palladium based catalysts with air as oxidant.

Catalyst (see Table 1)	Yield of phenyl acetate per pass, %	Space-time yield g l^{-1} h^{-1}	Other products, %
A	1.5	1.0	Phenol, trace
B	1.0	1.0	Phenol, 0.2
D	3.5	3.0[a]	Phenol, 0.5
F	1.0	1.0	Phenol, trace
H[b]	trace	—	—
I[c]	0	0	—

[a] Reported STY: 2.9 g l^{-1} h^{-1} with 0.75 % Pd and 0.75 % Au on silica, and 7.9 g l^{-1} h^{-1} with 2 % Pd and 2 % Au on silica. [b] Catalyst D with activated silica gel (0 – 1 mm) as carrier. [c] Catalyst D with activated carbon (3 – 5 mm) as carrier.

Table 3. Gas phase acetoxylation of anisole at 185 ± 5 °C over different palladium based catalysts (see Table 1) with air as oxidant.

Catalyst	Yield of nuclear acetate per pass, %	Space time yield, g l^{-1} h^{-1}	Nuclear acetates Isomer distribution *o*	*m*	*p*	Relative yield, %	Phenyl methoxymethyl ether, %	Other products, %
A	1.5	0.42	44	30	26	84	16	
B	1.0	0.28	38	33	29	67	16	Phenol, 17
C	0.1	0.03	38	32	30	87	13	
D	0.5	0.14	48	24	28	42	9	Phenol, 17; *m*-methoxyphenol, 6; PhOAc, 26
E	0.5	0.14	39	32	29	40	10	PhOH, 16; *m*-methoxyphenol, 11; PhOAc, 23
F	1.0	0.28	24	52	24	87	13	
G	0		—	—	—	—	—	

obtained by Arpe and Hörnig [9] (see Tables 1 and 2). We found, however, alumina in the form of pellets or granules to be superior to silica as a catalyst support and have therefore consistently used this material in this investigation. The yield of phenol and resulting resinification was especially less pronounced with this carrier. Also, it was found advantageous to use lower concentrations of palladium on the catalyst (1 % instead of 2 – 3 %). The optimum temperature was found to be in the range of 180 – 190 °C; at lower temperatures vaporization was not sufficiently fast and at higher temperatures side-reactions often occurred, sometimes with almost complete combustion of acetic acid and substrate and a spontaneous increase of reactor temperature to above 300 °C.

Next anisole was used as a substrate with a number of catalyst compositions modelled after those used by Arpe and Hörnig (Table 1, catalyst A – E). In this case bismuth or the combination silver-bismuth were found to be better promotors than gold, at least with respect to the yield of nuclear acetate obtained per pass (see Table 3). Alumina as pellets or granules and glass wool could be used as carriers, whereas silica or activated carbon did not support the acetoxylation process. As seen from the results given in Table 3, none of the catalysts A – E show any selectivity toward *meta* substitution. The isomer distribution in all five cases is close to the statistically expected one, 40:40:20.

It has been shown that the presence of co-oxidants, such as potassium dichromate [5] or nitric acid and derivatives thereof,[5,6,10,11] has a favorable effect on the homogeneous acetoxylation process and that *meta* selectivity is obtainable under such conditions.[6,12] We have suggested [1,2,4] that the *meta* selectivity of the homogeneous Pd(II) catalyzed reaction can be explained by electrophilic attack of a Pd(II) species on the aromatic substrate with formation of an oxypalladation adduct. The role of the cooxidant would then be to assist in the decomposition of the oxypalladation intermediate. Another possible role of the cooxidant in the homogeneous process would be to reoxidize Pd(0) to Pd(II) species.

Translating these ideas to the heterogeneous system, it was expected that impregnation of the catalyst with potassium dichromate would increase the *meta* selectivity. Firstly, the redox system Cr(III)/Cr(VI) would act as a mediator between O_2 and Pd(0) (it is known [13] that O_2 oxidizes Cr(III) to Cr(VI) on alumina) and hence possibly increase the concentration of Pd(II) on the catalyst surface. Secondly, Cr(VI) would assist in decomposing any oxypalladation adduct formed. As can be seen from Table 3, catalyst F, designed along these lines, does increase the *meta* selectivity for anisole acetoxylation compared to the others, even though the effect is fairly small. The possible correctness of the idea behind the incorporation of the

Table 4. Gas phase acetoxylation of aromatic compounds at 185 ± 5 °C over catalyst F (see Table 1).

Compound	Atmosphere	Yield of acetate per pass, %	Nuclear acetates Isomer distribution *o*	*m*	*p*	Relative yield, %	Other products, %
Anisole	Air	1.0	24	52	24	87	$PhOCH_2OAc$, 13
Chlorobenzene[a]	O_2	0.1	30	70	<1	70	PhOAc, 30
Toluene	Air	2 – 3	–	–	–	0	$PhCH_2OAc$, 99; PhCHO and $PhCH(OAc)_2$, traces
p-Xylene	O_2	1.5	–	–	–	25	4-$CH_3C_6H_4CH_2OAc$, 75
Mesitylene	O_2	1.2	–	–	–	0	3,5-$Me_2C_6H_3CH_2OAc$, 100
t-Butylbenzene	O_2	0.5	trace	66	34	100	PhOAc, trace
Isopropylbenzene	O_2	0.2	12	65	23	65	$PhC(OAc)Me_2$, 35
Ethylbenzene	O_2	0	–	–	–	–	Styrene
Naphthalene[b]	O_2	0	–	–	–	–	
Methyl benzoate[b]	O_2	0	–	–	–	–	

[a] Fluoro-, bromo-, and iodobenzene did not react under the reaction conditions employed. [b] At 245 ± 5 °C.

Cr(III)/Cr(VI) redox system in the catalyst was, however, further substantiated by the following experiment: The catalyst (F) bed was first oxidized by air at 300 °C (10 l/h for 10 h), and then the acetic acid/anisole solution introduced at 185 °C using a slow stream of *nitrogen*. The product from this run consisted of methoxymethyl phenyl ether (19 %) and nuclear acetates (81 %) in the *o:m:p* ratio of 17:62:21. Since the preoxidized catalyst is expected to contain a relatively high concentration of Pd(II) and Cr(VI) sites, it is obvious that the presence of these oxidation states favors *meta* orientation. Further evidence in this direction is provided by the ESCA results discussed below.

Catalyst F was then used for the acetoxylation of a number of aromatic substrates. It can be seen by inspection of Table 4 that nuclear acetoxylation is by no means a general reaction under these conditions, but that four monosubstituted benzenes (anisole, chlorobenzene, *t*-butylbenzene, and isopropylbenzene) do undergo acetoxylation with predominant *meta* orientation. For *p*-xylene, a substrate that previously has been shown [7] to undergo only side-chain acetoxylation in the gas phase, we were able to secure a 25 % relative yield of the nuclear acetate on catalyst F. Mesitylene gave side-chain acetate only.

While it has been shown above that it is possible to achieve *meta* selectivity in the gas phase process, it is not as high as in the homogeneous $Pd(OAc)_2/CH_3COOH/O_2$ reaction described by us earlier. On the other hand, later experience by us [13] and others [6] has revealed that attempts to increase the reaction rate, *e.g.*, by addition of nitrate ion, result in a decrease in *meta* selectivity. What appears to be a greater disadvantage of the gas phase reaction is the low space-time yield and the relatively fast inactivation of the catalyst due to resin formation. In spite of a great deal of experimentation with the catalyst composition, we have not been able to overcome these difficulties.

ESCA studies. Since the ESCA technique [14] is a sensitive method for the analysis of surface species, an ESCA investigation of two of the catalysts used in this investigation was performed in the hope that it would be possible to decide if the palladium metal of the reduced catalyst – the final step in the preparation of the catalyst is reduction by methanol – is transformed to Pd(II) during the reaction.

ESCA spectra of catalysts A and F, recorded before and after the catalytic reaction, revealed that charging of the samples made the exact external energy calibration impossible. It was, however, established from a reference spectrum [15] of a mixture of Pd(II) acetate and Pd metal that there is a shift between Pd(0) and $Pd(OAc)_2$ of 2.7 ± 0.5 eV (Fig. 1). A spectrum of the same mixture, applied on the carrier used in the catalysts, showed a shift of 2.9 ± 0.5 eV. In this experiment, it was noticed that Pd(II) acetate was partially transformed into Pd metal under the conditions prevailing during the recording of the spectrum.

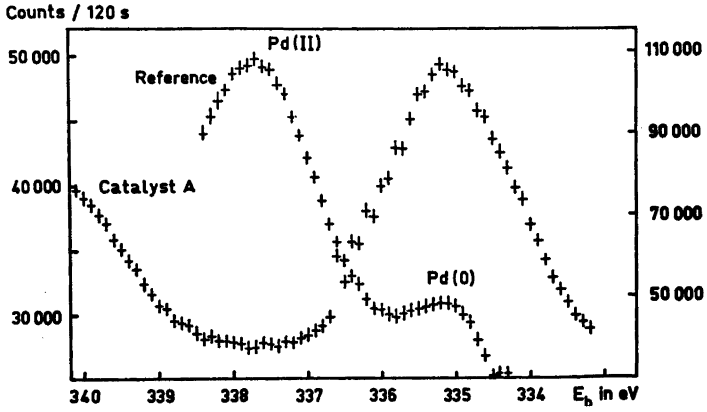

Fig. 1. ESCA spectra of (1) a reference mixture of Pd(II) acetate and Pd metal ($3d_{5/2}$ level) and (2) catalyst A.

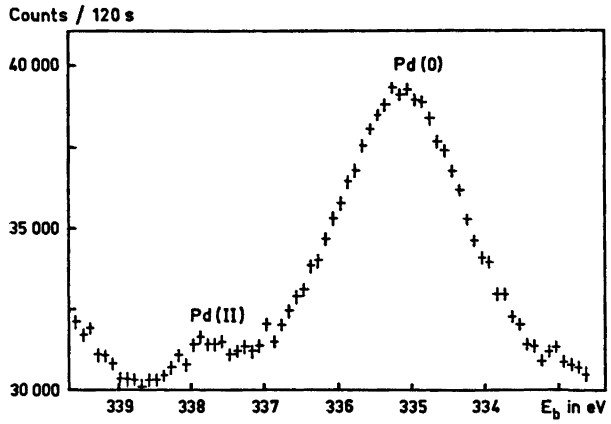

Fig. 2. ESCA spectrum of catalyst F.

Fig. 1 and 2 show printout spectra of catalysts A and F, respectively, after the reaction. In both cases the catalysts have been prepared in a way which excludes the presence of any Pd(II) on the catalyst before the reaction. Yet the spectrum of catalyst F, one which has been shown to give predominantly *meta* substitution and designed to do so by incorporation of a feature favoring formation of Pd(II), shows a signal shifted 2.8 eV from that of Pd(0). This signal must originate from Pd(II) generated in the course of the reaction. We thus conclude that the presence of Pd(II) species is a necessary requirement for the oxypalladation mechanism leading to anomalous substitution patterns both in the homogeneous and heterogeneous process. It also appears probable that there is another, more efficient acetoxylation mechanism possible in these systems, mediated *via* Pd(0) and leading to a statistical isomer distribution in nuclear acetoxylation or, if possible, to side-chain substitution. We have previously noticed [2] and commented [4] upon the fact that the appearance of Pd metal in the homogeneous process seems to be connected with the commencement of side-chain acetoxylation and would like to suggest that some type of dehydrogenation mechanism catalyzed by Pd(0) does occur both in side-chain and nuclear acetoxylation.

In a recent paper,[16] Evnin, Rabo, and Kasai have shown that it is possible to effect the vapor-phase oxidation of ethylene to acetaldehyde over a heterogeneous catalyst system. In the design of this catalyst, the same principle as that described above was used, *i.e.*, palladium metal was combined with an oxidizing agent, capable of reoxidizing Pd(0) to Pd(II) with high efficiency. In this particular case, vanadium pentoxide was used as the oxidizing agent.

EXPERIMENTAL PART

Materials. All chemicals used in this investigation were either purchased in the highest commercial quality available or prepared according to known procedures (see Parts I – III of this series). Carriers for catalysts were alumina pellets (1/8 in γ-Al_2O_3, activated), alumina granules (from Kebo, Stockholm, Sweden), silica (activated, 0 – 1 mm), carbon (activated 3 – 5 mm) and glass wool.

Preparation of catalysts. Catalysts A – G were prepared as follows: Alumina in the form of pellets or any of the other carriers used (100 g) was impregnated with *ca.* 100 ml of an HNO_3/H_2O (50:50) solution containing palladium acetate (2.1 g, corresponding to a Pd content of 1 % in the final catalyst) and the different compounds added as promotors. These were for A – C, $Bi(NO_3)_3.5H_2O$ (1.4 g); D, $Au(NO_3)_3$ (1.9 g) + $Bi(NO_3)_3.5H_2O$ (0.9 g); E, $AgNO_3$ (0.6 g) + $Bi(NO_3)_3.5H_2O$ (0.5 g); F, $AgNO_3$ (0.3 g) + $Bi(NO_3)_3.5H_2O$ (0.5 g) + $K_2Cr_2O_7$ (1.0 g); G, $Bi(NO_3)_3.5H_2O$ (1.4 g) + $K_2Cr_2O_7$ (1.0 g). The catalyst was first dried in a rotating film evaporator and then in the reactor tube at 200 °C for 30 min. Reduction of the catalyst was achieved by passing nitrogen, saturated with methanol at 25 °C, through the reactor for 2 h at 200 °C and 2 h at 400 °C at a flow rate of 5 l/h. The reduced catalyst was allowed to cool under nitrogen. Finally, the catalyst was impregnated with 50 ml of a water solution of

potassium acetate (1.5 g) and cadmium acetate (2.0 g) and dried in an oven at 150 °C.

Acetoxylation reactions. The catalytical reactions were carried out in a conventional flow reactor, consisting of a Pyrex tube (700 × 25 mm) which contained catalyst (100 g) and carrier (*ca.* 25 g) in the part (150 mm) adjacent to the inlet tube. This part of the reactor served to vaporize the liquid reactants before entering the catalyst zone. The tube was heated in an electrical oven, the temperature of which was controlled to within ± 5 °C.

The reaction was carried out in the following way: A solution of the substrate (0.05 mol) and a small amount of acetic anhydride (2 g) in acetic acid (20 g; molar ratio substrate/acetic acid = 1:6) was introduced into the reactor during 3 h *via* a fine dosage dropping funnel. Air (7 – 7.5 l/h) or oxygen (3 – 3.5 l/h) was flowing through the reactor at the same time. The product was condensed at – 20 °C and worked up by treatment with sodium bicarbonate solution and subsequent ether extraction (three times). The combined ether extracts were washed with water and dried with anhydrous magnesium sulfate and analyzed by GLC (Varian 1400 gas chromatograph, equipped with a disc integrator; column 2 m × 3 mm 5 % neopentylglycol succinate on Chromosorb W or 2 m × 3 mm 10 % Apiezon L on Chromosorb P). Internal standard was *m-t*-butylphenyl acetate, pentamethylbenzene, durene, or benzal diacetate. Products were identified by comparison with authentic specimens (GLC or GLC/MS).

ESCA studies. The ESCA measurements were carried out with an ES 100 electron spectrometer from AEI Scientific Apparatus Ltd., England. The pressure of the sample compartment was $10^{-6} - 10^{-7}$ Torr and the temperature 25 °C. The energy of the X-ray radiation was 1486.6 eV ($AlK_{\alpha 1,2}$) and the apparatus constant of the spectrometer 9.5 eV. The samples were deposited on nonconducting tape.

The spectra were recorded using both recorder and printer. The printout spectra (Fig. 1 and 2) were plotted from a printout giving the number of counts per 120 s in intervals of 0.1 eV.

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The Interpretation of an Abnormal Reaction Path Occurring during the Methylation of Certain Secondary Norbornanemethylamines with Formaldehyde in Acetic Acid

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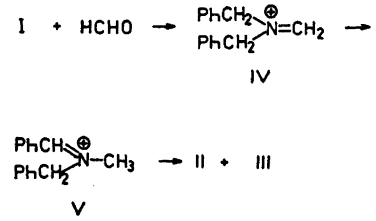
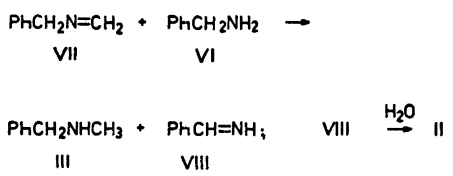
The carbon-nitrogen bond cleavage which occurs as a side reaction during the methylation of certain norbornanemethylamines with formaldehyde in acetic acid was investigated by employing deuterated and undeuterated reactants and by interpreting the NMR and mass spectra of the reaction products. It was shown that the pathway of the side reaction involves an intramolecular 1,3-hydride shift.

In the methylation of primary and secondary amines with formaldehyde, known as the Eschweiler-Clarke procedure,[1] the yields are best when the reaction is carried out in formic acid, although the reaction also proceeds in acetic acid.[2] In some cases carbonyl compounds [2-9] and secondary amines [2] are found among the by-products. Clarke *et al.*[2] suggest for the formation of the by-products benzaldehyde (II) and *N*-methylbenzylamine (III) by the methylation of dibenzylamine (I) a mechanism which proceeds *via* subsequent intermediate cations IV and V and the hydrolysis of V to the compounds II and III. These intermediate steps would include an intramolecular hydrogen shift (Scheme 1). On the other hand, Pine and



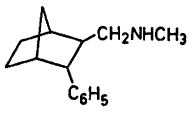
Scheme 1.

Sanchez [10] suggest that the reaction leading to aldehyde II during the methylation of benzylamine (VI) with formaldehyde in formic acid involves an intermolecular hydrogen shift between the Schiff base VII (from VI and formaldehyde) and VI. The hydrolysis of the subsequent benzylidene amine (VIII) leads to aldehyde II (Scheme 2).

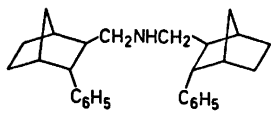


Scheme 2.

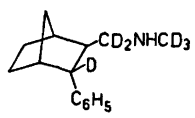
When studying the reaction of 2-norbornene with formaldehyde and secondary amines in acetic acid, it has been observed that the primary reaction products, secondary norbornanemethylamines, whose formation involves a 1,5-hydride shift, are methylated during the course of the reaction to the corresponding tertiary amines, and that these methylations include a side reaction which leads to carbonyl compounds and secondary amines.[11] Furthermore, when comparing the reaction products obtained from the reaction of 2-phenyl-2-norbornene, formaldehyde, and dimethylamine with those obtained from the corresponding reaction of 2-norbornene, the conclusion has been drawn that the formation of the main components found in the product mixture of the reaction follows a similar mechanism.[12] It



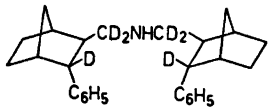
IX



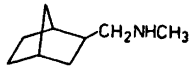
X



XI



XII

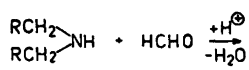


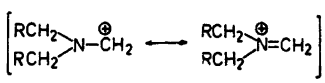
XIII

has not been investigated, however, if in the side reaction of the methylation the transfer of a hydrogen atom from the carbon atom adjacent to nitrogen is an intramolecular or an intermolecular process. To clarify this question, in the present study secondary amines IX and X (from 2-phenyl-2-norbornene, formaldehyde, and dimethylamine [12]), deuterated amines XI and XII (from 2-phenyl-2-norbornene, perdeuterioformaldehyde, and hexadeuteriodimethylamine), and secondary amine XIII (from 2-norbornene, formaldehyde, and dimethylamine [11]) were allowed to react with formaldehyde and perdeuterioformaldehyde in acetic acid, and the products were analysed by mass and NMR spectra.

RESULTS AND DISCUSSION

It is known that the reaction of a secondary amine with formaldehyde under acidic conditions leads to carbonium-immonium ion XIV (Scheme 3). This ion is considered to function as an intermediate species not only in the Mannich reaction [13] but also in the Leuckart-Wallach reaction, which includes the Eschweiler-Clarke methylation.[14] The reduction of ion XIV leads to the methylated product, the source of the hydride ion being formic acid, which is

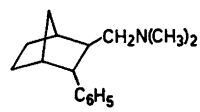




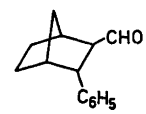
XIV

Scheme 3.

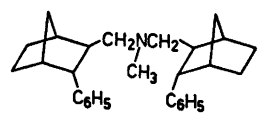
either added to the reaction mixture or is produced from formaldehyde by oxidation during the reaction. The reaction leading from ion XIV to a carbonyl compound and a secondary amine could possibly require either an isomerization suggested by Clarke *et al.*[2] (Scheme 1) or an intermolecular hydride shift analogous to that in the Sommelet reaction [3,15] as suggested by Pine and Sanchez [10] (Scheme 2).



XV



XVI

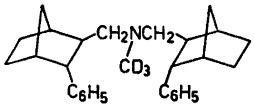


XVII

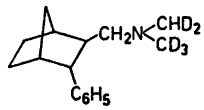
Earlier [12] the secondary amines IX and X have been methylated with formaldehyde in formic acid to the corresponding tertiary amines and the side reactions were not studied; now, the same reactions were performed in acetic acid and a more detailed characterization of the reaction products followed. After heating amine IX and formaldehyde (mol ratio 1:2) for 4 h, the reaction mixture contained tertiary amine XV, aldehyde XVI, and the starting compound IX. In the same way amine X gave, besides the normal methylation product XVII, also the cleavage products XVI and IX and the methylation product XV. These results distinctly show that during the reaction of secondary

amines IX and X with formaldehyde in acetic acid a cleavage of the carbon-nitrogen bond takes place besides methylation. Quite evidently hydrogen is released from the methylene carbon, adjacent to nitrogen, as hydride ion in the same fashion as in the Sommelet reaction of benzylamine.[16]

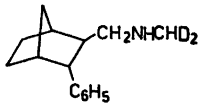
Since, according to Angyal,[3,15] methyleneimine produced from ammonia and formaldehyde or methylenebenzylamine (VII) (a Schiff base generated from benzylamine and formaldehyde) is likely to act as a hydride acceptor in the Sommelet reaction, which occurs when heating benzylamine with hexamethylenetetramine and yields benzaldehyde, also the carbonium-immonium ions of type XIV (generated from IX or X and formaldehyde) could function as hydride acceptors. To investigate this possibility amine X hydrochloride was heated with an excess of perdeuterioformaldehyde in acetic acid for 60 h so that both X and its cleavage product, a secondary amine, would be completely methylated. Upon diluting the reaction mixture with water, the methylated amine X precipitated as hydrochloride, was filtered off and liberated from its salt with potassium carbonate. The amine was identified by NMR and mass spectra to be compound XVIII. An amine containing five deuterium atoms and an aldehyde containing no deuterium were isolated from the filtrate. These compounds were assigned the structures XIX and XVI, respectively, by the aid of NMR and mass spectra. In order to identify the precursor of XIX, amine X and perdeuterioformaldehyde (mol ratio 1:2) were heated in acetic acid for only 4 h. The secondary amine isolated from the filtrate now contained only two deuterium atoms and was assigned the



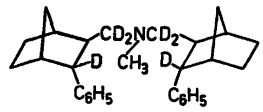
XVIII



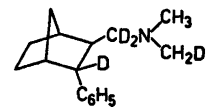
XIX



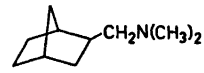
XX



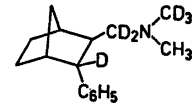
XXI



XXII



XXIII

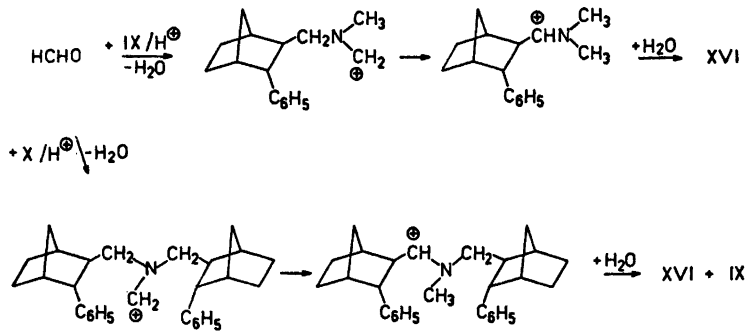


XXIV

structure XX. When the deuterated secondary amine XII (corresponding to X) was heated with an excess of formaldehyde in acetic acid for 60 h, the methylation product was, as expected, amine XXI and the methylated cleavage product was amine XXII.

The formation of XVIII from X and correspondingly XXI from XII indicates that the hydrogens of the methyl group in the actual methylation product originate from formaldehyde or its oxidation product, formic acid. The formation of amine XIX from X and perdeuterioformaldehyde, occurring by cleavage yielding XX and by subsequent methylation of XX with perdeuterioformaldehyde, and the corresponding formation of amine XXII from XII and formaldehyde are in accordance with the assumption that cations such as XIV function as acceptors of the hydride and the deuteride during the cleavage of the carbon-nitrogen bonds. In order to find out if the hydride transfer takes place intermolecularly, a mixture of amines XI and XIII was heated with formaldehyde in acetic acid. Amine XXIII, which is the methylation product of XIII, did not, according to the mass spectrum, contain any deuterium whereas compound XXIV is the methylation product of XI. This crossover experiment shows that the intermolecular hydrogen transfer from the carbon, adjacent to nitrogen, to the carbonium-immonium ion in the other molecule is out of question.

An intramolecular 1,3-hydride shift followed by a hydrolysis according to Scheme 4 seems to be the best explanation for the cleavage of the carbon-nitrogen bond which occurs as a side reaction during the methylation of secondary amines IX and X with formaldehyde in



Scheme 4.

acetic acid. This mechanism is analogous to that presented by Clarke *et al.*[3] who used the pathway in Scheme 1 to explain the side reaction taking place in the methylation of dibenzylamine with formaldehyde in formic acid.

EXPERIMENTAL

Preparation of the reactants. *N*-Methyl-exo-2-norbornanemethylamine (XIII), *N*-methyl-endo-3-phenyl-exo-2-norbornanemethylamine (IX), and 1,1′-bis(endo-3-phenyl-exo-2-norbornyl)dimethylamine (X) were prepared as described earlier.[11,12] To prepare the deuterated secondary amines XI and XII, 15.95 g (0.094 mol) of 2-phenyl-2-norbornene, 0.205 g of perdeuterioparaformaldehyde (equivalent to 0.064 mol of perdeuterioformaldehyde), and 10 g (0.114 mol) of hexadeuteriodimethylammonium chloride were allowed to react in acetic acid (30 ml) at 85 – 105 °C for 15 min. Addition of water and ether to the reaction mixture precipitated 1,1′-bis(endo-3-phenyl-exo-2-norbornyl-3*d*)di-(methyl-d_2)amine (XII) as hydrochloride, which was then liberated from its salt by potassium carbonate in a hot water-ethanol mixture. $C_{28}H_{29}D_6N$ (391.62). Mass spectrum: M^+ *m/e* 391. NMR spectrum (in C_6D_6): δ 7.21 (10 H, a singlet, the phenyl protons), 2.33 – 2.00 (4 H, the bridgehead protons), 1.80 (2 H, the endo-protons at C-2), and 1.65 – 0.87 ppm (12 H, the other norbornanering protons). *N*-Methyl-d_3-endo-3-phenyl-exo-2-norbornane-3*d*-methyl-d_2-amine (XI) was isolated from aqueous acetic acid and purified by the Hinsberg method. $C_{15}H_{18}D_5N$ (221.38). Mass spectrum: M^+ *m/e* 221 (9 %) and the base peak at *m/e* 49 (100 %) belonging to the fragment $CD_2NH^+\cdot=CD_2$. NMR spectrum (in C_6H_6): δ 7.21 (5 H, a singlet, the phenyl protons), 2.37 – 2.02 (2 H, the bridgehead protons), 1.78 (1 H, the endoprotons at C-2), and 1.70 – 1.00 ppm (6 H, the other norbornanering protons).

Reaction of N-methyl-endo-3-phenyl-exo-2-norbornanemethylamine (IX) with formaldehyde in acetic acid. Amine IX (0.173 g, 0.805 mmol) and 0.049 g of paraformaldehyde (equivalent to 1.63 mmol of formaldehyde) were heated in 1 ml of acetic acid in a boiling water bath for 4 h. Five ml of water and solid potassium carbonate were added to the reaction mixture until it was alkaline. The product was taken up in ether, the ether solution dried with potassium carbonate and the solvent evaporated. The residue (0.162 g) consisted of three components, which were identified by gas chromatography: endo-3-phenyl-exo-2-norbornanecarbaldehyde (XVI), *N*,*N*-dimethyl-endo-3-phenyl-exo-2-norbornanemethylamine (XV) and amine IX in the proportion 24:29:47. Aldehyde XVI was separated from the amines by extraction and amines IX and XV were isolated by preparative gas chromatography. The structures were confirmed by NMR and IR spectra and by the reference spectra.[12]

Reaction of 1,1′-bis(endo-3-phenyl-exo-2-norbornyl)dimethylamine (X) with formaldehyde in acetic acid. A mixture of 0.15 g (0.39 mmol) of amine X and 0.021 g of paraformaldehyde (equivalent to 0.7 mmol of formaldehyde) was heated in acetic acid (1 ml) for 4 h. The treatment of the reaction mixture as described above yielded 0.145 g of crude product, which in the gas chromatogram showed peaks for aldehyde XVI, tertiary amine XV, and secondary amine IX in the proportion 60:8:32. The unreacted starting material (X) and its methylation product, 1,1′-bis(endo-3-phenyl-exo-2-norbornyl)trimethylamine (XVII) did not elute from the column. After the bis-amines X and XVII were precipitated from the solution with hydrochloric acid, compounds XVI, XV, and IX were isolated and identified as described above.

Reaction of 1,1′-bis(endo-3-phenyl-exo-2-norbornyl)dimethylamine (X) with perdeuterioformaldehyde in acetic acid. A mixture of 4.5 g (10.7 mmol) of amine X hydrochloride and 2.5 g of perdeuterioparaformaldehyde (equivalent to 73.5 mmol of perdeuterioformaldehyde) in 40 ml acetic acid was heated under reflux for 60 h. The methylated X, liberated from its hydrochloride (sparingly soluble in water and ether) with potassium carbonate (the yield of hydrochloride 0.67 g) was identified by mass

and NMR spectra as 1,1′-bis(endo-3-phenyl-exo-2-norbornyl)trimethyl-1″,1″,1″-d_3-amine (XVIII), $C_{29}H_{34}D_3N$ (402.61). Mass spectrum: M^+ *m/e* 402 (1 %), M−171 (an abstraction of the endo-3-phenyl-exo-2-norbornyl radical) at *m/e* 231 (4 %) with the base peak at *m/e* 61 (100 %). NMR spectrum (in C_6D_6): δ 7.21 (10 H, a singlet, the phenyl protons), 2.84−2.60 (2 H, a multiplet, the exo-protons at C-3), 2.40−1.80 (8 H, a multiplet, the *N*-methylene and bridgehead protons), and 1.65−1.00 ppm (14 H, a multiplet, the other protons).

The neutral products and the amines were isolated by extraction from aqueous acetic acid. The main neutral component, endo-3-phenyl-exo-2-norbornanecarbaldehyde (XVI) (yield 1.5 g) did not contain any deuterium according to its mass spectrum. The amine portion (yield 0.8 g) contained *N,N*-dimethyl-d_5-endo-3-phenyl-exo-2-norbornanemethylamine (XIX) as the main component, $C_{16}H_{18}D_5N$ (234.39). Mass spectrum: M^+ *m/e* 234 (1 %) and the base peak $CD_3N^+\cdot(CHD_2)=CH_2$ at *m/e* 63 (100 %). NMR spectrum (in C_6D_6): δ 7.20 (5 H, a singlet, the phenyl protons), 2.71 (1 H, an approximate triplet, the exo-proton at C-3), 2.47−2.23 (2 H, a broad signal, the bridgehead protons), 2.15−1.87 (3 H, a multiplet, the protons of the *N,N*-dimethyl-d_5-aminomethyl group), and 1.75−1.09 ppm (7 H, the other protons).

Amine X hydrochloride (3.66 g, 9.51 mmol) and 0.65 g of perdeuterioparaformaldehyde (equivalent to 19.1 mmol of perdeuterioformaldehyde) were allowed to react in acetic acid (30 ml) for 4 h as described above. After precipitating the unreacted starting material X and the methylated amine as a hydrochloride, a secondary amine was recovered from the diluted aqueous acetic acid solution (yield 10 mg, 5 % of amine XIX as impurity). The amine was identified by mass spectroscopy as *N*-methyl-d_2-endo-3-phenyl-exo-2-norbornanemethylamine (XX), $C_{15}H_{19}D_2N$ (217.35). Mass spectrum: M^+ *m/e* 217 (2 %) and the base peak $CHD_2NH^+\cdot=CH_2$ at *m/e* 46 (100 %).

*Reaction of 1,1′-bis(endo-3-phenyl-exo-2-norbornyl-3*d*)di(methyl-*d_2*)amine (XII) with formaldehyde in acetic acid.* Amine XII hydrochloride (0.42 g, 1 mmol) and paraformaldehyde (0.15 g, equivalent to 5 mmol of formaldehyde) were heated in 7 ml of acetic acid for 60 h. The reaction mixture was treated as described above yielding the methylated XII, 1,1′-bis(endo-3-phenyl-exo-2-norbornyl-3*d*)trimethyl-1,1,1′,1′-d_4-amine (XXI), $C_{29}H_{31}D_6N$ (405.64). Mass spectrum: M^+ *m/e* 405 (1 %), M-172 (an abstraction of endo-3-phenyl-exo-2-norbornyl-3*d*) *m/e* 233 (7 %) and the base peak at *m/e* 63 (100 %). NMR spectrum (in C_6D_6): δ 7.23 (10 H, a singlet, the phenyl protons), 2.37−1.80 (9 H, the bridgehead protons, the C-2 endo-protons and the *N*-methyl protons at 2.09 as a singlet), and 1.70−0.83 ppm (12 H, the other protons). In the gas chromatogram the retention time of the main neutral component, which was isolated from aqueous acetic acid solution, is the same as that of endo-3-phenyl-exo-2-norbornanecarbaldehyde (XVI). The main amine component was identified by mass and NMR spectra as *N,N*-dimethyl-*d*-endo-3-phenyl-exo-2-norbornane-3*d*-methyl-d_2-amine (XXII), $C_{16}H_{19}D_4N$ (233.38). Mass spectrum: M^+ *m/e* 233 (3 %) and the base peak $CH_3(CH_2D)N^+\cdot=CD_2$ at *m/e* 61 (100 %). NMR spectrum (in C_6D_6): δ 7.22 (5 H, a singlet, the phenyl protons), 2.33 (2 H, a multiplet, the bridgehead protons), 2.08 (5 H, a singlet, the protons of *N,N*-dimethyl-*d* group), 1.89 (1 H, the endo-proton at C-2), and 1.55−1.07 ppm (6 H, the other protons).

Treatment of a mixture of N*-methyl-exo-2-norbornanemethylamine (XIII) and* N*-methyl-*d_3*-endo-3-phenyl-exo-2-norbornane-3*d*-methyl-*d_2*-amine (XI) with formaldehyde in acetic acid.* A mixture of 0.13 g (0.9 mmol) of amine XIII, 0.205 g (0.95 mmol) of amine XI, and 0.16 g of paraformaldehyde (equivalent to 5.09 mmol of formaldehyde) was heated in 1.6 ml of acetic acid for 9 h. The neutral products and the amines were separated by extraction. *N,N*-Dimethyl-exo-2-norbornanemethylamine (XXIII), the methylation product of amine XIII, distilled at 70 °C/10 Torr in a bulb tube oven and contained no deuterium according to the mass spectrum. The mass spectrum was the same as that of XXIII formed from XIII and formaldehyde.[11] The methylation product of amine XI distilled in a bulb tube oven at 120 °C/0.2 Torr and was identified by its mass spectrum as *N,N*-dimethyl-1,1,1-d_3-endo-3-phenyl-exo-2-norbornane-3*d*-methyl-d_2-amine (XXIV). $C_{16}H_{17}D_6N$ (235.40). Mass spectrum: M^+ *m/e* 235 and the base peak $CH_3(CD_3)N^+\cdot=CD_2$ at *m/e* 63 (100 %).

Apparatus. The apparatus employed is the same as used in the previous investigation.[12]

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Base-induced Conversion of Dichloromethanesulfonyl Chloride to Trichloromethanesulfinate

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Dichloromethanesulfonyl chloride (*1*) is converted to trichloromethanesulfinate (*3*) when treated with triethylamine or pyridine. The reaction is suggested to proceed *via* an intermediate sulfene *2*.

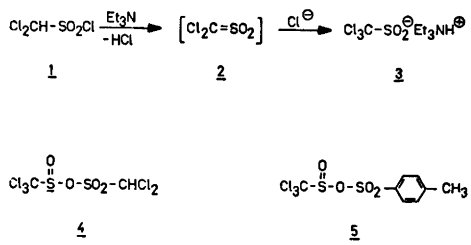
When trichloromethanesulfinate (*3*) is treated with triethylammonium bromide a halogen exchange is observed, as shown by a mass spectrometric investigation. This exchange reaction may also proceed *via* dichlorosulfene (*2*).

Mass spectra of trichloromethanesulfonyl chloride and trichloromethanesulfonyl bromide are discussed.

The conversion of sulfonyl chlorides to halosulfinates have not previously been observed. Intermediate halosulfinates have, however, been postulated [1] in the formation of sulfines from sulfonyl chlorides when treated with a base. This paper describes the reaction of dichloromethanesulfonyl chloride (*1*) when treated with triethylamine or pyridine. This reaction is shown to yield trichloromethanesulfinate (*3*).

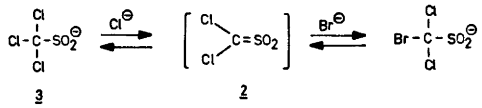
Dichloromethanesulfonyl chloride (*1*) was treated with an equivalent amount of triethylamine or pyridine in ethyl ether or benzene at room temperature to yield a product which separated during the reaction. This product was shown to be trichloromethanesulfinate (*3*), and was characterized by transforming it into the trichloromethanesulfinyl chloride [2] or into the crystalline morpholide.[3] The crystalline trichloromethanesulfonyl chloride [4] and the corresponding bromide [5] were also prepared by oxidation with chlorine or bromine in water.

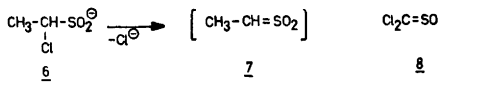
Chloromethanesulfonyl chloride is not converted to dichloromethanesulfinate when treated with triethylamine. This was shown by the fact that the corresponding dichloromethanesulfonyl chloride could not be detected in the reaction mixture after oxidation with chlorine in water. Consequently, the reaction of dichloromethanesulfonyl chloride seems to be a special case due to the effect of the two halogen atoms of the dichloromethyl grouping.



The conversion of dichloromethanesulfonyl chloride (*1*) to trichloromethanesulfinate (*3*) may proceed *via* an intermediate sulfene *2* [6] which, when attacked by a chloride ion, yields the sulfinate *3*. A second mechanism for the reaction should also be considered. This mechanism involves an intermediate sulfinyl sulfonate *4*, formed in a reaction of the intermediate sulfene *2* and dichloromethanesulfonyl chloride (*1*).[7] The sulfinylsulfonate *4* then decomposes in the presence of a base to the sulfinate *3* and the sulfene *2*. The sulfene *2* can react further in a chain reaction. Intermediate sulfinyl sulfonates, *e.g.* *5*, which lack α-protons, should not react further. However, in the presence of toluenesulfonyl chloride the conversion of dichloromethanesulfonyl chloride proceeds in the normal way, and the toluenesulfonyl chloride is recovered quantitatively. This experiment shows that a sulfinyl sulfonate does not seem to be an intermediate in the reaction.

It is of interest to note that there is a halogen exchange in trichloromethanesulfinate (*3*) when this compound is treated with triethylammonium bromide. This halogen exchange was demonstrated by a mass spectrometric investigation (see experimental), and may proceed *via* dichlorosulfene (*2*), which is in rapid equilibrium with trichloromethanesulfinate (*3*).





The formation of dichlorosulfene (*2*) from trichloromethanesulfinate (*3*) is analogous to the decomposition of 1-chloroethanesulfinate (*6*) to yield the intermediate methylsulfene (*7*).[8] Dichlorosulfine (*8*) has recently been prepared,[9,10] and was found to be a rather stable compound. It is therefore reasonable to predict that dichlorosulfene (*2*) is also stable. It is of interest to note that theoretical calculations predict that electron-donating substituents stabilize sulfenes.[11]

The Fourier transform ^{13}C NMR spectrum of the triethylammonium salt of trichloromethanesulfinic acid in a chloroform solution exhibits a signal at 115.6 ppm (downfield from TMS; benzene external standard $\delta_{(benzene)}$ 127.6 ppm) due to the carbon atom of the trichloromethyl group. This chemical shift is comparable with those of trichloromethanesulfinyl chloride (109.6 ppm) and trichloromethanesulfonyl chloride (106.8 ppm). The sp^2-hybridized carbon of dichlorosulfene (*2*) is expected to exhibit a chemical shift further downfield. If there is a rapid equilibrium between dichlorosulfene (*2*) and trichloromethanesulfinate (*3*), there seems to be very little contribution from the carbon species of dichlorosulfene and the equilibrium must be strongly shifted towards the sulfinate *3*.

It is known [12] that 2-chlorothiirane 1,1-dioxide is formed, when chloromethanesulfonyl chloride is treated with triethylamine in the presence of diazomethane, thus trapping the intermediate sulfene. However, when the base-induced conversion of dichloromethanesulfonyl chloride (*1*) was carried out in the presence of diazomethane no reaction with the suggested intermediate dichlorosulfene (*2*) and diazomethane could be observed.

Trichloromethanesulfonyl chloride and the corresponding bromide have been used for the halogenation of hydrocarbons.[5] The $Cl_3C-SO_2\cdot$ radical has been suggested as being the hydrogen abstractor in the halogenation reaction with trichloromethanesulfonyl chloride, whereas in the reaction with the corresponding bromide, the $Cl_3C\cdot$ radical is suggested as being the hydrogen abstractor.[5]

This difference between the sulfonyl chloride and the corresponding bromide is also reflected in the mass spectrometric fragmentation pattern of the two compounds. No molecular ion peaks are observed in the spectra. The mass spectrum of the sulfonyl chloride exhibits peaks at *m/e* 181 and 117 due to the $(Cl_3C-SO_2)\cdot^+$ and $(Cl_3C)\cdot^+$, respectively. A peak at *m/e* 298 must be an artefact due to radical dimerization to the bis(trichloromethyl) sulphone. The mass spectrum of the sulfonyl bromide, on the other hand, did not exhibit any trichloromethanesulfonyl fragment, $(Cl_3C-SO_2)\cdot^+$, at *m/e* 181. The parent peak was the trichloromethyl radical ion, $(Cl_3C)\cdot^+$, at *m/e* 117.

EXPERIMENTAL

All melting points are uncorrected. IR spectra were measured on a Perkin Elmer Model 421 infrared spectrophotometer. ^1H NMR spectra (TMS internal standard) were recorded on a Varian Model A-60 A instrument. ^{13}C NMR spectra were recorded on a Varian HA-100-D spectrometer. Mass spectra were obtained when using an LKB Model 9000 mass spectrometer (direct inlet).

Pyridinium and triethylammonium salts of trichloromethanesulfinic acid. A solution of pyridine (7.9 g, 0.1 mol) or triethylamine (10.1 g, 0.1 mol) in ethyl ether (25 ml) was added during 15 min to dichloromethanesulfonyl chloride [13] (18.3 g, 0.1 mol) in ethyl ether (100 ml). The mixture was stirred for 1 h at room temperature. The pyridinium salt thus formed was filtered off, washed with ethyl ether and dried *in vacuo*. Yield: 25 g (95 %); m.p. 115–117 °C. IR ($CHCl_3$) 1200 cm^{-1} (S=O); ^1H NMR ($CHCl_3$) δ 10.4–9.2 ppm (m, pyridinium protons).

The triethylammonium salt, prepared as described above for the pyridinium salt, was a

heavy oil. This oil was separated from the ether phase, and traces of ethyl ether dissolved in the oil were removed *in vacuo*. Yield: 27 g (95 %); IR (film) 1220 cm^{-1} (S=O); ^1H NMR ($CHCl_3$) δ 1.2 (t, 9, CH_3) and 2.8–3.2 ppm (two overlapping quartets, 6, CH_2, coupling with the CH_3 and N–H).

Trichloromethanesulfinyl chloride. Thionyl chloride (23.8 g, 0.2 mol) was added to the pyridinium salt of trichloromethanesulfinic acid (26.2 g, 0.1 mol) or its triethylammonium salt (28.4 g, 0.1 mol) in benzene (100 ml). The mixture was heated under reflux for 30 min and then cooled to room temperature. Insoluble salts were removed by filtration, and the solution evaporated *in vacuo*. The residue was distilled under reduced pressure to give trichloromethanesulfinyl chloride. Yield: from the pyridinium salt, 14 g (69 %), and from the triethylammonium salt, 13 g (64 %); b.p. 53–54 °C/9 mmHg (lit.[2] b.p. 36–38 °C/1 mmHg); m.p. 18 °C; n_D^{24} 1.5455; IR (film) 1200 cm^{-1} (lit.[2] 1190 cm^{-1}, S=O).

The trichloromethanesulfinyl chloride was further characterized as the corresponding morpholide: m.p. 88–89 °C (lit.[3] m.p. 85–87 °C). (Found: C 24.20; H 3.30; Cl 41.86; N 5.38; S 12.50. Calc. for $C_5H_8Cl_3NO_2S$: C 23.78; H 3.19; Cl 42.11; N 5.54; S 12.69).

Trichloromethanesulfonyl bromide. Triethylamine (10.1 g, 0.1 mol) was added to dichloromethanesulfonyl chloride (18.3 g, 0.1 mol) in benzene (100 ml), and the mixture was stirred at room temperature for 30 min. A solution of bromine (19.2 g, 0.12 mol) in water (200 ml) and benzene (100 ml) was then added in one portion to the trichloromethanesulfinate under vigorous stirring. The organic layer was allowed to separate after 15 min, washed with aqueous sodium bisulfite solution (10 %) to remove excess bromine, and then with water. The benzene solution was dried and evaporated *in vacuo* to give a crystalline residue. The crystals were dissolved in a minimum amount of ethanol. The product (trichloromethanesulfonyl bromide) was precipitated with water, filtered and dried *in vacuo*. Yield: 14.5 g (55 %); m.p. 138–140 °C (lit.[5] m.p. 138–139 °C); IR (CCl_4) 1400 and 1180 cm^{-1} (lit.[5] 1398 and 1177 cm^{-1}, $-SO_2Br$); mass spectrum (20 eV, direct inlet) *m/e* (rel. intensity) CCl_3^+· 117 (100), 119 (94.5) and 121 (27.9).

Trichloromethanesulfonyl chloride. The pyridinium salt (5.2 g, 0.02 mol) or the triethylammonium salt (28.4 g, 0.1 mol) of trichloromethanesulfinic acid, was added to a water solution which was continuously saturated with chlorine. The reaction was stopped when no more chlorine was consumed. The product, trichloromethanesulfonyl chloride, which separated as white crystals during the oxidation, was filtered off and washed with water. For further purification, the crystals were dissolved in a minimum amount of ethanol and precipitated with water, then filtered and dried *in vacuo*. Yield: from the pyridinium salt, 3 g (68 %), and from the triethylammonium salt, 14 g (64 %); m.p. 140–141 °C (lit.[4] 140–140.5 °C); mass spectrum (20 eV, direct inlet) *m/e* (rel. intensity) CCl_3^+· 117 (100), 119 (95.11), 121 (32.1); 149 (0.26), 151 (0.27), 153 (0.09); $Cl_3C-SO_2^+$· 181 (1.58), 183 (1.53), 185 (0.53); $Cl_3C-SO_2-CCl_2^+$· 263 (0.24), 265 (0.40), 267 (0.27), 269 (0.10); $Cl_3C-SO_2-CCl_3^+$· 298 (0.074), 300 (0.14), 302 (0.12), 304 (0.05).

Treatment of dichloromethanesulfonyl chloride with diazomethane in the presence of triethylamine. Dichloromethanesulfonyl chloride (16.5 g, 0.09 mol) in ethyl ether (100 ml) and triethylamine (9.1 g, 0.09 mol) in ethyl ether (100 ml) were simultaneously added dropwise, to a stirred and cold solution (–40 to 50 °C) of diazomethane [14] (0.09 mol) in ethyl ether (200 ml). The addition requires about 30 min. The reaction mixture was stirred for an additional 30 min. Diazomethane was not consumed, as shown by its characteristic colour. Acetic acid was added dropwise in order to react with the diazomethane. The ether solution was separated from an insoluble residue. The residue (18 g) was oxidized with chlorine in water to give trichloromethanesulfonyl chloride. The sulfonyl chloride was dissolved in a minimum amount of ethanol, and then precipitated with water, filtered off and dried *in vacuo*. Yield 9 g, m.p. 140–141 °C. The ether solution was washed with water and dried. Evaporation *in vacuo* gave a liquid residue (5 g).

Infrared spectrum (film) of this residue did not exhibit absorption typical of a sulfone (1300–1320 and 1140–1170 cm^{-1}). Distillation of the oily residue at reduced pressure gave a compound which was characterized as methyltrichloromethanesulfinate. B.p. 74–75 °C/9 mmHg (lit.[2] b.p. 38–40 °C/1 mmHg); n_D^{25} 1.5022; IR (film) 1190, 970, 795 and 820 cm^{-1} (lit.[2] IR 1190, S=O; 970, S–O–C; 795 and 822 cm^{-1}, C–Cl of Cl_3C-); ^1H NMR, δ 4.0 ppm (s, CH_3-).

The reaction of dichloromethanesulfonyl chloride in the presence of toluenesulfonyl chloride. Toluenesulfonyl chloride (5.2 g, 0.02 mol) in ethyl ether (25 ml) was mixed with triethylamine (2.75 g, 0.02 mol), and dichloromethanesulfonyl chloride (5 g, 0.02 mol) was slowly added. After 1 h at room temperature the ether phase was separated from the oily residue and evaporated *in vacuo* to yield 5.2 g unreacted toluenesulfonyl chloride. The recovered toluenesulfonyl chloride was identified with an authentic sample.

The reaction of dichloromethanesulfonyl chloride with half an equivalent triethylamine. Triethylamine (5.0 g, 0.05 mol) was slowly added to a solution of dichloromethanesulfonyl chloride (18.3 g, 0.1 mol) in ethyl ether (100 ml). The mixture was stirred at room temperature for 1 h. The heavy oil was allowed to separate from the ether solution. Evaporation of the ether solution *in vacuo* yielded half an equivalent unreacted dichloromethanesulfonyl chloride (9 g) which was identified with an authentic sample.

The reaction of dichloromethanesulfonyl chloride in the presence of triethylammonium bromide. Triethylamine (5 g, 0.05 mol) was added for 3 min to a solution of dichloromethanesulfonyl chloride (9.1 g, 0.05 mol) and triethylammonium bromide (0.1 g, 0.05 mol) in chloroform (100 ml). The mixture was stirred for 1 h at 25 °C. A solution of bromine (8.8 g, 0.055 mol) in chloroform (25 ml) and water (75 ml) was then added in one portion with vigorous stirring. The organic layer was allowed to separate after 10 min, and washed with aqueous sodium bisulfite (10 %) to remove excess bromine and then with water. The organic layer was dried and evaporated *in vacuo* to give a semicrystalline residue. Mass spectrum (20 eV, direct inlet) of the crude residue shows the presence of different halomethyl fragments at *m/e* (rel. intensity): $CCl_3^{+\cdot}$ 117 (100), 119 (96.4), 121 (30.7); $BrCl_2C^{+\cdot}$ 161 (48.9), 163 (78.1), 165 (35.0), 167 (5.1); $Br_2ClC^{+\cdot}$ 205 (5.8), 207 (11.7), 209 (9.5), 301 (2.9).

Treatment of trichloromethanesulfinate with triethylammonium bromide. Triethylamine (5.0 g, 0.05 mol) was added to a solution of dichloromethanesulfonyl chloride (9.1 g, 0.05 mol) in chloroform (100 ml). The solution was stirred for 15 min at 25 °C. Triethylammonium bromide (9.1 g, 0.05 mol) was added and the mixture was stirred for an additional hour. A solution of bromine (8.8 g, 0.055 mol) in chloroform (25 ml) and water (75 ml) was then added in one portion with vigorous stirring. The organic layer was allowed to separate after 10 min, and washed with aqueous sodium bisulfite (10 %) to remove excess bromine and then with water. The organic layer was dried and evaporated *in vacuo* to give a semicrystalline residue. Mass spectrum (20 eV, direct inlet) of the crude residue shows the presence of halomethyl fragments at *m/e* (rel. intensity): $CCl_3^{+\cdot}$ 117 (100), 119 (95.2), 121 (30.6); $BrCl_2C^{+\cdot}$ 161 (4.5), 163 (6.9), 165 (3.2), 167 (0.5).

Reaction of chloromethanesulfonyl chloride and triethylamine. Triethylamine (3.3 g, 0.033 mol) was slowly added to a cooled solution of chloromethanesulfonyl chloride (5 g, 0.033 mol) in ethyl ether (50 ml). The reaction mixture was stirred for 30 min at room temperature and then extracted with water (100 ml). The aqueous solution was saturated with chlorine and extracted with carbon tetrachloride. Dichloromethanesulfonyl chloride is not obtained in this experiment as shown by NMR analysis. A yield of less than 1 % is detectable which was determined by a reference solution of dichloromethanesulfonyl chloride in CCl_4.

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A *trans*-Coplanar Elimination of Sulfur Dioxide and Chloride Ion in 2-Chloroalkanesulfinates*

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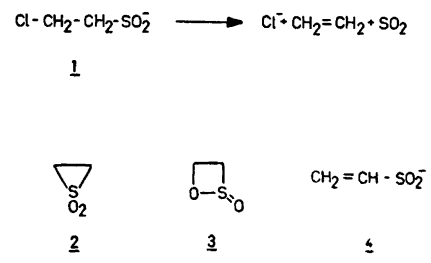
2-Chloroalkanesulfinates are shown to decompose into alkenes, sulfur dioxide, and chloride ions. The mechanism of the reaction is discussed. The reaction is shown to proceed *via* a *trans*-coplanar elimination. The possible implication of a 2-substituted alkanesulfinate in the base-promoted rearrangement of α-halosulfones (Ramberg-Bäcklund rearrangement) is discussed.

The decomposition of a 2-haloalkanesulfinate has not previously been studied. However, it is known [1] that the barium salt of 2-hydroxyethanesulfinic acid decomposes to yield ethylene and barium sulfite. During the course of this work the decomposition of perfluoroalkanesulfinates to yield perfluoroalkenes has been reported.[2] It is also of interest to note that a 2-halosulfinate has been discussed [3] as an intermediate in the base-induced rearrangement of α-halosulfones to alkenes (Ramberg-Bäcklund rearrangement).

In connection with a study of sulfur dioxide elimination reactions, 2-chloroethanesulfinic acid was treated with base. The sulfinate was found to decompose spontaneously to ethylene, chloride ion, and sulfur dioxide in good yield (Scheme I). In this paper the decomposition of 2-chloroalkanesulfinates will be discussed and evidence for a *trans*-coplanar elimination will be presented.

2-Chloroethanesulfinate (*1*) is readily available and has been used as a model compound for kinetic and mechanistic studies. This compound was prepared from the corresponding sulfinyl chloride which may be prepared by addition of thionyl chloride to ethylene according to a previous method.[4] A more convenient method for the preparation of 2-chloroethanesulfinyl chloride has now been developed. Treatment of ethylene sulfide and acetic anhydride in equivalent amounts with chlorine afforded 2-chloroethanesulfinyl chloride in good yield. The corresponding sulfinic acid was prepared by a careful hydrolysis with aqueous sodium hydrogen carbonate followed by acidification with sulfuric acid. The 2-chloroethanesulfinic acid was extracted with ethyl ether. The crude sulfinic acid, obtained after evaporation of the solvent, was found to be pure and was characterized by IR and NMR.

The decomposition of 2-chloroethanesulfinate (*1*) can be performed in water or in organic solvents. The rate of the decomposition in water with excess sodium hydroxide has been determined. The formation of ethylene was found to follow first order kinetics and the activation energy of the reaction was 127.8 ± 8.4 kJ mol^{-1}.



Scheme 1.

* Kempe, T. and Norin, T., presented in part at the "14de Nordiska Kemistmötet", Umeå, June 1971; "Vth Symposium on Organic Sulphur Chemistry", Lund, June 1972.

Different reaction mechanisms for the decomposition reaction can be formulated. Ethylene sulfone (*2*) can be ruled out as an intermediate since this compound was found to be stable under the reaction conditions used. A less probable intermediate is 1,2-oxathietane 2-oxide (*3*, β-sultine). β-Sultines are known [5] to decompose to alkanes and sulfur dioxide. However, they also react with alcohols to give 2-hydroxyalkanesulfinic acid esters.[5]

When the decomposition of 2-chloroethanesulfinate was carried out in methanol in the presence of triethylamine, no methyl 2-hydroxyethanesulfinate could be detected in the reaction product.

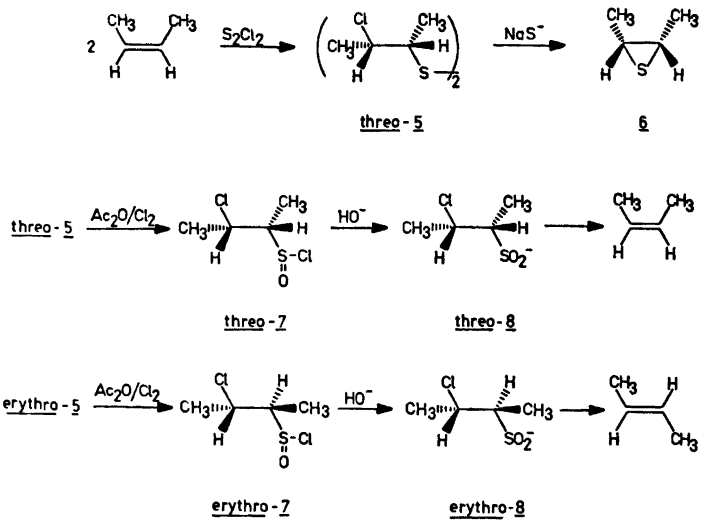
When the decomposition of 2-chloroethanesulfinate was performed in a deuterium oxide solution, no deuterium incorporation could be detected in the ethylene as shown by the mass spectrum of the 1,2-dibromoethane formed by bromination of the ethylene. Thus ethylenesulfinate (*4*) derived from the 2-chloroethanesulfinate by hydrogen chloride elimination, cannot be an intermediate in the reaction.

The most likely mechanism for the 2-halosulfinate decomposition is a *trans*-coplanar elimination of sulfur dioxide and chloride ion. In order to obtain additional information regarding this reaction its stereochemistry was investigated as follows.

The addition of sulfur monochloride to alkenes is known to proceed *via* a *trans*-addition yielding the corresponding 2-haloalkyl disulfides [6] (Scheme 2). Lautenschlaeger and Schwartz have recently shown [6] that the addition to *cis*-2-butene yields bis(*threo*-2-chloro-1-methylpropyl)disulfide (*threo-5*) which upon treatment with sodium sulfide gives the *cis*-2,3-dimethylthiirane (*6*). The corresponding *trans*-2-butene yields the *trans*-2,3-dimethylthiirane under similar conditions.

We have reacted *cis*-2-butene with sulfur monochloride. The disulfide (*threo-5*) thus obtained was oxidized with chlorine in acetic anhydride to yield the corresponding *threo*-2-chloro-1-methylpropanesulfinyl chloride (*threo-7*) by a known reaction.[7] This compound was hydrolysed with aqueous sodium hydroxide. The intermediate *threo*-2-chloro-1-methylpropanesulfinate (*threo-8*) rapidly decomposed to yield *cis*-2-butene. Less than 1 % *trans*-2-butene was present in the product as shown by gas chromatography.

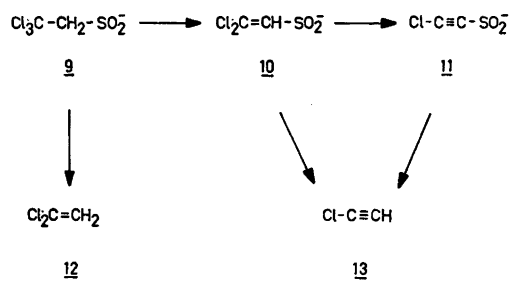
Similarly *trans*-2-butene yielded *erythro*-2-chloro-1-methylpropanesulfinyl chloride (*erythro-7*) which, without isolation, was treated with aqueous sodium hydroxide to yield *trans*-2-butene *via* the intermediate *erythro*-2-chloro-1-methylpropanesulfinate (*erythro-8*). Less than 1 % *cis*-2-butene was present in the product.



Scheme 2.

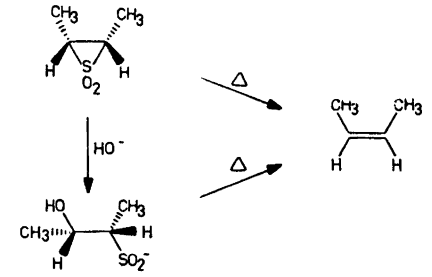
The 2-halosulfinate decomposition is thus a stereospecific reaction and the results presented above support a mechanism involving a *trans*-coplanar elimination.

A 2-chloroalkanesulfinate decomposition also occurs with 2,2,2-trichloroethanesulfinate (*9*, Scheme 3). When the sulfinyl chloride of *9* was hydrolysed with an equivalent amount of aqueous sodium hydroxide, 1,1-dichloroethylene (*12*) was formed. With an excess of sodium hydroxide, chloroacetylene (*13*) was isolated in addition to dichloroethylene (*12*). Two possible routes for the formation of chloroacetylene may be considered. In one the 2,2-dichloroethylenesulfinate intermediate (*10*) decomposes *via* a reaction mechanism similar to that for the 2-halosulfinate and in the other, further dehydrohalogenation of the 2,2-dichloroethylenesulfinate (*10*) gives 2-chloroacetylenesulfinate (*11*) which decomposes into chloroacetylene (*13*) and sulfur dioxide.[8]



Scheme 3.

There is strong evidence for the presence of intermediate thiirane 1,1-dioxides in the base-induced rearrangement of α-halosulfones (Ramberg-Bäcklund rearrangement).[9] The decomposition of this intermediate thiirane 1,1-dioxide to alkene and sulphur dioxide is a stereospecific reaction [9] (Scheme 4). Although this decomposition is known to be a thermal reaction it is not clear whether the base, in some cases, may take part in the decomposition. It has been established that ethylene sulfone upon treatment with alkali yields 2-hydroxyethanesulfinate.[1] Hence nucleophilic attack of the base on the intermediate thiirane 1,1-dioxide in the Ramberg-Bäcklund rearrangement should yield 2-substituted alkanesulfinates. This reaction has been discussed by Bordwell *et al.*[10] They propose that an intermediate 2-methoxyalkanesulfinate would appear to be stable under conditions whereby thiirane 1,1-dioxides are converted to alkenes. Therefore, they conclude that this mechanism does not operate in the decomposition of thiirane 1,1-dioxides in the presence of methoxide ions. However, there are no indications that alkanesulfinates with a leaving group in 2-position are stable under the conditions for a Ramberg-Bäcklund rearrangement and the formation of an intermediate 2-substituted alkanesulfinate cannot be excluded. This intermediate would then decompose by a reaction analogous to the stereospecific decomposition of 2-chloroalkanesulfinates (Scheme 4).



Scheme 4.

EXPERIMENTAL

All boiling points are uncorrected. IR spectra were measured on a Perkin Elmer Model 421 infrared spectrophotometer. NMR spectra (TMS internal standard) were recorded on a Varian Model A-60A instrument. Mass spectra were obtained using an LKB Model 9000 mass spectrometer.

2-Chloroethanesulfinyl chloride. A solution of ethylene sulfide (23.5 g, 0.39 mol) and acetic anhydride (40 g, 0.39 mol) was cooled to 5 °C. Chlorine was then passed into the solution at such a rate that the temperature remained between 10 and 15 °C. When no more chlorine was consumed excess chlorine and acetyl chloride was removed by evaporation *in vacuo* at room temperature. The residue was distilled at reduced pressure to give 2-chloroethanesulfinyl chloride; b.p. 42 °C/0.7 mmHg (lit.[4] b.p. 81 °C/9 mmHg); yield 46 g (80 %); IR (film) 1150 cm^{-1} (S=O); NMR ($CDCl_3$) δ 3.95 ppm (m, CH_2).

2-Chloroethanesulfinic acid. 2-Chloroethanesulfinyl chloride (14.7 g, 0.1 mol) was slowly added to an aqueous solution (100 ml) of sodium hydrogen carbonate (16.8 g, 0.2 mol) cooled to 5 °C. The mixture was stirred for 10 min and

then acidified with conc. sulfuric acid (10 g). The 2-chloroethanesulfinic acid thus formed was extracted with ethyl ether (200 ml). The solution was dried and the solvent evaporated *in vacuo* to give the oily 2-chloroethanesulfinic acid; yield 9 g (70 %); IR (film) 1080 (broad) cm^{-1} (SO_2H); NMR ($CDCl_3$) δ 3.2 (t, 2, CH_2) and 3.9 ppm (t, 2, CH_2).

Decomposition of 2-chloroethanesulfinic acid in the presence of sodium hydroxide. 2-Chloroethanesulfinic acid (8 g, 0.062 mol) was added to an aqueous sodium hydroxide solution (2 M, 100 ml). The reaction mixture was slowly heated to 60 °C and was then kept at that temperature for 10 min. The ethylene thus formed was passed through a chloroform solution (100 ml) containing bromine (16 g, 0.1 mol). The chloroform solution was treated with an aqueous sodium bisulfite solution (10 %) in order to remove unreacted bromine, washed with water and dried. Evaporation of the solvent afforded crude 1,2-dibromoethane; b.p. 131–132 °C/760 mmHg; yield 4 g (34 %); n_D^{22} 1.5360. The 1,2-dibromoethane was analysed by GLC-MS (column, 150 cm × 3 mm, packed with 5 % SE 30 on Chromosorb W 60/80 mesh, column temperature 70 °C), *m/e* (rel. intensity); $C_2H_4Br^+$ 107(100), 109(100); $C_2H_4Br_2^{+\cdot}$ 186(1.3), 188(2.6), 190(1.3).

Decomposition of 2-chloroethanesulfinyl chloride in the presence of D_2O/NaOD. 2-Chloroethanesulfinyl chloride (1 g, 6.8 mmol) was added to a sodium deuterium oxide solution (1 M, 15 ml) in deuterium oxide. The mixture was heated to ensure decomposition of 2-chloroethanesulfinate. The ethylene gas was passed through a chloroform solution containing bromine to give 1,2-dibromoethane. The excess of bromine was removed in the usual way. The 1,2-dibromoethane was analysed by GLC-MS as has been described above. No deuterated 1,2-dibromoethane could be detected in the reaction mixture. The mass spectrum of the product was identical with that of an authentic sample of 1,2-dibromoethane.

Decomposition of 2-chloroethanesulfinic acid in the presence of ethylene sulfone. The NMR spectrum of 2-chloroethanesulfinic acid in chloroform solution exhibits peaks at δ 3.2 (t, 2, CH_2), 3.9 (t, 2, CH_2) and 7.4 ppm (s, SO_2H). When excess pyridine is added to the sulfinic acid, gas is evolved. The peaks at δ 2.93 and 3.85 ppm of 2-chloroethanesulfinate decrease. 2-Chloroethanesulfinate disappeared after 18 min and no other peaks except that of ethylene (δ 5.22 ppm) could be detected in this region. Ethylene sulfone[11] (δ 3.12 ppm) was found to be stable in pyridine/chloroform solution under the condition used. No appreciable decrease of the peak at δ 3.12 ppm was observed after 1 h. 2-Chloroethanesulfinic acid and ethylene sulfone were mixed in a chloroform solution. Excess pyridine is then added and the decomposition of 2-chloroethanesulfinate is followed by NMR. When the 2-chloroethanesulfinate is completely consumed (*ca.* 20 min) the intensity of the peak from the ethylene sulfone (δ 3.12 ppm) is still undiminished.

Decomposition of 2-chloroethanesulfinate in the presence of methanol. Triethylamine (2 g, 0.02 mol) was added to a solution of 2-chloroethanesulfinic acid (2.5 g, 0.02 mol) in methanol (40 ml) at room temperature. The reaction mixture was then boiled for 30 min. The presence of sulfur dioxide could be detected by the characteristic smell of the gas. The volatile constituents in the solution were then evaporated *in vacuo* to give a crystalline residue which was washed several times with ethyl ether. The combined ether solutions were washed with water and dried. Evaporation of the solvent under reduced pressure did not afford any residue. The insoluble crystals which did not dissolve in ethyl ether was characterized as triethylammonium chloride which upon treatment with alkali gave triethylamine. This experiment shows that methanol does not react with any intermediate (*e.g.* β-sultine) during the decomposition of 2-chloroethanesulfinate and it is thus unlikely that any such intermediate is involved in the decomposition reaction.

Bis(2,2,2-trichloroethyl)disulfide was prepared essentially according to the method of Aichenegg.[12] Sulfur monochloride (81 g, 0.6 mol) was added dropwise over 2 h to a mixture of 1,1-dichloroethylene (250 g, 2.58 mol) and $FeCl_3$ (8 g). The reaction mixture was kept at room temperature overnight and was then diluted with 1 vol of acetone, followed by addition of sufficient water to give 2 layers. The organic phase was recovered and washed with water. The procedure was repeated until no more acetone was present in the organic phase, which was then dried and evaporated *in vacuo* to give a residue. Distillation at reduced pressure afforded bis(2,2,2-trichloroethyl)disulfide; b.p. 122 °C/0.5 mmHg (lit.[12] b.p. 145–150 °C/0.36–0.46 mmHg); yield 90 g (46 %); n_D^{25} 1.5750 (lit.[12] n_D^{20} 1.5780).

2,2,2-Trichloroethanesulfinyl chloride. A solution of bis(2,2,2-trichloroethyl)disulfide (32.8 g, 0.1 mol) and acetic anhydride (20.4 g, 0. 2 mol) was cooled to 5 °C. Chlorine was then passed into the solution at such a rate that the temperature remained between 10 and 15 °C. When no more chlorine was consumed, chlorine and acetyl chloride were removed by evaporation *in vacuo* at room temperature. The residue was distilled at reduced pressure to give 2,2,2-trichloroethanesulfinyl chloride; b.p. 56 °C/1.5 mmHg; yield 32 g (74 %); n_D^{25} 1.5446; IR (film) 1160 cm^{-1} (S=O); NMR (neat) δ_A 4.68 and δ_B 4.85 ppm (J_{AB} 14 Hz).

Decomposition of 2,2,2-trichloroethanesulfinyl chloride with an equivalent amount of base. An aqueous sodium hydroxide solution (1.6 M, 50 ml) was slowly added to a cooled mixture (5 °C) of 2,2,2-trichloroethanesulfinyl chloride (8.6 g, 0.04 mol) and water (150 ml). Carbon tetrachloride (50 ml) was then added to dissolve

the 1,1-dichloroethylene formed. The reaction mixture was then kept at room temperature for 2 h. The NMR spectrum of the carbon tetrachloride solution shows that 1,1-dichloroethylene was formed in 53 % of the theoretical yield (determined by using a reference solution of 1,1-dichloroethylene in CCl_4). 1,1-Dichloroethylene; IR (CCl_4) 1610 cm^{-1} (C=C); NMR (CCl_4) δ 5.5 ppm (s, $H_2C=$).

Decomposition of 2,2,2-trichloroethanesulfinyl chloride in excess base. To an aqueous sodium hydroxide solution (1 M, 200 ml) and carbon tetrachloride (5 ml) was slowly added 2,2,2-trichloroethanesulphinyl chloride (4.3 g, 0.02 mol). The reaction mixture was kept during 2 h at room temperature under nitrogen. Air must be excluded owing to the explosive nature of chloroacetylene in the presence of oxygen. The IR and NMR spectra of the carbon tetrachloride solution showed that chloroacetylene was formed together with 1,1-dichloroethylene. The yield of chloroacetylene was not determined. Chloroacetylene; IR (CCl_4) 3300 (CH) and 2100 cm^{-1} (C≡C); NMR (CCl_4) δ 1.78 ppm (s, CH). 1,1-Dichloroethylene was treated with a sodium hydroxide solution under the conditions described above. Chloroacetylene could not be detected in this experiment.

*Bis(*threo*-2-chloro-1-methylpropyl)disulfide from* cis*-2-butene and sulfur monochloride.*[6] *cis*-2-Butene (35 g) was added to a solution of $FeCl_3$ (1 g) in ethyl ether (150 ml). Sulfur monochloride (27 g, 0.2 mol) was then slowly added to the stirred solution over 30 min. *cis*-2-Butene was passed continuously through the reaction mixture to maintain an excess. The reaction mixture was stirred at room temperature for 3 h after addition of sulfur monochloride and then washed with water. The organic phase was dried and the solvent removed *in vacuo*. The residue was distilled under reduced pressure and a fraction was collected at b.p. 80–83 °C/0.2 mmHg; yield 19 g. Without further purification this fraction was used in the preparation of *threo*-2-chloro-1-methylpropanesulfinyl chloride (see below).

threo*-2-Chloro-1-methylpropanesulfinyl chloride.* A solution of bis(*threo*-2-chloro-1-methylpropyl)disulfide (18.5 g, 0.075 mol, a mixture of *meso* and *racemic* forms) and acetic anhydride (15.3 g, 0.15 mol) was cooled to 5 °C. Chlorine was passed into the solution at such a rate that the temperature remained between 10 and 15 °C. When no more chlorine was consumed, chlorine and acetyl chloride were removed by evaporation *in vacuo* at room temperature. The residue was distilled at reduced pressure to give *threo*-2-chloro-1-methylpropanesulfinyl chloride; b.p. 64–66 °C/2 mmHg; yield 15 g (57 %); n_D^{25} 1.5140; IR (film) 1150 cm^{-1} (S=O).

Decomposition of threo*-2-chloro-1-methylpropanesulfinyl chloride in the presence of sodium hydroxide.* *threo*-2-Chloro-1-methylpropanesulfinyl chloride (7 g, 0.04 mol) was added at room temperature to an aqueous sodium hydroxide solution (2 M, 100 ml). The butene gas thus formed was passed through a sodium hydroxide solution to remove sulfur dioxide and then collected over water in a measuring cylinder. No more gas evolution could be observed after 2 h at room temperature. The gas (820 ml) was analysed at GLC (column, 4.5 m × 3 mm, packed with 15 % dimethylsulfolane on GasChrom RZ 60/80 mesh, relative retention times *trans*- and *cis*-2-butene 1.00:1.08, column temperature 30 °C). *cis*-2-Butene was present in more than 99 % purity.

*Bis(*erythro*-2-chloro-1-methylpropyl)disulfide from* trans*-2-butene and sulfur monochloride.*[6] This compound was first prepared according to the procedure described for the corresponding *threo*-isomer. However, because of the slow reaction of *trans*-2-butene and sulfur monochloride under these conditions a modified procedure was used. *trans*-2-butene (*ca.* 35 g) was added to a solution of chloroform (130 ml) and pyridine (0.2 ml). Sulfur monochloride (20.5 g, 0.152 mol) was then added slowly to the chloroform solution which was cooled with water. The reaction temperature was not allowed to rise above 25 °C. An excess of *trans*-2-butene was passed continuously through the reaction mixture. After the addition, the reaction mixture was kept at room temperature for 2 h. The chloroform and excess *trans*-2-butene were removed *in vacuo*. It was not possible to distil the residue at reduced pressure (0.2 mm) owing to decomposition. The crude residue was therefore used for the preparation of *erythro*-2-chloro-1-methylpropanesulfinyl chloride (see below).

erythro*-2-Chloro-1-methylpropanesulfinyl chloride.* A solution of crude bis(2-chloro-1-methylpropyl)disulfide (37.5 g, 0.152 mol), prepared as described above from *trans*-2-butene and sulfur monochloride, and acetic anhydride (31 g, 0.304 mol) was cooled to 5 °C. Chlorine was then passed into the solution at such a rate that the temperature remained between 10 and 15 °C. When no more chlorine was consumed the excess chlorine and acetyl chloride was evaporated *in vacuo* at room temperature. The residue could not be distilled owing to decomposition. Therefore, the crude *erythro*-2-chloro-1-methylpropanesulfinyl chloride was used for the decomposition experiment (see below) without further purification.

Decomposition of crude erythro*-2-chloro-1-methylpropanesulfinyl chloride in the presence of sodium hydroxide.* Crude *erythro*-2-chloro-1-methylpropanesulfinyl chloride (7 g) was decomposed as described above for the corresponding *threo*-isomer to yield *trans*-2-butene (280 ml) of more than 99 % purity.

Kinetic procedures. The rates of the decomposition of 2-chloroethanesulfinate have been measured at 21 °C and 34 °C in excess 0.5 M aqueous sodium hydroxide solution. The rates for the appearance of ethylene was followed

by collecting the gas in a measuring cylinder over water (21 °C).

Run a at 21 °C. 2-Chloroethanesulfinyl chloride (1.1178 g, 7.6 mmol) was added to a sodium hydroxide solution (0.5 M, 120 ml) to give 2-chloroethanesulfinate. The solution was kept at 21 °C and the gas was collected. Totally, 161 ml ethylene was evolved over 1396 min, which corresponds to an 85 % yield (correction was made for water pressure and atmosphere pressure). The rate of the decomposition was calculated from the slope of a plot of $\log [a/(a-x)]$ *versus* time. (a = theoretical yield of ethylene; x = the ethylene gas evolved). The decomposition gave good first order kinetics up to 63 % reaction. The rate at 21 °C was $(16.1 \pm 1.5) \times 10^{-4}$ min^{-1}.

Run b at 34 °C. 2-Chloroethanesulfinyl chloride (1.1025 g, 7.5 mmol) was added to a sodium hydroxide solution (0.5 M, 120 ml). The solution was kept at 34 °C and the ethylene gas was collected. Totally, 171 ml ethylene was evolved over 225 min which corresponds to 90 % yield. The rate of the decomposition was derived as described in *run a*. The decomposition gave good first order kinetics up to 75 % reaction. The rate at 34 °C was $(14.7 \pm 0.7) \times 10^{-3}$ min^{-1}.

The activation energy 127.8 ± 8.4 kJ. mol^{-1} was obtained using the Arrhenius equation:

$$\log k_2/k_1 = (\Delta H_a/2.303R)[(T_2 - T_1)/T_2T_1]$$

where k is the rate constant, ΔH_a the activation energy, $R = 8.314$ J deg.$^{-1}$ mol^{-1} and T = absolute temperature.

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Non-equivalence of Methylene Protons in Some Nitroxide Radicals of the Type XYZC—CH_2—N(O·)-*tert*-Butyl and XYN—CH_2—N(O·)-*tert*-Butyl

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A number of nitroxide radicals of the type XYZC—CH_2—N(O·)-*tert*-butyl and one of the type XYN—CH_2—N(O·)-*tert*-butyl were found to exhibit ESR spectra with unequal splittings of the methylene protons together with temperature-dependent line width alternations. The origin of the non-equivalence and the line width alternations was shown to be connected with the presence of unsymmetrically related conformers produced by a steric hindrance combined with an unsymmetric center in position β to the nitrogen atom of the nitroxide group.

In connection with ESR studies on the trapping of short-lived free radicals by the nitroxide method,[1] it was observed that some acyclic nitroxides of the type XYZC—CH_2—N(O·)-*tert*-butyl and one of the type XYN—CH_2—N(O·)-*tert*-butyl gave rise to spectra exhibiting unequal splittings of the methylene protons.

In this paper it is shown that the non-equivalence of the methylene protons originates from the presence of unsymmetrically related conformers produced by a steric hindrance combined with an unsymmetric center in position β to the nitrogen atom of the nitroxide group. Non-equivalence of β-methylene** protons connected with the presence of a chiral atom has been observed for nitronyl nitroxide radicals,[3] nitroxide radicals,[4] and some nitro radicals.[5]

RESULTS

Primary short-lived radicals R—CH_2· (I) were produced in water or a slightly alkaline aqueous solution by the abstraction of a hydrogen atom from methyl groups of the parent compounds R—CH_3 (II) by photochemically generated OH radicals. The nitroxides (III) were subsequently formed in the reaction of the primary radicals with *tert*-nitrosobutane, eqn. 1.

$$\underset{\text{II}}{\text{R}-\text{CH}_3} \xrightarrow{\cdot\text{OH}} \underset{\text{I}}{\text{R}-\text{CH}_2\cdot}$$

$$\underset{\text{I}}{\text{R}-\text{CH}_2\cdot} + \textit{tert}\text{-nitrosobutane} \longrightarrow \underset{\text{III}}{\text{R}-\text{CH}_2-\text{N}(\text{O}\cdot)\text{-}\textit{tert}\text{-butyl}} \qquad (1)$$

The experimental details have been described elsewhere.[1]

5,5-Dimethylhydantoin, 5-methylhydantoin, isopropyl alcohol, 2-hydroxy-2-methylpropanoic acid, and *N*,*N*-dimethylformamide were found to give rise to the nitroxides *1*—*5*, which exhibit unequal β-methylene proton splittings, whereas *tert*-butyl alcohol and 2,2-dimethylpropanoic acid gave rise to the structurally similar nitroxides *6* and *7* which exhibited equivalent β-methylene proton splittings above room temperature.

The ESR spectra of the nitroxides *1*—*5* consisted of 3 × 4 lines indicating an interaction with the ^{14}N nucleus and two non-equivalent

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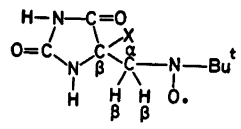
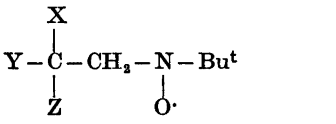
** The notation used for the atoms concerned is in conformity with the nomenclature used for radicals, *e.g.* by Briere *et al.*[2]



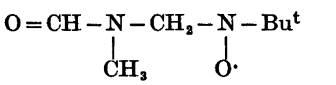
1, 2

1 X = CH_3; 2 X = H



3, 4, 6, 7

3 X = CH_3, Y = OH, Z = H; 4 X = CH_3, Y = OH, Z = COO^-; 6 X = CH_3, Y = CH_3, Z = OH; 7 X = CH_3, Y = CH_3, Z = COO^-.



5

protons, evidently those of the methylene group. The nitroxides *3* and *5* gave rise to further splittings, nitroxide *3* to a doublet caused by the hydrogen atom on the β-carbon atom, nitroxide *5* to a triplet by interaction with the ^{14}N nucleus of the amide group. The proton splittings of the nitroxides *1*, *3*, and *4* varied somewhat with the temperature in an inversed way, so that the large one decreased and the small one increased when the temperature was raised (Table 1). The spectra obtained with nitroxides *3*, *4* and *5* are shown in Figs. 1, 2, and *3*, respectively.

Line width alternations were observed with the nitroxides with the unequal β-proton splittings. The two inner lines of the 1:1:1:1 quartet caused by the two non-equivalent methylene

Table 1. Nitroxide radicals of the type R—CH_2—N(O·)-*tert*-butyl formed by trapping of the radicals R—$\dot{C}H_2$ produced in the reaction between photochemically generated OH radicals and a methyl group of the parent compounds. Scavenger: *tert*-nitrosobutane. Splitting constants in gauss. Solvent: H_2O.

Parent compound	Nitroxide radical	Temp. °C	a_N	$a_{H(2)}$	$a_{H(1)}$	$a_{H(2)}+a_{H(1)}$	a_X[a,b]
5,5-Dimethylhydantoin	*1*	+3	15.8	20.3	3.68	24.0	
»	*1*	+20	15.9	20.0	4.08	24.1	
»	*1*	+46	15.8	19.3	4.52	23.8	
»	*1*	+84	15.9	18.4	5.38	23.8	
»	*1*	+98	15.8	17.9	5.55	23.5	
5-Methylhydantoin	*2*	+20	16.1	26.0	0.72	26.7	
Isopropyl alcohol	*3*	−8	16.0	14.2	9.3	23.5	0.73
»	*3*	+25	16.3	13.3	8.9	22.2	0.74
»	*3*	+35	16.3	12.9	9.3	22.2	0.75
»	*3*	+49	16.4	12.6	9.8	22.4	0.76
»	*3*	+61	16.3	12.4	9.9	22.3	0.75
2-Hydroxy-2-methylpropanoic acid	*4*	−19	16.3	19.7	7.6	27.3	
»	*4*	−1.5	16.3	18.9	8.9	27.8	
»	*4*	+19	16.3	17.4	10.3	27.7	
»	*4*	+50	16.4	16.4	11.3	27.6	
»	*4*	+95	16.5	15.0	12.7	27.2	
N,*N*-Dimethylformamide	*5*	−16	15.4	10.3	9.3	19.6	2.35
»	*5*	0	15.4	10.1	9.1	19.2	2.35
»	*5*	+16	15.3	10.1	9.1	19.2	2.38
»	*5*	+35	15.4	10.0	8.9	18.9	2.43
»	*5*	+50	15.4	9.90	8.9	18.8	2.48
tert-Butyl alcohol	*6*	−25	16.3	11.0	11.0		
»	*6*	0	16.3	10.9	10.9		
»	*6*	+23	16.3	11.1	11.1		
»	*6*	+51	16.4	11.2	11.2		
»	*6*	+84	16.3	11.1	11.2		
2,2-Dimethylpropanoic acid	*7*	−20	15.8	21.6	5.82	27.42	
»	*7*	−9	16.2	22.0	5.60	27.60	
»	*7*	−1	16.1	13.5	13.5		
»	*7*	+23	16.3	13.5	13.5		
»	*7*	+72	16.3	13.0	13.0		

[a] Radical *3*. Doublet splitting from the proton on the β-carbon atom. [b] Radical *5*. Triplet splitting from the nitrogen atom of the amide group.

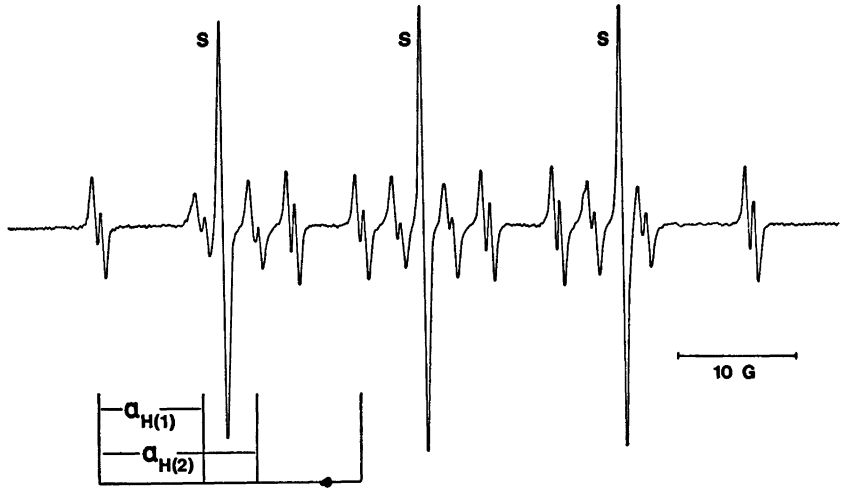


Fig. 1. ESR spectrum of the nitroxide radical *3* derived from isopropyl alcohol at +25 °C. Solvent: H_2O. S: symmetrical nitroxide radicals derived from the scavenger.

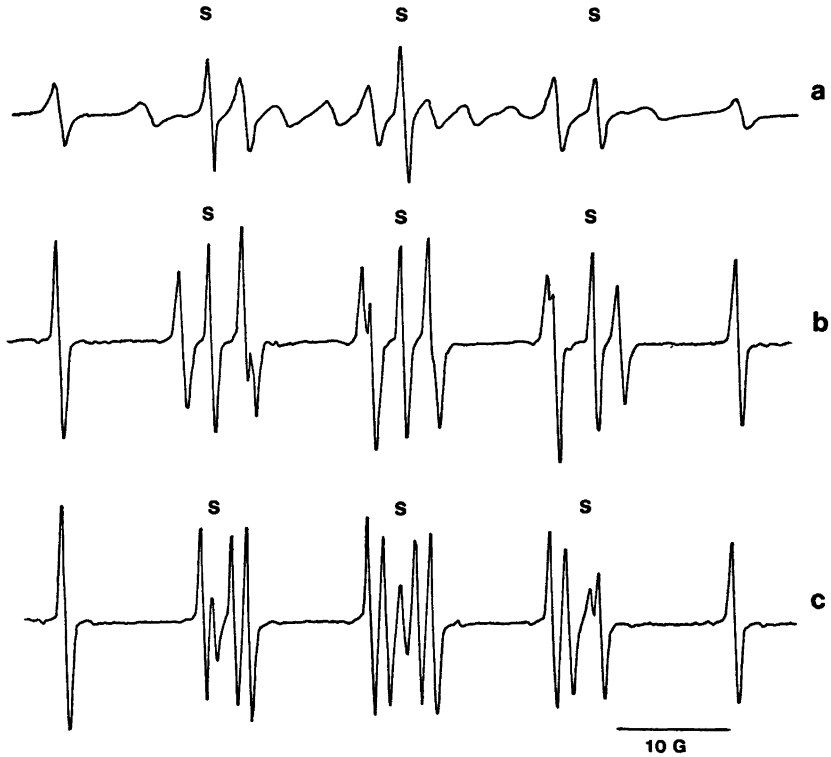


Fig. 2. ESR spectrum of the nitroxide radical *4* derived from 2-hydroxy-2-methylpropanoic acid. Solvent: H_2O + a small amount of alkali. S: symmetrical nitroxide radicals derived from the scavenger. *a* at −19 °C, *b* at +29 °C, and *c* at +83 °C.

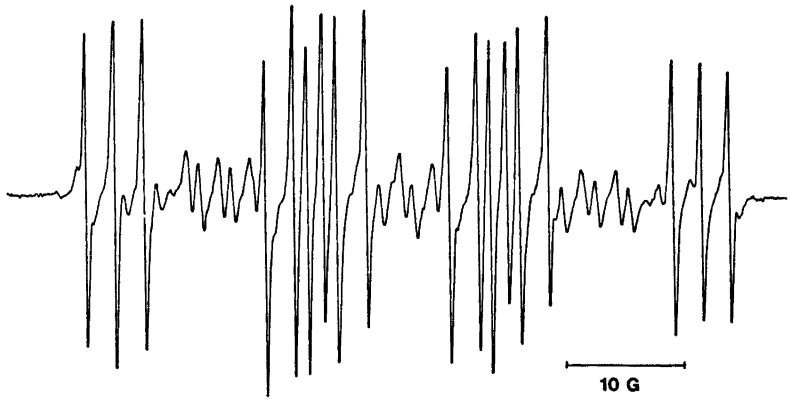


Fig. 3. ESR spectrum of the nitroxide radical *5* derived from *N*,*N*-dimethylformamide, at +35 °C. Solvent: H_2O.

protons broadened upon decreasing the temperature, but sharpened when the temperature was increased, so that all 12 lines became of equal height, as seen from the spectra in Fig. 2 obtained at different temperatures with nitroxide *4*, derived from 2-hydroxy-2-methylpropanoic acid. However, the spectra obtained with nitroxide *5* (Fig. 3) exhibited still very pronounced line width alternations, with broadening of the inner lines of the methylene quartets, even at the highest accessible temperature (+50 °C). The basic 12-line structure of the spectra was preserved for the nitroxides *1*–*5* in the entire experimentally accessible range of temperature, *i.e.* from −20 °C to about +90 °C.

Nitroxide *6* gave rise to 3×3 (1:2:1)-line spectra, indicating equivalence of the methylene protons. The center line of the 1:2:1 triplet became broadened already below +50 °C. Nitroxide *7* exhibited 3×3 (1:2:1)-line spectra above room temperature. Below room temperature, the center line of the 1:2:1 triplets broadened and below −10 °C there was a change into a 3×4 (1:1:1:1)-line spectrum, indicating nonequivalence of the methylene protons.

DISCUSSION

Most acyclic nitroxides of the type $R-CH_2-N(O\cdot)-R'$ hitherto described in the literature give rise to ESR spectra with equivalent methylene protons at room temperature. Many such nitroxides show line width alternations at lower

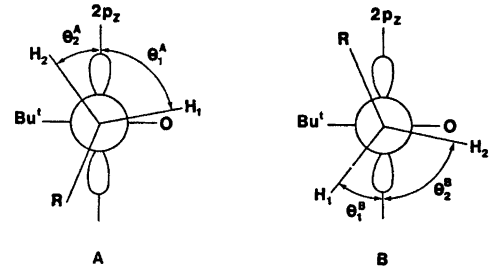


Fig. 4. Steric arrangement around the bond axis between the nitrogen atom of the nitroxide group and the α-carbon atom. The conformers A and B are symmetrically related, so that $\theta_1^A = \theta_2^B$ and $\theta_2^A = \theta_1^B$. The arrangement on the nitrogen atom was made planar.

temperatures due to the presence of a steric hindrance which introduces two symmetrically related conformers (A and B in Fig. 4).[6,7] This case was actually observed with nitroxide *6* below +50 °C. At even lower temperatures the rate of jumping between the conformers A and B became slow enough to allow the spectrometer to record a superposition spectrum of the individual conformers, the spectra of which are identical. The center line of the methylene triplets is now resolved into two separate lines, and the spectrum consists of 12 lines of equal intensity. Such an interpretation is evidently valid also for the spectra obtained with nitroxide *7* below −10 °C.

The results obtained with the nitroxides *1*–*5* cannot be explained by the mechanism outlined.

If this mechanism had been valid, the 12-line spectra would have exhibited a broadening of the inner lines of the methylene 1:1:1:1 quartet upon increasing the temperature, not a broadening upon decreasing the temperature which was actually observed. Furthermore, the 12-line spectra would have been changed into 9-line spectra with 1:2:1 methylene triplets in the high temperature range, a finding which was not observed for any of the nitroxides *1*–*5*.

The nitroxides *1*–*4* with unequal β-proton splittings all have a center of chirality in position β to the nitrogen atom of the nitroxide group, whereas no such center is present in the nitroxides *6* and *7* which show equivalent β-protons at high temperature. A hindered rotation around the bond axis between the α-carbon atom and the chiral β-atom introduces two conformers which are mutually unsymmetrical due to a steric interaction between the oxygen atom or the *tert*-butyl group on the nitrogen atom and the non-identical substituent groups XYZ on the chiral β-atom. The origin of the unsymmetrically related conformers in the case of radical *5* is less easily explained. The nitrogen atom of the amide group is evidently involved but it is not clear if the unsymmetry originates from the nitrogen atom as a chiral center, or indirectly from the steric behaviour of one of the substituent groups, possibly the COH group.

The mutually unsymmetrical conformers, A and B in Fig. 5, infer that the dihedral angles are related by the inequalities $\theta_1^A \neq \theta_2^B$ and $\theta_2^A \neq \theta_1^B$. Considering the splitting constant of a β-proton of nitroxides to be proportional to the cosine square of the dihedral angle concerned,[8] the non-equivalence of the β-protons is produced as follows. As a result of the unsymmetry conformers A and B will be represented by individual ESR spectra with non-identical splitting constants, as shown for one of the three methylene splittings in Fig. 6a and b. At low temperature, when the rate of jumping between conformations A and B is slow, the spectrometer will resolve all eight lines of the actual superposition spectrum of the two conformers (Fig. 6c). When the temperature is raised and the rate of interconversion increases past the limit of slow exchange, the lines coalescence in pairs into a 4-line spectrum, which gradually sharpens towards the situation shown in Fig. 6d.

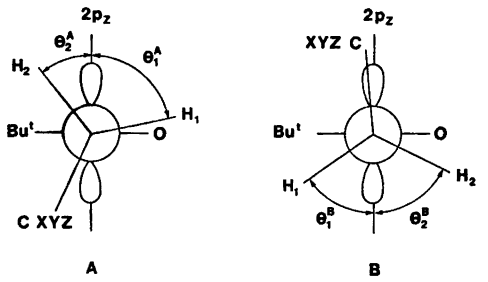


Fig. 5. Steric arrangement around the bond axis between the nitrogen atom of the nitroxide group and the α-carbon atom. The conformers A and B are unsymmetrically related, so that $\theta_1^A \neq \theta_2^B$ and $\theta_2^A \neq \theta_1^B$. The arrangement on the nitrogen atom was made planar.

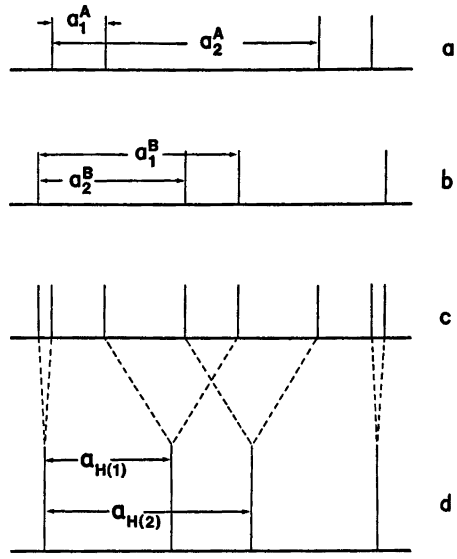


Fig. 6. Schematic ESR spectra of one of the three methylene splittings for nitroxides with the unsymmetrically related conformers A and B.
a and *b*: individual spectra corresponding to conformers A and B. *c*: actual spectrum at low jumping rate, *d*: at high rate.

This mechanism explains the origin of the four-line splittings produced by the methylene protons as observed with nitroxides *1*–*5*. Evidently the rate of interconversion between different conformations exceeds the limit of slow exchange in the whole range of the experimentally accessible temperatures. The broadening of the inner lines as compared with the

outer ones of the methylene quartet observed at low temperatures originates from the coalescence of pairs of lines in conformations A and B far more widely separated than the corresponding pairs of the wings. The continuous variation of $a_{(H1)}$ and $a_{(H2)}$ so that the sum $a_{H(1)} + a_{H(2)}$ remains constant as seen from Table 1, is believed to be connected with an unequal population of the two conformations A and B. The position of the inner lines of the methylene quartets formed by coalescence (see Fig. 6d) is a weighted mean of the positions of the parent lines in conformations A and B. An increase in the temperature will therefore move the inner lines towards the line position of the conformation, whose population was increased by the rise in temperature.

The interpretation made for the origin of the unequal β-methylene splittings of the nitroxides *1–5* is in some details similar to that proposed by Gilbert *et al.*[5] for the nitro radicals mentioned above, and seem to satisfactorily explain our findings.

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Organic Hydroxylamine Derivatives. XI.* Structural Analogues of γ-Aminobutyric Acid (GABA) of the Isoxazole Enol-betaine Type. Synthesis and the Crystal Structure of 3-Hydroxy-5-(3-aminopropyl)isoxazole Zwitterion

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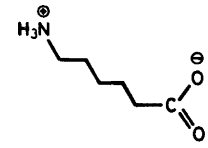
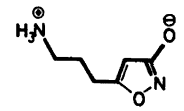
The synthesis and the crystal structure determination of 3-hydroxy-5-(3-aminopropyl)isoxazole zwitterion (V), which is a structural analogue of ε-aminocaproic acid, are described. The synthesis is based on methyl 3-(3-methoxyisoxazol-5-yl)propionate (I), which by conventional methods is converted into (V). The pK_A values of 3-hydroxy-5-(3-aminopropyl)isoxazole zwitterion have been determined to 5.37 ± 0.04 and 10.36 ± 0.04.

Crystals of 3-hydroxy-5-(3-aminopropyl)isoxazole monohydrate, $C_6H_{10}N_2O_2.H_2O$, are monoclinic, space group $C2/c$, $a = 23.45(1)$, $b = 5.768(1)$, $c = 13.483(4)$ Å, $\beta = 120.33(3)°$, $Z = 8$. The structure has been solved by a direct phasing technique using X-ray diffraction data, and has been refined by full-matrix least-squares methods. The final R value is 0.039 for 1218 independent observations. The molecule is a zwitterion, and the crystal structure is stabilized by a system of hydrogen bonds.

As part of our investigations of isoxazole enol-betaines, which are structural analogues of γ-aminobutyric acid (GABA) or closely related amino acids with GABA-like activity in the mammalian central nervous system, the crystal structure determinations of muscimol (3-hydroxy-5-aminomethylisoxazole zwitterion) [1,2] and homomuscimol (3-hydroxy-5-(2-aminoethyl)isoxazole zwitterion) [3] have been performed. The present paper describes the synthesis and the crystal structure determination of 3-hydroxy-5-(3-aminopropyl)isoxazole zwitterion (V), which is a structural analogue of ε-aminocaproic acid.[4] This synthetic amino acid exhibits a weak, GABA-like depressant activity when applied to feline spinal interneurones.[5] The biological properties of ε-aminocaproic acid are, however, multifarious, and rather conflicting results have been obtained after administration of this compound to different structures of the mammalian central nervous system (see Ref. 6). Investigations of the biological properties of 3-hydroxy-5-(3-aminopropyl)isoxazole will be initiated in the near future.



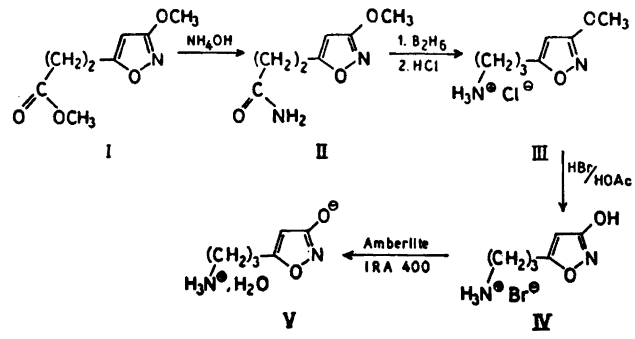
ε-Aminocaproic acid



3-Hydroxy-5-(3-aminopropyl)isoxazole zwitterion (V)

Fig. 1. Schematic drawings of ε-aminocaproic acid and 3-hydroxy-5-(3-aminopropyl)isoxazole.

* Part X. *Acta Chem. Scand. B 28* (1974) 533.



Scheme 1.

RESULTS AND DISCUSSION

The synthesis of 3-hydroxy-5-(3-aminopropyl)isoxazole zwitterion (V) is based on methyl 3-(3-methoxyisoxazol-5-yl)propionate (I), which is converted into 3-(3-methoxyisoxazol-5-yl)-propionamide (II). Subsequent reduction of (II) with diborane gives the corresponding primary amine, isolated from the reaction mixture as its hydrochloride (III). (III) is cleaved by hydrogen bromide in glacial acetic acid to 3-(3-hydroxyisoxazol-5-yl)propylammonium bromide (IV), from which the isoxazole enol-betaine (V) is isolated using a strongly basic ion exchange resin.

The structure determinations of (II – V) are based on IR, UV, and ^{1}H NMR spectroscopy and supported by elemental analyses. The spectroscopic data arising from the 3-methoxy- and 3-hydroxyisoxazole moieties of (II, III) and (IV), respectively, are in accordance with the general findings described by Jacquier *et al.*[7] The aromatic character of the isoxazole nucleus of (V) is evident from IR absorption bands at 1610 and 1520 – 1450 cm^{-1},[8] and absorptions over the range 3600 – 2000 and at 2170 cm^{-1} suggest ammonium salt character of (V). The UV maximum of (V) at 211 nm is in agreement with that observed (210 nm) for homomuscimol.[8] Some spectroscopic data of the compounds (II – V), which are all new, and the pK_A values of (V) are listed in Table 1.

The molecular structure of (V) is unambiguously confirmed by the results of the X-ray diffraction analysis. The dimensions and the conformation of the molecule are shown in Figs. 2a and 2b.

The isoxazole ring is planar within the limits of experimental error; Table 2 lists the displacements of some atoms from the least-squares plane through this ring. The least-squares plane through the planar *trans-zigzag* aminopropyl side chain makes an angle of 11.3° with the isoxazole ring plane (Table 2). The three hydrogen atoms are tetrahedrally arranged about the nitrogen atom. Their positions correspond to a 5 – 15° rotation about the C – N bond relative to that for a strictly staggered conformation.

Tables 3 and 4 and Figs. 2a and 2b show some

Table 1. IR and UV data of (II – V) and the pK_A values of (V).

	IR data [a] (cm^{-1})	UV data [b] λ_{max}(nm)	$\varepsilon \times 10^{-3}$	pK_A values [c]
II	3370(s), 3200(s), 1660(s), 1635(s), 1615(s), 1515(s), 1460(s)	211	6.86	
III	3700 – 2400(s), 2040(w), 1615(s), 1515(s), 1505(s), 1460(s)	211	5.58	
IV	3700 – 2100(s), 2000(w), 1620(s), 1525(s), 1465(s)	209	6.02	
V	3600 – 2000(s), 2170(m), 1710(w), 1610(s), 1520 – 1450(s)	211	6.16	5.37 ± 0.04 10.36 ± 0.04

[a] The IR spectra were recorded in the solid state (KBr). [b] The UV spectra were recorded in ethanol solutions. [c] The pK_A values were determined by titrations in aqueous solutions at 17 °C.

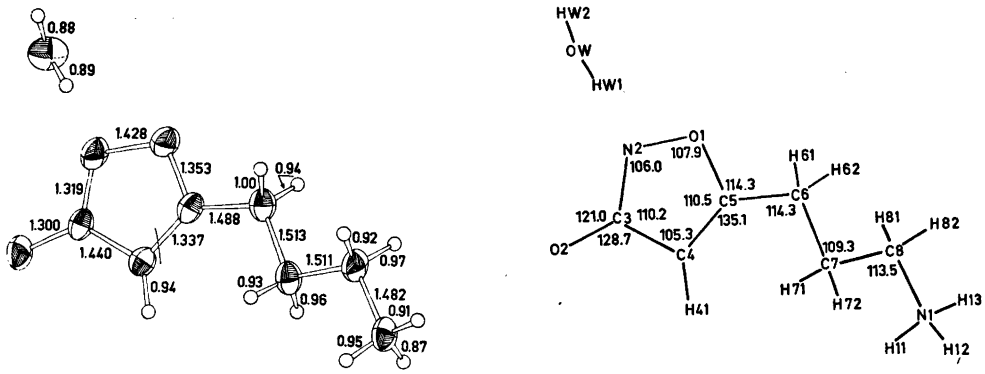


Fig. 2. (a) Bond lengths (Å), and thermal ellipsoids for the non-hydrogen atoms drawn to enclose 50 % probability. (b) The numbering of the atoms, and some bond angles (°).

Table 2. Distances of atoms from least squares planes (Å). The equations of the planes are in direct (unit-cell) space.

Atom	Deviation	Atom	Deviation
I. Isoxazole ring			
Equation:	$-10.1648x+0.7429y+13.3311z-0.2225=0$		
O(1)	−0.001	O(2)[a]	0.053
N(2)	−0.004	C(6)[a]	0.063
C(3)	0.006	C(7)[a]	0.176
C(4)	−0.007	C(8)[a]	0.471
C(5)	0.005	N(1)[a]	0.601
II. 3-Aminopropyl side chain			
Equation:	$14.1011x-0.6273y-13.3061z-1.9908=0$		
N(1)	−0.0003	C(6)	−0.0003
C(8)	0.0003	C(5)[a]	−0.2336
C(7)	0.0003		

[a] These atoms were not included in the calculation of the least squares plane.

*Table 3. Intra*molecular distances (Å). The estimated standard deviations ($\times 10^2$ for bonds to hydrogen, $\times 10^3$ for others) of the distances are given in parentheses.

O(1)−N(2)	1.428(2)	C(7)−H(71)	0.93(3)
N(2)−C(3)	1.319(3)	C(7)−H(72)	0.96(2)
C(3)−C(4)	1.440(3)	C(8)−H(81)	0.92(2)
C(4)−C(5)	1.337(3)	C(8)−H(82)	0.97(3)
C(5)−O(1)	1.353(2)	N(1)−H(11)	0.95(3)
C(5)−C(6)	1.488(3)	N(1)−H(12)	0.87(3)
C(6)−C(7)	1.513(3)	N(1)−H(13)	0.91(2)
C(7)−C(8)	1.511(3)	O(W)−H(W1)	0.89(2)
C(8)−N(1)	1.482(3)	O(W)−H(W2)	0.88(3)
C(3)−O(2)	1.300(2)	N(1)···N(2)	7.252(4)
C(4)−H(41)	0.94(2)	N(1)···O(1)	6.165(3)
C(6)−H(61)	1.00(2)	N(1)···O(2)	7.925(5)
C(6)−H(62)	0.94(3)		

Table 4. Valency angles (°). The estimated standard deviations (× 10 for angles not involving hydrogen) of the angles are given in parentheses.

C(5) – O(1) – N(2)	107.9(1)	H(61) – C(6) – H(62)	103(2)
O(1) – N(2) – C(3)	106.0(1)	C(6) – C(7) – H(71)	109(1)
N(2) – C(3) – C(4)	110.2(2)	C(6) – C(7) – H(72)	110(1)
N(2) – C(3) – O(2)	121.0(2)	C(8) – C(7) – H(71)	108(1)
C(4) – C(3) – O(2)	128.7(2)	C(8) – C(7) – H(72)	113(1)
C(3) – C(4) – C(5)	105.3(2)	H(71) – C(7) – H(72)	108(2)
C(4) – C(5) – O(1)	110.5(1)	C(7) – C(8) – H(82)	110(1)
C(6) – C(5) – O(1)	114.3(2)	C(7) – C(8) – H(81)	110(1)
C(6) – C(5) – C(4)	135.1(2)	N(1) – C(8) – H(82)	107(1)
C(5) – C(6) – C(7)	114.3(2)	N(1) – C(8) – H(81)	108(1)
C(6) – C(7) – C(8)	109.3(2)	H(82) – C(8) – H(81)	107(2)
C(7) – C(8) – N(1)	113.5(2)	C(8) – N(1) – H(11)	110(1)
C(3) – C(4) – H(41)	129(1)	C(8) – N(1) – H(13)	107(1)
C(5) – C(4) – H(41)	126(1)	C(8) – N(1) – H(12)	110(1)
C(5) – C(6) – H(61)	110(1)	H(11) – N(1) – H(13)	109(2)
C(5) – C(6) – H(62)	109(1)	H(11) – N(1) – H(12)	110(2)
C(7) – C(6) – H(61)	108(1)	H(13) – N(1) – H(12)	111(2)
C(7) – C(6) – H(62)	111(1)	H(W1) – O(N) – H(W2)	102(2)

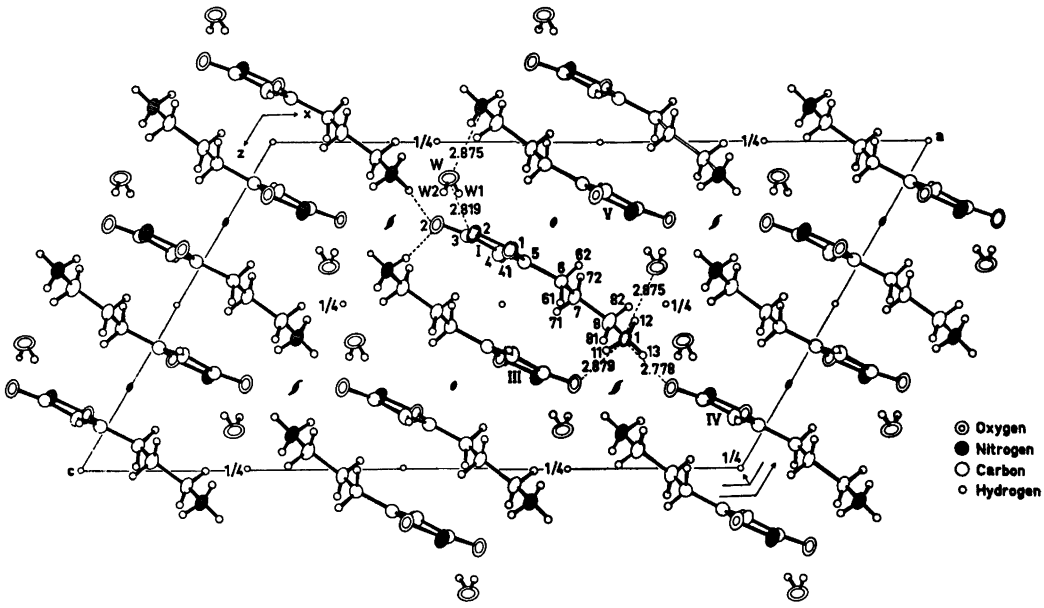


Fig. 3. Projection of the structure down the *b* axis. Some of the shorter *inter*molecular contacts are indicated.

calculated *intra*molecular distances and angles. The bond lengths and angles appear consistent with the general pattern of 3-hydroxy-5-(ω-aminoalkyl)isoxazole zwitterions.[2,3] The *intra*molecular distance $N(1)^+ \cdots O(2)^-$ is 7.925 Å.

The anisotropic thermal parameters of the non-hydrogen atoms are given in Table 6 and are drawn schematically in Fig. 2a.

The packing of the molecules in the crystal structure is determined by a system of hydrogen bonds. All hydrogen atoms that are covalently bonded to nitrogen or oxygen atoms are utilized

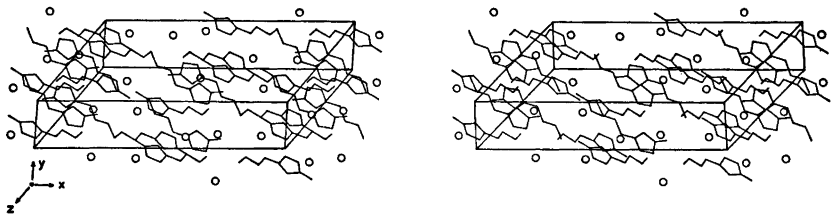


Fig. 4. Stereo diagram illustrating the molecular packing.

in the formation of hydrogen bonds. The zwitterions are inclined at about 13° to the ($\bar{1}01$) planes and, since they are approximately planar, form layers as shown in Figs. 3 and 4. Those zwitterions related by the *n*-glide plane are bound head to tail by the hydrogen bond $N(1)-H(13)\cdots O(2)_{IV}$, thereby leading to infinite chains in the [101] direction. These chains are interlinked on one side by double hydrogen bonds [$N(1)-H(11)\cdots O(2)_{III}$; $O(2)\cdots H(11)_{III}-N(1)_{III}$], formed between pairs of molecules related by a centre of symmetry. The spaces between these pairs of layers are filled with water molecules, each of which is linked by hydrogen bonds in directions almost parallel to the *b* [$O(W)-H(W1)\cdots N(2)_I$; $O(W)-H(W2)\cdots O(2)_{II}$] and *c* [$O(W)\cdots H(12)_V-N(1)_V$] axes to three different 3-hydroxy-5-(3-aminopropyl)-isoxazole molecules. In addition the $N(1)\cdots O(W)_{IV}$ distance is short, presumably because of the strong attractive interactions between the molecules. All other *inter*molecular contacts are greater than 3.2 Å. A summary of the geometry of the close contacts is shown in Table 5.

EXPERIMENTAL

The determination of melting points, the recording of IR, UV, and ^1H NMR spectra, and the performance of microanalyses were accomplished as described in a previous paper.[9]

The pH values were measured on a Radiometer pH meter 26, and the pK_A values were determined according to the method of Albert and Serjeant [10] as described previously.[3]

The computations were performed on a GIER computer and an IBM 360/75 computer using *INDIFF*,[11] a local version of *The N. R. C. 2A Picker Data Reduction Program*,[12] *The X-Ray System*,[13] and *ORTEP*.[14] The X-ray atomic scattering factors used were those of Cromer and Mann [15] for oxygen, nitrogen, and carbon, and of Stewart, Davidson, and Simpson [16] for hydrogen.

Synthesis

3-(3-Methoxyisoxazol-5-yl)propionamide (II). A mixture of 3.20 g (17 mmol) of methyl 3-(3-methoxyisoxazol-5-yl)propionate (I) [17] and aqueous ammonia (170 ml; ϱ 0.88) was stirred until a clear solution was obtained. This was allowed to stand at 25 °C for 18 h. Evaporation *in vacuo* afforded 3.0 g of crystalline product, which was recrystallized (acetone) to give 2.21 g (75 %) of (II) as colourless crystals, m.p. 142.0–143.0 °C. (Found: C 49.60; H 6.03; N 16.42. Calc. for $C_7H_{10}N_2O_3$: C 49.40; H 5.92; N 16.46). ^1H NMR data [$CDCl_3$-DMSO-d_6 (1:1)]: δ 7.5–7.1 and 6.8–6.5 (two broad signals, total 2 H, $CONH_2$); 5.75 (s, 1 H, $C=CH-C$); 3.84 (s, 3 H, $O-CH_3$); 3.1–2.7 (m, 2 H, $CH_2-CH_2-C=$); 2.6–2.2 (m, 2 H, $CO-CH_2-CH_2$).

3-(3-Methoxyisoxazol-5-yl)propylammonium chloride (III). To 100 ml of ice-cooled tetra-

Table 5. Close *inter*molecular contacts involving hydrogen and non-hydrogen atoms.

C–A–H	B	B equipoint	A–H Å	A···B Å	H···B Å	∠AHB deg.	∠CAB deg.
$O(W)-H(W1)\cdots N(2)_I$		(x,y,z)	0.89(2)	2.819(2)	1.94(2)	173(3)	
$O(W)-H(W2)\cdots O(2)_{II}$		$(x,y+1,z)$	0.88(3)	2.737(2)	1.86(3)	174(3)	
$C(8)-N(1)-H(11)\cdots O(2)_{III}$		$(1-x,-y,1-z)$	0.95(3)	2.879(3)	1.95(3)	168(1)	115.5(1)
$C(8)-N(1)-H(12)\cdots O(W)_V$		$(1-x,y-1,\frac{1}{2}-z)$	0.87(3)	2.875(3)	2.07(3)	153(2)	91.6(1)
$C(8)-N(1)-H(13)\cdots O(2)_{IV}$		$(\frac{1}{2}+x,\frac{1}{2}-y,\frac{1}{2}+z)$	0.91(2)	2.778(2)	1.90(2)	161(2)	94.0(1)
$C(8)-N(1)-H(12)\cdots O(W)_{IV}$		$(\frac{1}{2}+x,\frac{1}{2}-y,\frac{1}{2}+z)$	0.87(3)	3.073(3)	2.57(2)	118(1)	152.1(2)

hydrofuran containing diborane, externally generated[18] from 4.75 g (125 mmol) of sodium borohydride in diglyme (125 ml) and 25.5 g (180 mmol) of boron trifluoride etherate in diglyme (50 ml), was added during a period of 15 min a solution of 5.95 g (35 mmol) of (II) in tetrahydrofuran (125 ml). The mixture was refluxed for 19 h, and after cooling to 25 °C followed by careful addition of 6 M hydrochloric acid (50 ml) the solution was evaporated to dryness *in vacuo*. Upon addition of water (10 ml) and a 50 % aqueous solution of potassium hydroxide (40 ml) the mixture was extracted with two 50 ml portions of ether. The combined ether phases were dried and evaporated *in vacuo* to give 3.3 g of an oil. The oily product was dissolved in ethanol (13 ml) and upon addition of an ethanolic solution of hydrogen chloride, prepared from ethanol (100 ml) and acetyl chloride (12 ml), followed by addition of ether (18 ml) (III) was allowed to crystallize at 5 °C for 18 h to give 2.88 g (43 %) as colourless crystals, m.p. 129 – 131 °C (decomp.). (Found: C 43.40; H 6.80; N 14.65; Cl 18.43. Calc. for $C_7H_{13}ClN_2O_2$: C 43.65; H 6.80; N 14.54; Cl 18.41). ^{1}H NMR data [DMSO-d_6-$CDCl_3$ (3:2)]: δ 8.7 – 8.0 (broad, 3 H, NH_3^+); 5.93 (s, 1 H, C=C*H*–C); 3.87 (s, 3 H, O–CH_3); 3.1 – 2.5 (m, 4 H, NH_3^+–CH_2–CH_2–CH_2–C=); 2.3 – 1.7 (m, 2 H, CH_2–CH_2–CH_2).

3-(3-Hydroxyisoxazol-5-yl)propylammonium bromide (IV). 1.40 g (7.3 mmol) of (III) were dissolved in 11 ml of glacial acetic acid containing 43 % of hydrogen bromide. The solution was heated in an oil bath for 30 min, during which time the temperature rose to 90 °C. A further amount of 11 ml of the above mentioned reagent was carefully added, and the solution was refluxed (bath temperature: 90 – 100 °C) for 40 min. After cooling to 25 °C the reaction mixture was evaporated to dryness *in vacuo*. Recrystallization (methanol-ether) gave 1.10 g (68 %) of (IV) as colourless crystals, m.p. 193 °C (decomp.). (Found: C 32.30; H 5.02; N 12.60; Br 35.74. Calc. for $C_6H_{11}BrN_2O_2$: C 32.30; H 4.97; N 12.55; Br 35.82). ^{1}H NMR data (DMSO-d_6): δ 11.5 – 10.8 (broad, 1 H, O*H*); 8.5 – 7.4 (broad, 3 H, NH_3^+); 5.82 (s, 1 H, C=C*H*–C); 3.0 – 2.4 (m, 4 H, NH_3^+–CH_2–CH_2–CH_2–C=); 2.2 – 1.5 (m, 2 H, CH_2–CH_2–CH_2).

3-Hydroxy-5-(3-aminopropyl)isoxazole zwitterion (V). A solution of 900 mg (4.0 mmol) of (IV) in water (10 ml) was passed through a column containing an ion exchange resin [Amberlite IRA 400, (OH), 90 ml] using acetic acid (1 M) as an eluent. The fractions containing (V) were collected and evaporated *in vacuo* to give an oil. The oily residue was dissolved in water (15 ml), and upon addition of ethanol (35 ml) (V) was allowed to crystallize at 5 °C for 18 h to give 280 mg (43 %) as colourless crystals, m.p. 136 – 137 °C (decomp.). (Found: C 44.80; H 7.58; N 17.39. Calc. for $C_6H_{10}N_2O_2.H_2O$: C 44.99; H 7.55; N 17.49). [Found after drying of (V) over P_2O_5 (16 h; 75 °C; 0.05 mmHg): C 50.55; H 6.96; N 19.69. Calc. for $C_6H_{10}N_2O_2$: C 50.69; H 7.09; N 19.71]. ^{1}H NMR data [D_2O (sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard)]: δ 5.52 (s, 1 H, C=C*H*–C); 4.69 (s, 5 H, DO*H*); 3.2 – 2.8 and 2.8 – 2.4 (two t, total 4 H, NH_3^+–CH_2–CH_2–CH_2–C=); 2.2 – 1.5 (quintet, 2 H, CH_2–CH_2–CH_2).

X-Ray analysis

Colourless prismatic crystals of 3-hydroxy-5-(3-aminopropyl)isoxazole monohydrate were grown at room temperature by diffusion of absolute ethanol into an aqueous solution of the compound.

Crystal data. 3-Hydroxy-5-(3-aminopropyl)-isoxazole monohydrate, $C_6H_{10}N_2O_2.H_2O$, M = 160.18. Monoclinic, $a = 23.45(1)$, $b = 5.768(1)$, $c = 13.483(4)$ Å, $\beta = 120.33(3)°$, $U = 1574.0$ Å^3, $D_m = 1.35$ g cm^{-3}, $Z = 8$, $D_c = 1.352$ g cm^{-3}. Linear absorption coefficient for X-rays [λ (Mo$K\alpha$) = 0.7107 Å], $\mu = 1.17$ cm^{-1}. Number of electrons per unit cell, $F(000) = 688$. Space group $C2/c$ from systematically absent reflections: hkl when $h+k$ odd, $h0l$ when l odd, and from intensity statistics.

The unit-cell parameters were refined by least-squares techniques from the diffractometer-measured θ angles observed for 40 reflections well distributed in reciprocal space. The crystal density was measured by flotation in a chlorobenzene/bromobenzene mixture.

Data collection. Three-dimensional diffraction data were measured at room temperature on a Nonius three-circle automatic diffractometer using graphite monochromated Mo$K\alpha$ radiation. The ω scan technique with a scan speed of 1.2° min^{-1} was employed. Background counts were taken for half the scanning time at each of the scan range limits. One standard reflection was measured after every 25 reflections.

All the data were measured from a single crystal with approximate dimensions 0.25 × 0.32 × 0.30 mm. The crystal was mounted with [010] along the ϕ axis of the goniostat.

Out of the 1725 independent reflections measured in the range $2.5° \leq \theta \leq 27°$, 1231 had $I_{net} \geq 3.0\sigma(I)$, where $\sigma(I)$ is the standard deviation from counting statistics. These were regarded as observed reflections, whereas the remaining reflections were regarded as unobserved and excluded from the refinement procedure. Lorentz and polarization corrections were applied, but no absorption corrections were made owing to the low absorption coefficient.

Structure determination. The observed structure amplitudes were brought to an absolute scale by Wilson statistics and normalized structure amplitudes, $|E(hkl)|$, were calculated.

The phase problem was solved by direct methods using the programs of the X-Ray 72 System.[18] Of the highest thirteen peaks in an E-map based on 92 $E(hkl)$'s with $|E(hkl)| \geq 1.5$, eleven corresponded to all the "heavy" (nitro-

gen, oxygen, and carbon) atoms in the asymmetric unit. Individual atomic parameters of this model were refined, first with isotropic and then anisotropic thermal parameters using the full-matrix least-squares method. On convergence the R value was 0.093. The quantity minimized was $\sum w(|F_o|-|F_c|)^2$, where weights were initially taken as unity.

After having omitted from the data set the strongest observation, *i.e.* $\bar{4}04$, which was considered to be severely affected by extinction, a three-dimensional difference synthesis was computed. The subsequent map showed maxima with peak heights of $0.4-0.7$ e Å^{-3} in positions expected for all the hydrogen atoms, and there were no extraneous peaks greater than the lowest hydrogen atom. Introduction of the hydrogen atoms in the refinement, with the isotropic temperature factors of the connected non-hydrogen atoms as constant parameters reduced the R value to 0.050 for 1231 independent observations. Among the strongest reflections twelve were considered to be affected by extinction, and were omitted from the data set.

The least-squares refinement was completed with the introduction of a weighting scheme of the form: $w=1/(1+[(|F_o|-b)/a]^2)$, where $a=17.50$ e and $b=18.75$ e. On the last cycle of least-squares refinement shifts of all parameters were less than one tenth of their estimated standard deviations and the final R value is 0.039 for 1218 independent observations. Tables 6 and 7 list the final positional and thermal parameters for the non-hydrogen and hydrogen atoms, respectively. From these parameters the terminal set of structure factors, listed with the observed data in Table 8, was computed. Comparison of the 92 signs determined by direct methods with the corresponding phases computed from the refined structure shows that all had been correctly assigned.

Table 6. Final positional and thermal ($\times 10^4$ Å^2) parameters for non-hydrogen atoms. The estimated standard deviations of positional and thermal parameters ($\times 10^4$) are given in parentheses. The temperature expression is of the form:
$\exp[-2\pi^2(h^2a^{*2}U_{11}+k^2b^{*2}U_{22}+l^2c^{*2}U_{33}+2hka^*b^*U_{12}+2hla^*c^*U_{13}+2klb^*c^*U_{23})]$

	x/a	y/b	z/c	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
O(1)	0.4590(1)	0.6042(2)	0.3329(1)	250(6)	298(7)	407(7)	35(5)	121(5)	12(6)
N(2)	0.3897(1)	0.5642(3)	0.2821(1)	209(7)	365(9)	372(8)	64(6)	100(6)	21(7)
C(3)	0.3829(1)	0.3391(3)	0.2902(1)	214(8)	353(9)	231(8)	50(7)	94(6)	14(7)
C(4)	0.4464(1)	0.2268(3)	0.3439(2)	225(8)	291(9)	324(9)	44(7)	120(7)	31(8)
C(5)	0.4902(1)	0.3975(3)	0.3687(1)	230(8)	326(9)	226(7)	60(7)	105(6)	16(7)
C(6)	0.5635(1)	0.4121(4)	0.4282(1)	223(8)	380(10)	258(8)	−1(8)	107(7)	6(8)
C(7)	0.5986(1)	0.1838(4)	0.4761(2)	208(8)	385(11)	325(9)	2(8)	108(7)	8(8)
C(8)	0.6718(1)	0.2276(3)	0.5516(2)	198(8)	365(10)	353(9)	7(8)	102(7)	−26(8)
N(1)	0.7108(1)	0.0137(3)	0.6030(1)	199(7)	399(9)	326(8)	1(6)	104(6)	−16(7)
O(2)	0.3250(1)	0.2480(2)	0.2547(1)	193(6)	434(8)	395(7)	22(6)	113(5)	21(6)
O(10)	0.3025(1)	0.8774(3)	0.1127(1)	447(8)	381(8)	324(7)	19(7)	90(6)	29(6)

Table 7. Final positional and thermal ($\times 10^3$ Å^2) parameters for hydrogen atoms. The estimated standard deviations ($\times 10^3$) of the coordinates are given in parentheses.

	x/a	y/b	z/c	U_{iso}
H(41)	0.456(1)	0.068(4)	0.359(2)	28
H(61)	0.580(1)	0.525(4)	0.493(2)	29
H(62)	0.576(1)	0.479(4)	0.379(2)	29
H(71)	0.583(1)	0.120(3)	0.522(2)	31
H(72)	0.588(1)	0.078(4)	0.414(2)	31
H(81)	0.679(1)	0.327(4)	0.610(2)	31
H(82)	0.689(1)	0.300(4)	0.507(2)	31
H(11)	0.693(1)	−0.072(4)	0.641(2)	31
H(12)	0.711(1)	0.071(4)	0.550(2)	31
H(13)	0.752(1)	−0.060(4)	0.656(2)	31
H(W1)	0.331(1)	0.776(4)	0.162(2)	38
H(W2)	0.307(1)	0.996(4)	0.158(2)	38

Table 8. Observed structure amplitudes $|F_o|$ and calculated structure factors F_c (in electrons × 10). Within each group of reflections with constant k and l, the column list, from left to right, h, $10|F_o|$, and $10F_c$. Reflections considered unobserved or affected by extinction are marked with one or two asterisks, respectively.

	H,0,0		-26	67	68	-11	465	457	-9	570	583		H,1,14				H,2,4					
						-13	796	774	11	186	-188											
4	366	-342		H,0,10		15	335	339	-11	83	79	1	155	146	0	660	-667					
6	221	211				-15	45	27	13	79	-97	-1	124	120	2	454	462					
8**	1128	-1194	0	82	-81	-19	285	-289	15	129	129	-7	108	-106	-2	364	353					
10	655	648	2	228	-232	23	64	-55	-15	289	291	-9	159	158	-4	64	-77					
12	350	-337	-2	139	-128	-23	67	64	17	81	-77	-17	57	-51	6	352	-345					
14	551	-541	4	99	-98	-25	117	-120	-17	309	-309	-21	200	-199	-6	294	-278					
16	192	195	-4	85	-86	-27	129	128	-23	398	-401	-23	122	-122	8	334	349					
18	476	494	-6	481	-481				-25	90	-91	-25	78	-82	10	319	-324					
20	88	96	8	168	169		H,1,3		-29	79	-88				-10	105	-90					
22	56	-53	-8	259	252								H,1,15		12	57	-64					
24	95	95	10	188	191	1	663	-703		H,1,8					-12	258	-252					
			-10	520	527	-1	623	677				-1	108	95	14	109	115					
			12	170	-173	3	726	762	1	98	-92	-3	72	-75	-16	235	216					
	H,0,2		-12	385	-378	-3**	1321	1709	-1	170	-180	-5	76	-78	18	70	60					
			-14	289	302	5	514	-534	3	210	198	-11	155	-160	-18	179	-177					
			-18	226	227	-5**	940	-1053	-3	101	90	-13	136	-141	20	80	79					
0	499	-476	-20	493	493	7	131	-139	5	205	-204	-17	54	62	-20	127	128					
2	733	-789	-22	263	-263	-7	37	-40	-5	295	279	-19	101	106	-24	74	-72					
-2	427	454	-26	121	119	9	530	537	7	60	54				-26	201	199					
4	87	-83	-28	76	72	-9	69	56	-7	699	716		H,1,16									
-4	706	712				11	268	-264	-9	161	161						H,2,5					
6	739	-735		H,0,12		-11	404	-392	11	131	137	-7	93	-107								
-6	105	119				13	85	87	-11	148	148	-11	91	108	0	64	62					
8	737	-727	0	56	-61	-13	380	387	13	164	178	-13	181	181	2	451	455					
-8	221	202	2	223	213	15	188	190	-13	113	103	-15	153	152	4	166	-160					
10	376	-366	-2	117	-121	-15	78	66	15	154	-154	-19	82	90	-4	259	265					
-10	919	967	4	82	76	17	146	-141	-15	176	172				6	99	-85					
12	181	184	-4	135	125	-17	170	-170	19	195	-193		H,1,17		-6	141	-130					
-12	457	439	6	90	-96	-19	340	359	-21	49	-41				8	437	-449					
14	411	403	-6	183	-185	-21	337	347	-23	100	94	-15	103	-110	-8	112	-103					
-14	583	581	8	387	-396	-25	105	86	-25	144	155				10	470	-482					
16	107	103	-10	363	-367				-27	94	-91		H,2,0		-10	439	-427					
-16	570	-585	-12	316	-307		H,1,4								12	107	-113					
18	100	-91	-14	51	-62					H,1,9		0	213	-182	-12	396	-394					
-18	382	-372	-16	143	143	1	387	409				2	160	146	14	162	-166					
20	68	-68	-24	67	-68	-1	517	-537	1	356	358	4	537	535	-14	115	106					
-20	326	325	-26	131	-172	3	311	-317	-1	64	48	6	669	-599	16	71	59					
22	128	128				-3**	1269	-1533	-5	239	237	8	369	360	-16	90	-87					
-22	142	-144		H,0,14		5	362	371	7	164	164	10	281	280	18	82	70					
-24	120	115				-5	176	-159	-7	254	-239	12	477	-460	-20	164	-161					
			2	179	184	7	265	-264	11	147	150	14	457	457	-24	166	164					
	H,0,4		-2	204	200	-7	621	-662	-11	161	151	16	93	-103	-26	80	80					
0	476	-516	-4	80	-63	9	286	282	13	63	65	18	148	-152	-28	109	93					
2	672	688	8	219	217	-9	284	295	-13	101	-112	20	139	147								
-2**	1063	-1232	-10	67	-64	11	94	99	-15	84	-88	22	209	-196			H,2,6					
4	62	-49	-12	125	-123	-11	311	-302	-21	252	247											
-4**	1761	-3298	-14	180	-174	13	330	-334	-23	263	265		H,2,1		0	78	-69					
6	77	-73	-16	149	164	-13	160	-155	-25	184	-183				2	89	-99					
-6	273	-278	-20	102	-94	15	358	346	-27	93	-89	0	117	111	-2	215	-221					
8	303	299	-22	164	-174	17	137	-149				2	573	-583	4	325	-326					
-8	588	622	-24	130	-119	-17	89	75		H,1,10		-2	203	-206	-4	343	330					
10	230	235	-26	103	110	19	181	175				4	162	151	6	455	467					
-10	255	-255		H,0,16		-21	230	-230	1	186	-182	6	392	-381	-6	250	250					
12	424	425				-23	54	24	-1	119	111	-6	961	947	8	62	66					
-12	561	581	-6	66	69	-25	85	77	3	129	-124	8	50	-26	-8	222	-227					
14	674	-715	8	86	-83	-27	62	40	-3	122	115	-8	259	260	10	67	-88					
-14	259	-256	-10	84	93		H,1,5		-5	171	-169	10	424	403	-10	44	-41					
16	246	-249	-12	276	275				7	186	184	-10	288	271	12	164	159					
-16	293	278	-14	50	46	-1	277	281	9	53	49	12	292	290	-12	415	404					
18	87	102	-16	173	175	3	246	-249	-9	151	-153	14	600	619	14	141	-135					
-18	314	307	-20	96	-111	-3	443	-444	-11	189	191	-14	109	-96	-14	363	-367					
20	136	-139	-22	127	123	5	66	-79	-15	188	-188	16	211	218	16	85	52					
-20	410	-413				-5	150	158	-17	315	318	-16	238	234	-18	182	-188					
-22	199	-215		H,1,0		7	171	-163	-19	148	149	-20	118	-124	-20	357	-355					
						-7	65	-70	-21	272	272	-22	115	-117	-24	242	244					
	H,0,6		1**	984	1052	9	65	73	-25	62	-66	-24	125	-117	-28	107	115					
			3**	1101	1193	-9	256	-257	-29	81	-84		121	-127								
0	201	-195	5**	955	-1010	11	83	-87					H,2,2				H,2,7					
2	296	293	7	672	683	-11	483	485		H,1,11					0	465	-463					
-2**	1167	1353	9	127	-132	13	205	-213				0	155	138	-2	94	-82					
4	570	563	11	186	-181	-13	78	-69	-3	141	140	-2	292	-280	4	95	-82					
-4	282	-277	13	94	-71	15	151	-146	5	214	-217	4	415	396	-4	434	-438					
-6	784	-810	15	166	-162	-15	96	-89	-5	100	-97	-4	117	-116	6	64	-70					
8	213	219	17	401	403	-17	117	-115	7	84	93	6	138	137	8	60	52					
-8	223	206	19	40	3	19	75	79	-7	253	257	-6	291	282	-8	357	367					
10	179	-181	23	96	-92	-19	399	-402	9	114	110	-8	763	-716	10	131	-132					
-10	580	-588	25	66	65	-21	55	63	-9	351	347	10	473	-473	-10	79	-81					
12	323	-320				-23	135	141	-13	80	-81	-10	284	283	12	56	65					
-12	133	126		H,1,1		-27	85	89	-15	187	-186	12	107	-105	-12	274	284					
14	71	59							-17	75	-61	-12	195	183	14	114	110					
-14	470	-483	1	195	186		H,1,6		-23	68	76	14	61	46	-14	171	-170					
16	97	98	-1**	768	894				-25	137	137	-14	215	212	16	101	94					
-16	568	-563	3	546	-559	1	64	-62	-27	148	147	-16	335	338	-16	79	-71					
18	139	-141	-3	412	437	-1	232	235				18	174	178	-20	401	-414					
-20	345	350	-5	221	-224	3	210	-206		H,1,12		-18	193	-191	-22	279	-273					
-22	53	41	7	238	249	-3	253	265				20	123	-122	-24	111	-116					
-24	282	-274	-7	40	56	5	429	430	-3	81	67	-20	98	-106	-26	74	70					
-26	125	130	9	62	-62	-5	448	448	-5	103	106	-26	69	-65	-28	63	70					
-28	85	-82	-9	378	-346	7	266	273	7	148	-149											
			11	347	344	-7	691	-707	-7	102	-94		H,2,3				H,2,8					
	H,0,8		-13	453	445	11	215	-217	-11	267	-271	0	635	634	-2	101	-86					
			15	53	55	-11	97	79	-15	120	-113	2	444	432	-4	172	169					
0	268	280	17	496	519	13	58	-61	-17	48	-51	-2	527	-551	6	149	142					
2	98	-103	-17	162	164	-13	218	-203	-19	108	-115	4	411	410	-6	202	-198					
-2	447	-455	-21	195	-183	15	57	59	-21	56	55	6	450	449	8	65	53					
-4	443	460	-23	60	54	-15	227	-220	-23	116	120	-6	291	-306	-8	223	229					
6	222	-225	-25	87	-93	-17	619	-618	-25	64	-67	8	219	-221	10	77	79					
-6**	1068	1152				-19	57	60				-8	375	-366	12	91	-83					
8	362	-367		H,1,2		-23	68	68		H,1,13		16	90	88	-14	311	314					
-8	590	576				-25	80	73				-10	261	271	-18	74	60					
10	434	453	1	614	-620	-27	60	-60	3	103	-94	12	82	81	-20	140	-143					
-10	288	297	-1	538	-592	-29	92	99	-3	182	-178	-12	129	-144	-22	53	-62					
12	243	249	3	48	-33				-7	53	-60	-14	313	295	-24	60	61					
-12	194	-194	-3**	718	804		H,1,7		-9	154	-143	-16	414	414								
14	107	109	5	398	390				-11	286	281	18	211	-208			H,2,9					
-14	109	-117	-5	75	66	-1	130	-140	-15	150	-148	-18	367	376								
-16	101	103	7	214	-209	3	53	62	-17	152	150	20	66	-94	0	89	-93					
-18	82	-96	-7	149	151	-5	85	-83	-19	62	-52	-20	280	285	2	48	49					
-20	171	-178	9	617	-609	-5	515	-498	-21	59	67	-22	98	-96	-2	48	-43					
-22	166	173	-9	338	-329	-7	649	-665	-25	279	-279	-24	61	-58	4	422	427					
-24	140	133	11	338	-331	9	202	204														

Table 8. Continued.

	H,2,9		5	500	-489	5	159	166	-19	82	-79		H,4,6		3	178	-185
			-5	407	375	-5	298	305							7	95	87
-4	191	-199	7	269	250	7	125	-130	10	H,4,0		0	151	-155	9	70	62
6	217	209	-7	180	145	-7	143	145				2	125	125	11	42	51
-6	127	123	9	77	66	9	116	-113	0	77	-91	4	93	-98	15	90	83
8	183	178	-9	373	361	-9	67	80	2	389	-392	6	86	-96	17	81	-73
-8	79	-70	11	234	237	11	246	252	4	151	-145	-6	279	-280			
10	114	102	-11	89	-98	-11	152	159	6	122	132	8	168	-172		H,5,1	
-10	86	-80	13	622	643	-13	85	-89	8	212	-208	-8	241	243			
-12	313	315	-13	1336	1336	-15	92	-91	10	115	-114	10	59	62	1	126	-117
-14	86	82	15	285	281	-19	246	-250	12	192	187	-10	151	-148	-1	130	136
-16	98	87	-15	117	115	-21	384	-378	16	52	-50	14	60	-55	3	99	98
-18	59	-57	-17	156	-164				18	137	147	-14	403	407	-3	66	-63
-22	111	114	-19	103	-101		H,3,8		20	143	-135	-16	102	104	5	295	298
			21	77	-64							-18	146	146	-5	174	169
	H,2,10		-23	89	-85	1	81	-89		H,4,1		-20	123	129	7	156	-152
						-1	218	213				-22	89	-86	9	223	-221
0	287	282		H,3,2		3	72	83	0	168	-154		H,4,7		-9	359	-350
2	214	-209				-3	187	-187	2	279	-262				-11	56	66
4	98	-98	1	673	688	-5	168	-165	-2	235	220				13	62	61
-4	118	109	-1	195	-200	7	96	-94	4	119	117	0	59	-56			
6	98	94	3	215	-202	9	57	66	-4	409	397	2	210	215		H,5,2	
-6	119	116	-3	60	65	-9	76	-69	6	100	99	-2	173	-171			
8	98	90	5	99	-106	-11	75	89	8	107	-112	-4	196	-192	1	196	200
-8	417	-411	-5	342	-336	-13	354	-358	-8	205	-217	6	158	-166	-1	194	-188
-12	84	89	7	123	114	-17	56	50	-10	117	-124	-6	70	-79	3	72	73
-14	78	-75	-7	48	-59	19	93	90	12	81	85	-8	76	68	-3	120	115
-18	157	161	-9	234	234	-21	101	99	-12	227	224	10	45	48	5	183	184
-22	175	178	11	409	408	-23	187	-179	14	206	205	-14	76	83	-5	83	-80
-24	125	131	-11	301	-281	-25	110	106	-14	240	241	-16	75	77	7	214	229
-26	262	-262	13	146	-140	-27	77	60	16	150	155	-18	180	-185	-7	240	-235
			-15	243	-238				-16	88	-98	-20	151	-152	-9	63	-54
	H,2,11		-19	82	74		H,3,9		-18	76	-88	-22	57	-43	11	87	93
			-23	111	99				-20	65	50	-24	77	-61	-11	244	-249
0	135	133				1	70	-63	-22	67	74				13	81	-84
2	62	-65		H,3,3		-1	68	-76					H,4,8		-19	57	31
-4	175	164				3	178	177		H,4,2							
-6	250	241	1	654	667	5	292	301								H,5,3	
8	82	-74	-1	228	231	-5	244	-237	2	104	-91	6	68	71			
-8	122	114	3	235	206	7	255	260	-2	264	263	-6	329	-343	1	143	143
-10	81	83	-3	527	-537	-7	68	-56	4	329	337	8	66	-76	3	89	91
-12	213	-211	5	161	160	9	189	187	-4	322	-318	-8	73	-66	-3	55	43
-16	72	64	-5	262	-245	-9	206	206	6	278	-271	10	55	-48	5	231	-233
-20	66	54	7	268	257	-13	280	263	-6	196	182	-10	210	-206	-5	102	-114
-24	235	231	9	92	-93	-15	104	95	8	348	356	-12	99	-106	7	234	-232
-26	224	229	-9	416	-410	-17	189	-183	-8	76	-68	-16	167	-176	-7	179	174
			11	145	144	-19	186	191	10	172	185	-18	153	158	9	113	110
	H,2,12		-11	349	348	-23	91	-93	-10	164	-164	-22	72	67	-9	222	223
			13	65	51	-25	89	94	-12	240	-244				11	55	65
0	52	-36	-13	107	95	-27	76	-70	14	131	138		H,4,9		-11	297	-290
2	145	-130	15	313	-321				-14	201	-208				13	55	-62
-2	129	132	-15	117	119		H,3,10		16	117	-114	0	119	118	-13	155	-155
6	127	-130	-17	727	732				18	61	46	-2	122	114	15	116	108
-6	99	92	-19	70	68	1	67	73	-18	64	-59	4	100	-98	-15	115	98
-10	98	-90	-21	63	-66	-1	172	-173	-20	113	116	6	127	128	-19	72	56
-12	81	-82	-23	60	65	3	121	128				-6	218	-222			
-16	96	-100	-25	170	-158	-7	64	71		H,4,3		8	164	166		H,5,4	
-18	119	-127				9	93	-88				-10	229	230			
-20	63	63		H,3,4		-9	73	69	0	345	346	-12	114	111	1	130	132
-22	81	-84				-17	98	-100	2	161	157	-14	159	-160	-1	94	103
-24	174	172	1	212	217	-19	108	-107	-2	109	115	-16	97	-102	3	65	-62
			-1	88	73	-23	111	-115	4	86	-105	-20	132	131	-3	451	464
	H,2,13		3	75	-62				-4	155	-145	-22	116	108	5	91	-102
			-3	179	173		H,3,11		6	209	-216	-24	81	-73	-5	372	372
0	181	-182	5	81	69				-6	133	-135				-7	77	68
2	192	-190	-5	74	-77	3	101	100	8	116	123		H,4,10		9	57	-57
-2	94	-86	7	59	-72	-3	106	106	-8	77	74				11	81	-77
4	150	-146	-9	409	412	-5	147	147	10	229	238	0	130	-126	-11	190	-188
-4	154	163	11	97	85	-7	265	262	12	66	-54	-2	82	81	13	91	87
-12	79	-77	-13	161	156	-9	105	-96	-12	157	-158	4	190	196			
-14	54	55	-15	278	-272	-11	79	-83	14	94	-94	6	122	-115		H,5,5	
-16	157	-149	17	103	-95	-15	54	-41	-14	128	128	-8	131	139			
-18	69	-55	-17	78	89	-17	106	103	-16	146	154	-14	130	127	1	56	-60
-20	85	84	-19	196	198	-21	64	71	18	107	109	-16	161	-175	-1	141	-149
			-21	117	-117	-23	203	207	-18	138	129	-18	228	-237	3	98	-106
	H,2,14		-25	116	-112	-25	139	138	-20	109	105	-20	87	-79	-3	100	94
												-22	124	-130	5	160	149
-2	68	74		H,3,5			H,3,12			H,4,4					7	148	163
-4	115	-112											H,4,11		-7	149	-140
-8	81	-77	-1	325	333	1	165	159	0	287	286				9	124	-126
-12	226	229	3	123	134	-1	129	-119	2	249	-271	-2	112	-115	-9	57	65
-14	54	-53	-3	243	236	-5	81	-79	-2	231	234	-4	117	115	-11	118	118
-24	109	-114	7	361	-370	-7	58	65	4	105	109	-6	139	133	-13	143	139
			-7	219	-212	-9	205	204	-4	54	41	-8	76	70	-15	89	-91
	H,2,15		9	315	-325	-11	120	-123	6	162	161	-10	58	67			
			-9	356	-334	-17	182	185	-6	536	557	-16	102	-94		H,5,6	
-10	201	-205	11	418	-423	-21	116	-119	8	126	-122	-18	76	-74			
-12	102	-109	-11	206	-191				-8	129	-126	-22	161	167	1	217	-215
-16	64	56	13	143	-141		H,3,13		-10	136	127				3	98	-122
-20	69	-95	15	108	119				12	58	64		H,4,12		-3	91	-89
			-15	51	-62	1	260	-258	-12	268	272				5	84	-79
	H,2,16		-17	74	-80	-3	92	89	14	73	-75	0	104	95	-5	111	101
			-21	83	76	-5	93	-76	-14	202	-204	-2	53	-40	11	40	-68
-10	113	-113				-7	130	123	16	116	108	-4	106	-102	-11	184	189
-12	66	44		H,3,6		-9	52	60	-18	79	-80	-6	99	104	-15	166	177
-14	137	132				-11	93	-94				-8	57	-64	-17	103	106
			1	102	-112	-13	63	-56		H,4,5		-10	245	250			
	H,3,0		-1	171	169	-15	101	-107				-12	103	102		H,5,7	
			3	197	201	-17	92	-104	0	69	-55	-16	235	237			
3	146	-132	-3	312	-313	-19	52	51	2	161	-153				1	69	71
5	254	-249	5	111	-118	-21	75	81	-2	299	295		H,4,13		-1	175	-176
7	86	-98	-5	64	-69	-23	73	-78	-4	67	65				3	312	315
9	170	-163	7	303	-310				6	55	47	-4	120	-114	5	57	-49
11	280	279	-7	156	158		H,3,14		-6	281	-276	-6	58	-45	-5	85	-82
13	41	41	9	136	142				-8	271	-276	-10	100	106	7	79	-77
15	94	-90	13	92	93	-5	65	56	10	205	-202	-14	120	-131	9	62	45
21	157	160	-15	175	164	-19	94	94	-10	147	150	-18	111	100	-13	240	-248
23	64	-56	-19	131	131	-23	57	58	12	178	-178				-15	223	222
			-27	107	-101				-12	184	189		H,4,14				
	H,3,1						H,3,15		-16	214	-214					H,5,8	
				H,3,7					-18	182	-186	-12	64	-58			
1	307	-294				-7	113	-106	-20	105	114				-1	79	91
-1	584	-590	-1	209	-199	-9	111	-104	-24	57	-59		H,5,0		3	50	41
3	53	-58	3	362	-355	-11	57	-54							-3	164	-168
-3	759	740	-3	634	-660	-15	84	65				1	421	-421	5	81	66

Table 8. Continued.

	H,5,8		-2	82	-81	21	30*	26		H,1,17			H,2,14			H,3,10		
			4	169	-180	-21	29*	14										
-5	104	-110	-10	92	103				-13	18*	-34	0	41*	32	-3	34*	46	
-7	276	-277	-12	98	95		H,1,3		-17	10*	12	-6	9*	-19	5	29*	7	
-9	347	-361	-14	112	-98							-10	9*	15	-6	13*	27	
-11	80	71	-16	98	93	19	9*	0		H,2,0		-16	37*	20	7	10*	-10	
-15	111	107				21	41*	10				-18	14*	-7	-11	20*	-37	
-19	88	-102		H,6,7		-23	46*	-50	24	8*	0	-20	34*	-41	-13	6*	-13	
						-27	11*	13				-22	52*	-43	-15	25*	-18	
	H,5,9		0	73	68					H,2,1					-21	35*	38	
			4	65	40		H,1,4						H,2,15		-25	37*	-31	
-1	122	123	-6	166	177				-4	29*	11				-27	10*	45	
3	107	-111	-12	72	-68	15	9*	-20	-12	8*	-3	-4	10*	-9				
-5	76	68	-16	65	62	-19	33*	-34	18	9*	5	-8	53*	-53		H,3,11		
-7	55	-62				21	39*	-46	-18	43*	-52	-8	51*	-43				
-11	155	166		H,6,8					20	33*	-16	-14	40*	39	1	9*	-1	
-13	171	-174					H,1,5					-16	9*	2	-1	31*	56	
-15	79	-75	-2	107	-105					H,2,2		-22	10*	-24	5	33*	15	
-17	73	61	-4	76	-70	1	25*	-14							7	10*	-27	
			-6	183	-186	17	42*	-33	2	24*	-49		H,2,16		-13	9*	-11	
	H,5,10		-8	91	-82	-25	13*	-9	8	40*	-27				-19	27*	20	
			-10	99	-94				16	36*	-24	-8	51*	57				
1	108	107	-14	93	92		H,1,6		22	52*	52	-16	43*	-15		H,3,12		
-1	86	83							-22	28*	-32	-16	28*	42				
-3	81	81		H,6,9		9	37*	28	-24	31*	14	-20	28*	41	3	47*	16	
-9	64	-61				17	26*	39							-3	22*	19	
-15	81	-74	-6	84	-73	-21	35*	44		H,2,3			H,3,0		-13	42*	38	
-19	133	-143	-8	65	71				-4	6*	13	1	6*	20	-15	9*	32	
			-14	59	43		H,1,7		14	36*	13	17	33*	24	-19	9*	49	
	H,5,11								16	45*	-48	15	11*	-5	-23	38*	-21	
				H,7,0		1	8*	-26	-26	49*	-1				-25	24*	-41	
-1	141	-135				-3	7*	3					H,3,1			H,3,13		
-5	127	134	7	53	53	7	11*	-3										
-15	89	93				-13	26*	-14		H,2,4								
-17	89	-104		H,7,1		-19	38*	41				17	27*	12	-1	10*	-23	
-19	87	-120				-21	45*	3	4	39*	-44	15	44*	-48				
			-5	60	-48	-27	23*	-13	-8	7*	27	-21	34*	-20		H,3,14		
	H,5,12		-7	78	-56				-14	30*	-30							
							H,1,8		16	40*	-29		H,3,2		-3	10*	31	
-7	75	72		H,7,2					-22	9*	1				-7	42*	-35	
-9	148	149				9	47*	48				9	25*	-32	-9	18*	-11	
-11	109	116	3	78	-75	-17	28*	-31		H,2,5		-13	13*	1	-11	39*	-48	
-13	228	223	5	65	-37	-29	52*	-52				15	16*	-30	-13	28*	-1	
-15	58	-51	-7	100	107				-2	7*	-1	17	9*	13	-15	39*	-35	
							H,1,9		-18	14*	-18	-17	9*	11	-17	34*	18	
	H,6,0			H,7,3					-22	19*	-12	15	40*	-49	-21	41*	27	
						3	42*	-53				21	54*	58				
0	228	-233	3	175	-165	-3	8*	7		H,2,6		-21	30*	-31		H,3,15		
2	154	-151	-5	60	44	5	17*	14				-25	24*	-27				
4	71	-76	-7	59	-47	9	47*	-33	-16	25*	-7				-13	54*	-54	
6	114	-116				-9	19*	-46	-24	38*	30		H,3,3		-17	17*	1	
8	169	166		H,0,2		-17	25*	6	-26	19*	-17							
10	147	148				-19	8*	-2				-7	8*	17		H,4,0		
12	93	-86	-26	43*	-35	-29	37*	22		H,2,7		17	38*	32				
14	78	81										15	46*	-51	14	34*	-25	
				H,0,4			H,1,10		4	33*	-25							
	H,6,1								-6	31*	38		H,3,4			H,4,1		
			-24	9*	-2	-7	36*	28	-18	28*	-35							
0	100	-107	-26	22*	3	11	43*	-40				-7	8*	-1	-6	8*	-2	
2	69	80	-28	10*	4	-13	43*	31		H,2,8		9	39*	-42	10	26*	6	
-2	110	-109				-23	22*	23				-11	8*	-21	18	29*	-31	
4	59	-51		H,0,6		-27	8*	23	0	34*	-33	13	24*	-3	20	40*	37	
-4	129	136							2	41*	38	15	28*	-10				
6	80	82	6	41*	31		H,1,11		4	9*	-18	19	17*	-5		H,4,2		
-6	63	-49	-18	8*	5				-10	40*	20	-23	9*	-9				
8	61	-55				1	18*	-5	-12	8*	24				0	13*	-20	
10	121	-123		H,0,8		-1	8*	-11	14	10*	-8		H,3,5		12	34*	-18	
-10	71	-57				3	28*	14	-16	35*	-50				-16	9*	-43	
			4	28*	-24	-11	40*	41	-26	8*	10	1	43*	-35	-22	49*	-37	
	H,6,2		-28	10*	33	-19	34*	19	-28	10*	-11	8	31*	12				
						-21	25*	32				-5	38*	-46		H,4,3		
0	97	106		H,0,10		-29	51*	49		H,2,9		-13	8*	-21				
4	66	84										17	10*	8	-10	25*	-8	
-4	91	-92	6	48*	-46		H,1,12		12	10*	-23	-15	9*	41	16	35*	14	
8	89	86	-16	46*	-60				-20	32*	-27	-23	35*	18	-22	18*	-15	
-8	158	-165	-24	49*	-59	1	9*	-12	-24	9*	8	-25	55*	44				
10	65	51				-1	18*	-26	-26	12*	-2					H,4,4		
-10	64	43		H,0,12		3	37*	-39	-28	48*	-63		H,3,6					
12	65	66				5	23*	20							10	9*	30	
-12	61	-46	-6	9*	6	-13	39*	-33		H,2,10		-5	23*	-27	-16	47*	48	
-14	147	-136	-18	32*	-53	-27	28*	-42				11	20*	-32	-20	9*	-24	
			-20	26*	26				-2	8*	-33	-11	23*	35	-22	38*	-10	
	H,6,3		-22	48*	41		H,1,13		10	21*	-15	-13	28*	39	-24	24*	-24	
			-28	29*	4				-10	34*	23	15	54*	-53				
-2	83	-96				1	38*	-28	-16	42*	-46	-17	47*	45		H,4,5		
-4	124	-136		H,0,14		-1	39*	-27	-20	45*	40	-21	9*	4				
6	87	-80				5	10*	-23	-28	37*	30	-23	44*	-35	4	14*	-26	
-6	77	76	0	10*	12	-5	9*	12				-25	30*	-23	8	42*	32	
-8	62	59	-6	17*	28	-13	5*	-25		H,2,11					14	10*	5	
-14	92	-96	-18	23*	33	-23	9*	6					H,3,7		-14	9*	5	
						-27	23*	-35	-2	31*	7				-22	36*	48	
	H,6,4			H,0,16					4	36*	43	1	8*	-5				
							H,1,14		6	41*	39	13	19*	20		H,4,6		
2	152	146	-18	10*	28				-14	8*	-9	15	33*	32				
-2	226	225				-3	36*	-29	-18	38*	10	-17	46*	-47	-2	35*	-17	
4	73	-75		H,1,0		-5	19*	11	-22	49*	48	-23	28*	-26	-4	34*	-30	
-4	157	162				-11	5*	2	-28	10*	0	-25	19*	30	12	14*	-22	
6	192	-197	21	39*	-21	-13	18*	-11				-27	22*	-2	-12	40*	-14	
-6	106	104				-15	43*	52		H,2,12					-24	32*	-44	
-8	88	101		H,1,1		-19	44*	47					H,3,8					
-12	110	-114							4	10*	4					H,4,7		
-16	59	44	5	13*	-13		H,1,15		-4	47*	-58	5	45*	-57				
			-11	15*	-36				-8	43*	6	-7	13*	20	4	29*	-15	
	H,6,5		-15	16*	-3	-5	19*	-34	-14	9*	6	11	10*	-47	8	44*	28	
			19	9*	-12	-7	28*	11	-26	52*	44	13	46*	56	-10	19*	15	
2	85	-86	-19	9*	15	-15	37*	-18				-15	26*	-16	12	31*	31	
-2	59	47	21	29*	23	-21	25*	10		H,2,13					-12	30*	36	
4	123	129	23	46*	53	-23	5*	-19					H,3,9					
-6	118	120				-25	10*	-3	-6	48*	-30					H,4,8		
-8	137	-141		H,1,2					-8	43*	-13	-3	28*	16				
-12	76	71					H,1,16		-10	19*	10	11	37*	10	0	45*	41	
-16	68	78	13	21*	18				-22	28*	-12	-11	8*	8	2	26*	44	
			17	30*	-26	-9	9*	10	-24	10*	3	-21	45*	-52	-2	19*	-13	
	H,6,6		-17	20*	24	-17	42*	-44	-26	50*	-46				-4	24*	20	
			19	45*	-30	-21	35*	40										

Table 8. Continued.

H,4,8

-14	51*	-56	-8
-20	39*	-37	-10
-24	15*	42	-14
			-16

H,4,9

2	9*	-5	
-4	9*	-20	5
-8	25*	24	13
-18	44*	57	

H,4,10

			-7
2	35*	52	11
-4	15*	10	-13
-6	9*	-7	15
-10	9*	28	-15
-12	20*	-36	17
-24	10*	-19	-17
			-19

H,4,11

0	27*	-30	
2	24*	44	9
4	13*	2	-13
-12	9*	6	15
-14	9*	-8	-15
-20	34*	15	-17

H,4,12

-14	9*	1	-1
-18	32*	-28	-17
-20	10*	33	-21
-22	10*	-6	

H,4,13

			7
-2	29*	-40	-9
-8	42*	47	-13
-12	44*	-57	-15
-16	10*	-14	-17
-20	32*	30	-19
			-21

H,4,14

43*	-46	-5	
10*	-1	11	
24*	-24	13	
10*	5	-17	
		-19	
		-21	

H,5,0

30*	-30	
11*	-1	
		-1

H,5,1

			-7
43*	-49	9	
9*	-5	-9	
39*	53	-13	
18*	30	-19	
34*	17	-21	
50*	-45		
34*	31		
10*	25		
		-3	
		-7	
		-9	

H,5,2

29*	-12	-13	
9*	-39	-17	
10*	6	-19	
9*	-19	-21	
28*	32		

H,5,3

			1
9*	4	7	
9*	-9	-13	
54*	-41	-17	
		-21	

H,5,4

11*	-5		
38*	51	1	
9*	15	-3	
14*	11	5	
13*	-27	-9	
10*	9	-19	
10*	14	-21	

H,5,5

14*	-9	-5	
10*	-7	-7	
27*	41	-11	
36*	-51	-13	
32*	-2	-17	
10*	-26		

H,5,6

			-3
9*	1	-7	
14*	-42	-9	
9*	-6	-11	
45*	65	-13	
19*	-21		
41*	50		
26*	-17		
19*	-36	-5	

H,5,7

9*	-20	-8	
44*	50	12	
47*	69	-12	
51*	57	14	
9*	17	-14	
43*	-36		
55*	-57		

H,5,8

			2
			-2
9*	-17	6	
43*	26	-6	
35*	-20	-16	
24*	10		
10*	42		

H,5,9

			0
			2
27*	-28	4	
9*	-4	8	
17*	-8	10	
40*	-18	-10	
10*	-2	-12	
45*	60	-16	

H,5,10

32*	32	0	
35*	0	8	
8*	9	10	
38*	-7	-10	
15*	-46	-14	

H,5,11

43*	-20	0	
42*	52	-4	
33*	-34	6	
19*	-6	8	
16*	8	-10	
		-14	

H,5,12

10*	-11	
		0

H,6,1

			2
			-4
9*	21	6	
55*	55	-6	
21*	-42	-8	
10*	-11		
10*	0		

H,6,2

			2
			-2
37*	29	-4	
47*	-22	-8	
10*	39	-10	
25*	-31	-14	
32*	9		

H,6,3

			0
9*	-10	-12	
47*	29	-16	
9*	-5		
20*	16		
49*	-43		
33*	9	-2	
15*	-11	-4	
36*	21	-10	
		-12	

H,6,4

43*	45	1	
43*	35	3	
10*	-21	5	
22*	-27		
27*	-57		

H,6,5

		1	
		-1	
41*	32	3	
43*	-54	-3	
28*	46	5	
37*	24	7	
9*	-5		
38*	-38		

H,6,6

		1	
		-1	
40*	14	-3	
14*	-4	-5	
32*	-16	-9	
10*	9		
9*	1		
45*	40		

H,6,7

		1	
		-1	
43*	-3	-3	
39*	-32		
23*	30		
26*	30		
36*	-51	1	
28*	-33	-1	
		-3	
		-5	
		-7	

H,6,8

			0
20*	11		
52*	-31		
10*	-13		

H,6,9

			-3
			-5
			-7
10*	-17		
48*	16		
10*	-10		
36*	37		

H,7,0

		1	
		3	
		5	
8*	-2		
33*	8		
17*	-35		

H,7,1

		1	
		-1	
10*	-44	3	
46*	31	-3	
10*	25	5	
10*	-22	7	
46*	-39		
28*	17		

H,7,2

		1	
		-1	
29*	-40	-3	
10*	34	-5	
31*	14	-9	
15*	12		
10*	5		

H,7,3

		1	
		-1	
10*	-7	-3	
10*	-33	-5	
35*	-39	-9	
10*	-19		

H,7,4

		1	
		-1	
41*	-20	-3	
53*	26	-5	
34*	-42	-7	
40*	46		
32*	9		

H,7,5

		3	
		-3	
49*	55	-5	
31*	-38	-7	
10*	-10		

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Organic Hydroxylamine Derivatives. XII.* Structural Analogues of γ-Aminobutyric Acid (GABA) of the Isoxazole Enol-betaine Type. Synthesis of Some 3-Hydroxy-5-(1-aminoalkyl)isoxazole Zwitterions

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The syntheses of three 3-hydroxy-5-(1-aminoalkyl)isoxazole zwitterions (VIIIa–c), which are conformationally restricted analogues of γ-aminobutyric acid (GABA), are described. The syntheses are based on 3-methoxy-5-isoxazolecarboxylic acid (I), which is transformed into the final products (VIIIa–c) *via* the ketones and oximes (IVa–c) and (Va–c), respectively. The pK_A values of (VIIIa–c) have been determined.

Muscimol (3-hydroxy-5-aminomethylisoxazole zwitterion), which is a conformationally restricted analogue of the inhibitory synaptic transmitter γ-aminobutyric acid (GABA),[1,2] is a valuable model compound in the investigations of structure-activity correlations for GABA agonists.[3–6] The present paper describes the synthesis of three 3-hydroxy-5-(1-aminoalkyl)isoxazole zwitterions (VIIIa–c), which are structurally related to muscimol. The aim of this work is to make available some structurally closely related GABA analogues in which the geometry of the preferred conformations is systematically changed. The increasing size of the alkyl groups of the compounds through the series (VIIIa–c) may increase the energy barriers separating the accessible conformations of the respective molecules.

Biological investigations of the racemates (VIIIa–c) are in progress. When the results of these investigations are available, efforts will be made to obtain the optical isomers of pertinent compounds of the series (VIIIa–c) for an investigation of the stereospecificity of the GABA receptor(s).

The acid chloride (II) is a key compound in the syntheses of the products (VIIIa–c). An unpurified product of (II) has previously been prepared in an apparently rather low yield as an intermediate in a synthesis of muscimol.[7] Reaction of the carboxylic acid (I) with a dimethylformamide-thionyl chloride reagent, however, gave (II) in good yields and in a pure state. Attempts to synthesize the methyl ketone (IVa) by reaction of the acid chloride (II) with lithium dimethylcuprate according to the method of Posner and Whitten [8] gave (IVa) in rather low yields. Reaction of (II) with diethyl ethoxymagnesiummalonate gives compound (III), which is converted into (IVa). The ketones (IVb, c) are synthesized *via* the appropriately substituted bistetrahydropyranyl malonates, using a method analogous to that described by Bowman and Fordham.[9] The oximes (Va–c), prepared by a conventional method, are reduced by aluminium amalgam to give the corresponding primary amines (VIa–c) as described by Bowden *et al.* for an analogous reaction.[7] Cleavage of (VIa) with hydrogen bromide in glacial acetic acid gives the hydrobromide (VII), which is subsequently converted into the enol-betaine (VIIIa) using a strongly basic ion exchange resin. Likewise the amines (VIb, c) are transformed into the final products (VIIIb, c), respectively, without isolation of the intermediately formed hydrobromides.

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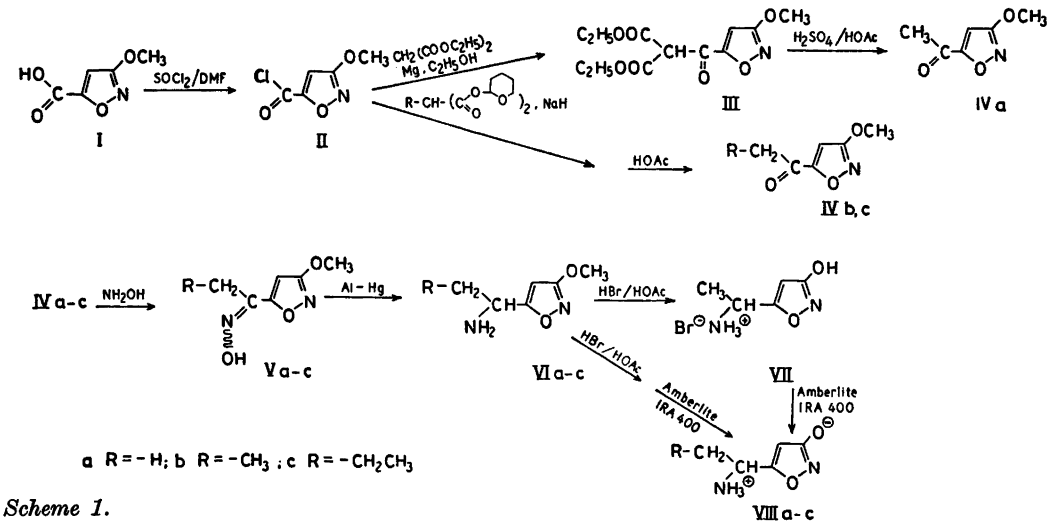

Scheme 1.

The structure determinations of (II)–(IVa–c), (VIa–c), (VII), and (VIIIa–c) are based on IR, UV, and ^{1}H NMR spectroscopy and supported by elemental analyses. The IR and ^{1}H NMR spectroscopic data originating in the 3-methoxy- and 3-hydroxyisoxazole moieties of (II)–(IVa–c), (VIa–c), and (VII), are in accordance with the general findings described by Jacquier *et al.*[10] The UV spectra of the compounds (II)–(IVa–c) are consistent with the presence of carbonyl groups in conjugation with the isoxazole nucleus. As revealed by IR, UV, and ^{1}H NMR spectroscopy compound (III) is largely in the enol form. The oximes (Va–c) are shown by ^{1}H NMR spectroscopy to consist of mixtures of the *Z*- and *E*-forms. Separation of the oximes (Va–c) in the isomeric forms and the determination of

Table 1. Some IR and UV data of the compounds (II)–(IVa–c), (VIa–c), (VII), and (VIIIa–c) and the pK_A values of (VIIIa–c).

	IR data (cm^{-1})	UV data [a] λ_{max}(nm)	$\varepsilon \times 10^{-3}$	pK_A [b]
II[c,e]	3145(m),1760(s),1605(s),1515(s)	242	9.5	
III [c]	3145(m),1760–1700(s, several bands),1605(m),1515(s)	245, 286	7.3, 6.8	
IVa [c]	3140(m),1700(s),1600(m),1510(s)	238	9.0	
IVb [d]	3125(m),1700(s),1600(s),1510(s)	237	8.8	
IVc [c]	3140(m),1700(s),1600(s),1510(s)	238	8.9	
VIa [c]	3450–3200(m),3145(m),1605(s),1520(s)	211	6.8	
VIb [c]	3450–3200(m),3150(m),1610(s),1515(s)	211	6.6	
VIc [c]	3600–3200(m),3145(w),1605(s),1520(s)	213	6.1	
VII [d]	3600–2400(s),1625(s),1510(s)	212	6.1	
VIIIa [d]	3145(m),3100–2000(s),2210(m), 1620(s),1520–1460(s, several bands)	211	6.0	4.69±0.04 8.45±0.01
VIIIb [d]	3200–2000(s),2200(m),1630(s), 1520–1440(s, several bands)	211	6.6	4.65±0.03 8.42±0.05
VIIIc [d]	3300–2000(s),2200(m),1640(s), 1540–1460(s, several bands)	212	6.6	4.69±0.03 8.49±0.04

[a] Unless otherwise stated the UV spectra were recorded in 99.9 % ethanol solutions. [b] The pK_A values were determined by titration in aqueous solutions at 17 °C. [c] The IR spectra were recorded using the film technique. [d] The IR spectra were recorded in the solid state (KBr). [e] The UV spectrum was recorded in cyclohexane solution.

their configuration have been performed and will be published in the near future. The spectroscopic properties of the zwitterions (VIIIa–c) are in accordance with those published for muscimol,[11] 3-hydroxy-5-(2-aminoethyl)-isoxazole zwitterion,[7,12] and 3-hydroxy-5-(3-aminopropyl)isoxazole zwitterion,[13] the structures of which have been established by X-ray diffraction analyses.[2,12,13] The pK_A values of muscimol (4.78 and 8.43)[11] and those determined for the enol-betaines (VIIIa–c) (*cf.* Table 1) are almost alike. This is an important fact since the compounds (VIIIa–c) together with muscimol are model compounds in the conformation-biological activity investigations.

Some spectroscopic data of the compounds (II–IVa–c), (VIa–c), (VII), and (VIIIa–c), which are all new, and the pK_A values of (VIIIa–c) are listed in Table 1.

EXPERIMENTAL

Unless otherwise stated the determination of melting points, the recording of IR, UV, and ^{1}H NMR spectra, and the performance of microanalyses were accomplished as described in a previous paper.[14] pH Values were measured on a Radiometer pH meter 26. Thin layer and column chromatographic procedures were accomplished using silica gel GF_{254} plates (Merck) and silica gel, 0.05–0.20 mm (Merck), respectively. The pK_A values were determined according to the method of Albert and Serjeant[15] as described in a previous paper.[12]

3-Methoxy-5-chloroformylisoxazole (II). To a mixture of 14.3 g (100 mmol) of 3-methoxy-5-isoxazolecarboxylic acid (I)[16] and thionyl chloride (160 ml) was added 5.1 g (70 mmol) of dimethylformamide. After reflux for 5 min the excess of thionyl chloride was removed *in vacuo* and the residue was extracted with six 30 ml portions of ether. The combined ether phases were concentrated *in vacuo* and the residue was distilled to give 11.1 g (69 %) of (II) as a pale yellow oil, b.p. 80–85 °C/14 mmHg. (Found: C 37.15; H 2.60; Cl 22.13; N 8.68. Calc. for $C_5H_4ClNO_3$: C 37.18; H 2.50; Cl 21.95; N 8.67). ^{1}H NMR data (CCl_4): δ 6.73 (s, 1 H, C=C*H*–C); 4.03 (s, 3 H, O–C*H*$_3$).

Ethyl 2-ethoxycarbonyl-3-oxo-3-(3-methoxyisoxazol-5-yl)propanoate (III). To a solution of 5.28 g (33 mmol) of diethyl malonate, 2.4 g (50 mmol) of ethanol and 150 μl of tetrachloromethane in ether (30 ml) was added 0.84 g (35 mmol) of magnesium. The mixture was refluxed for 2 h and filtered, and to the refluxing filtrate was added slowly and with stirring a solution of 4.83 g (30 mmol) of (II) in ether (20 ml). Reflux was continued for further 30 min, and after cooling to room temperature 60 ml of sulfuric acid (2 M) were added and the ether phase was isolated, dried, and concentrated *in vacuo* to give 7.8 g (91 %) of (III) as a yellow oil. An analytical sample was distilled at 0.01 mmHg to give (III) as a very viscous oil. (Found: C 50.32; H 5.32; N 5.19. Calc. for $C_{12}H_{15}NO_7$: C 50.52; H 5.30; N 4.91). ^{1}H NMR data (CCl_4): δ 12.7–12.3 (broad s, 0.4 H, enol-O*H*); 6.50 and 6.29 (two s, 0.4 H and 0.6 H, respectively, C=C*H*–C); 4.94 (s, 0.3 H, C*H*–C=O); 4.5–4.0 (m, 4 H, 2 × CH$_3$–C*H*$_2$–O); 3.96 and 3.93 (two s, total 3 H, C*H*$_3$–O); 1.5–1.1 (m, 6 H, 2 × C*H*$_3$–CH$_2$).

3-Methoxy-5-acetylisoxazole (IVa). A mixture of 7.8 g (27 mmol) of (III), glacial acetic acid (15 ml), concentrated sulfuric acid (2 ml), and water (10 ml) was refluxed for 3 h. After cooling to room temperature and after addition of water (60 ml) the solution was adjusted to pH 6 by addition of an aqueous solution of potassium hydroxide (10 M) and extracted with three 50 ml portions of ether. The combined ether extracts were dried, and evaporated *in vacuo*. Distillation of the residue afforded 3.0 g (78 %) of compound (IVa) as a colourless oil, b.p. 92–98 °C/15 mmHg. (Found: C 50.95; H 5.11; N 9.92. Calc. for $C_6H_7NO_3$: C 51.06; H 5.00; N 9.93). ^{1}H NMR data (CCl_4): δ 6.34 (s, 1 H, C=C*H*–C); 3.95 (s, 3 H, O–C*H*$_3$); 2.47 (s, 3 H, CO–C*H*$_3$).

3-Methoxy-5-(1-hydroxyiminoethyl)isoxazole (Va). A solution of 5.0 g (35 mmol) of (IVa), 5.3 g (39 mmol) of sodium acetate trihydrate, and 2.7 g (39 mmol) of hydroxylammonium chloride in aqueous ethanol (35 ml; 50 %) was refluxed for 30 min. Evaporation of the solution to *ca.* 15 ml followed by filtration afforded 5.0 g (90 %) of a colourless crystalline product. An analytical sample was recrystallized (water) to give (Va) as colourless crystals. The product was by thin layer chromatography shown to consist of two compounds with $R_F = 0.38$ and $R_F = 0.33$ (eluent: methylene chloride). (Found: C 45.95; H 5.19; N 18.02. Calc. for $C_6H_8N_2O_3$: C 46.15; H 5.16; N 17.94). ^{1}H NMR data of (Va) [$CDCl_3$-DMSO-d_6 (6:1)], which are consistent with a mixture (*ca.* 1:4) of the isomeric oximes: δ 11.37 (s, 1 H, N–O*H*); 6.73 and 6.04 (two s, 0.2 H and 0.8 H, respectively, C=C*H*–C); 2.91 (two coincident s, 3 H, O–C*H*$_3$); 2.10 (two coincident s, 3 H, C–C*H*$_3$).

3-Methoxy-5-(1-aminoethyl)isoxazole (VIa). To a solution of 5.0 g (32 mmol) of (Va) in aqueous methanol (150 ml; 50 %) aluminium amalgam was added. [The latter was prepared by treatment of 10.5 g (388 mmol) of aluminium strips with an aqueous mercuric chloride solution (300 ml; 5 %) for 30 s followed by washing with ethanol]. After stirring for 90 min at room temperature the mixture was filtered and concentrated *in vacuo* to give a colourless oil, which was distilled to give 3.1 g (67 %) of (VIa) as a colourless oil, b.p. 106–110 °C/16 mmHg. (Found: C 50.75; H 7.19; N 19.88. Calc. for $C_6H_{10}N_2O_2$: C 50.69; H 7.09; N 19.71).

^{1}H NMR data (CCl_4): δ 5.58 (s, 1 H, C=C*H*−C); 4.1−3.7 (partly overlapped q, $CH_3-CH-NH_2$), and 3.75 (s, CH_3-O) total 4 H; 1.52 (s, 2 H, NH_2); 1.35 [d(J=6 cps), 3 H, CH_3-CH].

3-Hydroxy-5-(1-aminoethyl)isoxazole hydrobromide (VII). A solution of 1.47 g (10.3 mmol) of (VIa) in glacial acetic acid (10 ml) containing 43 % of hydrogen bromide was heated to reflux for a total of 20 min. After reflux for 10 min an additional 10 ml of glacial acetic acid containing hydrogen bromide was added. After cooling to 25 °C the solution was evaporated to dryness *in vacuo*. The residue was dissolved in methanol (15 ml) and concentrated *in vacuo* to give an oil, which slowly crystallized. The product was recrystallized (ether−propan-2-ol) to give 1.57 g (72 %) of (VII) as slightly coloured crystals, m.p. 150 °C (decomp.). (Found: C 28.66; H 4.59; Br 38.15; N 13.47. Calc. for $C_5H_9BrN_2O_2$: C 28.73; H 4.34; Br 38.23; N 13.40). ^{1}H NMR data (DMSO-d_6): δ 9.0−8.2 (broadened s, 4 H, O*H* and NH_3^+); 6.17 (s, 1 H, C=C*H*−C); 4.54 [q (J=7 cps), 1 H, $CH_3-CH-NH_3^+$]; 1.51 [d (J=7 cps), 3 H, CH_3-CH].

3-Hydroxy-5-(1-aminoethyl)isoxazole zwitterion (VIIIa). A solution of 756 mg (36.0 mmol) of (VII) in water (10 ml) was passed through a column containing an ion exchange resin [Amberlite IRA 400, (OH), 110 ml] using acetic acid (1 M) as an eluent. Recrystallization of the crude product (water-ethanol) gave 290 mg (63 %) of (VIIIa) as colourless crystals, m.p. 213−215 °C (decomp.). (Found: C 46.70; H 6.26; N 21.67. Calc. for $C_5H_8N_2O_2$: C 46.87; H 6.29; N 21.87). ^{1}H NMR data [D_2O (sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard)]: δ 5.75 (s, 1 H, C=C*H*−C); 4.76 (s, 4 H, DO*H*); 4.48 [q (J=7 cps), 1 H, $CH_3-CH-NH_3^+$]; 1.59 [d (J=7 cps), 3 H, CH_3-CH].

3-Methoxy-5-propionylisoxazole (IVb). To a stirred solution of 18.9 g (225 mmol) of 3,4-dihydro-2*H*-pyrane and 75 µl for concentrated sulfuric acid in benzene (75 ml) 8.85 g (75 mmol) of methylmalonic acid was added in portions with cooling to < 30 °C. After stirring for 30 min potassium hydroxide (6.0 g) was added and stirring was continued for 30 min. The solution was decanted from inorganic material and concentrated to 30 ml *in vacuo* (bath temperature < 30 °C). This solution was dropwise added to a stirred suspension of sodium hydride (*ca.* 75 mmol, prepared from 3.6 g of a 50 % dispersion of sodium hydride in oil) in benzene (75 ml) with cooling to < 30 °C. To the clear solution 10.9 g (67.5 mmol) of compound (II) dissolved in benzene (40 ml) was slowly added. After stirring for 2 h acetic acid (7.5 ml) was added and the solution was refluxed for 45 min. Upon addition of ether (150 ml) the mixture was extracted with water (200 ml). The organic phase was dried and concentrated *in vacuo* to give 30 g of a yellow oil, which was subjected to column chromatography (silica gel: 570 g; eluent: methylene chloride). Sublimation of the product at 0.2 mmHg (bath temperature 50 °C) yielded 6.4 g (61 %) of (IVb) as pale yellow crystals. An analytical sample was recrystallized (ethanol-water) to give (IVb) as colourless crystals, m.p. 64.5−65.5 °C. (Found: C 54.30; H 5.91; N 9.05. Calc. for $C_7H_9NO_3$: C 54.19; H 5.85; N 9.03). ^{1}H NMR data (CCl_4): δ 6.30 (s, 1 H, C=C*H*−C); 3.91 (s, 3 H, CH_3-O); 2.85 [q (J=7 cps), 2 H, CH_3-CH_2-CO]; 1.17 [t (J=7 cps), 3 H, CH_3-CH_2].

3-Methoxy-5-(1-hydroxyiminopropyl)isoxazole (Vb). (Vb) was synthesized as described above for (Va) using 4.65 g (30 mmol) of (IVb), 4.5 g (33 mmol) of sodium acetate trihydrate, and 2.41 g (33 mmol) of hydroxylammonium chloride as starting materials. (Vb) (5.2 g; 97 %) was isolated as colourless crystals. An analytical sample was recrystallized (water-ethanol) to give (Vb) as colourless crystals. The product was by thin layer chromatography shown to consist of two compounds with R_F=0.47 and R_F=0.33 [eluent: methylene chloride-ethyl acetate (9:1)]. (Found: C 49.65; H 6.02; N 16.57. Calc. for $C_7H_{10}N_2O_3$: C 49.40; H 5.92; N 16.46). ^{1}H NMR data of (Vb) ($CDCl_3$) which are consistent with a mixture (*ca.* 1:2) of the isomeric oximes: δ 9.2−8.8 (broad s, 1 H, N−O*H*); 6.77 and 6.05 (two s, 0.3 H and 0.6 H, respectively, C=C*H*−C); 3.96 (two coincident s, 3 H, CH_3-O); 2.9−2.4 (m, 2 H, CH_3-CH_2-C); 1.4−0.9 (m, 3 H, CH_3-CH_2).

3-Methoxy-5-(1-aminopropyl)isoxazole (VIb). (VIb) was synthesized as described above for (VIa) using 3.6 g (21 mmol) of compound (Vb) and aluminium amalgam prepared from 6.86 g (254 mmol) of aluminium. The filtered reaction mixture was concentrated *in vacuo* to *ca.* 10 ml. Upon addition of concentrated hydrochloric acid (3 ml) the mixture was extracted with ether (10 ml). Upon addition of an aqueous solution of potassium hydroxide (3 ml; 10 M) the aqueous phase was extracted with two 5 ml portions of ether, which were combined, dried, and concentrated *in vacuo*. The residue was distilled to give 1.85 g (56 %) of compound (VIb) as a colourless oil, b.p. 120−124 °C/17 mmHg. (Found: C 54.10; H 7.91; N 17.89. Calc. for $C_7H_{12}N_2O_2$: C 53.83; H 7.74; N 17.94). ^{1}H NMR data (CCl_4): δ 5.53 (s, 1 H, C=C*H*−C); 4.0−3.5 (partially overlapped t, $CH_2-CH-NH_2$), 3.83 (s, CH_3-O), total 4 H; 1.9−1.3 (m, 2 H, CH_3-CH_2-CH); 1.36 (s, 2 H, NH_2); 0.90 (t, 3 H, CH_3-CH_2).

3-Hydroxy-5-(1-aminopropyl)isoxazole zwitterion (VIIIb). A solution of 416 mg (2.64 mmol) of compound (VIb) in glacial acetic acid (6 ml) containing 43 % hydrogen bromide was refluxed for a total of 10 min. After reflux for 5 min an additional amount of 6 ml of glacial acetic acid containing hydrogen bromide was added. The solution was concentrated *in vacuo* to give an oily residue, which was dissolved in water (10 ml). The solution was treated with activated charcoal and passed through a

column containing ion exchange resin [Amberlite IRA 400, (OH), 14 ml) using acetic acid (1 M) as an eluent. The fractions containing (VIIIb) were treated with activated charcoal and concentrated *in vacuo* to give 478 mg of an oily product. Crystallization from ethanol-methanol (1:1) afforded 96 mg (26 %) of (VIIIb) as colourless crystals, m.p. 167-168 °C (decomp.). (Found: C 50.60; H 7.17; N 19.66. Calc. for $C_6H_{10}N_2O_2$: C 50.69; H 7.09; N 19.71). ^{1}H NMR data [D_2O (sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard)]: δ 5.77 (s, 1 H, C=C*H*−C); 4.77 (s, 3 H, DO*H*); 4.30 [t (J=8 cps), 1 H, CH_2−C*H*−NH_3^+]; 2.1−1.7 (m, 2 H, CH_3−CH_2−CH); 0.95 (t, 3 H, CH_3−CH_2).

3-Methoxy-5-butyrylisoxazole (IVc). (IVc) was synthesized as described above for compound (IVb). As starting materials were used 6.6 g (50 mmol) of ethylmalonic acid, 12.6 g (150 mmol) of 3,4-dihydro-2*H*-pyrane, sodium hydride (*ca.* 50 mmol), and 7.3 g (45 mmol) of compound (II). The resulting mixture was subjected to column chromatography (silica gel: 400 g; eluent: methylene chloride to which increasing amounts of ethyl acetate were added). Distillation of the product gave 4.75 g (63 %) of (IVc) as a slightly coloured oil, b.p. 132−137 °C/8 mmHg. (Found: C 56.95; H 6.67; N 8.25. Calc. for $C_8H_{11}NO_3$: C 56.79; H 6.55; N 8.28). ^{1}H NMR data (CCl_4): δ 6.43 (s, 1 H, C=C*H*−C); 4.03 (s, 3 H, CH_3−O); 2.88 (t, 2 H, CH_2−CH_2−CO); 2.1−1.4 (m, 2 H, CH_3−CH_2−CH_2); 1.02 (t, 3 H, CH_3−CH_2).

3-Methoxy-5-(1-hydroxyiminobutyl)isoxazole (Vc). (Vc) was synthesized as described above for (Va) using 1.54 g (22 mmol) of hydroxylammonium chloride, 3.00 g (22 mmol) of sodium acetate trihydrate, and 3.38 g (20 mmol) of compound (IVc) as starting materials. (Vc) (3.5 g; 95 %) was isolated as colourless crystals. The product was by thin layer chromatography shown to consist of two compounds with R_F=0.65 and R_F=0.55 [eluent: methylene chloride-ethyl acetate (4:1)]. ^{1}H NMR data of (Vc) ($CDCl_3$), which are consistent with a mixture (*ca.* 4:7) of the isomeric oximes: δ 9.3−8.6 (broad s, 1 H, N−O*H*); 6.80 and 6.06 (two s, 0.4 H and 0.7 H, respectively, C=C*H*−C); 3.97 (two coincident s, 3 H, CH_3−O); 2.8−2.4 (m, 2 H, CH_2−CH_2−C); 1.9−1.2 (m, 2 H, CH_3−CH_2−CH_2); 0.98 (t, 3 H, CH_3−CH_2).

3-Methoxy-5-(1-aminobutyl)isoxazole (VIc). (VIc) was synthesized as described above for compound (VIb). As starting materials were used 2.7 g (15 mmol) of (Vc), and aluminium amalgam from 4.75 g (176 mmol) of aluminium. (VIc) (1.6 g; 64 %) was obtained as a colourless oil, b.p. 87−89 °C/0.5 mmHg. (Found: C 56.70; H 8.25; N 16.62. Calc. for $C_8H_{14}N_2O_2$: C 56.45; H 8.29; N 16.46). ^{1}H NMR data (CCl_4): δ 5.58 (s, 1 H, C=C*H*−C); 3.9−3.6 (partially overlapped t, CH_2−C*H*−NH_2), 3.86 (s, CH_3−O), total 4 H; 1.9−0.7 (m, 7 H, CH_3−CH_2−CH_2−CH); 1.40 (s, 2 H, NH_2).

3-Hydroxy-5-(1-aminobutyl)isoxazole zwitterion (VIIIc). (VIIIc) was synthesized as described above for compound (VIIIb) using 1.02 g (6.0 mmol) of (VIc). Crystallization from methanol gave 262 mg (28 %) of (VIIIc) as colourless crystals, m.p. 172.5−173 °C (decomp.). (Found: C 53.90; H 7.65; N 18.04. Calc. for $C_7H_{12}N_2O_2$: C 53.83; H 7.74; N 17.94). ^{1}H NMR data [D_2O (sodium 3-(trimethylsilyl)-propanesulfonate was used as an internal standard)]: δ 5.75 (s, 1 H, C=C*H*−C); 4.75 (s, 6 H, DO*H*); 4.35 (t, 1 H, CH_2−C*H*−NH_3^+); 2.1−1.6 (m, 2 H, CH_2−CH_2−CH); 1.6−1.1 (m, 2 H, CH_3−CH_2−CH_2); 1.1−0.7 (m, 3 H, CH_3−CH_2).

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The Role of Cyclic Adenosine 3′,5′-Monophosphate in the Synthesis of the Enzymes of γ-Aminobutyrate Breakdown in *Escherichia coli* K-12

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When *Escherichia coli* K-12 (W 3001) is grown on glucose the synthesis of the enzymes of the pathway of γ-aminobutyrate breakdown is repressed. The specific activity of the NADP-specific succinate semialdehyde dehydrogenase is greater than that of the NAD-specific enzyme.

The synthesis of the enzymes of γ-aminobutyrate breakdown is induced when *E. coli* K-12 is grown on γ-aminobutyrate. In this case, however, the NAD-specific succinate semialdehyde dehydrogenase has more activity than the NADP-specific enzyme. This may indicate the existence of two different enzymes.

When *E. coli* K-12 is grown on γ-aminobutyrate, each of glucose, 2-oxoglutarate, glutamate, and succinate repress both γ-aminobutyrate oxoglutarate transaminase and succinate semialdehyde dehydrogenase. Cyclic AMP does not prevent the repression caused by glucose, which does not therefore operate simply by depletion of endogenous cyclic AMP.

In 1968, cyclic adenosine 3′,5′-monophosphate (cyclic AMP) was found to prevent glucose induced "catabolite repression" [1] of β-galactosidase in *E. coli*.[2,3] The ability of cyclic AMP to prevent the glucose repression of enzyme synthesis is not restricted to β-galactosidase. Cyclic AMP has now been shown to stimulate the synthesis of many enzymes in various bacteria.[3,4,5] It is supposed that catabolite repression is a consequence of the depletion of intracellular cyclic AMP by glucose.[6–11]

The activities of the enzymes of γ-aminobutyrate breakdown are low when *E. coli* is grown on glucose; the enzymes are repressed by glucose.[12–15] However, Dover and Halpern [13] have shown for *E. coli* K-12 that these enzymes specifically can escape from this catabolite repression when γ-aminobutyrate, glutamate, or aspartate are used as nitrogen source instead of ammonium salts.

In this work the effect of exogenous cyclic AMP on the repression by glucose of the enzymes of γ-aminobutyrate breakdown in *E. coli* K-12 (W 3001) was studied.

MATERIAL AND METHODS

Cultivation of E. coli. A wild strain of *E. coli* K-12 (*E. coli* K-12, W 3001) was transferred with a platinum wire from an agar slant to an inoculum medium (10 ml) containing 1 % Difco yeast extract. 1 % Difco tryptone, and 0.5 % dipotassium monohydrogen phosphate, that had been autoclaved at 115 °C for 7 min. After 4 h incubation at 37 °C, the whole medium was poured aseptically into 250 ml of sterilized inoculum medium. After 16 h incubation at 37 °C without shaking, the cells were centrifuged (4 000 *g*, 10 min) at room temperature, washed twice with cold 0.9 % sodium chloride and suspended in a minimal medium containing 0.1 % ammonium chloride, 0.7 % disodium hydrogen phosphate, 0.3 % potassium dihydrogen phosphate, 0.5 % sodium chloride, 0.01 % magnesium sulfate heptahydrate, and 0.2 % D(+)-glucose. The suspension was shaken (250 rpm) in a rotatory shaker (New Brunswick G-10) at 37 °C, and the turbidity was measured with a Klett-Summerson colorimeter using filter No. 62 (590 – 660 nm). When the turbidity of the culture was about 150 colorimeter scale units, the washing and centrifugation were performed as described above. The cells were suspended in a minimal medium together with ammonium chloride as the nitrogen source and

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γ-aminobutyrate as the only carbon source. Additions to the culture (divided into three) were made as indicated on the figure legends. Cultivation and washing were performed as described above. The cells for the enzyme assays were harvested by centrifugation (5 ml, 7 000 *g*, 5 min) and washed twice with 0.9 % sodium chloride (+10 °C). The cell pellets were stored at 3 °C until subjected to ultrasonic vibration.

Ultrasonication. The samples were suspended in 2 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 0.1 % (v/v) 2-mercaptoethanol, and ultrasonicated (MSE Ultrasonic Disintegrator, 60 W) for 6 min (2 microns peak to peak) at 0 °C. The suspensions were then centrifuged at 7 000 *g* for 10 min.

Enzyme and protein assays. γ-Aminobutyrate oxoglutarate transaminase and succinate semialdehyde dehydrogenase were measured by the methods described before.[16]

β-Galactosidase was determined by the method published earlier,[17] except that the cells were disrupted by ultrasonication and not by toluene treatment. The two methods were compared and shown to give similar results.

The protein contents of the extracts were estimated by the Folin-Ciocalteau method.[18]

Table 1. The specific activities of the enzymes of the pathway of γ-aminobutyrate breakdown [(μmol NADH or NADPH formed) min^{-1} (mg protein) $^{-1}$]. in *E. coli* K-12 (W 3001) cells grown on glucose and γ-aminobutyrate. The cells for the assays were harvested in the middle of the exponential growth phase.

Enzyme	Carbon source: Glucose	Carbon source: γ-Aminobutyrate
γ-Aminobutyrate oxoglutarate transaminase (NADPH)	0.009	0.789
Succinate semialdehyde dehydrogenase (NADPH)	0.025	0.250
Succinate semialdehyde dehydrogenase (NADH)	0.003	0.602

RESULTS AND DISCUSSION

When *E. coli* K-12 (W 3001) is grown on glucose the specific activities of the enzymes of γ-aminobutyrate breakdown are low (Table 1). The NADP-specific succinate semialdehyde dehydrogenase is clearly much more active than the NAD-specific enzyme. On the other hand, when γ-aminobutyrate acts as carbon source, the activities of both these enzymes and that of γ-aminobutyrate oxoglutarate transaminase are high. It appears that the synthesis of these three enzymes is induced. In this cultivation, however, the specific

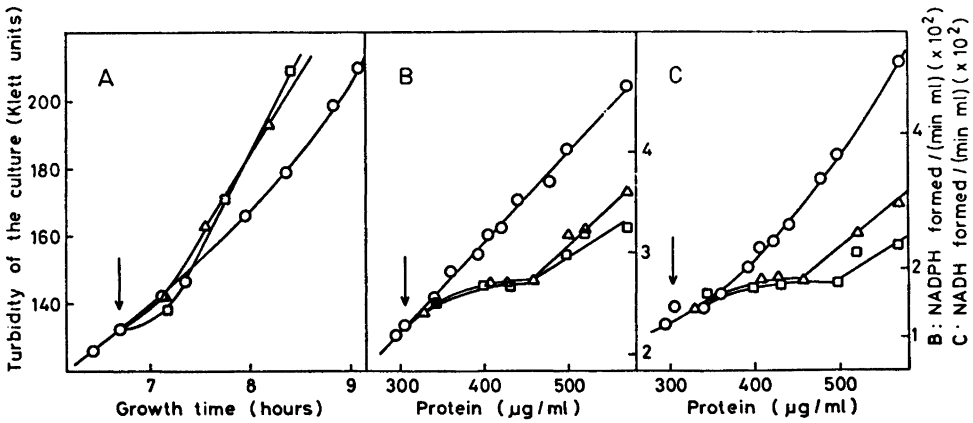

Fig. 1. Effect of glucose and cyclic AMP on the synthesis of γ-aminobutyrate oxoglutarate transaminase (B) and NAD-specific succinate semialdehyde dehydrogenase (C). The activities are expressed in (μmol NAD(P)H formed) ml^{-1} min^{-1}. *E. coli* K-12 (W 3001) culture growing on γ-aminobutyrate (0.25 %, ○) was divided into three. The following additions were made at the arrow: none (○); glucose (10 mM, △); and glucose (10 mM) plus cyclic AMP (5 mM) (□). In A, growth curves are marked by corresponding symbols.

activity of the NAD-specific succinate semialdehyde dehydrogenase is higher than that of the NADP-specific enzyme.

As mentioned above, several examples of catabolite repression caused by glucose are prevented by cyclic AMP. In the classic case of β-galactosidase,[2] glucose represses the synthesis of induced β-galactosidase, whereas exogenous cyclic AMP eliminates this repression. It is of interest whether a similar situation exists for the control of γ-aminobutyrate oxoglutarate transaminase and succinate semialdehyde dehydrogenase. Glucose causes the repression of these enzymes in *E. coli* K-12 (W 3001) grown on γ-aminobutyrate (Fig. 1). Cyclic AMP added together with glucose might prevent the repression if it were caused by depletion of endogenous cyclic AMP. When cyclic AMP is added with glucose, however, the differential rates of synthesis of the enzymes of γ-aminobutyrate breakdown, do not exceed the repressed values. Accordingly, the repression is not similar to the repression of β-galactosidase: it is unaffected by exogenous cyclic AMP, although exogenous cyclic AMP does prevent the repression by glucose of β-galactosidase in *E. coli* K-12 W 3001 grown on γ-aminobutyrate (Fig 2). As shown in Fig. 3, 2-oxoglutarate, glutamate, and succinate each repress γ-aminobutyrate oxoglutarate transaminase and succinate semialdehyde dehydrogenase.

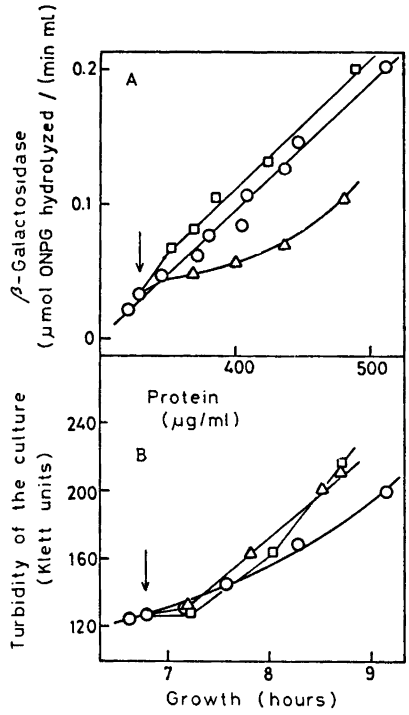

Fig. 2. Effect of glucose and cyclic AMP on the differential rate of β-galactosidase synthesis (A) in the culture growing on γ-aminobutyrate as the only carbon source (0.25 %, O) and corresponding growth curves (B). To induce β-galactosidase isopropyl-β-D-thiogalactoside (IPIG) (1.25 mM) was added to the growth medium 15 min before the arrow. At the arrow the culture was divided into three and the following additions were made: none (O); glucose (10 mM, △); and glucose (10 mM) plus cyclic AMP (5 mM) (□). In B, growth curves are marked by corresponding symbols.

The metabolism of γ-aminobutyrate is associated with nitrogen metabolism *via* the transamination of 2-oxoglutarate, and also provides succinate, whose role in the citric acid cycle is both energetic and biosynthetic. The control of this metabolism is therefore likely to be complex. We have shown here that repression of γ-aminobutyrate-oxoglutarate transminase and succinate semialdehyde dehydrogenase is caused by glucose and several Krebs cycle intermediates. It is particularly interesting that the repression by glucose appears not to be mediated merely by lowering the level of endogenous cyclic AMP. Dover and Halpern have previously shown that glucose repression of these enzymes is unusual in that the enzymes can escape from this control when γ-aminobutyrate is the sole nitrogen source.[18]

It has long been known that some enzymes of the citric acid cycle in bacteria are repressed when the culture is grown on glucose. It has also been reported that glutamate is an efficient corepressor.[19-20] In this case catabolite repression requires some other metabolite. This could explain why the enzymes of the citric acid cycle, like the enzymes of γ-aminobutyrate breakdown are not found to be under the control of cyclic AMP. Glutamate, however, is not necessarily the metabolite responsible for control of the enzymes in the metabolism of γ-aminobutyrates, although it is the dominant free amino acid in *E. coli*.[22]

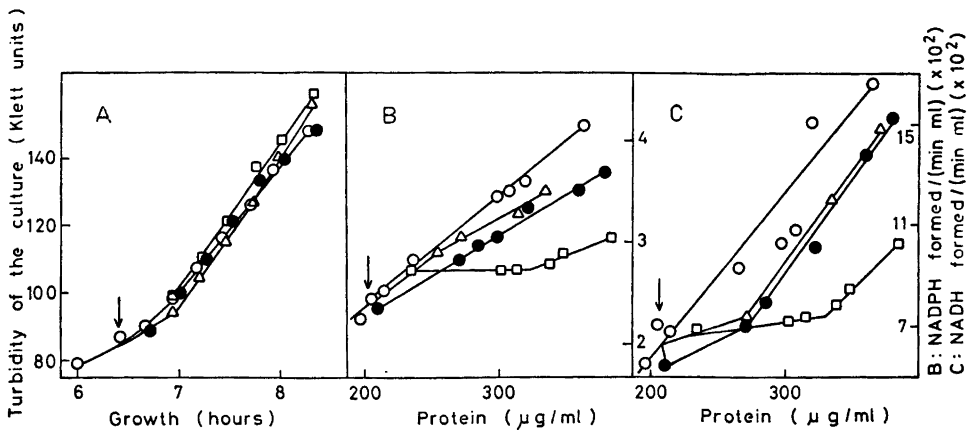

Fig. 3. Differential rate of synthesis of γ-aminobutyrate oxoglutarate transaminase (B) and NAD-specific succinate semialdehyde dehydrogenase (C). The activities are expressed in [μmol NAD(P)H formed] ml^{-1} min^{-1}. The growth curves are presented in A. *E. coli* K-12 (W-3001) culture growing on γ-aminobutyrate (0.25 %, ○) was divided into four. The following additions were made at the arrow: none (○); 2-oxoglutarate (10 mM, △); glutamate (10 mM, □); and succinate (10 mM, ○).

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Reactions of Nitrobenzyl Alcohol under Acidic Conditions. Possibilities of Intramolecular Nucleophilic Participation by the Nitro Group

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o-Nitrobenzyl alcohol was refluxed in toluene under acidic conditions. Three products were obtained: di-(*o*-nitrobenzyl) azoxybenzene-*o*,*o*'-dicarboxylate (*2*), 6*H*,12*H*-indazolo[2,1-*a*]indazole-6,12-dione (*3*), and benzidine-*m*,*m*'-dicarboxylic acid (*5*). *p*-Nitrobenzyl alcohol scarcely reacted under these reaction conditions. Possible reaction paths are discussed.

The study of the reactions of *ortho*-substituted nitrobenzene derivatives has revealed interesting interactions between the nitro group and the *ortho*-substituent.[1] These interactions often result in a reduction of the nitro group and an oxidation of the *ortho*-substituent.

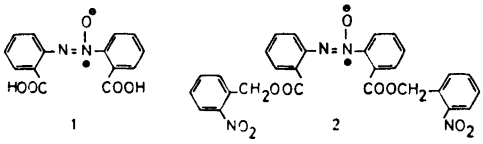
We have earlier studied the reactions between the nitro and methyl group in *o*-nitrotoluene under a variety of conditions,[2,3] and also the photochemical interaction between the nitro and hydroxyl group in 2-(*o*-nitrophenyl)ethanol.[4]

In continuation of our work on nitro group chemistry, some reactions of *o*-nitrobenzyl alcohol have been studied. Reactions under acidic conditions were believed to be particularly interesting. In the presence of strong acid, protonation of the hydroxyl group could be expected. However, the leaving of the water molecule would probably be assisted by the *o*-nitro group, thus providing interesting opportunities for reactions between the nitro group and the benzylic carbon atom.

The assistance by the nitro group was anticipated from the work of Noyce, Hartter and Miles [5] on acidic dehydration of 1,2-diarylethanols. They showed the reaction of 1-(*p*-nitrophenyl)-2-phenylethanol to be very slow, and indicated that this compound existed in solution as the protonated species. Further, the work of Spence and Tennant [6] and Sword [7] indicated that an *o*-nitro group assisted the ring opening of protonated epoxides.

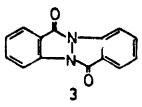
o-Nitrobenzyl alcohol was accordingly treated with *p*-toluenesulfonic acid in refluxing toluene. After a few hours, the *o*-nitrobenzyl alcohol was consumed and three main products were obtained. Two of these were neutral and the third was slightly acidic. The structures of these substances were elucidated, mainly by spectroscopic methods.

One of the neutral compounds had an IR spectrum indicating ester groups (1725 and 1740 cm^{-1} together with a band at 1260 cm^{-1}) and nitro groups (1525 and 1340 cm^{-1}) to be present, but no NH or OH groups. The NMR spectrum suggested the compound to contain benzylic protons. The mass spectrum indicated an elemental formula of $C_{28}H_{24}N_4O_9$. The high molecular weight, the two ester carbonyls, and the benzylic protons would be explained by a dicarboxylic acid esterified with *o*-nitrobenzyl alcohol. The elemental formula of such an acid would be $(C_{12}H_8N_2O)(COOH)_2$. One possible structure would be azoxybenzene-*o*,*o*'-dicarboxylic acid (*1*). Saponification of the isolated compound gave an dicarboxylic acid identical to *1*, [10] together with *o*-nitrobenzyl alcohol as the neutral product. The structure of the isolated compound was thus the di-*o*-nitrobenzyl ester of azoxybenzene-*o*,*o*'-dicarboxylic acid (*2*).



1 2

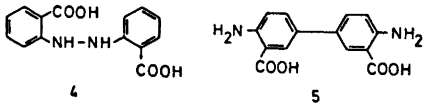
The other neutral compound gave an IR spectrum indicating carbonyl groups (1690 cm^{-1}) but no NH, OH, or NO_2 groups to be present in the molecule. The mass spectrum indicated an elemental formula of $C_{14}H_8N_2O_2$. The structure *3* (6*H*,12*H*-indazolo[2,1-*a*]indazole-6,12-dione) was in accordance with these data, and the isolated compound was identical to an authentic sample of *3*.[8]



3

The isolated acidic product was almost insoluble in neutral solvents, and gave a rather indistinct melting point. The IR spectrum suggested the product to contain primary amino groups together with carboxylic acid groups.

The structure of *2* showed the formation of a compound with a nitrogen-nitrogen bridge between the aromatic rings. One could thus imagine that compounds with more reduced nitrogen-nitrogen bridges could have been formed, for instance of the hydrazobenzene type. Under the acidic reaction conditions, a hydrazobenzene would probably undergo a benzidine rearrangement to give products with primary amino groups. From these considerations and from the IR spectrum, a possible structure for the acidic product would be benzidine-*m*,*m*′-dicarboxylic acid (*5*). Reaction of hydrazobenzene-*o*,*o*′-dicarboxylic acid (*4*) with *p*-toluenesulfonic acid gave a product identical to the one isolated from *o*-nitrobenzyl alcohol. This path of formation, the IR spectrum and m.p. properties [9] identified the compound as *5*, and also indicated that *4* was the primary product in the reaction of *o*-nitrobenzyl alcohol.

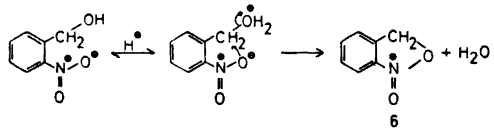


4 5

The structures of the isolated compounds (*2*, *3*, *5*) from the reaction of *o*-nitrobenzyl alcohol show a variety of reductions and oxidations to have taken place. From the products, it is obvious that intermolecular reactions have taken place, as neither *2*, *3* nor *5* are at the same oxidation stage as *o*-nitrobenzyl alcohol.

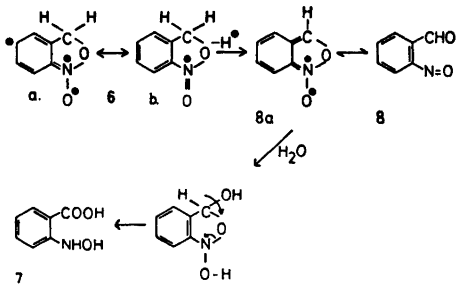
However, the reactions have probably not taken place solely by intermolecular processes, as *p*-nitrobenzyl alcohol was shown to react only with difficulty under the conditions used in the reaction of *o*-nitrobenzyl alcohol. The initial reaction of *o*-nitrobenzyl alcohol is therefore probably of intramolecular nature.

An intramolecular reaction might have started with a protonation of the hydroxyl group, followed by internal substitution reaction to give the cyclic intermediate *6*. Similar



6

intermediates have been postulated both in the ring opening of *o*-nitrophenylethylene oxides,[6,7] in the reactions of *o*-nitrobenzhydrol,[11] and in the solvolysis of *o*-nitrobenzhydryl bromide.[12] The intermediate *6* may then undergo internal electron shifts, for instance by the reactions postulated in Scheme 1, to produce *o*-hydroxylaminobenzoic acid (*7*) and *o*-nitrosobenzaldehyde (*8*) or the cyclic form *8a*. Compounds *7*



Scheme 1.

and *8* would probably be rather unstable under the reaction conditions, both towards oxidations and reductions, and towards condensation reactions. From the resonance formula *a* of

compound *6*, a nucleophilic attack on the aromatic ring seemed possible. Such attacks have indeed been observed with analogous structures when nucleophiles like chloride ions have been present in the reaction.[6,7,11,12] In the present reaction, with *p*-tosylate ions as the predominant nucleophile, such attack was neither expected nor found.

It should also be noticed that bimolecular formulas analogous to *6* can be written, and that the reaction of *o*-nitrobenzyl alcohol is not proven to be intramolecular.

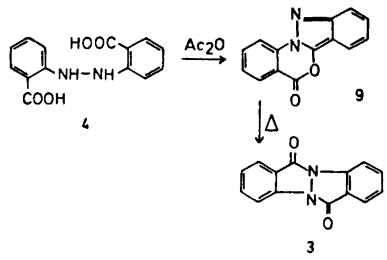
By further oxidation-reductions of compounds *7* and *8*, molecules with different combinations of oxidized benzylic carbon and reduced nitrogen atoms would be obtained, and by condensations of these molecules compounds *2*, *3*, and *5* could arise.

Benzidine-*m*,*m*′-dicarboxylic acid (*5*) is believed to originate from hydrazobenzene-*o*,*o*′-dicarboxylic acid (*4*). Both compounds *2* and *4* have been produced by condensation reactions between two nitrogen atoms. Azoxy- and hydrazobenzenes are interconvertible by various reduction and oxidation reactions.[13] If such interconversions have been important in their formation in the present reactions, or if they have been partly or solely formed by condensation reactions is not known at present.

Condensation products from the reduction of nitro compounds are usually formed under basic and not under acidic conditions.[13] However, the non-polar solvent used in the present reaction may explain the observed condensation reactions. In toluene, the protonation of the amines and hydroxylamines formed would be slow as compared to the reaction in more polar (*e.g.* aqueous) solvents, and the amines and hydroxylamines would therefore be more accessible to condensation reactions.

Of the two dicarboxylic acids formed by the reaction (*1*,*5*), only azoxybenzene-*o*,*o*′-dicarboxylic acid (*1*) was esterified by *o*-nitrobenzyl alcohol. Benzidine-*m*,*m*′-dicarboxylic acid (*5*) is hardly soluble in toluene and was therefore probably removed from the reaction before esterification could take place.

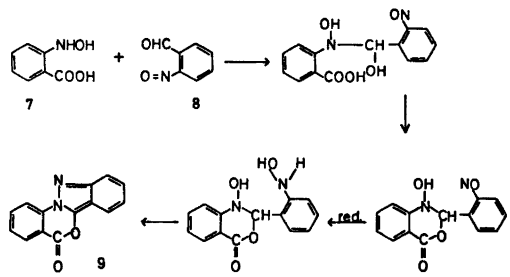
Several formation paths for compound *3* may be envisaged. Formally, the substance could be formed by condensation of *o*-hydroxylaminobenzoic acid (*7*) with *o*-aminobenzoic acid. However, not suprisingly, an experiment showed *o*-aminobenzoic acid to be precipitated as the *p*-toluenesulfonate under the reaction conditions, and substances *2* and *3* to be formed in the same ratio as when *o*-aminobenzoic acid was not added to the reaction. Compound *3* has earlier been made by heating of hydrazobenzene-*o*,*o*′-dicarboxylic acid (*4*) in acetic anhydride, followed by heating of the initially formed lactone (*9*).[8,14]



Under the present reaction conditions, hydrazobenzene-*o*,*o*′-dicarboxylic acid (*4*) gave benzidine-*m*,*m*′-dicarboxylic acid (*5*) as the major reaction product, with only traces of a substance which could have been *3*. The major part of *3* is therefore not formed from *4*.

However, lactone *9* was transformed to compound *3* when refluxed with *p*-toluenesulfonic acid in toluene, and the IR spectrum of a reaction mixture from *o*-nitrobenzyl alcohol indicated traces of lactone *9* to be present. The results thus indicate that compound *3* may have been formed from lactone *9*, but that hydrazobenzene *o*,*o*′-dicarboxylic acid (*4*) was not a precursor for *9*.

One possibility for the formation of *9* would be the reaction between one molecule of *o*-hydroxylaminobenzoic acid (*7*) and one of *o*-nitrosobenzaldehyde (*8*) (or the cyclic form *8*a).



Further investigations are necessary to determine the exact path of formation of the products *2*, *3* and *5*. Two points seem nevertheless to be clear from the present investigation. First, as *p*-nitrobenzyl alcohol reacted only slightly under the applied conditions, the *ortho* position of the nitro and hydroxymethyl groups is necessary for the reaction. Second, intermolecular reactions take place as the products are at different oxidation stages from *o*-nitrobenzyl alcohol.

One hypothesis fitting these two points is the one presented above: The reaction starts as an intramolecular reaction resulting in products with oxidized carbon atom and reduced nitrogen atom. Further intermolecular red-ox and condensation reactions then give the isolated products.

EXPERIMENTAL

The IR spectra were recorded on a Perkin-Elmer infrared spectrophotometer, Model 457. NMR spectra were recorded on a Varian A 60 spectrometer with TMS as in internal standard. The chemical shifts are given in δ-units. Mass spectra were recorded on an A.E.I. M.S. 902 mass spectrometer. The elemental formulas were obtained by peak matchings which gave *m/e* within ± 0.0006 mass units from the calculated values. Melting points are uncorrected.

Reaction of o-nitrobenzyl alcohol with p-toluenesulfonic acid. *p*-Toluenesulfonic acid (0.6 g) and toluene (*p.a.*) (35 ml) were refluxed with a water separator at the condenser. A toluene/water mixture (5 ml) was separated. *o*-Nitrobenzyl alcohol (0.3 g) was added to the solution and the mixture refluxed for 5 h. TLC indicated *o*-nitrobenzyl alcohol to diminish and two new compounds with $R_F > R_F$-nitrobenzyl alcohol and one with $R_F = 0$ (eluted with 10 % ether/chloroform) to be formed. After 5 h all of the nitrobenzyl alcohol had reacted. The mixture was left at 25 °C overnight and then filtered. The toluene solution contained 0.2 g which TLC indicated to consist of the two compounds with $R_F > R_F$-nitrobenzyl alcohol. Compound *3* (15 mg) crystallized from a chloroform solution of this mixture.

Attempts to separate the two compounds by dry column chromatography on silica gel or alumina were unsuccessful, and compound *2* was finally obtained pure by crystallization from chloroform/light petroleum. The 200 mg obtained from the toluene solution was estimated by TLC chromatography to contain *ca.* 150 mg of di-(*o*-nitrobenzyl)*o,o'*-azoxybenzene dicarboxylate (*2*) and *ca.* 50 mg of 6*H*,12*H*-indazolo[2,1-*a*]indazole-6,12-dione (*3*). More accurate estimations have not yet been possible.

Di-(*o*-nitrobenzyl)*o,o'*-azoxybenzenedicarboxylate (*2*) had m.p. 177 – 178 °C, IR (KBr): 1740, 1725, 1525, 1480, 1340, 1260, 1140, 1090, 1080, 865, 795, 770, 760, 740, 730, 720 cm^{-1}. NMR ($CDCl_3$): 16 protons with signals 7.15 – 8.20 (complex), 4 protons at 5.7 (singlet). Mass spectrum; *m/e* 556.1233 (2 %, $C_{28}H_{20}N_4O_9$), 540.1278 (2 %, $C_{28}H_{20}N_4O_8$), 420.0833 (2 %, $C_{21}H_{14}N_3O_7$), 404.0877 (2 %, $C_{21}H_{14}N_3O_6$), 376.0931 (50 %, $C_{20}H_{14}N_3O_5$), 136 (55 %), 78 (100 %).

Compound *3* had m.p. 296 – 297 °C (lit.[8] 300 – 300.8 °C), IR (KBr): 1690, 1620, 1480, 1470, 1365, 1320, 1210, 1150, 1130, 755, 670 cm^{-1}. Mass spectrum, *m/e* 236.0587 (100 %, $C_{14}H_8N_2O_2$), 208.0635 (21 %, $C_{13}H_8N_2O$, loss of CO from *m/e* 236 verified by *m** 183.32), 179.0612 (9 %, $C_{12}H_7N_2$), 118.0295 (11 %, C_7H_4NO).

In a new experiment, *o*-nitrobenzyl alcohol (8 g) and *p*-toluenesulfonic acid (16 g) in toluene (800 ml) were refluxed for 5 h. Substances *2* and *3* were obtained by crystallization of the toluene dissolved material (8.6 g).

The black material not dissolved in toluene was washed with water to remove *p*-toluenesulfonic acid. The undissolved material (1.3 g) had IR almost identical to benzidine-*m,m*-dicarboxylic acid (see below) and m.p. at 265 °C when the m.p. tube was put into the preheated (250 °C) m.p. apparatus. 1.1 g of the solid was dissolved on extraction with ethanol (2×100 ml) and 0.13 g of black powder remained. The undissolved black powder had an undistinguished IR and gave no indications of melting below 320 °C. This material is believed to be a polymer. The substance dissolved in ethanol (1.1 g) was refluxed with chloroform (25 ml), 0.25 g of it was dissolved. The undissolved material (0.8 g, 11 %) had IR and m.p. behaviour identical with benzidine-*m,m'*-dicarboxylic acid (see below). The chloroform-dissolved material consisted of at least seven compounds as judged by TLC. Two of these were compounds *2* and *3*, and one may have been azobenzene carboxylic acid. The chloroform fraction was not investigated further.

Saponification of di-(o-nitrobenzyl) o,o'*-azoxybenzenedicarboxylate (2).* *2* (50 mg) from the reaction of *o*-nitrobenzyl alcohol was dispersed in ethanol (10 ml) and sodium hydroxide (1 M, 1 ml) added. After stirring overnight at 25 °C, the reaction was complete. The neutral product (15 mg) was identical with *o*-nitrobenzyl alcohol and the acidic product (15 mg) with *o,o'*-azoxybenzenedicarboxylic acid m.p. 250 °C (decomp.) and IR (KBr): 3500 – 2000, 1690, 1610, 1490, 1420, 1310, 1290, 1280, 1090, 940, 800, 770, 755, 660, cm^{-1}. Mass spectrum *m/e*: 286.0584 (1 %, $C_{14}H_{10}N_2O_5$), 270.0636 (2 %, $C_{14}H_{10}N_2O_4$), 241.0616 (100 %, $C_{13}H_9N_2O_3$), 197.0709 (13 %, $C_{12}H_9N_2O$), 165 (13 %), 159 (13 %), 121 (68 %), 115 (29 %), 93 (58 %), 77 (32 %), 65 (65 %).

Reaction of o-nitrobenzyl alcohol in the presence of o-aminobenzoic acid. o-Nitrobenzyl alcohol (0.3 g), o-aminobenzoic acid (0.3 g), and *p*-toluenesulfonic acid (0.6 g) were refluxed in toluene (30 ml) for 5 h. A precipitate formed at once, and TLC indicated the reaction to be slower than the one without o-aminobenzoic acid; the ratio between products *2* and *3* was the same as before.

Reaction of hydrazobenzene-o,o'-dicarboxylic acid (4). *4* (1 g) and *p*-toluenesulfonic acid (2 g) were refluxed in toluene (100 ml) for 3 h. The toluene phase contained 15 mg which TLC indicated to consist partly of compound *3*. The crystalline part of the reaction mixture was washed with water to remove *p*-toluenesulfonic acid, and the residue dried. This compound had IR (KBr): 3510, 3400, 3400 – 2700, 1680, 1630, 1590, 1560, 1240, 815, 690 cm^{-1} and m.p. 275 °C when the m.p. tube was put into a preheated (250 °C) m.p. apparatus.

The result from the reaction was the same when *4* (0.1 g) was dissolved in refluxing toluene (100 ml) and added to a refluxing solution of *p*-toluenesulfonic acid (100 mg) in toluene (100 ml) and then refluxed for 3½ h.

Reaction of p-nitrobenzyl alcohol with p-toluenesulfonic acid. *p*-Toluenesulfonic acid (2 g) in toluene (100 ml) was refluxed with a water separator at the condenser. A toluene/water mixture (5 ml) was removed, and *p*-nitrobenzyl alcohol (1 g) added. The mixture was refluxed for 5 h. TLC of the product indicated only a slight reaction with trace of one product with $R_F > R_F$-nitrobenzyl alcohol to have been formed.

Reaction of the lactone 9. *p*-Toluenesulfonic acid (140 mg) and *9* [8] (25 mg) were refluxed in toluene (7 ml) for 5 h. IR and TLC showed *9* to have been transformed almost completely to *3*.

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Intramolecular Hydrogen Bonding in Aryl Substituted Aliphatic Alcohols

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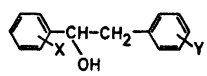
The internal hydrogen bonding in *ortho* substituted benzyl alcohols and in *ortho* and *para* substituted 1,2-diarylethanols (1-X-phenyl-2-Y-phenylethanol) (*1*) has been studied by IR spectroscopy and with chloro, methoxy, and nitro groups as substituents. The hydrogen bonds to these *ortho* groups in benzyl alcohols or in 1-(*o*-X-phenyl)-2-phenylethanols are non-existent or very weak, with the strongest bond to the nitro group. With the Y groups in *ortho* position in 1,2-diarylethanols (*1*), relatively strong hydrogen bonds to the *ortho* substituents (Y) are indicated. The results may be explained by steric relations in the molecule.

Intramolecular hydrogen bonding in *ortho* substituted phenols is well documented.[1,2] Many of these internal hydrogen bonds are particularly strong, *e.g.* in *o*-nitrophenol, and broad and strong absorptions are recorded in the OH region of the infrared spectra; a large frequency shift occurs as compared to the absorption found with nonbonded phenols.

In contrast to the *ortho* substituted phenols, no systematic study seems to have been carried out on *ortho* substituted benzylic alcohols or 2-arylethanols with the *ortho* substituent as hydrogen bond acceptor. Both these classes of substances have, however, been thoroughly studied from the point of view of internal hydrogen bonding to the π-bond system.[3–5]

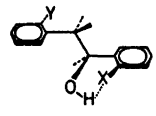
It was thus considered of interest to study some *ortho* substituted benzyl alcohols and arylethanols where the *ortho* group might serve as hydrogen bond acceptor. The aim of the study was to see if the *ortho* substituents would serve as acceptors and whether there existed a preference in acceptor site for the hydrogen bond, *i.e.* if the π-bond system or the *ortho* substituent would dominate as acceptor site.

It was considered of particular interest to study the 1,2-diaryl substituted ethanols of type *1* where competition between four acceptor sites for the hydrogen bonds is possible (*i.e.* the conformers I – IV) in addition to conformer V which has the hydroxyl group non-bonded.

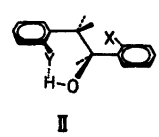


1

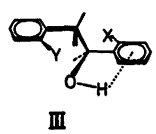
1a. X=H, Y=H
b. X=o-OCH_3, Y=H
c. X=H, Y=o-OCH_3
d. X=p-OCH_3, Y=o-OCH_3
e. X=Y=o-OCH_3
f. X=o-NO_2, Y=H
g. X=H, Y=o-NO_2
h. X=o-NO_2, Y=p-NO_2
i. X=p-NO_2, Y=o-NO_2
j. X=Y=o-NO_2
k. X=Y=p-NO_2
l. X=o-Cl, Y=H
m. X=H, Y=o-Cl
n. X=Y=o-Cl



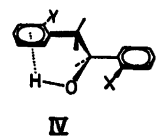
I



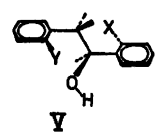
II



III



IV



V

Table 1. Hydroxyl stretching frequencies of benzyl alcohols, in cm^{-1}. Absorbance (A) as log I_0/I.

X	a_1	a_2	A_1/A_2
H[a]	3634	3614	–
H[b]	3635(sh)	3615	1:15
o-OCH_3 [a]	3636(sh)	3610	–
o-OCH_3 [b]	3630(sh)	3615	1:0.8
m-OCH_3 [12]	3636.2	3616.9	1:1.7
p-OCH_3 [12]	3636.0	3617.1	1:2.5
p-OCH_3 [b]	3635(sh)	3615	1:3
o-NO_2 [a]	3640	3597	–
o-NO_2 [b]	3634.0	3600	1:0.7
m-NO_2 [a]	3632	3617.2	–
m-NO_2 [b]	3635	3615	1:1
p-NO_2 [a]	3635	3619	–
p-NO_2 [b]	3635	3615(sh)	1:0.3
o-Cl [a]	3635.7	3615.3	–
m-Cl [a]	3632.4	3615.3	–
m-Cl [12]	3636.4	3617.0	1:1.3
p-Cl [a]	3632.1	3616.4	–
p-Cl [12]	3635.3	3617.1	1:1.4

[a] Private communication from v. R. Schleyer, P. [b] This work.

The substituents used in the study of the alcohols of type *1* may be classified according to their electronic properties. Two of the substituents utilized, the nitro and methoxy groups, would be expected to have opposite electronic effects on the aryl rings, but to function as good hydrogen bond acceptors.[6,7] The chloro substituent would be expected to give rise to only weak hydrogen bonds and to have electronic properties intermediate between those of the nitro and methoxy group.[1,2]

By using these substituents it was hoped to obtain some information concerning the preferred conformer or conformers I – V, both as the acceptor sites and the electron density in the π-bond systems varied. The study was carried out by IR spectroscopy.

RESULTS

The IR absorptions in the OH-region (*i.e.* 3300 – 3700 cm^{-1}) of benzyl alcohols are given in Table 1 and those of 1,2-diarylethanols in Tables 2a – 2c.

All spectra were recorded in dilute CCl_4 solution with concentrations below 10^{-2} M and for compounds *1h*, *1i*, and *1k* below 10^{-4} M. Continued dilution did not change the ratio between the various absorption bands. These concentrations are so low that intermolecular hydrogen bonds are usually broken.[7]

Some of the bands given in Tables 1 and 2 were only partly resolved. In such cases the exact frequencies of the absorptions making up

Table 2. Hydroxyl stretching frequencies in 1,2-diarylethanols (*1*), in cm^{-1}. Absorbance (A) as log I_0/I.

Substance	X	Y	ν_1	ν_2	A_1/A_2
(a) With methoxy substituents					
1a	H	H	–	3620	–
1b	o-OCH_3	H	3620	3590(sh)	1:0.2
1c	H	o-OCH_3	3620	3550(bd)	1:0.6
1d	p-OCH_3	o-OCH_3	3620	3550(bd)	1:0.4
1e	o-OCH_3	o-OCH_3	3615	3560(bd)	1:2.5
(b) With nitro substituents					
1f	o-NO_2	H	3610(bd)	–	–
1g	H	o-NO_2	3615	3560(bd)	1:0.14
1h	o-NO_2	p-NO_2	3625	3600(sh)	1:0.05
1i	p-NO_2	o-NO_2	3625	3560(bd)	1:0.12
1j	o-NO_2	o-NO_2	3615	3540(bd)	1:1.1
1k	p-NO_2	p-NO_2	3620	–	–
(c) With chloro substituents					
1l	o-Cl	H	3610	–	–
1m	H	o-Cl	3615	–	–
1n	o-Cl	o-Cl	3615	–	–

the registered band, and furthermore the absorbance (A) of each absorption, is uncertain [8] and must be used with caution. No "Curve Resolver" was available.

DISCUSSION

The IR absorption in the O–H stretching region of benzyl alcohol in dilute solution is usually interpreted as being contributed to by two conformers of the alcohol: one with a free hydroxyl group and one with a hydrogen bond to the π-bond system.[3-5] The spectrum (Table 1) shows the OH band of the hydrogen bonded conformer to be 20 cm^{-1} below that of the free conformer. This is a shift of the same magnitude as that between the two rotamers A and B of ethanol [8-9] (9 – 17 cm^{-1}) and it has been questioned if the band at 3615 cm^{-1} in benzyl alcohol is really due to a hydrogen bonded species.[10]

A B

From Table 1 it is evident that there are no strong internal hydrogen bonds in any of the studied benzyl alcohols. Of the three *ortho* substituted benzyl alcohols, *o*-methoxy-, *o*-nitro-, and *o*-chlorobenzyl alcohol; the methoxy and chloro compounds have ν_2 in the same position as in the corresponding *m*- or *p*-compounds. The *o*-nitrobenzyl alcohol has a rather strong band at 3600 cm^{-1}, 15 cm^{-1} lower than that of *m*-nitrobenzyl alcohol. From these frequencies, it seems probable that there is no or only a very weak hydrogen bond to the *ortho* substituents in *o*-methoxybenzyl alcohol and *o*-chlorobenzyl alcohol. For *o*-nitrobenzyl alcohol there appears to be a possibility of a hydrogen bond to the *o*-nitro group as judged from ν_2.

Judging from the spectral shifts of phenol or methanol in CCl_4 solutions containing nitrobenzene or anisole, the ether oxygen seems to give rise to stronger hydrogen bonds than the nitro group.[6] Further, a six membered ring would be formed by internal hydrogen bonding in *o*-methoxybenzyl alcohol and a seven membered ring in the *o*-nitrobenzyl alcohol. A six membered ring is usually favoured over a seven membered one both from enthalpy and entropy points of view. Both these circumstances would be expected to favour the formation of an internal hydrogen bond in *o*-methoxybenzyl alcohol as compared to *o*-nitrobenzyl alcohol.

There are, however, two other factors which would favour formation of the internal hydrogen bond in *o*-nitrobenzyl alcohol. One is the electron withdrawing property of the nitro group as opposed to the electron donating ability of the methoxy group. This would make the hydroxyl proton in *o*-nitrobenzyl alcohol more acidic than that in *o*-methoxybenzyl alcohol, and thus the nitrobenzyl alcohol a better hydrogen donor than the methoxybenzyl alcohol.

The other factor is of steric nature. The strength of some types of hydrogen bond is dependent on the angle O–H···O with maximum strength for an angle of 180°.[7] The angle O–H···O may be larger in the seven membered ring of *o*-nitrobenzyl alcohol than in the six membered one of *o*-methoxybenzyl alcohol, thus favouring the internal hydrogen bond in *o*-nitrobenzyl alcohol over the one in *o*-methoxybenzyl alcohol. These points will be further discussed in the following section on 1,2-diarylethanols.

The studied 1,2-diarylethanols (*1a – 1n*) can be divided into two groups, according to whether the *ortho* substituent is present in the 1-aryl or in the 2-aryl ring. The OH stretch absorptions of 1,2-diphenylethanol (*1a*) and of 1,2-di-*o*-substituted arylethanols (*i.e. 1e* and *1j*) are also reported in Table 2.

The substances with *o*-X-substituents (*i.e. 1b, f, h, l*) have IR absorption in the OH region (Table 2) similar to the corresponding *ortho* substituted benzyl alcohols (Table 1); *i.e.* *o*-methoxy-, *o*-nitro-, and *o*-chlorobenzyl alcohol, respectively. Some points are nevertheless worth of discussion.

No band at 3640 cm^{-1} could be detected for 1-(*o*-methoxy)-2-phenylethanol (*1b*), thus indicating a larger proportion of hydrogen bonded conformer than for *o*-methoxybenzyl alcohol. Replacing a hydrogen of *o*-methoxybenzyl alcohol with a benzyl group would slightly decrease the proton donor ability of the hydroxyl group. This electronic effect can therefore not be the reason for the decrease of the contribution of the free conformer.

In *1b* there is a possibility of hydrogen bonding to the 2-phenyl ring (*i.e.* IV) in addition to the 1-aryl ring (III). This may explain the apparent absence of the free conformer (V). However, the larger CH_2-Ph group placed on the hydroxyl bearing carbon may have severe consequences for the population of the various conformers, and may give increased importance to the one with hydrogen bonding to the 1-aryl ring (III) as compared to *o*-methoxybenzyl alcohol. This substitution at the hydroxyl bearing carbon may also result in a large portion of conformer I with a hydrogen bond to the *o*-methoxy group. The shoulder at 3590 cm^{-1} may be interpreted as a band of this species.

The result from the 2-aryl-1-(*o*-nitrophenyl)-ethanols (*1f*, *h*) have some bearing on these problems. 1-(*o*-Nitrophenyl)-2-phenylethanol (*1f*) has a relatively broad absorption centered at 3610 cm^{-1}, and no observable band at 3640 cm^{-1}. The IR absorption thus gives no indication of a free hydroxyl group (*i.e.* no V). The same possibilities exist for hydrogen bonding in *1f* as in *1b: i.e.*, to the aryl rings and to the *ortho* substituent. From the discussion above on the methoxy case and from the results from substances *1f* and *1h*, some indications of the preferred acceptor site of the hydrogen bond may be obtained. Both in *1f* and *1h* there may exist a hydrogen bond to the *o*-nitro group. However, the IR spectra (Table 2b) indicate that such bonds are not stronger and do not account for a larger proportion of the conformers than in *o*-nitrobenzyl alcohol. This type of hydrogen bond therefore seems not to be predominant in substances with *o*-X-substituents, at least not with $X=NO_2$. Further, the frequency of the main peak in *1f* is at 3610 cm^{-1} and that of *1h*, where a *p*-nitro group has been substituted in the 2-phenyl ring, is at 3625 cm^{-1}. This is the expected direction of the frequency shift if an important conformer of *1f* was one with hydrogen bonding to the 2-phenyl ring (*i.e.* IV). By substituting a *p*-nitro group into the 2-ring, a weaker bond to this ring may be expected,[6] resulting in an IR absorption at a higher frequency. The proportion of this conformer is probably determined largely by steric effects, as a hydrogen bond giving rise to an absorption near 3600 cm^{-1} in any case is rather weak. The results from the substances with *o*-X-substituents thus indicate the conformer IV with hydrogen bonding to the 2-aryl ring to be of importance in this group. This conclusion is in accordance with the one of v. R. Schleyer *et al.* on the hydrogen bonding in 2-arylethanols,[5] and is confirmed by the spectrum of 1,2-di(*p*-nitrophenyl)ethanol (*1k*) (Table 2b) which shows a single band at 3620 cm^{-1}. The band at 3635 cm^{-1} in the spectrum of *p*-nitrobenzyl alcohol indicates a large fraction of non-bonded conformer. The absence of such a band in the spectrum of 1,2-di(*p*-nitrophenyl)ethanol shows the importance of hydrogen bonding to the 2-aryl ring.

The substances with *o*-Y-substituents (*1c*, *d*, *g*, *i*) give a different IR absorption pattern from the ones discussed above, as all four substances have a rather broad band in the region 3550–3560 cm^{-1}.

This is a larger frequency shift than those discussed above and indicates stronger hydrogen bonds than those found with the compounds with *o*-X-substituents and for the benzyl alcohols in Table 1.

The only possible reason for these relatively strong hydrogen bonds must be that the *ortho* substituent (Y) in the 2-aryl ring is acting as a hydrogen bond acceptor (conformer II). From the discussions above on benzyl alcohols and diarylethanols with *o*-X-substituents no such strong hydrogen bonds could be expected to the 1-or 2-aryl rings (*i.e.* conformers III and IV).

The reason for the stronger hydrogen bond to the *o*-Y-substituents as compared with the *o*-X-substituents must be a more favourable geometry for the hydrogen bond in conformer II than in I. For 1-aryl-2-(*o*-methoxyphenyl)ethanols (*1c*, *d*) this conformer results in a seven-membered ring, and for 1-aryl-2-(*o*-nitrophenyl)-ethanols (*1g*, *i*,) in an eight-membered ring. In both cases one would expect a larger $O-H\cdots O$ angle (closer to 180°) than for the corresponding 1-*o*-substituted arylethanols or *o*-substituted benzyl alcohols, where six- and seven-membered rings, respectively, results from hydrogen bonding to the *o*-substituents.

These results may thus explain in part, why *o*-nitrobenzyl alcohol and not *o*-methoxybenzyl alcohol has a hydrogen bond to the *ortho* substituent (see above). When the methoxy group is part of a seven membered ring system (*e.g. 1c*) it becomes part of an even stronger hydrogen bond than the nitro group in *o*-nitrobenzyl

alcohol does. When the nitro group is involved in an eight-membered ring system, (*e.g. 1g*) a stronger hydrogen bond results than for the seven-membered ring system. The results thus stress the importance of the ability of the angle O – H···O to approach 180°.

From the intensity ratios A_1/A_2 it is nevertheless indicated that the conformer II may not be of great percentage for the species discussed so far.

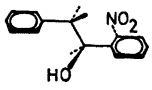
A different picture emerges when one inspects the spectra of 1,2-di(*o*-methoxyphenyl)-and 1,2-di(*o*-nitrophenyl)ethanol (*1e* and *1j*). These two compounds provide a relatively broad band in the region 3540 – 3560 cm^{-1} of the spectra, indicating a hydrogen bond to the *ortho* substituent in the 2-aryl ring. However, for these two compounds, the band in this region has a larger intensity than those discussed above, indicating a relatively larger proportion of the conformer II.

The reason for this might be the changed electron density in the 1-aryl ring as compared to the substances discussed above. In *1j* with a nitro substituent in the 1-aryl ring, this might give conformer III diminished importance, thus increasing the relative importance of conformer II. However, in substance *1i*, there is a *p*-nitro group in the *1*-phenyl ring which would give approximately the same electron density in this ring as in substance *1j* with two *o*-nitro groups. Nevertheless, the spectrum of *1i* indicates a far smaller proportion of conformer II than that of compound *1j*.

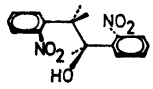
Changes in the electron density of the 1-ring can thus not be invoked to explain the increase of conformer II in compound *1j* as compared to *1g* and *1i*. For 1,2-di-(*o*-methoxyphenyl)ethanol the change in electron density in the 1-aryl ring cannot be the explanation for the increase in importance of conformer II. In this case, the 1-aryl ring was made a better hydrogen acceptor by the introduction of the *o*-methoxy group, and should thus have decreased the importance of conformer II as compared to *1c* and *1e*.

An explanation of the apparently increased importance of conformer II for the di-*o*-substituted compounds *1e* and *1j* therefore has to be sought in the changed steric conditions.

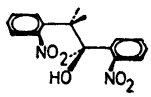
In the solid state, X-ray studies by Sivertsen [12] showed that for compounds *1f* and *1j* the hydroxyl group is placed *transoid* to the *o*-nitro group at the 1-aryl ring.



1f



1j



1j

Further, in *1j*, the nitro-group in the 2-aryl ring is placed *cisoid* to the hydroxyl group.

Inspection of models suggests that these conformations are important even in dilute solution. Except for a small hydrogen bonded portion, the *o*-nitro group of the 1-aryl ring in both *1f* and *1j* will probably be *transoid* to the hydroxyl group. For *1j*, this has consequences for the conformation of the 2-aryl group as there will be repulsion between the two nitro groups if these are in the *cisoid* conformation. The conformation with the nitro groups *transoid* to each other will therefore probably be preferred in solution as well as in the solid state. This means that the hydroxyl group and the nitro group in the 2-aryl ring will be *cisoid* to each other, favourable for hydrogen bonding, and resulting in an increase in conformation II for substance *1j* as compared to compound *1g*. The same type of argument may be used to explain the increased portion of conformer II in 1,2-di(*o*-methoxyphenyl)ethanol (*1e*) as compared to 1-phenyl-2-(*o*-methoxyphenyl)ethanol (*1c*).

This reasoning is based on the assumption that the proportion of various conformers is determined by the steric relations in the molecule and not by the presence of the hydrogen bond. This assumption is not unreasonable when one remembers that a hydroxyl group stretch frequency of 3540 cm^{-1} probably corresponds to an enthalpy of the hydrogen bond of only a few kcal/mol.[7]

The chlorosubstituted 1,2-diarylethanols did not give any indications of hydrogen bonding to the chlorine atoms as shown by the IR data (Table 2c), although a hydrogen bond to the

2-aryl group probably is as important for these alcohols as for those discussed above.

In conclusion, the hydrogen bonds to the *ortho* substituent in *o*-chlorobenzylalcohol and *o*-methoxybenzylalcohol are either non-existent, or of the same strength as those to the π-bond system. In *o*-nitrobenzyl alcohol on the other hand such a hydrogen bond is not excluded. The same is the case with the corresponding 1-(*o*-X-phenyl)-2-arylethanols (*1*). With 1-aryl-2-(*o*-Y-phenyl)ethanols, there exists a relatively strong hydrogen bond to the *o* substituent (Y) in the 2-aryl ring when the substituent is nitro or methoxy. The populations of the various conformers seem to be mainly determined by steric effects and not by the presence or absence of a hydrogen bond.

EXPERIMENTAL

The infrared spectra were recorded in dilute CCl_4 solution. The CCl_4 was dried by refluxing over P_2O_5 and pipetted on to the sample. The spectra were run in 1 cm quartz cells or a 10 cm cell equipped with NaCl windows. The spectra were recorded on a Perkin-Elmer infrared spectrophotometer, Model 457. The resolution of the instrument is 4 cm^{-1} in the OH stretch region. The spectra were recorded in the slow scanning mode, with a minimum slit opening. The various benzyl alcohols studied were all available commercially. The synthesis of the substances *1g*, *1j*, and *1k* have been reported earlier.[13,14]

1-(o-Nitrophenyl)-2-phenylethanol (1f). This substance was made from *o*-nitrostilbeneoxide by refluxing with *p*-toluenesulfonic acid followed by $NaBH_4$ reduction of the resulting ketone. This procedure is analogous to the one used for the synthesis of 1-(*p*-nitrophenyl)-2-phenylethanol.[15] *1h* had m.p. 76–77 °C, IR (KBr): 3350 (broad), 1525, 1340, 1040, 750, 700, 545 cm^{-1}. NMR ($CDCl_3$): δ 7.2–8.2 (9 H, m, aromatic H), 5.3–5.6 (1 H, X part of ABX pattern, $-CHOH-CH_2-$), 2.5–3.4 (2 H, AB part of ABX pattern, $-CHOH-CH_2$), 2.3 (1 H, s, $-OH$).

The synthesis of 1-(*o*-nitrophenyl)-2-(*p*-nitrophenyl)ethanol (*1h*) and 1-(*p*-nitrophenyl)-2-(*o*-nitrophenyl)ethanol, (*1i*) will be described elsewhere.[16]

General procedure for the preparation of chloro and methoxy substituted 1,2-diarylethanols. All the chloro and methoxy derivatives of 1,2-diphenylethanol were prepared by Grignard reactions. The reaction mixture was poured onto a mixture of ice and acetic acid. The ether layer was separated and washed with 10 % sodium bicarbonate solution and water and dried over potassium carbonate. The solvent was removed under reduced pressure and the product was purified by various methods.

1,2-Di(o-methoxyphenyl)ethanol (1e) was synthesised by Grignard reaction of *o*-methoxybenzyl chloride and *o*-methoxybenzaldehyde. *1e* had m.p. 80–82 °C, IR (KBr): 3400, 1605, 1590, 1500, 1470, 1440, 1250, 1190, 1110, 1055, 1040, 755, 740 cm^{-1}. NMR ($CDCl_3$): δ 6.8–7.5 (8 H, m, aromatic H), 5.23 (1 H, t, J=6Hz, $-CHOH-CH_2-$), 3.84 (3 H, s, $-OCH_3$), 3.80 (3 H, s, $-OCH_3$), 3.14 (2 H, d, J=6Hz, $-CHOH-CH_2-$), 2.9 (1 H, s, $-OH$).

2-(o-Methoxyphenyl)-1-phenylethanol (1c) was made by Grignard reaction from *o*-methoxybenzyl chloride and benzaldehyde and gave colourless prisms, m.p. 66–68 °C, on crystallization from ligroin/diethyl ether. NMR ($CDCl_3$): δ 2.53 (1 H, s, $-OH$, exchanged by D_2O), 2.9–3.15 (2 H, deformed d, $-CHOH-CH_2-$), 3.76 (3 H, s, $-OCH_3$), 4.75–5.1 (1 H, deformed t, $-CHOH-CH_2-$), 6.65–7.45 (9 H, broad m, aromatic H). Mass spectrum *m/e:* 228 (M^+), 122, 102, 91, 79.

1-(p-Methoxyphenyl)-2-(o-methoxyphenyl)-ethanol (1d) was made by Grignard reaction from *o*-methoxybenzyl chloride and *p*-methoxybenzaldehyde. 1-(*p*-Methoxyphenyl)-2-(*o*-methoxyphenyl)ethanol crystallized from ligroin/chloroform as tiny, white needles, m.p. 61–62 °C. NMR ($CDCl_3$): δ 2.45 (1 H, s, $-OH$, exchanged with D_2O), 2.9–3.1 (2 H, deformed d, $-CHOH-CH_2-$), 3.73 (3 H, s, $-OCH_3$, 3.76 (3 H, s, $-OCH_3$), 4.7–5.05 (1 H, deformed t, $-CHOH-CH_2-$), 6.7–7.35 (8 H, broad m, aromatic H).

1-(o-Chlorophenyl)-2-phenylethanol (1l) was prepared by Grignard reaction from benzyl chloride and *o*-chlorobenzaldehyde. Recrystallization from ligroin/chloroform gave 1-(*o*-chlorophenyl)-2-phenylethanol as colourless short prisms, m.p. 70–72 °C. NMR ($CDCl_3$): δ 2.62 (1 H, broad s, $-OH$, exchanged with D_2O), 2.8–3.0 (2 H, deformed d, $-CH(OH)-CH_2-$), 5.05–5.35 (1 H, m, $-CH(OH)-CH_2-$), 6.4–7.35 (9 H, m, aromatic H), IR (KBr): 3150–3500 (broad), 3015, 2950, 1600, 1590, 1500, 1040, 700–780 cm^{-1} (several bands).

2-(o-Chlorophenyl)-1-phenylethanol (1m) was prepared from *o*-chlorobenzyl chloride and benzaldehyde. Recrystallization of the product from ligroin/chloroform yielded 1-phenyl-2-(*o*-chlorophenyl)ethanol, m.p. 68–70 °C. NMR ($CDCl_3$): δ 2.48 (1 H, broad s, $-OH$, exchanged with D_2O), 2.9–3.15 (2 H, deformed d, $-CH(OH)-CH_2-$), 4.7–4.95 (1 H, deformed t, $-CH(OH)-CH_2-$), 6.95–7.35 (9 H, m, aromatic H). IR (KBr): 3200–3500 (broad, $-OH$), 3015, 2945, 1600, 1580, 1480 1030, 680–780 cm^{-1} (several bands).

1,2-Di-(o-chlorophenyl)ethanol (1n) was prepared from *o*-chlorobenzyl chloride and *o*-chlorobenzaldehyde. This yielded a pale yellow crystalline mass, which on recrystallization from ligroin/chloroform gave 1,2-di-(*o*-chlorophenyl)-ethanol as colourless cubes, m.p. 82–84 °C.

NMR ($CDCl_3$): δ 2.22 (1 H, broad s, $-OH$, exchanged with D_2O), 2.8–3.45 (2 H, AB-part of an ABX-system, $-CH(OH)-CH_2-$), 5.25–5.55 (1 H, X-part of an ABX-system, $-CH(OH)-CH_2-$), 7.05–7.6 (8 H, m, aromatic H). IR (KBr): 3500–3100 (broad), 3050, 2940, 1695, 1675, 1480, 1035, 765 cm^{-1}.

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Mass Spectra of *N*-Phosphorylated Imino Compounds

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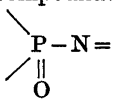
The mass spectra of diethyl phosphor(isocyanatidate) (I), diethyl phosphor(isothiocyanatidate) (II), diethyl phosphoro(thionylamidate) (III), diethyl *N*,*N*-dichlorophosphoramidate (IV), and diethyl phosphorazidate (V) have been recorded and interpreted with the aid of high resolution measurements and the metastable defocusing technique.

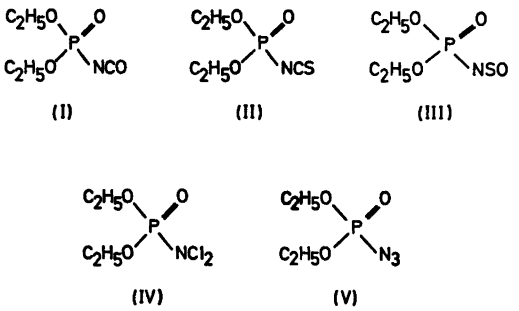
Characteristic for the fragmentations are P—N bond cleavage and degradation in the ethoxy groups. A general fragmentation scheme for the compounds is given.

Recently we have described the general fragmentation pattern of various esters of phosphoramidic acid $(RO)_2P(O)NH_2$.[1]

Continuing our mass spectrometric investigations of phosphorus compounds containing a P—N bond, we have studied some compounds in which the phosphinylimino system  is present. The purpose of this investigation is to examine to what extent mass spectrometry can be used for identification of these types of compounds, and to compare the degradation patterns of *N*-phosphorylated imino compounds with nitrogen connected to different groups.

We shall discuss here the mass spectra of the following compounds: Diethyl phosphor(isocyanatidate) (I), diethyl phosphor(isothiocyanatidate) (II), diethyl phosphoro(thionylamidate) (III), diethyl *N*,*N*-dichlorophosphoramidate (IV), and diethyl phosphorazidate (V).

The mass spectra of the *N*-phosphorylated imino compounds investigated are characterised by fragmentation in the ethoxy groups in agreement with the findings for phosphoramidic acid esters.[1] In addition to these fragmentations,



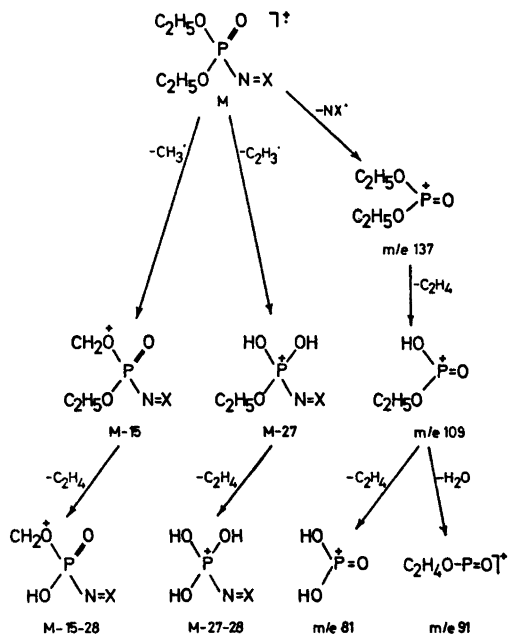
P—N bond cleavage is of importance for the degradation of the molecular ion for all the compounds investigated.

P—O bond breaking from the molecular ion is seen for all compounds except diethyl *N*,*N*-dichlorophosphoramidate, but in all cases the fragments formed are of lower intensity than for the corresponding fragments in diethyl phosphoramidic acid ester.

A generalized fragmentation scheme is given as Scheme 1 (X: C=O, C=S, S=O, Cl_2, or N_2). The P—O bond breaking (loss of C_2H_5O and C_2H_4O) is omitted from the scheme. Variations from the scheme will be mentioned in the discussion.

DISCUSSION

Diethyl phosphor(isocyanatidate) (I) fragments in accordance with the general scheme with the only exception that it splits off HNCO instead of NCO from the molecular ion. No fragmentations take place in the NCO part of the molecule which is in accordance with findings for aliphatic isocyanates.[2]



Scheme 1.

Diethyl phosphor(isothiocyanatidate) (II) fragments similarly to the isocyanate (I), but the ion M − HNCS is of low intensity whereas M − NCS is abundant. The formation of $HNCS^{+\cdot}$ (*m/e* 59) which is general for aliphatic isothiocyanates [8] is also of importance for this phosphor(isothiocyanatidate).

Diethyl phosphoro(thionylamidate) (III) shows no peak at *m/e* 137 corresponding to loss of NSO from the molecular ion, but a metastable peak at *m/e* 94.3 indicates that the P − N bond is cleaved. Furthermore *m/e* 137 is shown by the defocusing technique to be precursor for *m/e* 109 [$C_2H_5OP(O)OH$], so the fragmentation of this thionyl compound is in accordance with the general scheme. No cleavage in the NSO group like loss of SO, as reported for the aromatic thionylamines [4] or loss of SO or HSO as reported for the aliphatic thionylamines [5] is observed. Another degradation involving complex rearrangements results in the formation of the base peak *m/e* 73 with the composition C_2H_3NS.

Diethyl *N,N*-dichlorophosphoramidate (IV) follows only one of the three degradation routes given in scheme 1, namely P − N bond cleavage followed by degradation in the ethoxy groups. It is remarkable that no fragmentation in the ethoxy groups is observed from the molecular ion as this degradation is of importance for both the imino compounds investigated and for diethyl phosphoramidate.[1]

Diethyl phosphorazidate (V) fragments according to the general scheme following all the three routes. No loss of N_3 from the molecular ion like the "normal" azide degradation [6] is observed. This is in accordance with results found for sulphonylazides [7] where loss of N_3 is shown to be of minor importance.

Loss of N_3 is observed from *m/e* 124 and *m/e* 106.

Table 1. Exact mass measurements.

Compound	*m/e*	Composition	Compound	*m/e*	Composition
I	124	CH_3NO_4P	III	73	C_2H_3NS
	136	$C_2H_3NO_4P$		81	H_2O_3P
	124	CH_3NO_4P		99	H_4O_4P
				109	$C_2H_6O_3P$
				126	HNO_3PS
II	59	CHNS	V	81	H_2O_3P
	81	H_2O_3P		91	$C_2H_4O_2P$
	91	$C_2H_4O_2P$		106	$C_2H_3O_3P$
	109	$C_2H_6O_3P$		109	$C_2H_6O_3P$
	122	$CHNO_3PS$		124	$H_3N_3O_3P$
	137	$C_4H_{10}O_3P$		137	$C_4H_{10}O_3P$
	151	$C_3H_6NO_2PS$			

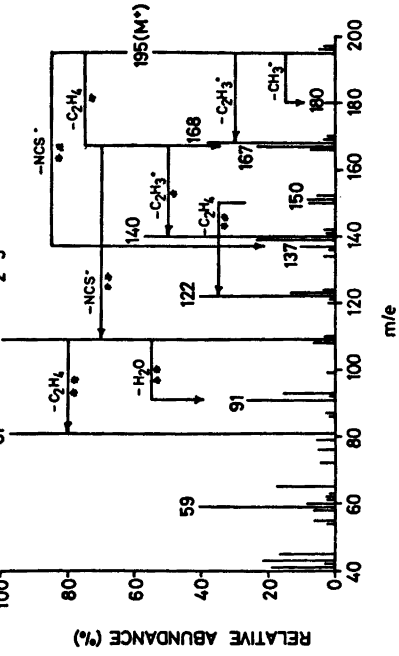


Fig. 2. Mass spectrum of diethyl phosphor(isothiocyanatidate) (II).

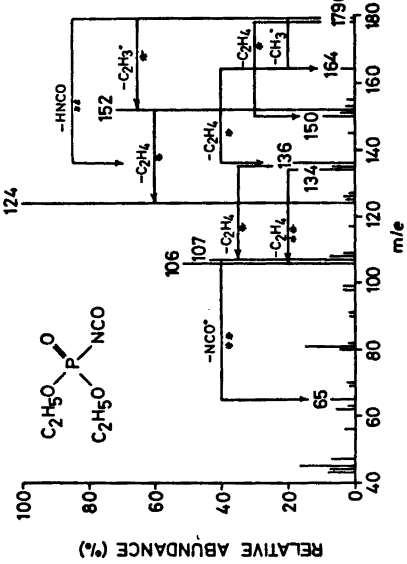


Fig. 1. Mass spectrum of diethyl phosphor(isocyanatidate) (I).

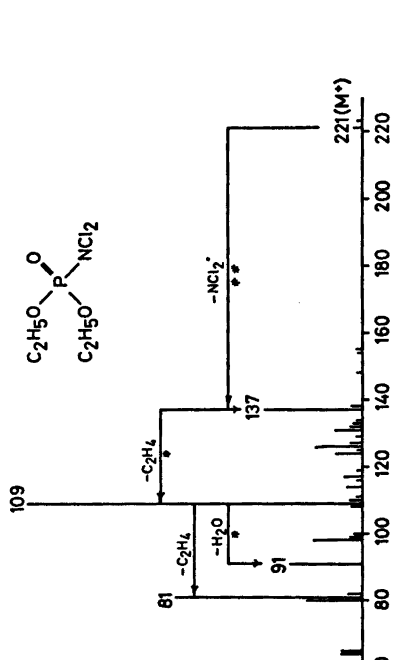


Fig. 4. Mass spectrum of diethyl *N,N*-dichlorophosphoramidate (IV).

Fig. 3. Mass Spectrum of diethyl phosphoro(thionylamidate) (III).

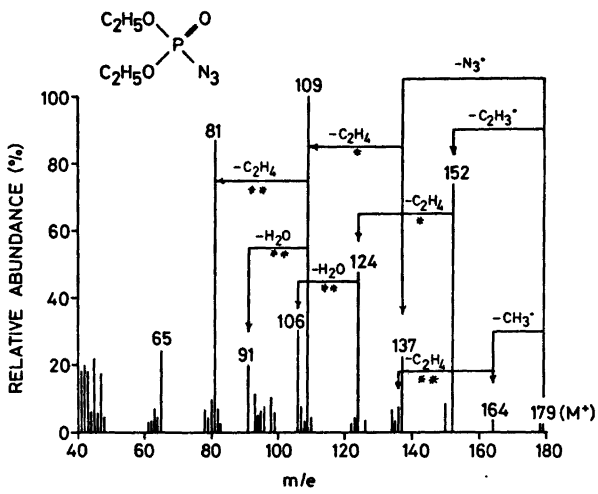


Fig. 5. Mass spectrum of diethyl phosphorazidate (V).

CONCLUSION

As is evident from the discussion above electron impact induced fragmentations of the phosphinylimines investigated are dominated by degradations in the ethoxy groups, namely loss of C_2H_4, C_2H_3· and CH_3·. In addition P – N bond cleavage in the molecular ion is of importance. The phosphinylimines follow, with minor exceptions, three main degradation routes (Scheme 1), a fact which might be useful for characterisation of similar compounds.

EXPERIMENTAL

The mass spectra were recorded on an AEI MS-902 mass spectrometer. The samples were introduced through the heated glass inlet system below 100 °C. All the decompositions given are, unless otherwise noted, supported by accompanying metastable peaks or verified by metastable defocusing (indicated by two asterisks in the figures). The elemental compositions of all characteristic ions have, when necessary, been determined by high resolution mass measurements (Table 1).

The measurements were performed on analytically pure compounds.

Diethyl phosphor(isocyanatidate) (I) was prepared by the action of oxalyl chloride on diethyl phosphoramidate.[8] B.p. 95 °C/15 mmHg, n_D^{20} 1.4170, yield 62 %.

Diethyl phosphor(isothiocyanatidate) (II) was synthesized from the corresponding phosphorochloridate and potassium thiocyanate in acetone solution.[9] B.p. 58 °C/0.12 mmHg, n_D^{20} 1.4795, yield 60 %.

Diethyl phosphoro(thionylamidate) (III) was obtained by the action of thionyl chloride on diethyl phosphoramidate.[10] B.p. 76 °C/0.1 mmHg, n_D^{20} 1.4578, yield 72 %.

Diethyl *N,N*-dichlorophosphoramidate (IV) was prepared by chlorination of the acetate-buffered aqueous solution of diethyl phosphoramidate.[11] B.p. 59 °C/0.03 mmHg, n_D^{20} 1.4622, yield 83 %.

Diethyl phosphorazidate (V) was prepared from diethyl phosphorochloridate and sodium azide in acetone solution according to Scott *et al.*[12] B.p. 70 °C/4 mmHg, n_D^{20} 1.4268, yield 85 %.

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Production of Carbohydrate by the Marine Diatom *Chaetoceros affinis* var. *Willei* (Gran) Hustedt. III. Structural Studies of the Extracellular Polysaccharide

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Methylation and periodate oxidation studies have been carried out on the extracellular polysaccharide from cultures of *Chaetoceros affinis* var. *Willei* (Gran) Hustedt. It is apparent from the results obtained that the polysaccharide is highly branched. The results from periodate oxidation concomitant with the methylation studies indicate that the main part of the rhamnose is located in the outer part of the molecule, whereas the fucose and galactose are present both in the outer and inner parts. Apart from being present as end groups the rhamnose is 1,2-linked, galactose 1,4- and 1,3- (major) and some of the fucose 1,3-linked while the main part is present at branch points.

It is well known that many planktonic algae excrete considerable amounts of organic material into the surrounding medium, and that this material in many cases at least partly consists of carbohydrates. Very little is known about the nature of these carbohydrates. Recently, the production of soluble, extracellular polysaccharides has been reported from several diatom species. Allan *et al.*[1] studied eight species, and found significant amounts of soluble polysaccharide in the medium in all cases. The amounts were very small (less than 5 mg/l or 0.2 mg per 10^8 cells), with the exception of one species, *Nitzschia frustulum*, which in standard one-liter culture in enriched seawater produced 15.6 mg/l (1.3 mg per 10^8 cells) in 7 days, and in mass cultures over two weeks were reported to give as much as 150 mg/l (9 mg per 10^8 cells), depending upon conditions like salinity and nutrient levels. Hydrolysis of the polysaccharide gave rhamnose (24 %), mannose (34 %), galactose (8 %), and two unidentified components (14 and 20 %). The composition of the polysaccharide depended upon salinity.

In a study of *Chaetoceros affinis* (clone CH 1), Myklestad and Haug [2] reported the production of an extracellular polysaccharide in amounts of 16 – 40 mg/l (10 – 20 mg per 10^8 cells). The production took place in the stationary growth phase, after the main production of glucan was finished. Later studies [3] have shown that two other *Chaetoceros* species, *C. curvisetus* (CH 24) and *C. decipiens* (CH 40), also produce substantial amounts of extracellular polysaccharides, while in a number of other species, only very small amounts of extracellular polysaccharide can be detected. Both the amounts and the composition of the extracellular polysaccharide strongly suggest that in the case of the three *Chaetoceros* species mentioned above, the polysaccharide is excreted into the medium, and that it is not a case of leakage from dead or dying cells. The function of the polysaccharide in the life of the diatom is unknown. These extracellular polysaccharides all gave fucose, rhamnose, galactose, and sulfate on hydrolysis.

The present paper describes structural studies of the extracellular polysaccharide produced by *Chaetoceros affinis*.

RESULTS AND DISCUSSION

The cultivation of the diatom and isolation of the extracellular polysaccharide has been de-

Table 1. Composition of polysaccharide before and after periodate oxidation.

	Carbohydr. content [a] mg	Wt. ratio rha:fuc:gal	Am. of each sugar; mg (calculated)
Start. material	35.0	35:39:26	12.2:13.6:9.1
Prod. after 1st. periodate oxidation	15.8	12:64:24	1.9:10.1:3.8
Prod. after weak acid hydr.	13.1	7:68:25	0.9:9.0:3.3
Prod. after 2nd. periodate oxidation	8.5	4:75:21	0.3:6.4:1.8

[a] Determined by the phenol-sulfuric acid method.[5]

scribed previously.[2,4] The polysaccharide had $[\alpha]_D = -74°$, a carbohydrate content [5] of 80 %, $-SO_3Na$ was 8.9 % [6] corresponding to an equivalent weight of 1157. Potentiometric titration of the polysaccharide gave an equivalent weight of 850–900, which, if the only anion present is sulfate, was calculated to correspond to 11.4–12.1 % sulfate. The polysaccharide moved as a single, anionic compound in free-boundary electrophoreses both at pH 2 and pH 7, with approximately the same mobility at both pH values.[4]

Analysis for phosphate gave negative results, but small amounts of protein (1.5 % of the sample) were present.[4]

Complete acid hydrolysis of the polysaccharide and analysis by GLC of the derived alditol acetates [7] showed the presence of rhamnose, fucose, and galactose in the proportions 35:39:26 (Table 1). No other sugars were detected. The presence of small amounts of arabinose and traces of other sugars reported previously [4] was most probably due to contamination.

Periodate oxidation [8] was carried out as described under "Experimental". The polysaccharide reduced 0.59 mol of periodate per C_6-anhydro-unit (Table 2). Complete acid hydrolysis of the derived polyalcohol and analysis of the derived alditol acetates by GLC showed that the sugars rhamnose, fucose, and galactose were present in the polyalcohol in the proportions 12:64:24. From Table 1 it can be seen that the periodate cleaved 87.5 % rhamnose, 25.7 % fucose and 58.3 % galactose of the total of the respective sugars in the starting material.

After weak acid hydrolysis followed by dialysis and another periodate oxidation, the polymer reduced 0.36 mol of periodate per C_6-anhydro-unit (Table 2).

Table 1 shows that of the original carbohydrate content of the polysaccharide, 24 % carbohydrate still remains after the second periodate oxidation. This polyalcohol contains the sugars rhamnose, fucose, and galactose in the proportions 4:75:21, which is equivalent to 2.5, 47, and 19.8 % left of the respective sugars present in the starting material.

These results show that most of the rhamnose is vulnerable to periodate oxidation, and must be present mainly in the outer part of the

Table 2. Periodate consumption.

Periodate oxidation	Wt. carbohydr.[a] mg (mmol)	$NaIO_4$ added mg (mmol)	Volume acetate buffer pH 4 ml	Periodate consumption mol IO_4/C_6-anhydr.
1st. oxidation	35.0 (0.22)	106.5 (0.50)	10	0.59
2nd. oxidation	13.1 (0.08)	33.5 (0.16)	10	0.36

[a] Determined by the phenol-sulfuric acid method.[5]

Table 3. GLC analysis of the methylated alditol acetates.

	Retention time[a] Column 1	Column 2	Area of peaks % of total Column 2
2,3,4-Tri-*O*-methylrhamnose	0.42	0.46	12.8
2,3,4-Tri-*O*-methylfucose	0.64	0.59	4.4
3,4-Di-*O*-methylrhamnose	0.91	0.87	17.0
2,4-Di-*O*-methylrhamnose	[b]	0.93	4.3
2,4-Di-*O*-methylfucose	1.12	1.01	4.6
2,3,4,6-Tetra-*O*-methylgalactose	1.26	1.15	7.6
2-*O*-Methylrhamnose	1.55	1.35	1.2
2-*O*-Methylfucose	1.65	1.41	5.0
4-*O*-Methylfucose	2.08	1.71	11.2
Fucose	2.20	1.90	16.2
2,4,6-Tri-*O*-methylgalactose	2.20	1.96	10.6
2,3,6-Tri-*O*-methylgalactose	2.45	2.10	5.0
Di-*O*-methylgalactose	3.60	2.70	small

[a] The retention times are given relative to 2,3,4,6-tetra-*O*-methyl-D-glucitol 1,5-diacetate. [b] Hidden in the neighbouring peaks.

polysaccharide molecule. Had it been present in the interior part, smaller fragments containing considerable amounts of carbohydrate would have passed through the dialysis bag during dialysis; this did not take place.

Methylation studies. Both the original polysaccharide and the product after the second periodate oxidation were methylated, [9] hydrolysed and converted into the partially methylated alditol acetates.[7] The mixtures were analysed on GLC (columns 1 and 2), and the identification of the products, confirmed by mass-spectrometry, is presented in Tables 3 and 4. The retention times obtained correspond quite well with those obtained by Lönngren and Pilotti.[10] For the derivatives of the original polysaccharide, the areas under the peaks are given in Table 3, allowing a semiquantitative estimation of the relative amounts of the methylated derivatives.

The methylation results show that all three sugars are present as end groups, rhamnose being responsible for the main part, followed by galactose and fucose as the minor part.

The rhamnose residues which are not present as end groups are mainly 1,2-linked as shown by the large proportion of 3,4-di-*O*-methylrhamnose and were accordingly vulnerable to periodate attack, in good agreement with the periodate results described above. A small proportion of rhamnose appears to be 1,3-linked and the possibility of this sugar occurring as branch points is indicated by the presence of small amounts of 2-*O*-methylrhamnose.

The galactose residues in the chain are mainly 1,3-linked, giving rise to 2,4,6-tri-*O*-methylgalactose. A smaller amount is 1,4-linked, and these residues, together with the end groups were oxidised in the first periodate treatment. The possibility of galactose forming a few branch points in the molecule is indicated by traces of di-*O*-methylgalactose.

Fucose, however, forms the main part (> 90 %) of the branch points in this poly-

Table 4. GLC analysis of the methylated alditol acetates from the product obtained after 2nd. periodate oxidation.

Identity	Retention times[a]	
2,3,4,-Tri-*O*-methylrhamnose	0.43	S[b]
2,3,4-Tri-*O*-methylfucose	0.6	S
Unknown	0.84	S
3,4-Di-*O*-methylrhamnose	0.90	S
2,4-Di-*O*-methylfucose	1.02	L
2,3,4,5-Tetra-*O*-methylgalactose	1.18	S
2-*O*-Methylrhamnose	1.35	S
2-*O*-Methylfucose	1.42	M
4-*O*-Methylfucose	1.73	L
2,4,6-Tri-*O*-methylgalactose	1.89	L

[a] See Table 3. [b] Relative size of peaks: L = large, M = medium, S = small.

saccharides, being to a large extent recovered as free fucose and 2- and 4-*O*-methylfucose. A small proportion of the fucose was recovered as 2,4-di-*O*-methylfucose, showing the presence of 1,3-linked fucose residues in the molecule.

The methylation of the polyalcohol obtained after the reduction of the product remaining after the second periodate oxidation gave 2,4,6-tri-*O*-methylgalactose and 2,4-di-*O*-methylfucose as the main products, indicating that the Smith degradation had removed some of the side chains attached to the fucose residues. The polymer was, however, still branched, indicated by the presence of mono-*O*-methylfucose.

The polysaccharide was not desulfated before methylation. No systematic investigation of the Hakomori methylation procedure on sulfated polysaccharides has been carried out, but complete methylation by this procedure of highly sulfated polysaccharides has proved impossible,[18] indicating that the methylation procedure does not lead to desulfation. The presence of free fucose and some of the methylderivatives indicating three substituents may, thus, originate from sulfated sugar residues and not from branch points. This is indicated by the results given in Table 3; the area under the peaks corresponding to free fucose and to mono-*O*-methylfucose and rhamnose and di-*O*-methylgalactose is considerably larger than the area under the peaks corresponding to end groups. The possibility of incomplete methylation should, however, also be considered. The position of sulfate is not known, but a band at 850 cm^{-1} in the IR spectrum indicates that it is present on an axial, secondary hydroxyl group.[11,12] This can be C-4 on D-galactose in $^4C_1$ conformation, C-2 on L-rhamnose in $^1C_4$, or C-4 on L-fucose in $^1C_4$ conformation. A small band at 820 cm^{-1} was also present. This indicates sulfate on a primary hydroxyl group, which, in this polysaccharide, would be C-6 on D-galactose.

The results described above show the extracellular polysaccharide produced by the diatom *C. affinis* to be a highly branched polymer of a complex structure. The rhamnose residues occur mainly in the outer part of the molecule, and the major part of the end groups of the molecule are rhamnose, although fucose and galactose also occur as end groups. The branch points in the molecule are fucose residues.

EXPERIMENTAL

Hydrolysis. The sample was dissolved in 90 % formic acid [13] and heated in a sealed tube for 6 h at 100 °C. After dilution with 5 volumes of water, the mixture was heated for another 2 h. The formic acid was removed by several evaporations to dryness after addition of methanol.

Preparation of alditol acetates (7). After hydrolysis the sugars were reduced to the corresponding alditols using $NaBH_4$. A mixture of acetic anhydride and pyridine (1 + 1) was added, followed by heating at 100 °C in sealed tubes for 1 h. 2 ml water was added and heating continued in a boiling waterbath for another 15 min. The derived alditol acetates were extracted with chloroform and analysed by GLC.

Gas liquid chromatography (GLC) of the partially methylated alditol acetates was performed on 0.3 × 200 cm stainless steel column in a Varian 1400 gas chromatograph, using 3 % ECNSS-M on Varaport-30 (column 1) at 165 °C and 0.3 × 400 cm glass column using 3.5 % OV-225 on Varaport-30 (column 2) at 185 °C.

Alditol acetates of the sugars were analysed as described by Myklestad *et al.* [4]

Carbohydrate contents were measured by the phenol-sulfuric acid method [5] using a mixture of rhamnose, fucose, and galactose in the proportions 35:39:26 as standard.

The proportions of sugars present in the hydrolysates were determined by GLC.

Sulfate content was estimated by potentiometric titration and after Antonopoulos method. [6]

IR-spectra were recorded in a Perkin-Elmer 257-spectrometer.

Dialysis was always carried out against distilled water.

The *polysaccharide* was prepared as described elsewhere.[2,4]

Periodate oxidation was performed in acetate buffer at pH 4.0, using excess periodate. The reaction was followed by withdrawing aliquots of 50 µl, which were diluted to 50 ml and OD read at 223 nm.[6] The reaction was complete after 4 h and excess periodate destroyed by adding ethylene glycol. The polyaldehyde was reduced to the corresponding polyalcohol with $NaBH_4$. Excess $NaBH_4$ was destroyed and the solution neutralized with acetic acid. The product was dialysed and freeze-dried.

Weak acid hydrolysis (1 N H_2SO_4 for 4 h at room temperature) was carried out in order to cleave acetal-linkages. The hydrolysate was neutralised with Na_2CO_3 and the final products freeze-dried after dialysis.

This polysaccharide was subjected to another periodate oxidation like the one mentioned above. The reaction was followed by withdrawing samples of 100 µl which were diluted to 25 ml. Carbohydrate contents and sugar-ratios were measured after all the above-mentioned steps.

Methylation.[14] The polysaccharide (10 mg) was freeze-dried, dried *in vacuo* over P_2O_5 overnight and dissolved in 1 ml dimethyl sulfoxide (DMSO) in a McCarthey-bottle.[9] Methylsulfinyl sodium in DMSO (1.5 ml, 2 M)[15] was added and the reaction mixture was stirred vigorously for 4 h. Methyl iodide (0.5 ml) was added while cooling, and stirring continued for another 2 h. The reaction mixture was then poured into water and dialysed against distilled water several times. The methylated product was hydrolysed, the partially methylated sugars converted into the corresponding partially methylated alditol acetates, and analysed by GLC on columns 1 and 2. The identity of the peaks were confirmed by GLC (column 2) combined with mass-spectrometry,[16] carried out on a combined GLC-MS instrument, type Varian CH7. Instrumental details are published elsewhere.[17]

Methylation of the product obtained after *the second periodate oxidation.* The polymer was methylated, converted to the partially methylated alditol acetates as described above, and analysed by GLC on column 2.

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N-Quaternary Compounds. XXXVII.[1] Syntheses and Reactions of Pyrid-2-thiones with α-Bromoacrylic Acid

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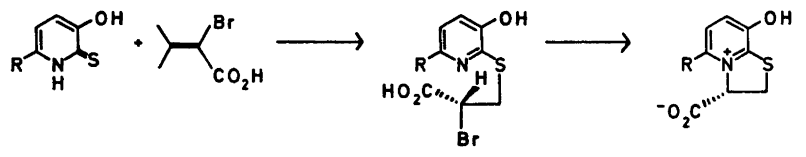
Syntheses of 6-ethyl- and 6-isopropyl-3-hydroxypyrid-2-thiones are described. The syntheses involve a series of reaction steps from a common α-picolinic acid precursor. The relative reactivities of some pyrid-2-thiones towards α-bromoacrylic acid have been investigated.

Further studies of the reaction between pyrid-2-thiones and α,β-unsaturated carbonyl compounds (Scheme 1) required for comparative purposes preparation of pyrid-2-thiones substituted in the 6-position. In previous studies the pyridine has carried a methyl group or a hydrogen atom in the 6-position.[2,3] Syntheses of the ethyl (*1c*) and isopropyl (*1d*) pyrid-2-thiones are herein described. These have been used for investigation of the relative reaction rate as Michael addends to α-bromoacrylic acid.

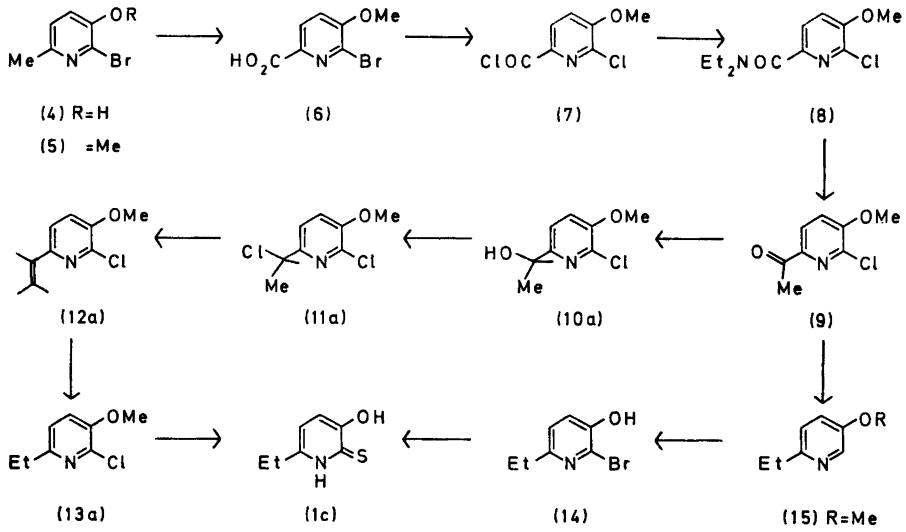
3-Hydroxypyridines are key intermediates in the syntheses of pyrid-2-thiones. Electrophilic substitution such as halogenation goes preferentially into the 2-position.[4] The halogen thus introduced is in an activated pyridine position and is replaceable by nucleophilic sulfur. In the present work 3-hydroxy-6-methylpyridine was brominated almost quantitatively in the 2-position (*4*) in aqueous HBr on addition of hydrogen peroxide. Thiation to (*1b*) was carried out by means of potassium hydrogen sulfide [4] or phosphorus pentasulfide.[5,6] In the case of the 3-alkoxy derivatives (5), choice of experimental conditions can be made such that thiation alone or thiation with concurrent cleavage of the alkoxy group occur.[6] The latter has been made use of in the below syntheses (Schemes 2 and 3).

In the syntheses of 6-substituted 3-hydroxypyridines substituted furfurals or furoic acids are convenient starting points [7] provided the desired 5-substituted furans are readily accessible. As several steps are involved in the syntheses of the desired compounds from these sources, we have instead looked at readily available pyridines. The synthesis of 3-hydroxy-6-ethylpyridine (*16*) from 3-nitro-6-chloropyridine was first repeated.[8] The chlorine was nucleophilically displaced by means of the sodium salt of diethyl methylmalonate followed by decarboxylations to 3-nitro-6-ethylpyridine. After reduction, the amino group was diazotised and the diazonium salt hydrolysed to (*16*). In our hands the diazotisation and hydrolysis proved preparatively unsatisfactory. Instead another synthetic route was worked out in which the bromo acid (*6*) (Scheme 2) is a common starting material for both the ethyl and



(1a) R=H (2) (3a) R=H
(b) =Me (b) =Me
(c) =Et (c) =Et
(d) =i-Prop. (d) =i-Prop.

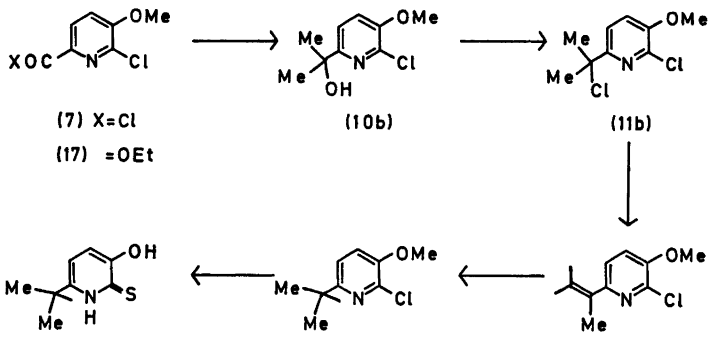
Scheme 1.



Scheme 2.

sopropyl series. For oxidation of the 6-methyl group by potassium permanganate the phenolic OH-group was protected by *O*-methylation. The choice of methyl as the *O*-protecting group was directed by the findings mentioned above that alkyl-aryl ethers are readily cleaved by the thiating agent and that the methoxy group is not affected by the reagents used in the intermediate steps. It was noticed during the preparation of the acid chloride from the acid (*6*) by means of thionyl chloride that partial halogen interchange took place in the pyridine ring. The halogen interchange was reduced but not overcome by heating the acid for a short time in thionyl chloride. The further reactions for the syntheses of the pyrid-2-thiones according to Schemes 2 and 3, may be run on products with partially interchanged halogens. For analytical purposes, however, the reactions herein described (Scheme 2) were run on the chloro compounds. Complete chlorine-bromine interchange was achieved (*7*) by heating the acid chloride of (*6*) in thionyl chloride for 4 d. Amination followed by the Grignard reaction on the amide (*8*) furnished the methyl ketone (*9*). The reaction with methylmagnesium iodide in ether was carried out at ice-bath temperature. The crude product contained about 10 % of the isopropyl alcohol (*10b*) which was removed on crystallisation. Clemmensen reduction of the ketone (*9*) resulted in hydrogenolysis of the chlorine (*15*). HBr was used to cleave the ether (*15*) and the hydroxy derivative (*16*) was brominated and thiated as described above. In a preferential alternative method the ketone (*9*) was reduced to the hydroxy derivative (*10a*) by means of sodium borohydride. Acid catalysed water elimination from (*10a*) was found difficult to effect. Instead (*10a*) was chlorinated by means of thionyl chloride. HCl elimination from (*11a*) to the vinyl pyridine (*12a*) was achieved by means of potassium *tert*-butoxide. The double bond was saturated by hydrogenation at atmospheric pressure over platinum oxide without significant hydrogenolysis of the chlorine. The thiation of the ethyl derivative (*13a*) was carried out by means of phosphorus pentasulfide under conditions which gave concurrent cleavage of the alkyl-oxygen ether linkage and formation of (*1c*).

A very similar approach to the synthesis of the isopropylpyrid-2-thione (*1d*) was used (Scheme 3) starting from the 6-carboxylic acid (*6*). The latter was initially converted to the ethyl ester (*17*) which was subjected to the Grignard reaction. This reaction required heating in ether which led to extensive dehalogenation in the pyridine ring. With the reactive acid chloride (*7*), however, the reaction proceeded smoothly at ice-bath temperature to the tertiary hydroxy compound (*10b*). As for the ethyl analogue (*10a*) acid catalysed water elimination



Scheme 3.

from (*10b*) was unsatisfactory and (*10b*) was chlorinated by means of thionyl chloride. In aqueous potassium hydroxide (*11b*) was converted to the propene (*12b*) which was selectively reduced to the isopropyl derivative (*13b*) on catalytic hydrogenation. Thiation by means of phosphorus pentasulfide finally furnished the hydroxy-thione (*1d*).

In the Michael addition of pyrid-2-thiones to α-bromoacrylic acids a methyl group in the pyridine 6-position causes a rate enhancement relative to the desmethyl analogues.[2] The overall reaction rates for the thiones (*1*) were investigated by competitive reactions in ethyl acetate with α-bromoacrylic acid in the cold. The precipitated HBr-salts of the dihydrothiazolo[3,2-*a*]pyridium products (*3*) were collected when the total yield corresponded to about 15 % and analysed by NMR (100 MHz). Slight solubility differences and partially overlapping of NMR signals made the figures obtained no more than qualitative. With reference to the standard (*1b*) the relative reaction rate for the desmethyl analogue (*1a*) was 0.3 and the rates for the other 6-substituted pyridines (*1c*, *1d*) approximately as for the standard (*1b*).

The overall reaction (Scheme 1) involves firstly adduct formation (*2*) and then cyclisation (*3*). The intermediate adduct (*2*) was not seen. In the cyclisation of (*2*) increasing steric interaction in the series (*1b*) to (*1d*) would be expected between the 6-substituent and the substituents on the side-chain carbon atom involved in the cyclisation reaction. In the adduct formation steric interaction from the 6-substituents is presumably not very different within the series and the alkyl groups exert similar inductive activation. The closely related overall reaction rates for the alkyl derivatives therefore suggest that the intramolecular cyclisation reaction is considerably faster than the intermolecular adduct formation. The rate differences for the two steps are such that the steric decrease in the cyclisation rate with increasing bulkiness of the substituents does not decrease the rate of cyclisation below that of adduct formation.

EXPERIMENTAL

All NMR spectra were determined on a Varian 60 MHz A-60 A spectrometer.

2-Bromo-3-hydroxy-6-methylpyridine (4). 3-Hydroxy-6-methylpyridine (21.8 g, 0.2 mol) was dissolved in 48 % HBr (100 ml, 0.88 mol) and the solution cooled to 0 – 5 °C and kept at this temperature during the ensuing reaction. Hydrogen peroxide (15 %, 50 ml, 0.22 mol) was added slowly with stirring to the cooled solution. The stirring was continued for another 2 h at 0 – 5 °C after the addition was complete. The precipitated product was then filtered off, the filtrate concentrated and the new precipitate collected. The combined product was dissolved in water and the solution neutralised with ammonia. The title compound was precipitated and obtained in more than 90 % yield; and having properties as previously described.[4]

2-Bromo-3-methoxy-6-methylpyridine (5). 2-Bromo-3-hydroxy-6-methylpyridine (188 g, 1 mol) and methyl iodide (200 g, 1.4 mol) were added to 1 M sodium methoxide in methanol (1 l). The mixture was heated under reflux overnight, more of the 1 M sodium methoxide solution (250 ml) and methyl iodide (50 g, 0.35 mol) added and the reaction mixture heated under reflux for another 3 h. The precipitated salt was filtered off from the cold reaction mixture, the filtrate evporated, the residue suspended in 0.1 M NaOH (300 ml) and the title compound extracted into ether. The etheral solution was washed and dried and yielded the title compound in 74 % yield (149 g) on evaporation; properties as previously described.[9]

2-Bromo-3-methoxypyridine-6-carboxylic acid (6). Potassium permanganate (47.4 g, 0.3 mol) was added gradually over 4 h to a suspension of 2-bromo-3-methoxy-6-methylpyridine (20.2 g, 0.1 mol) in water (1 l) at 70 °C. Towards the end of the reaction the temperature was raised to 85 – 90 °C. Methanol (20 ml) was then added to decompose any excess potassium permanganate and the reaction mixture filtered warm. The manganese dioxide filtered off was washed 3 – 4 times with boiling, dilute methanol (1:1) and the combined washings and filtrate concentrated to about half its volume before acidification with HCl. The white carboxylic acid precipitated was recrystallised from dilute ethanol (1:5); yield 16.5 g (71 %), m.p. 245 – 247 °C. (Found: C 36.31; H 2.61; N 6.01. Calc. for $C_7H_6BrNO_3$: C 36.24; H 2.61; N 6.04), τ(TFA) 5.6 (OMe), 1.1 and 1.6 (H-4, H-5).

2-Chloro-3-methoxypyridine-6-carbonyl chloride (7). A solution of 2-bromo-3-methoxypyridine-6-carboxylic acid (11.6 g, 0.05 mol) in thionyl chloride (300 ml) was heated under reflux for 4 d. The solution was then evaporated and the residue triturated with acetonitrile. There remained 6.0 g (58 %) of the title compound sufficiently pure for further reaction. For elemental analysis part of the product was recrystallised from acetonitrile, m.p. 126°C. (Found: C 40.82; H 2.45. Calc. for $C_7H_6ClNO_2$ C 40.81; H 2.45), τ($CDCl_3$) 6.0 (OMe), 1.9 and 2.7 (H-4, H-5).

*2-Chloro-3-methoxypyridine-6-*N,N*-diethylcarboxamide (8).* Diethylamine (5.5 g, 0.075 mol) was added dropwise with stirring to an ice-cold solution of 2-chloro-3-methoxypyridine-6-carbonyl chloride (5.1 g, 0.025 mol) in toluene (250 ml). The reaction mixture was left overnight, the precipitated salt removed by filtration and the filtrate evaporated. Recrystallisation of the residue from diisopropyl ether gave the amide m.p. 60 – 62 °C, yield 5.5 g (90 %). (Found: C 54.40; H 6.15. Calc. for $C_{11}H_{15}ClN_2O_2$: C 54.44; H 6.23), τ($CDCl_3$), 6.1 (OMe), 6.5 and 8.8 (*N*-Et_2), 2.4 and 2.8 (H-4, H-5).

2-Chloro-3-methoxy-6-acetylpyridine (9). A Grignard solution in anhydrous ether (50 ml), prepared from magnesium turnings (0.5 g, 0.022 mol) and methyl iodide (3.1 g, 0.022 mol), was added dropwise to an ice-cold, stirred solution of 2-chloro-3-methoxypyridine-6-*N*,*N*-diethylcarboxamide (4.8 g, 0.02 mol) in anhydrous ether (200 ml). The reaction was run under nitrogen. The reaction was stirred for 1 h after the addition had been completed and the reaction mixture then poured onto ice with some acetic acid. The ether layer was separated, the aqueous layer extracted with ether and the etheral solutions dried and evaporated. The product thus obtained contained about 10 % of the corresponding tertiary alcohol (*10b*). The latter was readily removed by crystallisation from ethanol in which the ketone is very little soluble in the cold; yield 2.3 g (62 %), m.p. 103 – 104 °C. (Found: C 51.98; H 4.42. Calc. for $C_8H_8ClNO_2$: C 51.77; H 4.34), τ($CDCl_3$) 7.3; τ(CH_3CO) 6.0 (OMe), 2.0 and 2.7 (H-4 and H-5).

2-Chloro-3-methoxy-6-(1-hydroxyethyl)pyridine (10a). Sodium borohydride (0.6 g, 0.015 mol) was added gradually to a methanolic solution (50 ml) of 2-chloro-3-methoxy-6-acetylpyridine (0.9 g, 0.005 mol). The solution was evaporated after 1 h, the residue triturated with water and the pH adjusted to about 7 before chloroform extractions. Evaporation of the dried chloroform extracts gave the title compound 0.8 g, 90 %), m.p. 35 – 37 °C. The product was sufficiently pure for elemental analysis. (Found: C 51.25; H 5.42. Calc. for $C_8H_{10}ClNO_2$: C 51.21; H 5.37), τ($CDCl_3$) 8.5 (3 H, d, CH_3C), 6.1 (OMe), 5.2 (H, q, CH-Me), 2.8 (2 H, broad singlet, H-4 and H-5).

2-Chloro-3-methoxy-6-(1-chloroethyl)pyridine (11a). 2-Chloro-3-methoxy-6-(1-hydroxyethyl)-pyridine (0.8 g, 0.004 mol) was kept in thionyl chloride (30 ml) overnight. Evaporation left the title compound. The chlorination is quantitative and the product can be used directly in the next step. For analysis part of the product was dissolved in chloroform, this solution washed well with water and dried before evaporation. The residual material, after recrystallisation from diisopropyl ether, had m.p. 51 – 53 °C. (Found: C 46.48; H 4.36. Calc. for $C_8H_9Cl_2NO$: C 46.63; H 4.40), τ($CDCl_3$) 8.2 (3 H, d, CH_3C), 6.1 (OMe), 4.9 (H, q, CH – Me), 2.6 and 2.8 (H-4 and H-5).

2-Chloro-3-methoxy-6-methylpyridine (13a). A solution of 2-chloro-3-methoxy-6-(1-chloroethyl)pyridine (0.8 g, 0.004 mol) in 0.5 M potassium *tert*-butoxide in *tert*-butanol (16 ml) was heated at 80 °C for 90 min. Acetic acid (4 ml) was added to the cold reaction mixture until acid pH. The *2-chloro-3-methoxy-6-vinylpyridine (12a)* thus obtained was not isolated but the solution subjected to hydrogenation over platinum oxide at room temperature and atmospheric pressure for 3 h. The catalyst was then removed and the solution evaporated to dryness at reduced pressure. Water was added to the residual material, pH adjusted to about 7 and the title compound extracted into chloroform. Evaporation of the washed and dried chloroform extracts left an oily material, 0.4 g (58 %). For elemental analysis a portion of this material was dissolved in anhydrous ether, and HBF_4 in ether added dropwise. The white, semicrystalline tetrafluoroborate was fully solidified on trituration with acetone; m.p. 166 – 168 °C. (Found: C 36.80; H. 4.30. Calc. for $C_8H_{10}ClNO.HBF_4$: C 37.05; H 4.25), τ(NMR of oily material in $CDCl_3$) 8.7 and 7.3 (Et), 6.1 (OMe), 2.8 and 2.9 (H-4, H-5).

3-Hydroxy-6-ethylpyridine (16). Zinc amalgam was prepared from granulated zinc (5 g) and mercuric chloride (0.6 g) in water (20 ml) and conc. HCl (1 ml). The water solution was decanted after 5 min and dilute HCl (1:1) solution (60 ml) added. 2-Chloro-3-methoxy-6-acetylpyridine (2.4 g, 0.013 mol) was next added and the reaction mixture heated under reflux

for 3 h. The reaction mixture was then neutralised and the compound extracted into ether. The ether was evaporated leaving 3-methoxy-6-ethylpyridine. The latter was dissolved in 48 % HBr in acetic acid (25 ml) and the solution heated for 5 h. Evaporation left the HBr salt which was dissolved in a minimum amount of water and the solution neutralised when the title compound was slowly precipitated in 20 % yield; properties as described.[5]

2-Chloro-3-methoxy-(2-hydroxy-2-propyl)pyridine (10b). A solution of methylmagnesium iodide was prepared from magnesium turnings (2.0 g, 0.08 mol) and methyl iodide (11.4 g, 0.08 mol) in anhydrous ether (100 ml) under nitrogen. The acid chloride (*7*) (4.1 g, 0.02 mol) was then added dropwise with stirring to the Grignard solution cooled by ice-water. After completion of the addition the reaction was heated under reflux for 2 h before being poured onto ice containing a little acetic acid. The ether layer was separated, the aqueous layer was extracted with ether and the combined etheral solutions were dried and evaporated. The residual oily material was distilled; yield 3.3 g (82 %), b.p. 116–118 °C/0.15 mmHg. For elemental analysis a portion was crystallised from diisopropyl ether, the crystalline material being collected at −20 °C; m.p. 30–32 °C. (Found: C 53.25; H 5.96. Calc. for $C_9H_{12}ClNO_2$: C 53.61; H 6.00), τ($CDCl_3$) 8.5 (CMe_2, s), 6.1 (OMe), 2.6 and 2.8 (H-4, H-5).

2-Chloro-3-methoxy-6-isopropenylpyridine (12b). A solution of 2-Chloro-3-methoxy-6-(2-hydroxy-2-propyl)pyridine (4.0 g, 0.02 mol) and thionyl chloride (3.5 g, 0.03 mol) in benzene (50 ml) was heated under reflux for 30 min before the benzene was evaporated. The residual halide was added to aqueous 0.5 M potassium hydroxide (80 ml) and the suspension heated under reflux for 2 h. The cold reaction mixture was extracted with ether, the dried ether extracts evaporated and the residual oil distilled; yield 2.6 g (71 %), b.p. 88–90 °C/0.05 mmHg. (Found: C 58.66; H 5.43. Calc for $C_9H_{10}ClNO$: C 58.87; H 5.49), τ(CCl_4) 7.9 (CH_3–C), 6.2 (OMe), 4.3 and 4.9 (=CH_2), 2.8 and 3.0 (H-4, H-5).

2-Chloro-3-methoxy-6-isopropylpyridine (13b). A solution of 2-chloro-3-methoxy-6-isopropenylpyridine (2.2 g, 0.012 mol) in methanol (25 ml) was hydrogenated over 5 % palladium on charcoal (1/2 teaspoon) at 0.38 MPa* and room temperature. The catalyst was filtered off after 4 h, the solvent evaporated and the residual oil distilled; yield 1.6 g (72 %), b.p. 78–84 °C/0.1 mmHg. (Found: C 57.88; H 6.66. Calc. for $C_9H_{12}ClNO$: C 58.20; H 6.47), τ($CDCl_3$) 8.7 (6H, d, CMe_2) and 7.1 (H, m, CH–CMe_2), 6.1 (OMe), 2.8 and 2.9 (H-4, H-5).

3-Hydroxy-6-isopropylpyrid-2-thione (1d). 2-Chloro-3-methoxy-6-isopropylpyridine (5.6 g, 0.03 mol) and phosphorus pentasulfide (6.7 g, 0.03 mol) were ground well together and the mixture heated at 140 °C for 10 h. The reaction mixture was then decomposed on warming with water and the resultant suspension neutralised with conc. ammonia and extracted several times with chloroform. The residue, after evaporation of the dried chloroform extracts, was dissolved in boiling carbon tetrachloride and some hexane added. The yellow title compound crystallised out from this solution on standing; yield 3.5 g (68 %), m.p. 110–112 °C. (Found: C 56.45; H 6.57; S 19.08. Calc. for $C_8H_{11}NOS$: C 56.77; H 6.55; S 18.95), τ(TFA) 8.6 (6H, d, CMe_2) and 6.9 (H, m, CH–CMe_2), 2.1 and 2.6 (H-4, H-5).

3-Hydroxy-6-ethylpyrid-2-thione (1c) was prepared from (*13a*) and phosphorus pentasulfide as the isopropyl analogue (*1d*) above. The crude thione in chloroform was filtered through a short silica gel column before evaporation. Recrystallisation from toluene gave m.p. 150–151 °C; yield 43 %. (Found: C 53.94; H 6.13. Calc. for C_7H_9NOS: C 54.19; H 5.85), τ($CDCl_3$) 8.7 and 7.3 (Et), 3.0 and 3.5 (H-4, H-5).

In another experiment 3-hydroxy-6-ethylpyridine (*16*) was brominated as described for the 6-methyl analogue (*4*) above and the crude bromo compound (*14*) subjected to thiation as above using phosphorus pentasulfide. The yield in the thiation was 60 %.

8-Hydroxy-5-isopropyldihydrothiazolo[3,2-a]pyridinium-3-carboxylate (3d). 3-Hydroxy-6-isopropylpyrid-2-thione (1.7 g, 0.01 mol) and α-bromoacrylic acid (3.0 g, 0.02 mol) in ethyl acetate (66 ml) were stirred at room temperature for 24 h. The precipitated title compound as HBr salt was then filtered off (1.9 g, 59 %). Due to the case of decarboxylation the salt was not attempted recrystallised but washed well with ethyl acetate before elemental analysis; m.p. 195–197 °C (decomp.) (Found: C 41.00, H 4.40. Calc. for $C_{11}H_{14}NO_3S.HBr$: C 41.20; H 4.38, τ(TFA) 8.5 (6H, d, CMe_2) and 6.7 (H, m, CH–CMe_2), 5.7 (2 H-2), 3.5 (H-3), 2.0 and 2.5 (H-7, H-6).

8-Hydroxy-5-ethyldihydrothiazolo[3,2-a]pyridinium-3-carboxylate (3c) *as HBr salt* was formed from 3-hydroxy-6-ethylpyrid-2-thione and α-bromoacrylic acid in 65 % yield under the above reaction conditions in ethyl acetate. Due to the ease of decarboxylation the crude product after being washed well with ethyl acetate was subjected directly for elemental analysis; m.p. 179 °C (decomp.). (Found: C 38.98, H 4.26. Calc. for $C_{10}H_{11}NO_3S.HBr$: C 39.23; H 3.95), τ(TFA) 8.5 and 7.0 (Et), 5.7 (2 H-2), 3.5 (H-3), 2.0 and 2.5 (H-7, H-6).

* 1 MPa ≈ 10.2 kg cm^{-2}.

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An X-Ray and Vapour Pressure Study on Lecithin—Cholesterol—Water Interactions

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The molecular interactions in the lamellar mesomorphous phase of the lecithin-cholesterol-water system have been examined by X-ray diffraction and vapour pressure measurements. Cholesterol is found to have a condensing effect on the molecules of egg lecithin. This effect is accompanied by an increase in the thickness of the lipid layer and a decrease in the thickness of the water layer. The water binding capacity of egg lecithin is only slightly affected by cholesterol. The effect of cholesterol on hydrated egg lecithin seems mainly to be restricted to the hydrocarbon region.

Cholesterol is a prominent constituent in biological structures, *e.g.*, membranes and may also play a part in the development of pathological structures as atherosclerotic lesions and gall-stones. Cholesterol by itself insoluble in water can be solubilised in the mesomorphous phase of the lecithin-water system.[1]

A significant feature of the molecular interactions between cholesterol and lecithins is the condensing effect of cholesterol in mixed monolayers with most lecithins above their chain melting temperature.[2] This reduction in the mean molecular area was also found to be valid for bulk systems from calculations of X-ray data.[1]

Cholesterol has on the contrary been shown to cause a considerable increase in the surface area of lecithin in sonicated vesicles.[3]

Ladbrooke *et al.* applied differential scanning colorimetry (DSC) to cholesterol-lecithin suspensions and found that cholesterol inhibits the chain motion of lecithin above the gel-liquid crystal transition temperature and concluded that the transition vanishes at a 1:1 mol ratio.[4] The deuteron NMR [5] and ESR spin label method [6] have provided more details of the molecular interaction. It has been found that the restriction of the lecithin chain motion is smaller at the free nonpolar ends of the chains than in the region near the glycerol backbone. The lecithin phosphate group and cholesterol hydroxyl appear to be juxtaposed and may be hydrogen bonded.[7]

Regarding the interaction between mixtures of lecithin-cholesterol and water, DSC measurements indicate that the bound water is a maximum at 50 mol % cholesterol.[8] Other authors, on the contrary, have found decreasing affinity for water with increasing cholesterol concentration.[9] No vapour pressure measurements on lecithin-cholesterol mixtures seem to have been reported.

MATERIALS AND METHODS

The cholesterol used (Merck *p.a.*) was recrystallized three times from 1,2-dichlorethane. It was found to be chromatographically pure by TLC and GLC. The egg lecithin was isolated in this laboratory.[10] It was proved to be chromatographically and spectroscopically pure by TLC and IR-spectroscopy. The water was distilled twice in an apparatus of silica.

The lipid mixtures were prepared by dissolving cholesterol and lecithin in ampouls and the solvent was then removed in a stream of nitrogen. The last traces of solvent were removed *in vacuo* and then an appropriate amount of water was added. The ampouls were sealed under nitrogen and placed in a thermostated water bath to equilibrate over a period of ten days. After the X-ray diffraction measurements the water contents were checked with a Karl Fisher titration.

The X-ray diffraction measurements were conducted with an apparatus previously de-

scribed.[11] The Cu$K\alpha$ radiation used was Ni-filtered and the incident X-ray beam was collimated by a slit system. The scattering angle was measured with a Rigaku-Denki low-angle goniometer. No slit corrections were done.

The adsorption of water vapour by egg lecithin-cholesterol mixtures was measured by keeping the lipid mixtures in weighing bottles over standard saturated salt solutions in vacuum. All measurements were performed at a temperature of (25 ± 1) °C.

RESULTS

The X-ray diffraction measurements were limited to the region of the lamellar mesophase in the egg lecithin-cholesterol-water system. To make sure that the samples belonged to this phase they were examined by a polarizing microscope after the X-ray measurements. In order to make the results as surveyable as possible the cholesterol concentration at three constant lecithin/water ratios (9:1, 7.5:2.5, 6:4) was varied. The variations in long spacings, D, with cholesterol for the three different lecithin to water ratios are shown in Fig. 1. For the lecithin/water ratio of 9:1 the maximum solubilising capacity is reached at about 20 % cholesterol concentration then the curve stops.

Starting with the D values and the corresponding lecithin, cholesterol, and water percentages one can calculate a number of parameters in the system studied. In the calculations the following considerations were accounted for. From the molecular volumes and volume fractions of this egg lecithin [10] and cholesterol the mean molecular volumes for

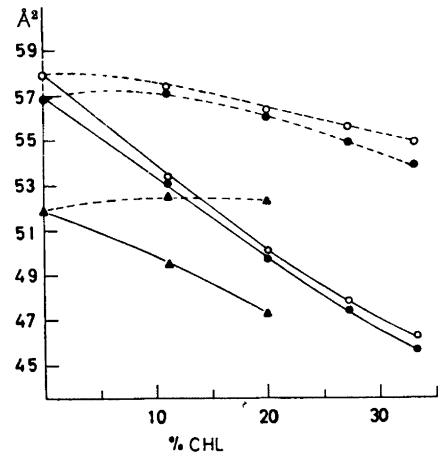

Fig. 2. The mean molecular surface area (S_M), continuous lines, and partial molecular area of lecithin (S_L), broken lines, as a function of the dry weight percentage of cholesterol. Each curve is relative to a fixed lecithin to water ratio of 9:1 (▲), 7.5:2.5 (●), and 6:4 (○), respectively.

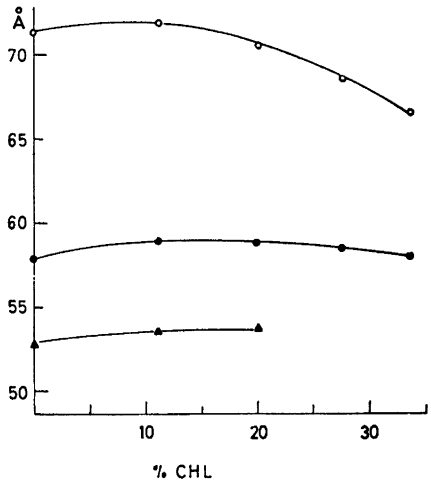

Fig. 1. The long spacings (D) as a function of a dry weight percentage of cholesterol. Each curve is relative to a fixed lecithin to water ratio of 9:1 (▲), 7.5:2.5 (●), and 6:4 (○), respectively.

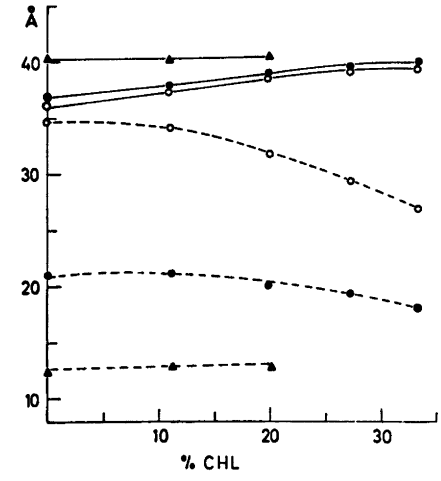

Fig. 3. The thickness of the lipid layer (d_L), continuous lines, and the thickness of the water layer (d_w), broken lines, as a function of the dry weight percentage of cholesterol. The curves represent lecithin to water ratios of 9:1 (▲), 7.5:2.5 (●), and 6:4 (○), respectively.

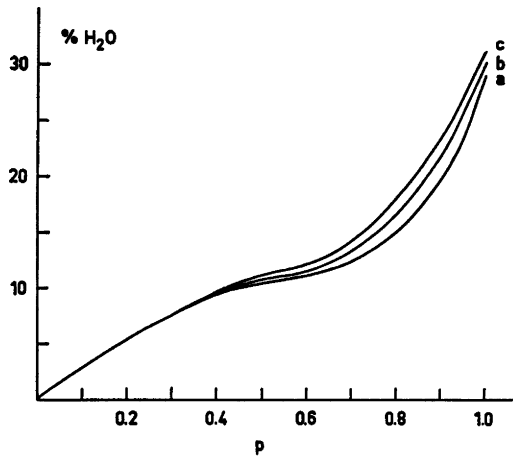

Fig. 4. Adsorption of water vapour by egg lecithin in mixtures with cholesterol. The weight percentage of adsorbed water based on water and lecithin weight only is plotted against the relative vapour pressure (p). Curve a 0 % cholesterol; b 15 % cholesterol, and c 30 % cholesterol.

mixtures of them (V_{LC}) are calculated. The mean molecular volume (V_L) of the lipidic layer exclusive the phosphoryl choline group is calculated from the molecular volumes of the glycerohydrocarbon part [10] and cholesterol and the volume fractions of them. The cholesterol molecules are assumed to occupy a practically constant area in the mixed layers of the lamellar phase whatever the molar fraction is. From monolayer measurements the surface area of cholesterol has been calculated to 37.5 Å² per molecule.[14] Starting with the related values, the following parameters can be estimated.

1. The mean molecular surface area, S_M, in Å² per molecule. $S_M = V_{LC}/(D/2 \times \phi_{LC})$, where $D/2$ is one half of the long spacing and ϕ_{LC} equals volume fraction of lecithin and cholesterol.

2. The surface area, S_L, per lecithin molecule in Å² per molecule. $S_L = S_M - (X_c \times 37.5)/X_L$, where X_C and X_L are mol fractions of cholesterol and lecithin, respectively.

3. The thickness, d_L, in Å of the lipid layer: $d_L = 2V_L/S$.

4. The thickness in Å of the water layer, d_w, including the phosphoryl choline group: $d_w = D - d_L$.

The experimental D values and the calculated parameters are summarized in Figs. 1, 2, and 3.

The results from the vapour pressure measurements are presented in Fig. 4. To make the curves more commensurable the water percentages are calculated on water and lecithin weight only. A slight increase in water adsorption with increasing cholesterol concentration is noted.

DISCUSSION

The most striking changes when cholesterol is incorporated into the hydrated lecithin bilayers are the reduction in the surface area of lecithin (see Fig. 2) and the corresponding increase in lipid layer thickness (see Fig. 3). It is still under discussion whether these cholesterol effects are caused by inhibition of chain motions or by orientation of their long axes more nearly perpendicular to the plane of the bilayer or by both these effects. NMR studies have shown that cholesterol severely restricts the lecithin chain motions.[5] Spin label studies indicate that lecithin which makes an angle of about 30°, with the normal to the lamellar phase, is made more nearly perpendicular by the addition of cholesterol.[15] It may be that the reduction in surface area and increase in lipid layer thickness is due to both the effects mentioned.

Although the reduction in surface area of lecithin by cholesterol is significant it is not of the same order of magnitude as in mixed films. For a 1:1 mixed monolayer the surface area of lecithin is at the collapse point *ca.* 44 Å²/molecule compared with *ca.* 55 for the same molar proportions at 6:4 hydration (see Fig. 2) in the bulk system. This discrepancy may be explained by the fact that the conditions are not identical. In a monolayer the paraffinic ends are in contact with air, whereas in a bulk system they face other paraffinic ends.

The vapour pressure measurements show that the water binding capacity of lecithin is not much altered by the incorporation of cholesterol. This indicates that the configuration of the lecithin phosphoryl choline group is not much altered by the interaction with the cholesterol hydroxyl. NMR studies have indicated a reduction in the $N(CH_3)_3$ mobility. The proportion of bulky hydrophilic end groups

decreases, however, when cholesterol is incorporated and therefore there is more space for water and greater freedom for hydrogen bondings.

The results from the vapour pressure measurements favour the opinion that the lecithin-cholesterol interaction is mainly hydrophobic. The most noticeable apolar effect is the reduction in the molecular area. In the light of the present study this condensing effect mainly found in membrane model studies with monolayers may have been overstressed. The situation with paraffinic ends facing each other is the same for both lamellar bulk systems and biomembranes. Therefore the much smaller condensing effect calculated from X-ray measurements may be relevant for membranes too. The main effect of cholesterol in natural structures seems more likely to be the chain melting effect thus regulating the physical state of the hydrocarbon region.

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A Calorimetric Study of *peri* Strain in 1,8-Dimethylnaphthalene and 1,4,5,8-Tetramethylnaphthalene

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Enthalpies of combustion, utilizing a micro combustion calorimetric technique, and enthalpies of sublimation have been determined for 1,8-dimethylnaphthalene and 1,4,5,8-tetramethylnaphthalene. Standard enthalpies of formation for the compounds in the gaseous state, $\Delta H_f°(g)$, have been derived and are (26.0 ± 0.7) and (19.5 ± 0.9) kcal mol^{-1}, respectively. The calculated strain energies caused by *peri* interactions in the two compounds are 6 and 15 kcal mol^{-1}, respectively.

Substitution in the 1,8-positions in naphthalene is generally referred to as *peri* substitution. Due to the structure of the naphthalene molecule such substituents come closer together than if they were *ortho*. As was pointed out in the review by Balasubramaniyan [1] *peri* interactions in naphthalene early aroused considerable interest among chemists, an interest that seems to be increasing rather than decreasing.

Thermochemical methods have not been utilized in the study of *peri* interactions in true naphthalene derivatives until quite recently. In two independent investigations [2,3] the energy of combustion was determined for crystalline 1,8-dimethylnaphthalene. In Ref. 2 energies of combustion were also reported for the 2,3-, 2,6-, and 2,7-isomers. From these measurements enthalpies of formation for the compounds in the crystalline state are derived. However, in studies of intramolecular interactions gas phase enthalpies of formation generally are those that contain the pertinent information. Enthalpy of vaporization (sublimation) determinations therefore are an essential part of such investigations. The development of a micro-bomb (4.5 cm^3) combustion calorimeter [3] now makes it possible to perform a full series of combustion measurements on 50 mg or less of pure material, yet with adequate accuracy. Calorimetric enthalpy of vaporization measurements for compounds with vapour pressures at 298 K extending down into the 10^{-5} Torr* region [4] in most cases have become a routine procedure in our laboratory.

In this paper enthalpy of combustion and sublimation measurements are reported for 1,8-dimethylnaphthalene (1,8-DMN) and 1,4,5,8-tetramethylnaphthalene (1,4,5,8-TMN). One of the questions asked was whether the first pair of *peri* methyl groups in the 1,8-DMN molecule would make it more "costly", or perhaps less so, to introduce the second *peri* pair in the 4,5-positions. Or, would the stepwise *peri* dimethyl substitutions in naphthalene cause constant increments in the energy?

The so called *ortho*-effect for a pair of methyl groups in benzene has been investigated previously by combustion calorimetry,[5,6] in combination with calorimetric measurements of enthalpies of vaporization.[7] The calculated mean value for the strain energy introduced by two *ortho* methyl groups is of the order of 0.6 – 0.7 kcal mol^{-1}.** The magnitude of the expected increased strain due to the much closer proximity of *peri* methyl groups should be an interesting piece of information derivable from the calorimetric measurements.

EXPERIMENTAL

Compounds. The benzoic acid used in the calibration experiments was National Bureau of Standards SRM 39i.

* 1 Torr = (101.325/760) kPa.

** 1 cal = 4.184 J.

The 1,8-dimethylnaphthalene (DMN) sample has been accounted for earlier.[3] 1,4,5,8-Tetramethylnaphthalene (TMN) was synthesized by Dr. Hans Sternerup (Department of Organic Chemistry, Chemical Center, Lund) from *p*-xylene and γ-valerolactone as described in Ref. 8. About 2.5 % of impurities remained after two recrystallizations from methanol. Running the material through a neutral aluminium oxide column, with pentane as eluent, did not markedly improve the purity. Subsequent recrystallization (twice) from hexane reduced the total amount of GLC detectable impurity to 0.13 mol %. Carbowax and silicone (SE-30) columns were used in the GLC studies. Mass spectrometric analysis, coupled with GLC, identified the impurities as an isomeric tetramethyl- and a trimethyl-naphthalene, present in approximately equal amounts. The NMR spectrum was fully consistent with the assumed 1,4,5,8-arrangement of the four methyl groups. The purified material was pressed into a pellet, which was then broken up and ground in an agate mortar to ascertain the escape of traces of solvent (hexane) that might be trapped in the crystalline material.[9] The melting temperature, determined on a Perkin-Elmer DSC-2 differential scanning calorimeter was 405.$_7$ K. For the combustion measurements 3.5 mm diameter pellets were made. The mass loss from such a pellet was less than 1 μg h^{-1}.

A Perkin-Elmer DSC-2 differential scanning calorimeter was used to determine enthalpies of fusion. High purity indium was used as calibration standard and naphthalene was included in the series for comparison. The enthalpies of fusion obtained for naphthalene, DMN, and TMN were 18.8$_5$, 15.8, and 23.9 kJ mol^{-1}, respectively. McCullough *et al.*[10] obtained 18.98 kJ mol^{-1} for naphthalene.

Combustion calorimetry. The micro combustion calorimeter as well as the calorimetric procedure were identical to those described in Ref. 3. Details of the combustion measurements on DMN have already been given together with the resulting standard energy of combustion for the crystalline material.[3] In the combustion experiments on TMN the initial pressure of oxygen, $p^i(O_2)$, varied around 35 atm;* for DMN it was 31 atm. Five experiments were performed on TMN; in one a small correction for unburned carbon had to be applied. The energy equivalent of the calorimetric system was determined by combustion of benzoic acid; series III in Ref. 3. The constancy of the calibration was checked by performing electrical calibrations in three of the runs on TMN; (for an account of the agreement between benzoic acid and electrical calibrations *cf.* Ref. 3).

All weighings of sample were made on a Mettler M5 micro balance, which had been carefully calibrated against standard weights,[3] calibrated at the National Bureau of Standards. The weighings have been reduced to masses and molar masses computed from the 1969 table of atomic weights.[11] The corrections to standard states, ΔU_Σ, were calculated using a computer program based on the procedure by Hubbard *et al.*[12] The estimated values for c_p and $(\partial v/\partial T)_p$ were the same as those in Ref. 3. The final overall precision of the ΔU_c° mean values was estimated as recommended by Rossini.[13] The reference temperature of the combustion experiments is 298.15 K.

Vaporization calorimetry. The enthalpies of sublimation at 298.15 K were measured using the Morawetz calorimeter.[4] Five determinations were made on DMN following the procedure described in Ref. 4, with around 13 mg evaporated per experiment.

Heating the vaporization calorimeter to temperatures above ~335 K was not considered "safe" and TMN therefore could not be melted directly into the evaporation pan. Furthermore, the compound could only be sublimed into the calorimeter at a very slow rate. Instead TMN was melted into a thin-walled gold cup that was then "soldered" to the evaporation chamber with gallium. Four test experiments on octadecane yielded (153.6 ± 1.1) kJ mol^{-1} (mean and standard deviation of the mean); literature values are 153.1 [14] and 152.7 [15] kJ mol^{-1}. When three successful vaporization experiments had been performed on TMN the calorimeter as such failed and further measurements had to be postponed. The sublimation enthalpy reported here is the mean value from these three determinations. Around 2.5 mg of TMN had evaporated per experiment. In the evaluation of the overall uncertainty of the enthalpy of sublimation for TMN, the standard deviation of the measured mean value, 0.40 kJ mol^{-1}, was raised to 0.60 kJ mol^{-1} to allow for a possible systematic error in this particular series of measurements.

RESULTS AND DISCUSSION

The results from the combustion experiments on TMN are given in Table 1, with Δu_c° referring to unit mass of sample. For DMN the corresponding mean value and standard deviation of the mean is Δu_c°(DMN) = −(41323 ± 5) J g^{-1}.[3] The Δu_c° values refer to the idealized combustion reaction in which all reactants and products are in their thermodynamic standard states at 298.15 K. Table 2 gives the standard molar energies, ΔU_c°, and enthalpies, ΔH_c°, of combustion for the compounds in the crystalline state at 298.15 K. Also listed in Table 2 are the molar enthalpies of sublimation, ΔH_{subl}, and derived enthalpies of formation, ΔH_f°, for the compounds in the crystalline and gaseous states (298.15 K). The enthalpies of formation at

* 1 atm = 101.325 kPa.

Table 1. Energy of combustion measurements at 298.15 K of 1,4,5,8-tetramethylnaphthalene. ε(calor) = (583.45 ± 0.07) J K^{-1}. For explanation of symbols, *cf.* Ref. 12.

m'/mg	8.023	8.426	8.282	7.923	7.893
ε^i(cont)/J K^{-1}	0.261	0.261	0.263	0.262	0.262
$\Delta\theta$/K	0.58016	0.60908	0.59876	0.57277	0.57046
ΔU_{ign}/J	0.239	0.236	0.255	0.237	0.159
ΔU(C)/J[a]	0	0	0	0.198	0
ΔU_Σ/J	0.188	0.198	0.196	0.186	0.186
$-\Delta u_c^\circ$(comp)/J mg^{-1}	42.156	42.142	42.146	42.170	42.143

Mean and std. dev. of the mean: $\Delta u_c^\circ = -(42151 \pm 5)$ J g^{-1}

[a] Correction for unburned carbon: 0.033 J μg^{-1}.

Table 2. Results and derived quantities at 298.15 K. The uncertainties given are twice the final overall standard deviation of the mean. 1 cal = 4.184 J.

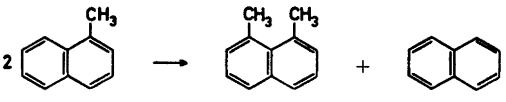
	1,8-Dimethyl-naphthalene	1,4,5,8-Tetramethyl-naphthalene
ΔU_c°/kJ mol^{-1}	−6455.8 ± 2.6	−7767.7 ± 2.8
ΔH_c°/kJ mol^{-1}	−6463.2 ± 2.6	−7777.6 ± 2.8
ΔH_f°(c)/kJ mol^{-1}	26.1 ± 3.0	−18.2 ± 3.4
ΔH_{subl}/kJ mol^{-1}	82.7 ± 0.3	99.8 ± 1.4
ΔH_f°(g)/kJ mol^{-1}	108.8 ± 3.0	81.6 ± 3.6
ΔH_f°(g)/kcal mol^{-1}	$26.0_1 \pm 0.7_2$	$19.5_0 \pm 0.8_7$

298.15 K for gaseous carbon dioxide and liquid water used in the calculations are from Ref. 16.

The selected standard enthalpy of formation for gaseous naphthalene [17] becomes $35.9_9 \pm 0.2_5$ kcal mol^{-1} * when two recent determinations of its enthalpy of sublimation [18,19] are taken into account. In the series naphthalene, 1,8-DMN, 1,4,5,8-TMN the increments in the gaseous enthalpies of formation are −(10.0 ± 0.7) and −(6.5 ± 1.0) kcal mol^{-1}, respectively.** This means that the di-*peri* substitution causes a strain that is $3._5$ kcal mol^{-1} larger than what might have been predicted on the basis of results for the mono-*peri* compound alone. Since the structures of these particular compounds are not yet known this result cannot be rationalized in terms of, *e.g.*, molecular deformations.

The absolute values of the strain introduced by the methyl-methyl *peri* interactions can be evaluated in a number of more or less equivalent ways. For instance, the average increment in the gas phase standard enthalpy of formation for methyl substitution in the series benzene, toluene, *m*-xylene, 1,3,5-trimethylbenzene [5–7] is equal to −7.87 kcal mol^{-1}. This increment, combined with the enthalpy of formation of naphthalene, can be used to calculate enthalpies of formation for unstrained di- and tetramethylnaphthalenes. The values obtained are $20._3$ and $4._5$ kcal mol^{-1}, respectively, implying that the *peri* strain in 1,8-DMN is $5._7$ kcal mol^{-1}, whereas it amounts to as much as $15._0$ kcal mol^{-1} in 1,4,5,8-TMN.

The enthalpy change for the isomerization reaction



is a direct measure of the strain in 1,8-DMN, since 1-methylnaphthalene as well as naphthalene in this context are to be considered as

* To facilitate comparisons with other results in the literature the discussion of the final results will be in terms of *calories* rather than the SI unit *Joule*.

** Unless otherwise stated the uncertainties are twice the final overall standard deviation of the mean. The enthalpy of formation differences can be calculated almost directly from the differences in combustion and vaporization enthalpies and therefore the overall uncertainties in these quantities were used in such calculations.

unstrained.* The *peri* strain energy calculated in this way is ($6._2 \pm 1._4$) kcal mol^{-1}. The similarly calculated *peri* strain in 1,4,5,8-TMN is ($15._8 \pm 2._8$) kcal mol^{-1}.

Added in proof. A recent study of the crystal structure of 1,8-DMN by D. Bright, I. E. Maxwell and J. de Boer (*J. Chem. Soc. Perkin Trans. 2* (1973) 2101), in connection with strain energy minimization calculations, shows that the repulsive interaction between the *peri* methyl groups is reduced mainly by bond-angle distortion at the junction between the methyl groups and the naphthalene nucleus. The inner angles are increased from the ideal 120° to almost 125°. The naphthalene skeleton, however, remains planar and there is no detectable out-of-plane displacement of the methyl groups. These are found to be in the fixed orientation, where four of the methyl hydrogens face each other in pairs, and the remaining two practically lie in the plane of the aromatic rings. The *peri* 4,5-hydrogens are found to be bent towards one another, such that the distance between them is reduced to 2.17 Å (from 2.43 Å). The results from the strain energy minimization calculations are in good agreement with the observed molecular geometry, except that they do not reproduce the "bending" of the *peri* 4,5-hydrogens.

Considering the general features of the 1,8-DMN structure, it seems reasonable that the second *peri* dimethyl substitution should be more costly than the first, in agreement with the calorimetric studies. A comparison between the numerical values of the minimization-calculated and the thermochemically derived strain energies is not meaningful, since the reference systems are not directly comparable.

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* The enthalpy of formation of gaseous 1-methylnaphthalene, ($27.9_3 \pm 0.6_4$) kcal mol^{-1},[20] is based on energy of combustion measurements on the compound in the liquid state combined with an estimate of its enthalpy of vaporization, with the uncertainty in the estimate accounted for in the overall uncertainty figure.

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X-Ray Crystallographic Studies on Cycloheptadithiophene Compounds and Similar Systems I. The Crystal Structure of Dithieno[2,1-*b*;4,5-*b*′]tropylium Perchlorate

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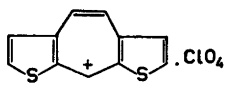
The crystal structure of a dithienotropylium perchlorate



has been investigated by X-ray diffraction methods. The substance is monoclinic, space group $P2_1/n$ with four formula units $C_{11}H_7S_2 \cdot ClO_4$ in a unit cell of dimensions $a = 6.819$, $b = 12.032$, $c = 14.677$ Å and $\beta = 93.60°$. The intensity data were collected by means of an integrating Weissenberg camera and the intensities were estimated visually. The structure was refined to a final R factor of 0.08 for 882 observed reflections. The crystal structure is built up from layers of dithienotropylium cations interleaved by perchlorate ions. As for many other organic salts the oxygen atoms of the perchlorate ion are disordered. The dithienotropylium cation is essentially planar; the largest deviations from the best plane are 0.05 Å and occur for the two sulfur atoms. A few short intermolecular distances exist, one of them being a sulfur–perchlorate oxygen distance of 3.03(3) Å.

The aromaticity of a number of cycloheptadithiophene compounds and also corresponding dithioborepines has been investigated by Gronowitz and co-workers.[1,2] They found that the dithieno[2,1-*b*;4,5-*b*′]tropylium ion was unusually stable, having a pK_{R^+} value of 6.65, while the dibenzotropylium ion has a pK_{R^+} value of only −1.5. They suggested that one of the factors contributing to the higher stability of the thiophene derivative was the absence of *peri* effects between the sulfur atoms and the 9-hydrogen, allowing the three rings to be almost coplanar. Ring strain effects were also assumed to be smaller in the thiophene case. It was therefore of great interest to obtain a detailed knowledge of the bonding distances within the various molecules and ions. One way to obtain the information wanted is to study the solid state of the compounds by means of X-ray diffraction methods. The present and a few following papers will report the results as obtained from studies on single crystals of the various substances.

The present paper deals with the crystal structure of dithieno[2,1-*b*;4,5-*b*′]tropylium perchlorate. For the sake of brevity the actual substance will be called dithienotropylium perchlorate in the following.

X-RAY DIFFRACTION WORK

A small single crystal, kindly supplied by Dr. B. Yom-Tov, was mounted with [001] as rotation axis and the layers 0–10 were registered by means of an integrating Weissenberg camera using Cu$K\alpha$ radiation. The symmetry was found to be monoclinic. The following conditions limiting possible reflections were found:
hkl, no conditions; $h0l$, $h+l=2n$; $0k0$, $k=2n$.
These conditions are characteristic of the space group $P2_1/n$ (No. 14).*

* Orientation different from that given in the International Tables. General point position 4(*e*): x,y,z; $\frac{1}{2}-x, \frac{1}{2}+y, \frac{1}{2}-z$; $\bar{x},\bar{y},\bar{z}$; $\frac{1}{2}+x, \frac{1}{2}-y, \frac{1}{2}+z$.

Table 1. Crystal data for dithienotropylium perchlorate.

Dithienotropylium perchlorate $C_{11}H_7S_2.ClO_4$.
F.W. = 302.7

Monoclinic, $P2_1/n$, $a = 6.819(2)$, $b = 12.032(4)$, $c = 14.667(6)$ Å, $\beta = 93.60(3)°$, $V = 1201(1)$ Å^3.
$Z = 4$.

$D_m = 1.79$, $D_x = 1.68$ g cm^{-3}, $\mu(\mathrm{Cu}K\alpha) = 64.0$

The multiple film technique was used and the intensities were estimated visually using a calibrated scale. The number of observed independent reflections were 882. As the crystal was small and as the value of the linear absorption coefficients was 64.0 cm^{-1}, no absorption correction was performed.

X-Ray powder diffraction photographs were recorded in a Guinier-Hägg focusing camera with $\mathrm{Cu}K\alpha_1$ radiation and potassium chloride ($a = 6.2909$ Å) added as an internal standard. The lattice parameters were obtained with the aid of least-squares calculations. The density of the crystals was determined by flotation. Some crystal data of the compound are given in Table 1.

DETERMINATION AND REFINEMENT OF THE STRUCTURE

The positions of the eight sulfur and four chlorine atoms of the unit cell were deduced from a three-dimensional Patterson function. Using conventional procedures the positions of all non-hydrogen atoms of the cation $C_{11}H_7S_2^+$ and of one of the oxygen atoms (O1) of the perchlorate group were immediately found from difference Fourier maps. As the rest of the perchlorate oxygen atoms did not turn up it was concluded that the perchlorate ion is disordered. The disorder may either be such that the perchlorate ion "rotates" with Cl – O1 as an axis or such that the remaining three oxygen atoms occupy sets of preferred orientations.

Successive least-squares refinements followed by difference Fourier calculations revealed the positions of five more oxygen atoms, *viz.* O2 – O6. The atoms O1, O2, O3, and O4 form one tetrahedron and the atoms O1, O5, and O6 form part of another tetrahedron of oxygens around chlorine. The position of the missing oxygen atom (O7) of the last tetrahedron was deduced by geometrical considerations and was afterwards found to correspond to a peak of moderate height in the last difference Fourier maps.

In a least-squares refinement the isotropic temperature factors found for the oxygen atoms O2 – O7 indicated the occupancy numbers to be 0.6 for O2 – O4 and 0.4 for O5 – O7. No further refinement of the occupancy numbers was made.

A refinement of the positional and thermal parameters of all atoms, using anisotropic temperature factors resulted in a conventional R factor of 0.088. A stereo picture of the perchlorate ion as obtained from the calculations is given in Fig. 1.

Difference Fourier maps based on positional and thermal parameters obtained above revealed the positions of the seven hydrogens of the organic molecule. A least-squares refinement of all atoms using fixed isotropic temperature factors ($B = 4.0$ Å^2) for the hydrogen atoms gave an R factor of 0.080. Thus a small improvement in the discrepancy factor was attained when introducing the positions of the hydrogen atoms in the calculations. However, not all atoms converged at this calculation. Therefore a new refinement was performed where the positions of the hydrogens were fixed at the values obtained above. All parameters of the non-hydrogen atoms now converged. Only small shifts in the parameters were obtained and the R factor did not change.

Fig. 1. Ortep stereoscopic view of the two preferred orientations of the perchlorate ion in dithienotropylium perchlorate.

Table 2. Analysis of the weighting scheme used in the last cycle of least-squares refinement. The averages $w(|F_o|-|F_c|)^2=\overline{w\Delta^2}$ are normalized. $w=(8.0+|F_o|+0.03|F_o|^2)^{-1}$.

Interval $\|F_o\|$	$\overline{w\Delta^2}$	Number of reflections	Interval $\sin\theta$	$\overline{w\Delta^2}$	Number of reflections
0 – 7.9	1.04	89	0.00 – 0.46	1.51	205
7.9 – 10.0	0.98	87	0.46 – 0.59	1.18	206
10.0 – 11.8	0.84	88	0.59 – 0.67	0.82	161
11.8 – 13.7	0.83	88	0.67 – 0.74	1.12	110
13.7 – 16.1	0.74	89	0.74 – 0.79	0.82	78
16.1 – 18.9	0.74	88	0.79 – 0.84	1.03	54
18.9 – 22.7	0.75	88	0.84 – 0.89	1.03	32
22.7 – 26.8	0.81	88	0.89 – 0.93	0.90	22
26.8 – 36.1	1.78	88	0.93 – 0.97	1.51	11
36.1 – 194.4	1.49	88	0.97 – 1.00	0.08	2

Table 3a. Final positional and thermal parameters for dithienotropylium perchlorate. Standard deviations are given within parentheses. All atoms are situated in the general point position 4(*e*) in $P2_1/n$. Anisotropic temperature factors have been used for all non-hydrogen atoms. Their isotropic equivalents are given within brackets. The β_{ij} values are listed separately (Table 3b). Notations of the atoms, *cf.* Fig. 2. The occupancy numbers of the oxygen atoms are 1.0 for O1, 0.6 for O2 – O4 and 0.4 for O5 – O7.

Atom	10^4x	10^4y	10^4z	B(Å^2)
S1	5642(4)	3451(2)	5934(2)	[4.879]
S8	9155(4)	1134(2)	6422(2)	[4.282]
Cl	5404(4)	221(2)	2190(2)	[4.147]
C2	6472(19)	3816(10)	4917(10)	[5.523]
C3	5310(17)	3526(9)	4161(8)	[4.641]
C4	2142(15)	2548(9)	3794(7)	[4.150]
C5	457(16)	1968(9)	3938(8)	[4.253]
C6	7925(15)	1004(9)	4760(9)	[4.200]
C7	7473(16)	719(10)	5592(9)	[4.683]
C9	2288(15)	2289(8)	5947(7)	[3.784]
CII	3602(16)	2835(8)	5383(9)	[3.722]
CIII	3577(14)	2935(8)	4442(8)	[3.576]
CVI	9728(15)	1591(9)	4747(8)	[3.980]
CVII	569(14)	1745(8)	5631(7)	[3.287]
O1	5114(17)	257(10)	1248(8)	[8.768]
O2	6191(46)	−730(18)	2500(25)	[6.619]
O3	6137(45)	1283(17)	2583(13)	[7.188]
O4	3411(30)	263(24)	2416(17)	[9.254]
O5	7507(38)	448(39)	2275(23)	[7.361]
O6	5334(125)	−832(51)	2629(28)	[9.083]
O7	4718(66)	1094(30)	2647(30)	[8.274]

The hydrogen atoms

Atom	10^3x	10^3y	10^3z	B(Å^2)
H2	779(19)	425(10)	472(9)	4.0
H3	566(18)	371(10)	352(10)	4.0
H4	234(17)	286(10)	309(9)	4.0
H5	−25(18)	159(10)	344(10)	4.0
H6	721(18)	94(9)	408(10)	4.0
H7	636(20)	44(11)	537(9)	4.0
H9	259(18)	233(11)	653(10)	4.0

Table 3b. Anisotropic thermal parameters. The temperature factor expression used is $\exp[-(h^2\beta_{11}+k^2\beta_{22}+l^2\beta_{33}+2hk\beta_{12}+2hl\beta_{13}+2kl\beta_{23})]$. The β-values have been multiplied by 10^4.

Atom	$10^4\beta_{11}$	$10^4\beta_{22}$	$10^4\beta_{33}$	$10^4\beta_{12}$	$10^4\beta_{13}$	$10^4\beta_{23}$
S1	230(7)	73(2)	81(2)	5(4)	−29(3)	−14(2)
S8	254(8)	84(2)	41(2)	11(3)	14(2)	−3(1)
Cl	230(7)	79(2)	44(2)	3(3)	5(2)	9(1)
C2	282(34)	92(10)	81(11)	24(15)	40(13)	23(7)
C3	306(34)	72(9)	51(9)	26(14)	8(12)	−4(6)
C4	262(31)	95(10)	44(8)	64(15)	−12(10)	20(6)
C5	245(29)	77(9)	45(9)	−4(12)	−16(10)	0(6)
C6	215(28)	72(9)	55(9)	−20(12)	−18(10)	−2(6)
C7	262(30)	99(10)	45(9)	26(14)	1(10)	9(7)
C9	277(28)	71(8)	31(7)	19(12)	1(10)	−6(5)
CII	261(28)	47(7)	55(9)	36(11)	−11(11)	−15(5)
CIII	211(26)	60(8)	40(9)	16(11)	−1(9)	7(5)
CVI	225(27)	75(9)	47(9)	44(13)	1(10)	−9(6)
CVII	249(26)	66(8)	25(7)	19(11)	−14(9)	−5(5)
O1	606(41)	196(12)	64(8)	−28(18)	−37(12)	6(7)
O2	660(92)	62(14)	234(35)	47(33)	−150(40)	73(16)
O3	621(87)	118(18)	95(13)	−166(36)	−63(27)	10(11)
O4	388(57)	233(28)	169(19)	60(38)	161(27)	52(21)
O5	176(57)	267(49)	164(27)	−142(46)	14(29)	9(28)
O6	1909(425)	284(73)	50(22)	−491(150)	−94(67)	78(28)
O7	609(138)	161(37)	216(40)	−88(59)	20(56)	−151(33)

The atomic scattering factors used were those suggested by Hanson, Herman, Lea and Skillman.[3] In the last refinement the expression $w=(8.0+|F_o|+0.03|F_o|^2)^{-1}$ was used for the calculation of the weights. The final weighting scheme is given in Table 2 and the final positional and thermal parameters in Table 3. On request to the author lists of observed and calculated structure factors are available from the Division of Inorganic Chemistry 2.

Table 4. Pertinent distances and angles for the perchlorate group in dithienotropylium perchlorate. *E.s.d.*'s are given within parentheses.

Orientation I Atoms	Distance (Å)	Orientation II Atoms	Distance (Å)
Cl−O1	1.39(1)	Cl−O1	1.39(1)
Cl−O2	1.33(3)	Cl−O5	1.46(3)
Cl−O3	1.47(3)	Cl−O6	1.43(7)
Cl−O4	1.42(3)	Cl−O7	1.35(5)
Atoms	**Angle (°)**	**Atoms**	**Angle (°)**
O1−Cl−O2	114(2)	O1−Cl−O5	99(2)
O1−Cl−O3	113(1)	O1−Cl−O6	118(2)
O1−Cl−O4	99(1)	O1−Cl−O7	116(2)
O2−Cl−O3	119(2)	O5−Cl−O6	102(5)
O2−Cl−O4	109(2)	O5−Cl−O7	101(3)
O3−Cl−O4	101(2)	O6−Cl−O7	115(3)

DESCRIPTION AND DISCUSSION OF THE STRUCTURE

The crystal structure of dithienotropylium perchlorate is built up from layers of dithienotropylium cations interleaved by perchlorate ions. The cation layers extend in each unit cell in the planes $z = 0$ and $z = \frac{1}{2}$. The chlorine atoms of the perchlorate ions are situated close to the planes $z = \frac{1}{4}$ and $z = \frac{3}{4}$. The perchlorate oxygen atoms are disordered probably in such a way that two orientations are preferred (*cf.* Fig. 1). Pertinent distances and angles for the perchlorate ion are summarized in Table 4.

As has been stated previously [4] the dithienotropylium cations are nearly planar, the largest deviations from the best plane being about 0.05 Å. A detailed analysis of the planarity of the cation is given in Table 5. Fig. 2 shows the

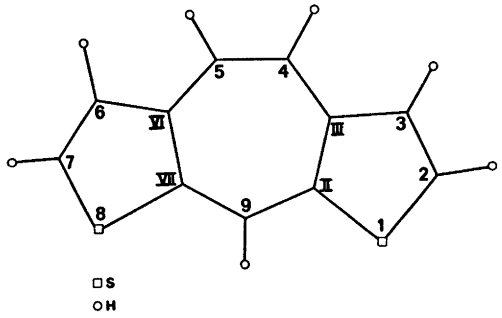


Fig. 2. Applied numbering of the atoms of the dithienotropylium cation.

applied numbering of the atoms of the dithienotropylium cation. The *e.s.d.*'s of the positions of the sulfur atoms are ~0.004 Å and of the carbon atoms ~0.013 Å. It is seen from Table 5 that for each of the separate rings, the thiophene rings and the tropylium ring, respectively, no deviation exceeds 2.0σ for the respective atom. Thus each of the rings now discussed may be considered to be planar. Taking the ring system of the cation as a whole, the carbon atoms deviate at most 3σ from the best plane whereas the sulfur atoms deviate about 10σ. Nearly the same result is obtained if the two sulfur atoms are included in the least-squares calculations of the best planes.

To sum up, each separate ring in the dithienotropylium cation may be considered to be planar but the ring as a whole shows a small deviation from planarity.

Fig. 3 shows the dithienotropylium cation projected perpendicularly on its best plane.

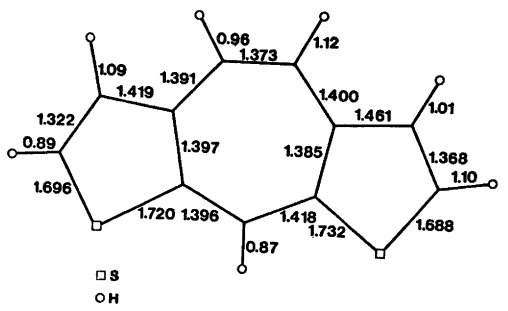


Fig. 3a. Pertinent distances within the dithienotropylium cation. Mean values of the *e.s.d.*'s are 0.012 Å for C–S distances, 0.016 Å for C–C distances and 0.14 Å for C–H distances.

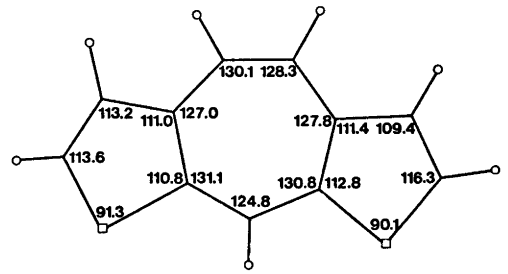


Fig. 3b. Pertinent angles for the non-hydrogen atoms of the dithienotropylium cation. Mean values of *e.s.d.*'s are 0.6° for angles with S at the centre and 1.0° for other angles formed between nonhydrogen atoms.

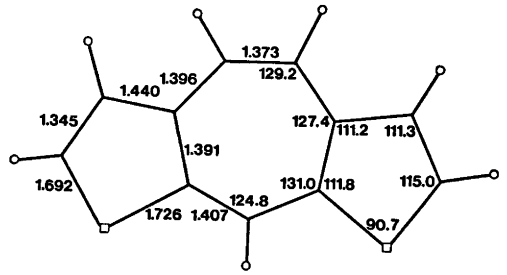


Fig. 3c. Mean values of distances and angles for the two halves of the dithienotropylium cation. For *e.s.d.*'s, *cf.* Table 6.

Table 5. Best least-squares planes through the S1 thiophene ring, the S8 thiophene ring, the tropylium ring and the complete dithienotropylium cation (*cf.* Fig. 2). Distances (Å) of all non-hydrogen atoms to the best plane of the respective ring are given. All non-hydrogen atoms are used to determine the best planes, except for the complete ion where the sulfur atoms are excluded from the calculations. The best planes are calculated according to Blow [10] and the result is given in the form: $lx'+my'+nz'+p=0$. All atoms used for the computations are ascribed unit weights.

Plane	l	m	n	p
S1 ring	0.4963	−0.8671	−0.0435	2.3335
S8 ring	0.5027	−0.8605	−0.0824	2.5419
Tropylium ring	0.5138	−0.8551	−0.0688	2.4369
Total cation	0.5059	−0.8599	−0.0681	2.4452

Atom	Distance to the plane	Atom	Distance to the plane
S1 ring		S8 ring	
S1	−0.006	S8	0.007
C2	−0.003	C6	−0.003
C3	−0.002	C7	−0.003
CII	0.006	CVI	0.009
CIII	−0.003	CVII	−0.010
Tropylium ring		Total cation	
C4	0.004	C2	0.011
C5	−0.011	C3	0.021
C9	0.002	C4	−0.007
CII	−0.015	C5	−0.009
CIII	0.013	C6	−0.006
CVI	0.002	C7	0.011
CVII	0.006	C9	−0.004
		CII	−0.033
		CIII	−0.007
		CVI	0.011
		CVII	0.012
		S1	−0.047
		S8	0.041

Interatomic distances and angles are given in the drawings (Figs. 3a – c)

It is seen from Figs. 3a and 3b that the cation, besides being nearly planar, also has an approximate mirror plane passing through the atom C9 and the midpoint of the line C4 – C5 (*cf.* Fig. 2). Whereas the differences between equivalent distances are at most possibly significant, the angles show one significant difference $\Delta/\sigma=2.5$. The author has, however, chosen to neglect this difference. Mean values and their *e.s.d.*'s have been calculated for the two halves of the cation, which implies an overestimation of the *e.s.d.*'s hitherto used by a factor of about 1.2 (Table 6, Fig. 3c).

Though the precision of the present investigation is not so high, a few conclusions may be drawn from Table 6. The annealed formal double bonds of the thiophene rings (CIII – CII, CVI – CVII) are on the average slightly longer than the other formal double bonds of the ring (C2 – C3, C6 – C7) (*cf.* Fig. 2). The sulfur-tropylium carbon distances (S1 – CII, S8 – CVII) are on the average slightly longer than the thiophenic ones (S1 – C2, S8 – C7). It might be mentioned that the same situation is met with in the crystal structure of the related compound bis-9-(diethieno[2,3-*b*;3′,2′-*f*]borepinyl)ether. (The structure will soon be published by the present author.) Similar disparities of the C – S bonds in thiophene derivatives have, among others, been discussed by Goldberg and Shmueli.[5] They state that the longer of the C – S bonds is that connecting sulphur to the

Table 6. Mean values of distances and angles for the two nearly identical halves of the dithienotropylium cation. *E.s.d.*'s are given within parentheses. A comparison is also made between the distances of the averaged thiophene rings in the present compound and of the thiophene ring in α-thiophenecarboxylic acid.[6] The latter molecule is oriented so that the carbon atom outside the thiophenic ring corresponds to the atom C9 in the present compound.

Atoms	Distance (Å) Mean value	Corresponding value in α-thiophene-carboxylic acid
S1 – C2 S8 – C7	1.692(11)	1.701(10)
C2 – C3 C7 – C6	1.345(15)	1.363(12)
C3 – CIII C6 – CVI	1.440(12)	1.414(11)
CIII – CII CVI – CVII	1.391(14)	1.362(10)
CII – S1 CVII – S8	1.726(9)	1.693(7)
CII – C9 CVII – C9	1.407(12)	
CIII – C4 CVI – C5	1.396(16)	
C4 – C5	1.373(18)	

Atoms	Angle (°) Mean value	
CII – S1 – C2 CVII – S8 – C7	90.7(5)	92.0(4)
S1 – C2 – C3 S8 – C7 – C6	115.0(8)	111.8(7)
C2 – C3 – CIII C7 – C6 – CVI	111.3(9)	111.9(5)
C3 – CIII – CII C6 – CVI – CVII	111.2(8)	112.4(7)
CIII – CII – S1 CVI – CVII – S8	111.8(6)	111.8(6)
C9 – CII – CIII C9 – CVII – CVI	131.0(8)	
CII – CIII – C4 CVII – CVI – C5	127.4(8)	
CIII – C4 – C5 CVI – C5 – C4	129.2(9)	
CII – C9 – CVII	124.8(11)	

carbon atom that seems to be more actively engaged in the π-delocalization (*viz.* the tropylium carbon atoms in the present compound).

The mean values of the distances and angles in the thiophene groups of the present compound are also compared to the corresponding values published for α-thiophene-carboxylic acid [6] in Table 6. It is seen that the averages of the sulfur-tropylium carbon distances are slightly longer than the sulfur – carbon distances in the carboxylic acid. No explanation can at the moment be given for the difference found in the angle at the non-fused thiophenic α-position in the present compound and in the carboxylic acid.

The positive charge of the C9 atom of the present compound is supposed to be delocalized over the whole cation, the β-thiophenic positions (C3, C6) being less positive than the other atoms.[4] More or less ionic interactions would therefore be expected between the perchlorate oxygen atoms and all of the non-hydrogen atoms of the dithienotropylium cation. Fig. 4 shows C – O contacts less than 3.2 Å and S – O contacts less than 3.3 Å for one cation. As seen, the C – O contacts fall in the range 3.06 – 3.11 Å. They are thus slightly shorter than or equal to the sum of the van der Waals radii, 3.1 Å. A somewhat similar situation arises in the crystal structure of succinylcholine perchlorate.[7] Here the positive charges of the quaternary ammonium groups are delocalized from the nitrogen atoms and weak ionic interactions occur between the carbonyl carbon atoms and the oxygen atoms of the perchlorate group. The corresponding C – O distances are 3.05 Å.

One of the distances between sulfur and a perchlorate oxygen atom in the present compound is quite short, 3.03 Å. However, S – O distances of the same magnitude, 3.02 and 3.04 Å, are found in the crystal structure of 5*H*,8*H*-dibenzo[*d*,*f*][1,2]-dithiocin-1,1-dioxide.[8] Likewise, a short S – O distance, 3.04 Å, exists in the crystal structure of phenylamino-2-phenyl-5-thiazolinone-4.[9] In the compounds just discussed the interactions do not seem to be of an ionic character.

Thus if there are ionic interactions between the atoms of the dithienotropylium cation and the perchlorate oxygen atoms, these interactions are weak.

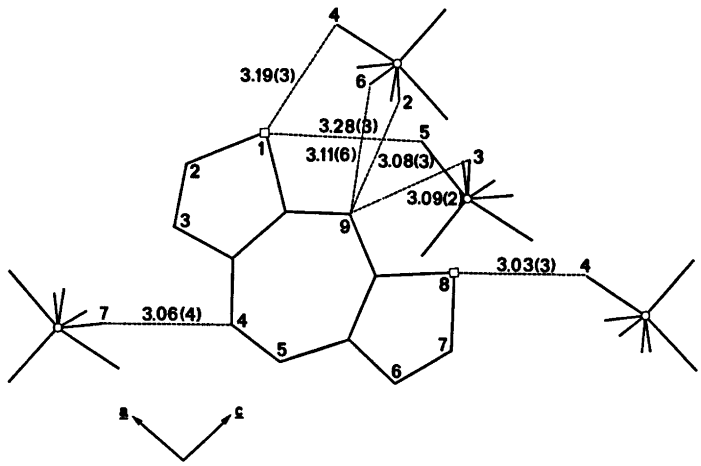


Fig. 4. The atoms of dithienotropylium perchlorate are projected on the *ac* plane along the *b* axis. Short contacts (Å) between the atoms of one dithienotropylium cation and the oxygen atoms of neighbouring perchlorate groups are indicated by dashed lines. The perchlorate oxygen atoms at the ends of the dashed lines are numbered according to Table 3a. *E.s.d.*'s in the distances are given within parentheses.

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Short Communications

A Study of the Ca—Na Salt of α-D-Galacturonic Acid by X-Ray Diffraction

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The studies of α-D-galacturonic acid derivatives, previously initiated with a structure determination of the methyl α-D-galacturonic acid methyl ester,[1] have been continued with an investigation of the Ca—Na salt, some preliminary results of which are given here.

Ca—Na-α-D-galacturonate was prepared by careful neutralization of an aqueous solution of α-D-galacturonic acid with $Ca(OH)_2$ to pH 6. The solution was then evaporated to crystallization and the product recrystallized from water at approximately 40 °C.**

Crystal data are: $2Ca^{2+}+2Na^{+}(C_6H_9O_7)_6^{-}.12H_2O$, hexagonal space group $P6_3$, cell dimension (with estimated standard deviations in parentheses) $a = 13.493(2)$ Å, $c = 9.655(2)$ Å, measured density 1.665 g cm^{-3}, calculated density 1.636 g cm^{-3}.

1077 reflexions with intensity greater than twice the background were observed on an automatic Picker FACS-1 diffractometer, using Mo$K\alpha$ radiation and the $\omega/2\theta$ scanning mode with max. $2\theta = 50°$. The Ca and Na positions were obtained from a 3-dimensional Patterson map. The C and O atoms were found by successive Fourier calculations. Full matrix least squares refinement,[2] with isotropic temperature factors for the Ca, O, and C atoms, has yielded the conventional R index of 0.091. A projection of the molecule along the c axis is shown in Fig. 1.

The Ca atoms occupy positions on the 3-fold axes and are caged by O(5) and O(7) from the galacturonate molecule and O(w1) from a water molecule, with Ca—O distances of 2.83, 2.39, and 2.43 Å, respectively. Due to the multiplicity of the axis the coordination around Ca is 9-fold. The Na atom on the 6_3 axis has O(2) and O(3) from the ring as nearest neighbours at distances of 2.36 and 2.50 Å, respectively. The coordination of Na is six-fold. Further refinement is in progress.

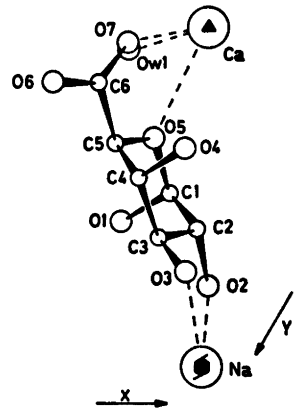

Fig. 1. Projection of the molecule along the c axis.

One of us (S. T.) is indebted to the Norwegian Agency for International Development (NORAD) for a fellowship. The work is part of a thesis (S. T.) for the degree Lic. Techn. at the University of Trondheim—Norges Tekniske Høgskole (NTH).

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** We originally planned to make the Ca salt but the product turned out to be the Ca—Na salt in low yield, the Na atoms probably coming from contamination. We later crystallized the Ca—Na salt from equal amounts of $Ca(OH)_2$ and NaOH and the crystals are identical to those previously obtained.

The Mutarotation of D-Glucose and Its Dependence on Solvent. III. The Existence of α- and β-D-Glucofuranose in *N,N*-Dimethylformamide

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Few authors have hitherto been concerned with the behaviour of D-glucose in *N,N*-dimethylformamide (DMF). Kuhn and Grassner [1] assume that the mutarotation almost exclusively is due to a pyranose-furanose conversion, while Jacin *et al.*[2] from their GLC investigations conclude that no anomerization had occurred during their experimental time. Our polarimetric observations are not compatible with any of the above statements. The plot of ln $([\gamma_t]-[\gamma\infty])$ against time always showed a significant deviation from a straight line at the experimental temperatures 20, 30, and 40 °C.[3] The rate of rotational change in thoroughly purified DMF (see Experimental) is extremely low, but was found to be very sensitive to the degree of purity. The shape of the mutarotation curves for both α- and β-D-glucose in DMF is similar to that described for the corresponding anomers of D-galactose in water.[4] Also the "thermal" mutarotation was found to be complex in analogy with that found for D-galactose in water.[5,6]

The observed deviation from a simple logarithmic law can be explained by the formation of the furanose anomers. In a preliminary communication [7] we have described the presence of D-glucofuranose, detected as trimethylsilyl

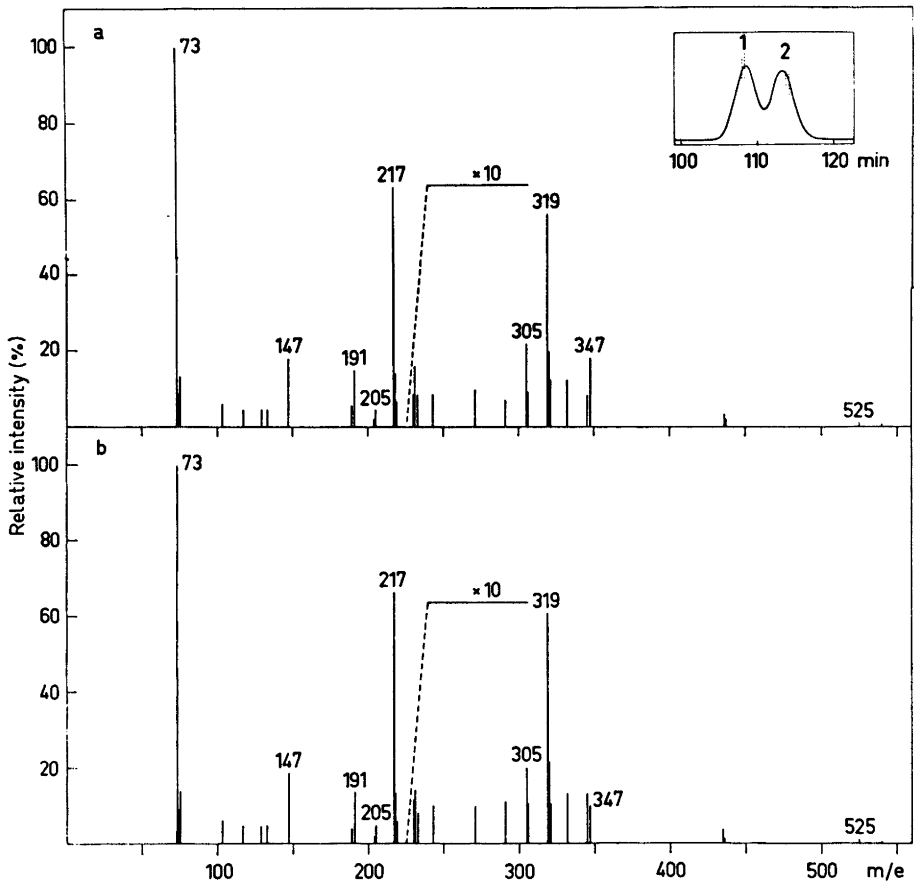

Fig. 1. Gas chromatographic separation and mass spectra of the two fastest moving persilylated tautomers of D-glucose. The partial mass spectra a and b are taken from peaks 1 and 2, respectively. The scan periods are indicated by broken lines in the chromatogram.

(TMS) derivative, in the equilibrium mixture (70 °C). We now report the existence of both α- and β-D-glucofuranose in this mixture. Using OV-17 as the stationary phase the previously described mixture of TMS derivatives was resolved into two minor peaks (1 and 2) and two major peaks (3 and 4). The relative retention times of these peaks were 0.76, 0.79, 1.00, and 1.65, respectively. The peaks 3 and 4 were, by the same methods as described earlier,[7] found to represent the α- and β-pyranosides, respectively. The gas chromatographic separation of peaks 1 and 2 in the GCMS analysis is shown in Fig. 1 along with partial mass spectra of these components. The two spectra (M^+ 540, 0.1 %) are nearly identical and show the characteristic differences from those of the pyranoses.[8,9] To establish which of peaks 1 and 2 represents the α- and which the β-anomer a separation was made by GLC on a preparative scale. To avoid a large α-pyranoid peak a mixture obtained by tautomerization of β-D-glucose at 70 °C for 1.5 min was used for this purpose. As the retention times of the two furanoid forms differ very little, they appeared as one broad peak under the preparative conditions. An NMR spectrum of a fraction collected from the first half of this peak showed a strong singlet at δ 5.04 and a weak doublet at δ 5.34, both being in the region ascribed to the anomeric protons. A fraction collected from the last half of the peak showed the same singlet and doublet, but both had approximately the same intensity. With the possible dihedral angles between neighbouring *cis*-hydrogens and between neighbouring *trans*-hydrogens in furanoid rings [10] the Karplus equation leads to the conclusion that the singlet only can be attributed to a β-furanoside. The α-furanoside should give a doublet. We judge our observations to indicate that peaks 1 and 2 represent the β- and α-furanoid form of TMS-glucose, respectively.

The total amount of furanose at 70 °C is calculated to be about 4.5 % of the total sugar content. This furanose proportion was found to develope in the first minutes of the mutarotation. This is in agreement with the many observations that in the ring formation of a sugar a five-membered ring is kinetically favourable while normally the six-membered ring is thermodynamically most stable. The mutarotation of D-glucose may have a complex character also in other organic solvents. It is of interest in this connection to note that the behaviour of D-glucose in ethylene glycol has been found to be "anormal".[11]

Experimental. The D-glucose products and *N*,*N*-dimethylformamide were those described earlier.[3] Purification of the DMF was obtained by filtration through Molecular Sieves (Union Carbide, 4A and 5A).

The polarimeter and the thermostats were as described in part I.[3] For the analytical studies a Hewlett Packard 5700A gas chromatograph equipped with a flame-ionization detector was used, while a Varian Aerograph 200 with a thermal conductivity detector was used for the preparative separation. The GLC-mass spectrometry was carried out on an LKB 9000 mass spectrometer. The column packing material was 10 % OV-17 on Chromosorb W (60 – 80 mesh, HMDS-treated for GLC and AW-DMCS for GCMS). For the analytical GLC an aluminium column (3 m × 3 mm) was maintained at 145 °C. The preparative GLC was carried out on a stainless steel column (1.5 m × 6 mm) at 150 °C and the separation for the mass spectrometer on a glass column (4 m × 6 mm) at 150 °C. The ion source block was maintained at 250 °C, the molecular separator at 190 °C, and the ionizing energy was 70 eV.

The NMR spectra ($CDCl_3$) were recorded on a Varian HA-100-15-D spectrometer operating at 98 MHz. Tetramethylsilane (TMS) was used as internal standard.

The mutarotation experiments were carried out as described previously.[3] For the preparation of the TMS derivatives the solutions (1 g/100 ml), still kept at the mutarotation temperature, were treated with trimethylchlorosilane and hexamethyldisilazane in the proportions described by Sweeley *et al.*[12] The reaction mixtures were stored for 8 h at room temperature in order to obtain full silylation, as short silylation time gave extra peaks in the chromatogram. The precipitated salts were removed by decantation, and the solutions were concentrated *in vacuo*. The sugar derivatives were extracted with hexane prior to injections into the gas chromatograph.

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Some Benzylseleno- and β-Phenylethylseleno-substituted Alkanoic Acids

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The title compounds have been prepared for two reasons. The original purpose was to test their activity in preventing dietary liver necrosis in rats (factor-3-effect) [1-4] and to study the influence of structural factors, *e.g.* the distance between the selenium atom and the carboxyl group, and the presence of substituents in the benzene nucleus. This part of the work has been carried out in collaboration with Professor Klaus Schwarz * and the results have in part been published.[5,6]

The compounds have also found use for studying the NMR-effects of the isotope ^{77}Se.

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These investigations were carried out in collaboration with Professor Salo Gronowitz and his group * and the results will be published elsewhere.

The present paper describes 38 acids (compounds I – IV and higher homologues). Three of these acids have been mentioned in earlier publications.[7,8] Since compounds of the types II and IV were found to be of minor interest regarding the factor-3-effect, only a limited number of these acids were prepared. The data are summarized in Tables 1 – 4.

$C_6H_5-CH_2-Se-CH_2-COOH$ (I)
$4\text{-}CH_3-C_6H_4-CH_2-Se-CH_2-COOH$ (II)
$4\text{-}Br-C_6H_4-CH_2-Se-CH_2-COOH$ (III)
$C_6H_5-CH_2-CH_2-Se-CH_2-COOH$ (IV)

In earlier papers, a number of naphthylmethylseleno acids [9] and nitro-substituted acids [10] have been reported.

Experimental. Diseleno-dicarboxylic acids were reduced in alkaline solution to selenol-substituted acids. These were not isolated but directly reacted with the appropriate chlorides or bromides. For the reduction, four methods have been used:

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Table 1. (Benzylseleno)-alkanoic acids.

Acid	m.p. °C	Calculated C	H	Se	Found C	H	Se
Acetic	71 – 72	47.17	4.40	34.46	47.29	4.45	34.45
					47.28	4.44	–
2-Propionic	68 – 69	49.39	4.97	32.47	49.46	5.01	32.34
3-Propionic	75 – 76	49.39	4.97	32.47	49.41	5.02	32.51
					49.40	4.97	32.37
3-Butyric	70 – 71	51.37	5.49	30.70	51.28	5.52	30.58
					51.39	5.43	30.55
4-Butyric	47 – 48	51.37	5.49	30.70	51.42	5.53	30.83
					51.43	5.50	–
2-Isobutyric	94 – 95	51.37	5.49	30.70	51.40	5.53	30.62
3-Isobutyric	46.5 – 47.5	51.37	5.49	30.70	51.50	5.52	30.66
					51.55	5.48	30.54
5-Valeric	45 – 46.5	53.14	5.95	29.11	53.04	5.99	29.20
					53.09	6.01	29.19
6-Caproic	40 – 41	54.74	6.36	27.68	54.48	6.36	27.68
					54.55	6.35	27.67
7-Oenanthic	49.5 – 50.5	56.19	6.74	26.40	56.18	6.77	26.26
					–	–	26.25
8-Caprylic	51 – 52	57.50	7.08	25.20	57.23	7.25	25.07
					57.22	7.33	25.03
9-Pelargonic	59.5 – 60.5	58.71	7.39	24.12	58.67	7.38	24.25
					58.68	7.40	24.21
10-Capric	61 – 62	59.81	7.68	23.13	59.95	7.78	22.94
					59.80	7.85	22.96
11-Undecanoic	67 – 68	60.83	7.94	22.22	60.79	7.98	22.14
					61.00	8.01	22.15

Table 2. (4-Methylbenzylseleno)-alkanoic acids.

Acid	m.p. °C	Calculated C	H	Se	Found C	H	Se
Acetic	94.5 – 95.5	49.39	4.97	32.47	49.54	4.91	32.29
					49.58	4.98	32.32
3-Propionic	76 – 77	51.37	5.49	30.70	51.62	5.55	30.61
					51.62	5.39	30.59
4-Butyric	57 – 58	53.14	5.95	29.11	52.83	5.87	29.39
					52.69	5.84	29.01
5-Valeric	(55) 60[a]	54.74	6.36	27.68	57.74	6.09	27.65
					57.48	6.27	27.60
8-Caprylic	65.5 – 67	58.71	7.39	24.12	58.67	7.42	23.98
					58.71	7.48	23.95

[a] Polymorphism.

Table 3. (4-Bromophenylseleno)-alkanoic acids.

Acid	m.p. °C	Calculated C	H	Se	Found C	H	Se
Acetic	99 – 100.5	35.09	2.95	25.63	35.03	2.93	25.48
					35.15	2.99	25.46
2-Propionic	71 – 72	37.29	3.44	24.52	37.22	3.43	24.48
					37.28	3.43	24.44
3-Propionic	81 – 82	37.29	3.44	24.52	37.31	3.41	24.46
					37.27	3.42	24.49
2-Butyric	77.5 – 78.5	39.31	3.90	23.49	39.22	3.85	23.41
					39.46	3.89	23.50
3-Butyric	53.5 – 54.5	39.31	3.90	23.49	39.50	3.92	23.49
					39.34	3.87	23.46
4-Butyric	70 – 71	39.31	3.90	23.49	39.41	3.92	23.46
					39.45	3.95	23.51
3-Isobutyric	64.5 – 65.5	39.31	3.90	23.49	39.42	3.92	23.48
					39.46	3.90	23.42
5-Valeric	63.5 – 64.5	41.16	4.32	22.55	41.29	4.38	22.50
					41.16	4.29	22.53
6-Caproic	58 – 59	42.88	4.71	21.68	42.70	4.64	21.60
					42.87	4.68	21.56
5-(3-Methyl)-valeric	43 – 44.5	42.88	4.71	21.68	42.92	4.71	21.57
					42.95	4.78	21.49
7-Oenanthic	63 – 64.5	44.46	5.06	20.88	44.48	5.09	20.74
					44.60	5.06	20.73
8-Caprylic	64 – 65.5	45.93	5.40	20.13	45.92	5.42	20.15
					45.80	5.42	20.07
9-Pelargonic	69 – 71	47.30	5.71	19.44	47.29	5.77	19.34
					47.30	5.78	19.27
10-Capric	72.5 – 74	48.58	6.00	18.79	48.51	6.04	18.79
					48.64	6.01	18.78
11-Undecanoic	75 – 77	49.78	6.27	18.18	49.87	6.37	18.28
					49.92	6.38	18.06

(1) Reduction with formaldehyde-sulphoxylate (rongalite) in aqueous ammonia.[9]

(2) Reduction with zinc powder in aqueous ammonia.[9]

(3) Reduction with sodium amalgam: the solution of the diselenide acid is stirred with the amalgam and the halogen compound is gradually added.[8]

(4) The diselenide acid is converted to mercury selenolate by shaking with metallic mercury in a suitable solvent.[11,12] The mercury selenolate acid is dissolved in slightly alkaline

Table 4. (β-Phenylethylseleno)-alkanoic acids.

Acid	m.p. °C	Calculated C	H	Se	Found C	H	Se
Acetic	71.5 – 72.5	49.39	4.97	32.47	49.44	5.02	32.46
					49.35	4.97	32.54
3-Propionic	46.5 – 47.5	51.37	5.49	30.70	51.45	5.50	30.72
					51.51	5.45	30.68
5-Valeric	37.5 – 38.5	54.74	6.36	27.68	54.75	6.40	27.62
					54.64	6.38	27.60
9-Pelargonic	44 – 45	59.81	7.68	23.13	59.89	7.69	23.10
					59.76	7.64	23.09

solution and the mercury is precipitated by adding an equivalent quantity of sodium sulphide ($Na_2S.12H_2O$) and shaking for some hours.[7]

Method 1 is generally most convenient and has been used for the majority of the compounds. Methods 3 and 4 can be used when foreign ions (zinc, sulphite) are not desirable. The procedure has been described in more detail in earlier papers.[7–9] A further example is given below.

11-(Benzylseleno)-undecanoic acid. Diseleno-11,11′-diundecanoic acid (6.7 g, 0.0125 mol) is placed in a strong flask or bottle of about 300 ml. Concentrated aqueous ammonia (75 ml) and water (25 ml) is added; since the ammonium salt of the acid is sparingly soluble in water, ethanol must be added with shaking to obtain a clear solution. Rongalite in slight excess is then added; if the solution is still yellow after 15 – 20 min, additional small amounts are added until the solution is quite colourless. The calculated amount of benzyl chloride (3.2 g, 0.025 mol), dissolved in 50 ml ethanol is finally added and the mixture is shaken until the milky suspension is practically clear. If the solution is yellow (due to partial oxidation to diselenide by the halogen compound), it is decolourised by adding some rongalite, after which a little benzyl chloride is added. Sometimes this operation must be repeated. A moderate excess of benzyl chloride is not harmful since it is converted to benzylamine by the ammonia. The solution is left to stand overnight.

The ethanol is then evaporated by a fan or a current of air, ammonia is added to strongly alkaline reaction and the solution is diluted to about 300 ml and finally shaken with ether to remove non-acidic impurities. The dissolved ether is removed by a current of air and the acid is precipitated by excess hydrochloric acid. The acid is obtained as a rapidly crystallising oil and recrystallised, first from formic acid (diluted with a little water) and then from ligroin or carbon tetrachloride. M.p. 67 – 68 °C. The yield of crude product is practically 100 %. Analyses see Table 1.

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Comparisons of Circular Dichroism in Liquid Solution and in the Crystal State with Reference to Conformations of 3,3′-Bithienyls

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The electronic structures as well as the nuclear arrangements in bithienyls have been the subject of recent studies by several workers, in the gas, liquid, and solid states.[1–7] However, in contrast to the corresponding biaryls,[8–10] which have a higher *quasi*-symmetry, no complete theory has been developed for the origin of the optical activity of the resolvable 3,3′-bithienyls. Consequently, the use of circular dichroism (CD) has been in general restricted to decisions between the two possible absolute configurations, on an empirical basis.[7] A correlation of the CD with a more precise structural assignment requires either a bridged compound [11,12] in which the conformation can be estimated from molecular models, or an independent structure determination.

In this paper we wish to report that the CD spectrum of 4,4′-dibromo-2,2′-dicarbomethoxy-3,3′-bithienyl (I) and that of 2,2′-dibromo-4,4′-dicarbomethoxy-3,3′-bithienyl (II), as obtained by the randomised crystal powder technique [13] on crystal fractions previously used in an X-ray structure determination,[4] closely resemble the corresponding solution CD spectra (Figs. 1 – 2).

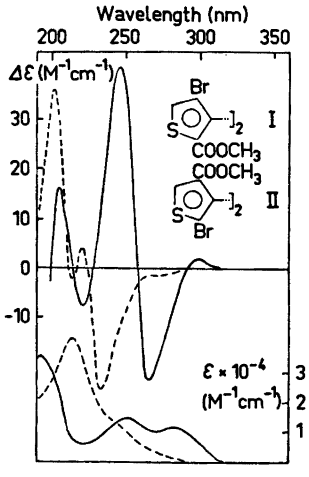


Fig. 1. Liquid solution (acetonitrile) CD and UV spectra of $R(+)$I ——— and $R(-)$ II – – –.

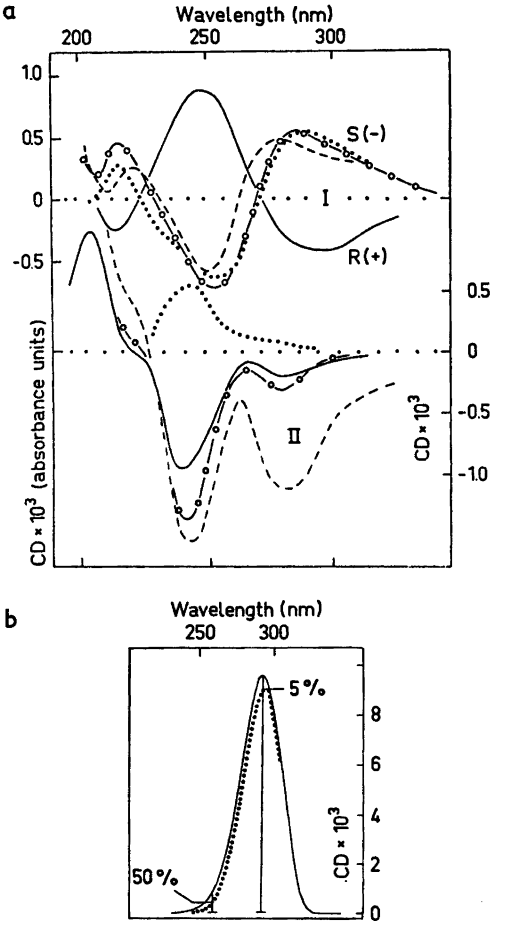
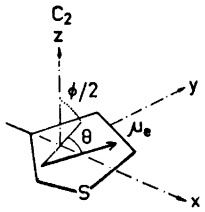


Fig. 2. a. CD spectra (selected from some 50) representative of cases of varying confidence: *Above:* $R(+)$ I ——— (3.0×10^{-3} %, KCl), S(−) I · · · (2.0×10^{-3} %, KCl), − O − (1.5×10^{-3} %, KCl), – – – (0.5×10^{-3} %, KCl). *Below:* $R(-)$ II – – – (3.7×10^{-3} %, KBr), − O − (0.7×10^{-3} %, KBr), ——— (0.5×10^{-3} %, KCl), S(+) · · · (0.3×10^{-3} %, KBr). b. Depolarisation test: A camphersulphonic acid/PVA standard [17] following the specimen in the light path · · · only standard ———. The decrease (50 % at 260 nm) is a measure of the percentage of the light intensity which is not acting as in a circularly polarized mode.

Before we may have confidence in an ordinary "pressed KBr disc" CD spectrum, it is necessary to minimize the influence of two principal errors, *viz.* the optical artifacts due to anisotropic CD or refraction (birefringence) of the crystallites, and those due to a stress birefringence induced by pressing the disc. The

first error is minimized by the randomisation procedure, while the second is detected by measuring the depolarisation.[13] An additional effect seems to be the scattering due to different refractive indices in substance and matrix. Preliminary experiments indicate that more reliable results are obtained by extrapolation to a zero substance concentration (*cf.* Fig. 2). On this basis Fig. 2 yields the following CD features for the *R*-configurations of I and II, in absorbance units (wavelengths, nm, in parentheses); I: −0.2 (225), +0.6 (250), −0.5 (275); II: +0.7 (200), ±? (230), −0.9 (240), −0.3 (275).



By analogy with, *e.g.*, biphenyls, a tentative conclusion which may be drawn from the great similarity between these features and the spectra in Fig. 1a, is that on the average the same structures of I as well as of II exist in both the solution and the crystal, *viz. cisoid* conformations with a dihedral angle $\phi = 75 - 90°$ in I and $60 - 70°$ in II.[4]

This conclusion is correct as long as any of the CD bands considered is due to a transition polarised at an angle θ (in the present notation) fairly close to zero, and if the CD is governed by a dipole-dipole coupling [14] ("exciton") mechanism. Such an origin of the CD may be of predominant importance considering the high oscillator strengths of the transitions involved and the probably small electronic exhcange between the thiophene nuclei.

The dipole coupling between the two equivalent transition moments μ_{e1}, μ_{e2}, directed as denoted above, gives rise to two components A and B (point group C_2) with rotational strengths given by eqn. (1) and at relative energies determined by (2).[15] It is thus obvious that CD bands due to transitions with $|\theta| > 35.3°$ will not depend in sign or energy on a conformational change ($0 < \phi < 180°$). However, it is most improbable that this is the trivial explanation for the

$$R_A = -R_B = \frac{\pi\nu}{2c} r_{12}\, \mu_e^2 \cos^2\theta \sin\phi \qquad (1)$$

$$\nu_A - \nu_B = \frac{2\mu_e^2}{hcr_{12}^3}(1 - 2\cos^2\theta\cos^2(\varphi/2) - 3\sin^2\theta) \qquad (2)$$

similarities between Fig. 1 and Fig. 2. Firstly, there is evidence for an intensive low-energy transition with $a = +30°$,[15] implying a "conformational" CD sign change due to eqn. (2) when ϕ exceeds 131.8°. Secondly, with a low $\nu_A - \nu_B$ the A and B components mutually cancel to a great extent, leading to an apparent CD spectrum whose features are very sensitive to changes in R, and consequently also to conformational changes.

Experimental details are to be described elsewhere.[13,16] KBr was found more suitable than KCl giving birefringence free discs with high transmission down to 230 nm. Close to 200 nm KCl was superior to KBr with respect to transparency. Discs with depolarisations (defined in Fig. 2) exceeding 5 % were discarded.

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The Conformation of Aliphatic Ethers

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The observation that the unique conformation of 1,5,9,13-tetraoxacyclohexadecane [1] and the stable crystal conformation of poly(trimethylene oxide) [2] are such that in each $-O-CH_2CH_2-CH_2-O-$ unit both CO-bonds are *anti* and both CC-bonds *gauche* of the same sign ($ag\pm g\pm a$) suggests that the 1,4-CH···O interaction may be attractive, whereas the the 1,4-CH···CH interaction must be even more repulsive than in hydrocarbons.[3]

It was of interest to verify this in simpler molecules and to check whether the relatively acidic character of the α-CH is a necessary condition for such an attractive interaction. We have therefore examined a series of dialkyl ethers and dimethoxypropanes where such interactions are possible between ether oxygen and either a non-acidic γ-hydrogen or an acidic α-hydrogen (Table 1). It was expected that any interaction would be reflected in an abnormal averaged chemical shift of the protons involved, in a relatively small vicinal α,β coupling constant, and in a relatively low dipole moment of the 1,3-dimethoxypropanes.

Chemical shift. The measured chemical shift values are shown in Table 1. For the methyl groups in propyl- and isobutyl ethers no significant chemical shift change due to interaction with or closeness to ether oxygen is noticeable, if the more distant methyl group in the butyl ether is taken as the reference. Of course, any interaction would be weak and averaged over three (or six) protons, so at most one can only conclude that such interaction is not demonstrated in the chemical shift.

In dimethoxypropane the α-methylene groups, which are those that would be involved in the interaction, appear about 0.10 ppm lower than α-methylene in the mono-ethers. This is a significantly larger effect than the 0.02 ppm that would be expected [4] merely by introducing an ether function in the γ-position.

For 1,3-dimethoxy-2,2-dimethylpropane no conclusion can be drawn, since the relatively large upfield shift of the α-methylene groups, due to the branching at the β-carbon,[5] dominates the picture. Similar upfield shifts are observed for methyl isobutyl ether (Table 1) and for methyl neopentyl ether.[5]

Table 1. NMR spectral data for simple ethers.

Solvent	Chemical shifts (δ)				Observed $J_{H\alpha H\beta}$ 58 °C	35 °C	−58 °C[a]	Expected $J_{H\alpha H\beta}$ Pure conformers			Statistical average
	$O(CH_2-CH_2-CH_3)_2$							*a*	*g*		
CCl_4	3.27	1.55	0.90		6.15	6.25	6.5	7.5	5.75		6.33
$CDCl_3$	3.35	1.57	0.92		6.50	6.62	7.0				
	$CH_3-O-CH_2-CH_2-CH_3$										
CCl_4	3.25	3.25	1.53	0.90	6.38	6.38	6.5	»	»		»
$CDCl_3$	3.32	3.32	1.57	0.90	6.50	6.62	6.75				
	$CH_3-O-CH_2-(CH_2)_2-CH_3$					6.3					
CCl_4	3.23	3.28	1.4	0.91				»	»		»
	$CH_3-O-CH_2-CH(CH_3)_2$							*a(g)*	*g(g)*		
CCl_4	3.23	3.03	1.75	0.87	6.25	6.45	6.5	7.5	4.0		6.33
$CDCl_3$	3.31	3.13	1.84	0.91	6.5	6.5	6.7				
	$(CH_3-O-CH_2-)_2CH_2$							*aa*	*ag*	*gg*	
CCl_4	3.25	3.35	1.73		6.2	6.2	6.12	7.5	6.63	5.75	6.5
$CDCl_3$	3.31	3.43	1.85		6.45	6.38	6.25				
	$(CH_3-O-CH_2-)_2C(CH_3)_2$										
CCl_4	3.25	3.03	0.83								
$CDCl_3$	3.31	3.13	0.88								

[a] −30 °C for CCl_4.

Vicinal coupling constants. Since the α-methylene protons give rise to a simple triplet* (or doublet) in a region well separated from the other resonances, except the single line of the methoxy group, the average α,β-vicinal coupling constant can be observed directly. For each conformer as well as for any mixture of conformers, an averaged coupling constant can be estimated, provided reliable values of *gauche* and *anti* H – H coupling constants are known. These have been shown [6,7] to depend additively on substituent electronegativities. Thus for "freely rotating" systems, with three identical conformers, the averaged vicinal coupling constant (representing *anti* and *gauche* couplings in the ratio of 1:2) has the values 8, 7.26, and 7 Hz for CH_3CH_3, $CH_3CH_2CH_3$, and CH_3CH_2OR, respectively.[6] Assuming additivity, we therefore expect the averaged α,β-coupling constant for $CH_3CH_2CH_2OR$ to be 6.26 Hz. From studies with rigid systems, such as *t*-butylcyclohexanols [8] and substituted 1,3-dioxans,[9] the best values for the corresponding individual coupling constants are $J_{anti} = 11$ Hz and $J_{gauche} = 4$ Hz, which gives 6.33 Hz as the average value for equally populated conformers, in excellent agreement with the value derived above. For the unbranched aliphatic mono-ethers, the calculated α,β-vicinal coupling constant is then 7.5 Hz if only the *anti*-conformer is populated, and 5.75 Hz for averaging between only two enantiomeric *gauche*-conformers. For methyl isobutyl ether the "double *gauche*" conformer, if it were populated exclusively, should have $J = 4$ Hz and the two "*gauche-anti*" conformers alone an averaged $J = 7.5$, but again the average value for equally populated conformers is 6.33 Hz. For each of four pure conformers of 1,3-dimethoxypropane (Fig. 1) the vicinal coupling constant can also be simply calculated (7.5, 6.63, 5.75, and 5.75 for *aa*, $ag^{\pm}$, $g^{\pm}g^{\pm}$, and $g^{\pm}g^{\mp}$) but since the $g^{\pm}g^{\mp}$ conformer is sterically forbidden, we get the average value 6.5 Hz for a statistical 1:4:2 population of the other three conformers, taking symmetry number and chirality into account.[10]

Before comparing these calculated values, summarized in Table 1, with the experimentally observed coupling constants, the expected influence of solvent and temperature on conformer population must be considered. Hydrogen bonding of chloroform to ethers has been demonstrated,[11] and this could influence the values of the coupling constants by altering the electronegativity of ether oxygen and/or the conformer populations. Since diethyl ether has the same coupling constant in $CDCl_3$ and CCl_4, any observed increase of the α,β-vicinal coupling constant in going from CCl_4 can be simply taken as the result of a decreased *gauche* conformer population.

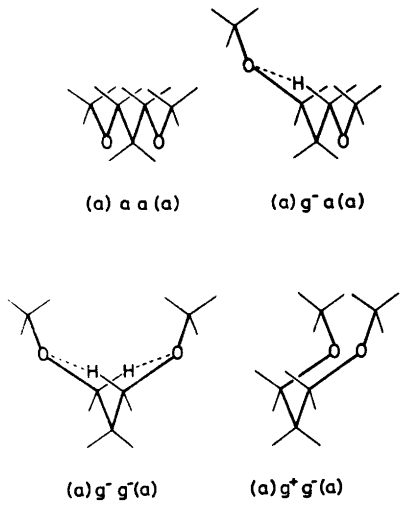

Fig. 1. Conformers of 1,3-dimethoxypropane.

When the observed coupling constant is close to the value expected for the statistical population of equi-enthalpic conformers, the direction of change observed on cooling may still reveal whether *gauche* or *anti* is enthalpy-preferred. All spectra were therefore recorded also at temperatures higher and lower than the usual probe temperature (Table 1).

Conclusions. For the normal unbranched mono-ethers, the numerical values of the averaged α,β-vicinal coupling constants show that there can be no important enthalpy difference between *gauche* and *anti* in CCl_4 solution, but the clear decrease observed as equal population is approached at higher temperature suggests that *anti* is slightly preferred. In $CDCl_3$ solution, the interaction of $CDCl_3$ with ether oxygen clearly favours the *anti*-conformers, as shown both by the numerical values and the temperature variation of the coupling constant. One can therefore conclude that the 1,4-*gauche* CH···O interaction is not attractive, but slightly repulsive when CH has no acidic character.

For the branched methyl isobutyl ether the same conclusions can be drawn.

1,3-Dimethoxypropane on the other hand has a significantly low coupling constant in CCl_4 solution and must consist at room temperature of roughly equal quantities of the *ag* and $g^{\pm}g^{\pm}$ forms. Since the *ag* form is statistically favoured, this means that the $g^{\pm}g^{\pm}$ conformer is lowest in enthalpy. The temperature variation of the

* Such a "deceptivelty simple" AA'XX' spectrum (Abraham, R. J. *The Analysis of High Resolution NMR Spectra*, Elsevier 1971) is a perfect triplet only when the *anti* and *gauche* conformers are equally populated, but even when a sligt broadening of the middle line betrays some enthalpy difference, the average vicinal coupling constant can still be extracted (=half the separation of the two outer lines).

coupling constant supports this conclusion. In $CDCl_3$ solution the situation is qualitatively similar, but the preference for *gauche* is much less pronounced. We thus conclude that the 1,4-*gauche* CH···O interaction is attractive, and competitive with the chloroform-ether interaction, when CH is made sufficiently acidic by the adjacent ether oxygen. Also the chemical shift data led to this conclusion. The dipole moment measured in benzene solution is 1.57 D (and 1.35 D for 1,3-dimethoxy-2,2-dimethylpropane) and this is significantly lower than calculated for randomly oriented ether groups ($1.3\ D \times \sqrt{2} = 1.8$ D) or for the *aa*-conformer (2.6 D), and so supports a general *gauche* preference. The even lower dipole moment of the 2,2-dimethyl derivative shows that there is no competition for the ether oxygen from the non-acidic methyl protons.

Experimental. Dipropyl ether was bought from Fluka and was used without purification. The methyl alkyl ethers were prepared [12] by gently heating the corresponding alcohol with dimethyl sulfate and distilling until the vapour temperature reached 100 °C. The distillate was passed through an alumina column and redistilled from sodium metal. Methyl propyl ether, b.p. 39–40 °C; methyl butyl ether, b.p. 70–71 °C; methyl isobutyl ether, b.p. 59–60 °C.

1,3-Dimethoxy-2,2-dimethylpropane was prepared [13] from neopentyl glycol by reaction with sodium hydride and methyl iodide in dry tetrahydrofuran and the product purified on an alumina column.

Dimethoxypropane [14] was prepared by the action of dimethyl sulfate on the dry disodium salt of trimethylene glycol (from sodium methoxide and trimethylene glycol in methanol). The distillate was twice redistilled from solid sodium hydroxide, passed through an alumina column and finally distilled from sodium hydride; b.p. 106–107 °C.

No impurity signals were apparent in the NMR spectra of the ethers so prepared.

The NMR spectra were recorded on a Varian HA 100 instrument for solutions containing about 7 % of the ether and TMS as internal reference. The dipole moments were determined as described previously.[15]

We thank the Science Research Council, London, for a NATO-fellowship to D.G.T.G.

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The Use of Tetrabutylammonium Azide in the Curtius Rearrangement

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The conversion of a carboxylic acid chloride to the corresponding azide is traditionally carried out with sodium azide.[1] The insolubility of the latter in common organic media makes it desirable to use a water-containing solvent mixture, *e.g.*, with aromatic acid chlorides. With lower aliphatic acid chlorides, their sensitivity to hydrolysis precludes the use of protic solvents, and heterogeneous conditions have to be accepted, leading to irreproducible results.

The use of hydrazoic acid and pyridine is described in a recent communication.[2] Acyl azides can thus be prepared under mild and homogeneous conditions. The same is the case when tetramethylguanidinium azide is used.[3] However, the latter two methods require the

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The use of hydrazoic acid and pyridine is described in a recent communication.[2] Acyl azides can thus be prepared under mild and homogeneous conditions. The same is the case when tetramethylguanidinium azide is used.[3] However, the latter two methods require the

Table 1. Preparation and rearrangement of acyl azides from tetrabutylammonium azide.

Starting chloride	Solvent [a]	Per cent N_2 evolved	Product isocyanate, b.p. (°C) found; lit.[b]	Per cent yield of aniline derivative	Aniline derivative m.p. (°C) found; lit.[b]
Acetyl	B	84	Methyl, 38; 40	52	150; 149–150
Propionyl	B	93	Ethyl, 61.5; 59.8	61	98–99; 100
Butyryl	T	93	Propyl, 88–90; 88	73	115.5–116.5; 116–116.5 [c]
Isovaleryl	B	90	Isobutyl, 102; 104–106	86	151–152; 151–152
Pivalyl	T	97	*t*-Butyl, 85.5; 84.6	60	167.5–168.5; 167–168
4-Chlorobutyryl	T	87	3-Chloropropyl, 60–61 at 2.7 kPa; 54.6–54.8 at 2.1 kPa	78	123–124; 127
Benzoyl	T	96	Phenyl, 158–162; 55 at 1.7 kPa	89	238–239; 237–237.5

[a] B, benzene; T, toluene. [b] Literature values from *Beilstein* unless otherwise indicated. [c] See ref. 8.

handling of hydrazoic acid with its concomitant hazards.

The present paper describes a method of azide synthesis, in which the azide ion is readily extracted as an ion-pair with tetrabutylammonium ion from an aqueous solution into an organic phase.

Tetrabutylammonium azide has been obtained in pure, crystalline form after extraction into methylene chloride, followed by evaporation of the solvent. This azide has earlier been prepared from hydrazoic acid and tetrabutylammonium hydroxide.[4–6] The azide, which apparently can be safely handled in dry form, is soluble in a number of organic solvents. Metathesis with carboxylic acid chlorides can thus be carried out in a one-phase system. The resulting acyl azides can be directly used in subsequent reactions.

In the present work, a number of acyl azides have been prepared and made to rearrange to isocyanates by heating. The reactions were followed by the nitrogen evolution. The isocyanates formed were characterized by conversion to substituted ureas with aniline.

It is possible to perform reactions with the isocyanates in the same solution that was used in the Curtius rearrangement. Final separation from the tetrabutylammonium chloride should ordinarily cause no problems, since the salt is readily removed from organic solvents by water.

Results obtained with different acyl chlorides are summarized in Table 1.

Experimental. Tetrabutylammonium azide. A solution containing 0.2 mol of tetrabutylammonium hydroxide was prepared by addition of 50 ml of 10 M sodium hydroxide solution (an excess) to 67.9 g (0.2 mol) of tetrabutylammonium hydrogen sulphate in 100 ml of water. A solution of 26 g (0.4 mol) of sodium azide in 50 ml of water was added and tetrabutylammonium azide extracted with 100 ml of dichloromethane. It is important to have an excess of sodium hydroxide in order to avoid extraction of free hydrazoic acid, which might make the subsequent operations very dangerous. The organic layer (upper phase), 170 ml, was separated off and the aqueous phase again extracted with 100 ml of dichloromethane (lower phase). The combined organic phases were evaporated *in vacuo* at 40 °C to yield 59.8 g (theor. 56.9 g) of crude tetrabutylammonium azide as a colourless oil. Residual water was removed by azeotropic distillation with benzene or toluene. The solutions thus obtained were used directly in the following step. If so desired, the dry tetrabutylammonium azide can be obtained in quantitative yield as a crystalline, hygroscopic solid by evaporation of the solvent *in vacuo*. Recrystallization from toluene gives colourless crystals, m.p. 80 °C, lit.[6] 80 °C (decomp.).

Curtius rearrangement. A dry solution of 0.2 mol of tetrabutylammonium azide (the above batch) in 150 ml of benzene or toluene (see Table 1) was placed in a round-bottomed flask equipped with a reflux condenser, a thermometer, a stirrer and a dropping funnel. To this solution the equivalent amount (0.2 mol) of freshly distilled acyl chloride in 150 ml

of benzene or toluene was added in portions. The temperature was kept below 25 °C by cooling in ice water. The mixture was left for 3 – 4 h and then heated, a gas burette being attached to the top of the reflux condenser. Nitrogen evolution started at 50 – 90 °C. The gas evolution was allowed to proceed at a brisk rate by gradual heating, giving a total reaction time of less than 4 h. The isocyanate was separated from the solvent by distillation through a 30 cm stainless steel spiral column having 5 mm internal diameter. Because of the instability of isocyanates, yields were variable but usually better than 50 %.

To obtain a better determination of the yields, the Curtius rearrangement was repeated (on a 7 mmol scale). After the nitrogen evolution had ceased, a slight excess of aniline was added to the reaction mixture. The resulting *N*-alkyl-*N'*-phenyl urea was freed from tetrabutylammonium chloride by washing the solution with water. The organic solvent was evaporated and the resulting product recrystallized from aqueous ethanol. The yields of the urea derivatives obtained are given in Table 1. The reaction with aniline is assumed to be quantitative,[7] and the recrystallization losses are small. The identity was further verified by ^{1}H NMR.

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Reinvestigation of the Synthesis of Thiete 1,1-Dioxide

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The four-membered cyclic unsaturated title compound was needed as a synthetic intermediate in our work. Dittmer and Christy [1,2] first prepared it as indicated in Fig. 1. Since

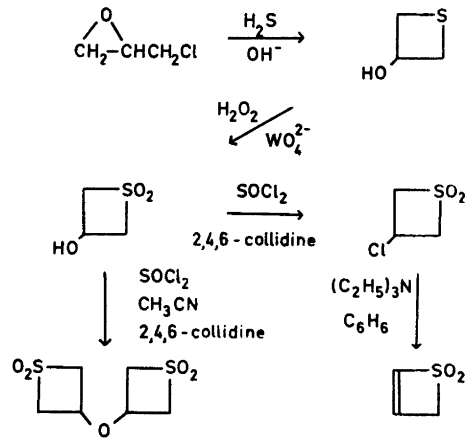

Fig. 1. Synthetic route to thiete 1,1-dioxide and bis-(3-thietanyl 1,1-dioxide) ether.

the yield in the first step was only 39 %, and since the second step involved a reportedly [8] hazardous evaporation of peracetic acid, an alternative synthetic route was later described.[3] This starts with a 2 + 2 cycloaddition of sulfene to *N*,*N*-dimethylvinylamine. The latter compound is very unstable, requiring low temperature and oxygen-free conditions, and we did not deem a large-scale preparation attractive.

Upon reinvestigation of the original procedure, we found that the first step is much more conveniently carried out in potassium hydroxide solution than with barium hydroxide.[1] The reaction of epichlorohydrin with hydrogen sulfide in basic solution was first described by Sjöberg.[4] He obtained 30 % yield with potassium hydroxide, but a much higher yield, 65 %, is actually secured by continuous extraction of the aqueous phase with ether.

The second step, oxidation of 3-thietanol to the sulfone, can advantageously be carried out with 30 % aqueous hydrogen peroxide and a catalytic amount of sodium tungstate [5] instead of the usual mixture of aqueous hydrogen peroxide and acetic acid.[1]

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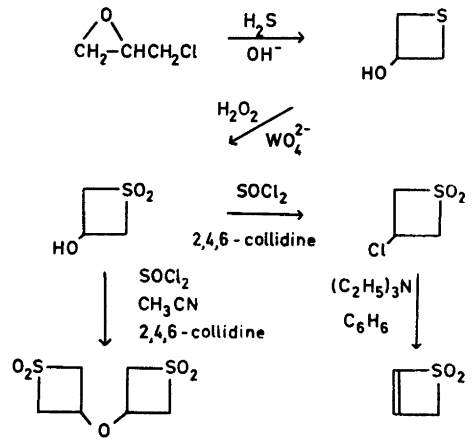

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Upon an attempted preparation of 3-chlorothietane 1,1-dioxide in acetonitrile as solvent, the hitherto unreported bis-(3-thietanyl 1,1-dioxide) ether was obtained instead of the chloro compound. This ether was characterized through its spectral data (IR, 60 MHz ^{1}H NMR, and high-resolution MS). The analogous sulfide is known.[6]

It is possible that in acetonitrile, the 3-thietanol 1,1-dioxide anion can compete with chloride ion in the decomposition of the chlorosulfite ester initially formed from 3-thietanol 1,1-dioxide and thionyl chloride, since the alcohol is soluble in acetonitrile. The desired chloro compound is formed only under "dry" conditions.

Experimental. 3-Thietanol. A solution of 200 g of potassium hydroxide (3 mol, assuming 15 % moisture content) in 900 ml of water was saturated with hydrogen sulfide. Epichlorohydrin, 143 g (1.54 mol), was added dropwise during 2 h with good stirring and simultaneous introduction of hydrogen sulfide. The temperature gradually rose to 60 °C in this process. A slow stream of hydrogen sulfide was introduced for 1 h, and the mixture left overnight. It was then exhaustively extracted with ether, a continuously working apparatus being used. The ether was removed *in vacuo* and the remainder distilled to yield 91 g of 3-thietanol (65 % yield), b.p. 95 – 97 °C at 3.3 kPa (25 mmHg).

3-Thietanol 1,1-dioxide. Of the preceding compound, 90 g (1 mol) was dissolved in 500 ml of water containing 2 g of sodium tungstate and about 0.5 ml of glacial acetic acid; pH 4 – 5. While stirring well and maintaining the temperature at 20 °C with a glass cooling coil, 250 ml of 30 % hydrogen peroxide (appr. 2.5 mol) was added during 1 h. The reaction mixture was watched for 5 h and cooled as required to maintain the temperature at 20 °C. It was then left overnight. After decantation from a small amount of a sticky, white by-product, excess peroxide was destroyed with a pinch of palladium on carbon. After a negative peroxide test, the colourless solution was evaporated *in vacuo* at a temperature not exceeding 40 °C. *Since explosions have been reported to occur in the similar preparation using acetic acid-hydrogen peroxide,[2] we earnestly urge the reader to take necessary precautions.* The evaporation of 1 mol batches has been carried out four times without incidents.

The remainder after evaporation consisted of a colourless crystalline material and a small amount of a likewise colourless oil. The crystals were brought into solution with boiling ethyl acetate and decanted from the oil. Upon cooling, large crystals were obtained, m.p. 100 °C, lit.[1] 99.5 – 102 °C, yield 75 g (61 %).

3-Chlorothietane 1,1-dioxide. The directions in Ref. 1 were closely followed. With 20 g batches, the yield was typically 77 %, lit.[1] 86 %, m.p. 135 – 137 °, lit.[1] 136.5 – 137.5 °C.

Thiete 1,1-dioxide. Also this compound was prepared according to Ref. 1. Recrystallization from ether containing a few per cent ethanol gave a fraction m.p. 49 – 50 °C, lit.[1] 48 – 50 °C, representing 63 % yield, lit.[1] 81 %. From the mother liquor, a second crop was secured, m.p. 45 – 50 °C, 27 % yield.

Bis-(3-thietanyl 1,1-dioxide) ether. 3-Thietanol, 5 g (0.041 mol) and redistilled 2,4,6-collidine, 5 g (0.041 mol) were dissolved in 50 ml of dry acetonitrile. A solution of 9.8 g (0.082 mol) of thionyl chloride in 25 ml of acetonitrile was added dropwise during 30 min with stirring and cooling in ice. A crystalline precipitate was formed during the addition but disappeared when the mixture was heated in a boiling water bath for 30 min. The resulting clear solution was evaporated at aspirator vacuum, and the solid remainder triturated with cold water. A greyish, insoluble material was filtered off and recrystallized from boiling water with charcoal decolourization. White needles were obtained, yield 0.6 g (13 %), m.p. 165 – 165.5 °C (Kofler Hot Stage). One further recrystallization did not change the m.p. The 60 MHz ^{1}H NMR spectrum in deuterated dimethyl sulfoxide consisted of a complex pattern at δ 4.1 – 5.0 and another one at δ 5.2 – 5.6, the integrals of which were in the ratio 4:1. The IR spectrum (KBr disc) had two broad bands, characteristic of sulfones, at 1 120 – 1 160 and 1 300 – 1 340 cm^{-1}, and a band with peaks at 1 210 and 1 230 cm^{-1} which can be ascribed to an ether structure. In the region above 1 450 cm^{-1}, only two bands at 2 970 and 3 040 cm^{-1}, typical of C – H stretching, were apparent but no hydroxyl band. The high-resolution mass spectrum was obtained with an AEI MS 902 instrument, using 50 eV electrons for ionization and a heated direct insertion probe. The fragmentation pattern was found to be very dependent on the probe temperature. A very low intensity M + 1 peak appeared at 150 °C (not visible at lower temperatures), measured mass 227.008, rel. height 0.03 % ($C_6H_{11}S_2O_5$ = 227.005). No parent peak was visible. Fragment peaks were apparent at 168.9634 mass units, 4.12 % ($C_3H_5S_2O_4$ = 168.9629); 148.0202, 0.59 % ($C_5H_8SO_3$ = 148.0194); 123.0119, 1.03 % ($C_3H_7SO_3$ = 123.0116); 104.9983, 14.0 % ($C_3H_5SO_2$ = 105.0010); 94.0085, 8.1 % ($C_2H_6SO_2$ = 94.0088); 78.9854, 6.3 % (CH_3SO_2 = 78.9853); 75.9986, 10.7 % (C_2H_4SO = 75.9989); and 63.9623, 100 % (SO_2 = 63.9619). The $C_3H_5S_2O_4$ peak is thought to result from a rearrangement procedure.

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preciated. Mr. P. Bergmark assisted in the preparative work and is also thanked.

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Alkyl Cyanates. XV. Reaction of Alkyl and Aryl Cyanates with Grignard Reagents. Product Formation

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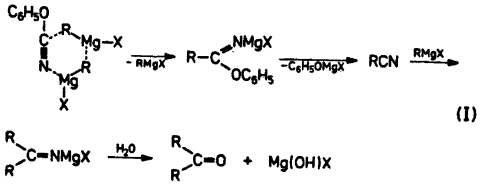
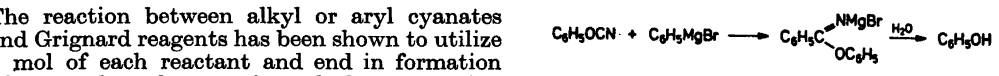
The reaction between alkyl or aryl cyanates and Grignard reagents has been shown to utilize 1 mol of each reactant and end in formation of a complex of magnesium alcoholate or phenolate, nitrile, and diethyl ether. The same complex is obtained directly by mixing the components. Evidence for imido ester salts as intermediates in the reaction between alkyl or aryl cyanates and Grignard reagents has not been obtained.

The reaction between Grignard reagents and aryl cyanates has been described by Martin and Rackow[1] and by Grigat, Pütter and Mühlbauer.[2,3] Martin and Rackow found that nitrile was formed and that 2 mol of Grignard reagent to 1 mol of phenyl cyanate were necessary for the reaction to go to completion. The second mol or molecule of Grignard reagent was assumed to act as a Lewis acid facilitating the nucleophilic attack on the cyanate. However, the nitrile formed partly reacts with the excess Grignard reagent yielding a ketone after hydrolysis, according to Martin and Rackow (eqn. I).[1] The experiments were carried out at −20 °C.



(I)

Grigat *et al.* investigated the reaction at −50 °C and isolated benzonitrile in 61 % yield using 1 mol of phenylmagnesium bromide to 1 mol of phenyl cyanate. The reaction was formulated as eqn. II.



(II)

The magnesium salt of phenyl imidobenzoate was assumed to be an intermediate although no evidence for this was presented. The reaction between phenylmagnesium bromide and 4-methylphenyl cyanate proceeded similarly.

The reaction between ethyl cyanate and Grignard reagents is briefly mentioned by Martin *et al.*[4] With cyclohexylmagnesium bromide and benzylmagnesium chloride nitrile was formed as described above with the aromatic cyanates. However, ethylcyclohexane and propylbenzene, respectively, were also found to be reaction products. With phenylmagnesium bromide only benzonitrile was identified.

At the time that these results were published we had some unpublished data and now wish to report on a more detailed investigation.

Addition of pentylmagnesium bromide in ether to an equimolar amount of isobutyl cyanate at −80 °C gave a white precipitate (*vide infra*). After warming to room temperature the supernatant ether solution was analysed by gas chromatography and found to contain approximately 20 % of the theoretical amount of capronitrile. Only traces of isobutyl alcohol were present. After hydrolysis of the reaction mixture the total yields of capronitrile and isobutyl alcohol were 72 % and 73 %, respectively.

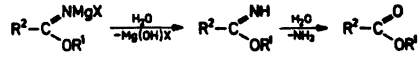
The addition of phenylmagnesium bromide to an equimolar amount of isobutyl cyanate at −80 °C proceeded similarly, and benzonitrile

and isobutyl alcohol were isolated in 87 % and 89 % yields, respectively, after hydrolysis. Furthermore, the gas-chromatrographic analysis showed that some benzene (8 %) was also formed.

When 2 mol of phenylmagnesium bromide were added to 1 mol of isobutyl cyanate at −80 °C and the mixture was warmed to room temperature a further reaction with the benzonitrile formed took place. After hydrolysis diphenylketimine, $(C_6H_5)_2C=NH$, could be isolated in 70 % yield besides an 84 % yield of isobutyl alcohol, even though diphenylketimine is liable to form benzophenone on further hydrolysis. Benzophenone was the only product isolated by Martin and Rackow and by Grigat *et al.*

To test the generality of these reactions a number of experiments were carried out in which the organic moieties of the cyanate and the Grignard reagent were varied. As shown in Table 1 the yields in most cases were high. The low yields of ethanol isolated in the reactions between ethyl cyanate and Grignard reagents are assumed to be due to the high solubility of ethanol in water from which ethanol is extracted. Secondary cyanates are known to undergo elimination reactions with basic reagents [5] and this may explain the low yields of alcohol and nitrile isolated from the reactions of isopropyl cyanate with aliphatic Grignard reagents. In no cases were alkylation products detected in the reaction mixture as found in the investigation of Martin *et al.*[4]

We have most carefully tried to obtain evidence for the intermediacy of the halomagnesium imidate proposed by Martin and Rackow and by Grigat *et al.* (eqns. I and II). On hydrolysis the imidate salt should yield an imido ester or eventually a further hydrolysis product (eqn. III).



(III)

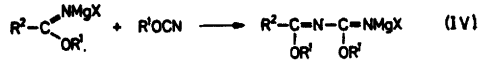
However, despite careful scrutiny we have not observed any of these products, either in the final reaction mixture or when the reaction was interrupted before completion. We have furthermore not observed reaction products of the possible imidate salt and a second mol of cyanate, which would be similar to those

Table 1. Yields of reaction products in the reaction between alkyl and aryl cyanates and Grignard reagents.

R^1OCN	R^2MgX	Molar ratio R^1OCN/R^2MgX	Yield [a] R^1OH %	R^2CN %	$R^2{}_2C=NH$ %
C_2H_5OCN	CH_3MgBr	1:1	48	62	–
»	C_6H_5MgBr	1:1	51	71	–
$(CH_3)_2CHOCN$	CH_3MgBr	1:1	46	38	–
»	C_4H_9MgBr	1:1	58	57	–
»	*t*-C_4H_9MgCl	1:1	45	34	–
»	C_6H_5MgBr	1:1	90	79	–
$(CH_3)_2CHCH_2OCN$	CH_3MgBr	1:1	79	61	–
»	C_2H_5MgCl	1:1	87	92	–
»	C_4H_9MgBr	1:1	90	87	–
»	*t*-C_4H_9MgCl	1:1	72	68	–
»	$C_5H_{11}MgBr$	1:1	73 [b]	72 [b]	–
»	C_6H_5MgBr	1:1	89 [b]	87 [b]	–
»	»	1:2	84 [b]	~0 [b]	70 [b]
C_6H_5OCN	CH_3MgBr	1:1	86	71	–
»	C_4H_9MgBr	1:1	100	86	–
»	*t*-C_4H_9MgCl	1:1	87	92	–
»	C_6H_5MgBr	1:1	92	81	–

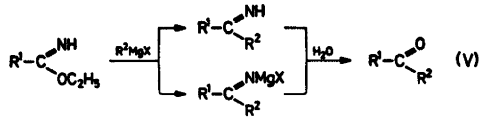
[a] The yields are calculated from the amount of cyanate employed and were obtained from gas chromatograms by electronic integration unless another means is indicated. [b] Yields determined by conventional work-up.

obtained in reactions between some nitriles and Grignard reagents (eqn. IV).[6,7]



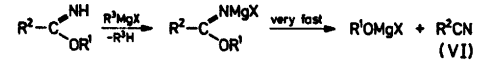
(IV)

It seems evident that the halomagnesium imidate should be formed in a reaction between an alkyl imido ester and a Grignard reagent. This reaction has been commented on only briefly, but general reaction schemes (eqn. V) were proposed.[8,9]



(V)

In a reinvestigation we have found, however, that nitrile is the isolable reaction product (eqn. VI).[10]



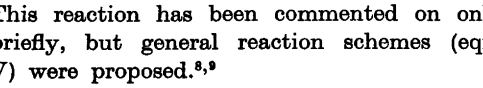
(VI)

Obviously combination of the nitrile with excess Grignard reagent followed by hydrolysis will lead to the observed ketone.

Thus the reaction between alkyl imido ester and Grignard reagent in fact yields the same products as are obtained from cyanates and Grignard reagents. It is also important to note that we have not been able to isolate the halomagnesium salt that obviously should be first formed, which again parallels the reaction between cyanate and Grignard reagent.

The white precipitate formed on mixing cyanate with Grignard reagent (*vide supra*) appears to be a complex with the composition $R^1OMgX.R^2CN.\frac{1}{2}(C_2H_5)_2O$. This composition was obtained from the integrals of the nuclear magnetic resonance spectrum of the complex ($R^1=R^2=C_6H_5$) dissolved in $(CD_3)_2SO$. In the infrared spectrum obtained in paraffin oil an absorption is observed at 2263 cm^{-1} which is assigned to benzonitrile coordinated to the magnesium atom. In infrared spectra of the complexes $C_6H_5CN \rightarrow AlCl_3$ and $C_6H_5CN \rightarrow SnCl_4$ the absorptions are observed at 2280 cm^{-1} and 2258 cm^{-1}, respectively.[11,12] Uncoordinated benzonitrile absorbs at 2235 cm^{-1} in paraffin oil. The product obtained on mixing phenoxymagnesium bromide and benzonitrile in diethyl ether had the same characteristics as those described for the complex formed from phenyl cyanate and phenylmagnesium bromide.

Further evidence for the composition of the complex was obtained by adding a different nitrile to the reaction mixture before hydrolysis. It could be shown by means of GLC that an exchange of nitrile in the complex with the nitrile added had taken place (eqn. VII).



(VII)

It has not been possible to obtain a satisfactory elemental analysis of the white complex owing to its hygroscopic nature.

In conclusion, the reaction between alkyl or aryl cyanates and Grignard reagents has been shown to proceed with 1 mol of each reactant despite the statement of Martin and Rackow.[1] The presence of a halomagnesium imidate on the route to the final products has not been shown, but on the other hand the data obtained do not exclude this intermediate. The final reaction product is in all cases a complex between magnesium alcoholate or phenolate, nitrile, and diethyl ether, which can also be made directly by mixing the components.

The further details of these reactions can be ascertained only from kinetic experiments, the results of which are presented in following papers.[13,14]

EXPERIMENTAL

All Grignard reagents were prepared in diethyl ether distilled from lithium aluminum hydride directly into the glass apparatus. This solvent was used in all reactions. The glass apparatus had been flamed and filled with argon. The magnesium used (monosublimed, Dow Chemical Corp.) was washed with anhydrous diethyl ether. Every precaution was taken against oxygen and moisture. The halides used in the preparations of the Grignard reagents were distilled and their purity checked gas-chromatographically. The molarity of the Grignard reagents was determined by titration with standard acid and the content of halogen by titration with standard silver nitrate. The content of halogen was never more than 4 % higher than the content of Grignard reagent. The different concentrations were obtained by dilution of *ca.* 2 M standard solution.

Alkyl cyanates were prepared from 5-alkoxy-1,2,3,4-thiatriazoles [5,15] and aryl cyanates from

phenols and cyanogen chloride in the presence of triethylamine.[16] The purity of the cyanates was checked by infrared and nuclear magnetic resonance spectroscopy and elemental analysis.

Infrared spectra were obtained on a Perkin-Elmer 337 grating spectrometer and nuclear magnetic resonance spectra on a Varian A-60 A instrument.

A. The reaction between isobutyl cyanate and pentylmagnesium bromide. To 50 ml of 2 M isobutyl cyanate cooled in an acetone/dry ice bath was added 50 ml of 2 M pentylmagnesium bromide with stirring over a 30 min period. After approximately 10 % of the Grignard solution had been added a white precipitate was formed. After the addition was finished the reaction mixture was allowed slowly to reach room temperature (30 min), which caused transformation of the solid into a very viscous liquid. A sample of the supernatant ether solution was analysed by means of gas chromatography and found to contain capronitrile corresponding to approximately 20 % of the theoretical amount, but only traces of isobutyl alcohol. After hydrolysis with 50 ml of 4 M hydrochloric acid saturated with sodium chloride a sample of the ether phase was again analysed gas-chromatographically and now found to contain capronitrile and isobutyl alcohol, both in approximately 75 % yield based on isobutyl cyanate. The water phase was extracted twice with 20 ml of ether, the combined extracts dried over magnesium sulfate and the ether partially removed by evaporation *in vacuo* at 10 °C. Distillation at atmospheric pressure yielded 3 fractions: *1.* 80 – 103 °C, 0.7 g; *2.* 103 – 108 °C, 5.0 g (68 %); *3.* 160 – 163 °C, 6.5 g (73 %); residue 1.0 g. The first fraction was found on gas chromatography to contain ether and isobutyl alcohol; the second was almost pure isobutyl alcohol and the third almost pure capronitrile. The products were identified by comparison of their infrared spectra with those of authentic samples. The residue was a tar of unknown composition.

The gas-chromatographic analyses were performed on a Perkin-Elmer 116 E gas chromatograph; column SE 30; column temperature 117 °C; injection temperature 200 °C; detector temperature 100 °C; carrier gas helium; flow rate approximately 100 ml/min.

B. The reaction between isobutyl cyanate and phenylmagnesium bromide (molar ratio 1:1). This experiment was carried out analogously to the reaction between isobutyl cyanate and pentylmagnesium bromide. After drying the combined ether extracts and evaporation of the ether, the remaining liquid was shown by gas chromatography to contain benzene in an amount corresponding to 8 % of the phenylmagnesium bromide used. The following fractions were obtained by distillation: *1.* 10 mmHg, bath temperature 40 °C, 6.6 g (89 %); *2.* 1 mmHg, bath temperature 80 °C, 9.0 g (87 %); residue 0.2 g (black tar). Fractions 1 and 2 were found to consist, of isobutyl alcohol and benzonitrile, respectively, by gas chromatography and infrared spectroscopy.

C. The reaction between isobutyl cyanate and phenylmagnesium bromide (molar ratio 1:2). To 50 ml of 2 M isobutyl cyanate cooled in an acetone/dry ice bath was added 100 ml of 2 M phenylmagnesium bromide with stirring during 30 min. The reaction mixture was then warmed to room temperature and stirred for additional 24 h. Water (100 ml) was added, the phases separated and the water phase extracted twice with 30 ml of ether. After drying ether was removed *in vacuo* and the residue distilled: *1.* 1 mmHg, bath temperature 30

Table 2. Conditions for the gas-chromatographic analysis for determining yields of the reaction between cyanates and Grignard reagents.

Column	Column temp. °C	Determination of yield of
Carbowax 1500	59	Ethanol and acetonitrile
Polypropylene glycol	59	Ethanol and valeronitrile
Carbowax 1500	59	2-Propanol and acetonitrile
Polypropylene glycol	55	2-Propanol and valeronitrile
»	57	2-Propanol and pivalonitrile
»	95	2-Propanol and benzonitrile
»	85	2-Methyl-1-propanol and acetonitrile
»	90	2-Methyl-1-propanol and valeronitrile
»	50	2-Methyl-1-propanol and pivalonitrile
»	50→120 [a]	Phenol and acetonitrile
»	50→120 [a]	Phenol and valeronitrile
»	50→120 [a]	Phenol and pivalonitrile
»	135	Phenol and benzonitrile

[a] Temperature programming (5 min at 50 °C and increase of the temperature to 120 °C at 10 °C per min).

°C, 6.2 g (84 %); *2.* 1 mmHg, bath temperature 125 °C, 12.6 g (70 %); residue 0.5 g (black tar). Fraction 1 was identified as isobutyl alcohol by infrared spectroscopy and fraction 2 as diphenylketimine by infrared spectroscopy and elemental analysis (Found: C 85.91; H 6.22; N 7.29. Calc. for $(C_6H_5)_2C=NH$: C 86.20; H 6.08; N 7.74).

D. Determination of yields by electronic integration of gas chromatograms in reactions of cyanates with Grignard reagents. 40 ml of 1 M cyanate was mixed with 40 ml of 1 M Grignard reagent by flowing the two solutions together in a stainless steel T-tube of diameter 0.6 mm. After mixing the reaction mixture was sprayed into a glass bulb cooled in ice/water. The reagents were cooled to −50 °C before mixing. The reaction mixture was hydrolysed with 20 ml of 4 M hydrochloric acid saturated with sodium chloride and the ether and water phases separated. The water was extracted 3 times with 30 ml of ether and the combined ether phases were dried over magnesium sulfate. A known amount of *o*-xylene (internal standard) was added to the filtered ether solution and the mixture diluted to 200 ml in a graduated flask. The gas chromatograms (conditions are given in Table 2) of these solutions were integrated electronically and yields of alcohol, phenol, and nitrile determined from standard curves (g_x plotted against $g_s A_x/A_s$*). Gaschromatographic analyses were performed on a Pye Unicam 104 chromatograph in connection with a Varian aerograph 477 integrator and a Victor digitmatic digital recorder. The flow rate was 45 ml/min.

E. The reaction of phenyl cyanate with phenylmagnesium bromide. To 20 ml of 0.2 M phenyl cyanate was added slowly 2.4 ml of 1.67 M phenylmagnesium bromide with stirring at room temperature. The ether solvent was evaporated and the residue washed 3 times with 5 ml of anhydrous ether and kept at 1 mmHg and 35 °C for 1 h.

The nuclear magnetic resonance spectrum of the white product dissolved in $(CD_3)_2SO$ indicated a content of phenolate, benzonitrile, and diethyl ether in the ratio 2:2:1.

The infrared spectrum of the product in paraffin oil had a characteristic band at 2263 cm^{-1}. Benzonitrile dissolved in paraffin oil absorbs at 2235 cm^{-1}.

F. The reaction of phenoxymagnesium bromide with benzonitrile. 0.02 mol (1.88 g) of phenol and 0.02 mol (2.06 g) of benzonitrile were dissolved in 50 ml of diethyl ether. To this solution was added at room temperature 9.72 ml of 2.057 M methylmagnesium bromide. A white precipitate was formed at once. The ether was evaporated *in vacuo* and the residue washed 3 times with 5 ml of anhydrous ether and kept at 1 mmHg and 35 °C for 1 h.

The nuclear magnetic resonance spectrum of the product in $(CD_3)_2SO$ showed a content of phenolate, benzonitrile and diethyl ether in ratio 2:2:1.

The infrared spectrum of the product dissolved in paraffin oil had an absorption band at 2263 cm^{-1}.

G. Exchange of nitrile formed in the reaction of a cyanate with a Grignard reagent with a different nitrile. 50 ml of 2 M isobutyl cyanate and 50 ml of 2 M pentylmagnesium bromide were allowed to react as described above. After warming to room temperature 20 % of the theoretical amount of capronitrile was shown to be present in the supernatant ether by means of GLC. Valeronitrile (0.1 mol, 8.3 g) was added to the reaction mixture, and after 10 min with stirring the contents of caponitrile and valeronitrile in the ether phase were 36 % and 76 %, respectively, of the theoretical. After hydrolysis with 4 M hydrochloric acid saturated with sodium chloride the contents of capronitrile, valeronitrile and isobutyl alcohol in the ether phase were 75 %, 100 %, and 75 %, respectively, of the theoretical. The conditions for the gas-chromatographic analysis are described in *A*.

A similar experiment was carried out on the mixture from the reaction between phenyl cyanate and phenylmagnesium bromide. The benzonitrile formed was exchanged with *p*-tolunitrile.

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* g_x grams of alcohol, phenol or nitrile; g_s grams of internal standard. A_x and A_s are the corresponding areas found by integration.

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Studies on a Soluble Dipeptidase from Pig Intestinal Mucosa. Enzymatic Properties

OVE NORÉN

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The kinetics of a soluble dipeptidase (glycyl-L-leucine dipeptidase, EC 3.4.13.2) purified from pig intestinal mucosa were studied under stable assay conditions. The pH-optima for the reaction of L-alanyl-L-alanine and glycyl-L-leucine with the enzyme were settled to 8.4 and 8.0, respectively. In the reaction with these two substrates the enzyme followed Michaelis-Menten kinetics and the K_m-values found were 0.74 mM and 2.1 mM for L-alanyl-L-alanine and glycyl-L-leucine. Several L-amino acids inhibited the enzyme and a kinetic study on the L-leucine inhibition showed it to be competitive in nature. The soluble dipeptidase was also inhibited by SH-compounds, EDTA, and SH-reagents while glycerol and divalent metal ions only slightly influenced the activity of the enzyme. DFP had no inhibitory effect.

Only a few intestinal exopeptidases have hereto been purified.[1-4] We have in a preceding report described a preparative purification procedure of a soluble dipeptidase from pig intestinal mucosa (glycyl-L-leucine dipeptidase, EC 3.4.13.2) together with its specificity.[5] Earlier studied proteolytic enzymes of the gastrointestinal tract followed the Michaelis-Menten kinectics and showed a distinct dependence of common enzyme inhibitors.[6] The objective of this investigation was to put the purified soluble dipeptidase in relation to these well-characterized proteolytic enzymes by studying these parameters.

MATERIALS AND METHODS

Enzyme. The soluble dipeptidase was prepared as described earlier.[5] The enzyme had a specific activity of 1400 units of activity per mg protein. In order to avoid repeated freezing and thawing the enzyme was stored in small samples at −20 °C in 0.07 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl, 4 mM 2-mercaptoethanol, and 12.5 % (w/v) glycerol. The concentration of the enzyme was about 600 units of activity per ml. Under these conditions the enzyme was found to be stable for several months. One of the stored samples was used for each experiments.

Chemicals. Glycyl-L-leucine was purchased from Sigma Chem. Co., St. Louis, U.S.A., and L-alanyl-L-alanine was obtained from Cyclo Chem. Corp., Los Angeles, U.S.A. The purity of the two dipeptides was tested by thin layer chromatography (precoated cellulose plates, 0.1 mm, Merck, Darmstadt, Germany; butanol-acetic acid-water, 4:1:1 by vol.; ninhydrin-visualization). Amino acids were obtained from Mann Res. Labs., New York, U.S.A. DFP and sodium *p*-hydroxymercuribenzoate (PHMB) were purchased from Sigma Chem. Co., St. Louis, U.S.A. All other chemicals used were of analytical grade and de-ionized and glass destilled water was used throughout.

Assays. Dipeptidase activity was assayed according to one of the following procedures. *Fixed time procedure* followed in general the assay method of Josefsson and Lindberg [7] with the modifications of Sjöström.[8]

The dipeptides and amino acid solutions were prepared in 0.05 M Tris-HCl buffer solution at proper pH (pH-meter 28, Radiometer, Copenhagen, Denmark) in their suitable concentrations (12.3 mM and 8.5 mM for glycyl-L-leucine and L-alanyl-L-alanine, respectively, unless otherwise stated). The incubation mixtures, containing an enzyme concentration of about 0.2 units of activity per ml, were incubated at 25 °C for 10 min unless otherwise stated. In experiments where compounds with disturbing UV-absorbance were present the dipeptidase activity was assayed by using the 2,4,6-trinitrobenzenesulfonic acid (TNBS-) reagent as described earlier.[5] *Continuously recording procedure*, as described by Sjöström [8] was used for the determination of the kinetic coefficients of the enzyme. The molar extinction

coefficient of the peptide bond of the substrates was determined at each wavelength used.

Unit of dipeptidase activity. One unit of soluble dipeptidase activity is defined as the amount of enzyme hydrolyzing 1 μmol glycyl-L-leucine (12.3 mM) per min in 0.05 M Tris-HCl buffer (pH 8.0) at 25 °C.

RESULTS AND DISCUSSION

Stability of the enzyme. Stability studies were performed, using glycyl-L-leucine as substrate and the fixed time procedure, to assure stable assay conditions for the soluble dipeptidase. Solutions of varying enzyme concentrations (2 – 20 units of activity per ml) were prepared in two different buffers (0.05 M Tris-HCl, pH 8.0, and 0.1 M sodium phosphate, pH 7.8) and stored at 0 °C. Samples were withdrawn at once after the preparation of the solutions and then for each 30 min interval during a 2 h period and assayed for the dipeptidase activity. No decrease of enzyme activity was observed. Likewise, an enzyme solution (0.5 units of activity per ml) in 0.05 M Tris-HCl buffer (pH 8.0) stored at 25 °C and assayed for its activity at 6 min interval during a 36 min period showed no loss of activity. The stability of the soluble dipeptidase was also studied at different pH-values (0.05 M Tris-HCl buffer, pH 7.0, 8.0, and 9.0). At each pH a series of incubation mixtures with varying enzyme concentrations (0.82 – 0.13 units of activity per ml) were prepared and assayed for their activity, using incubation times for the separate mixtures inversely proportional to their enzyme concentrations (5 – 30 min). At each pH the various solutions showed the same percentage of glycyl-L-leucine hydrolysis, indicating no loss of activity during the time of incubation.

pH-Optima of the enzyme. The influence of pH on the reaction rate was studied in the pH range 6.9 – 8.9 (0.05 M Tris-HCl buffer) with glycyl-L-leucine and L-alanyl-L-alanine as substrate and using the fixed time procedure. pH-Measurements of the different reaction mixtures at the start and at the end of the incubation period made in parallel experiments showed a change of less than 0.03 pH-units. The pH-activity curves for the enzyme are presented in Fig. 1. The pH-optimum, 8.0, for the dipeptidase reaction with glycyl-L-leucine is in good accordance with the values earlier reported for pig intestinal

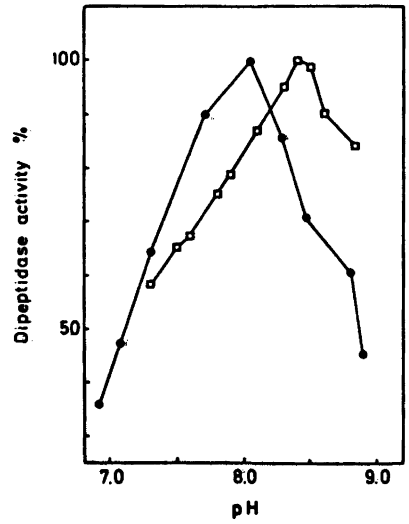

Fig. 1. pH-Activity curves for the soluble dipeptidase. Substrate: □ L-Alanyl-L-alanine; ● glycyl-L-leucine.

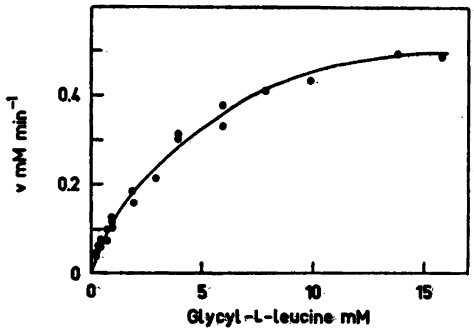

Fig. 2. Dipeptidase activity as a function of glycyl-L-leucine concentration.

mucosa homogenate[7] and for the purified monkey intestinal dipeptidase.[8] The pH-optimum for L-alanyl-L-alanine was found to be 8.4.

Kinetics of the enzyme. Experiments using the fixed time procedure were performed to determine the type of kinetic behaviour of the enzyme. The rate of hydrolysis of glycyl-L-leucine was measured in 0.05 M Tris-HCl buffer (pH 8.0) at different substrate concentrations (0.25 mM to 16 mM). The reactions were not allowed to proceed to more than 15 % of hydrolysis. The velocities calculated from these experiments were plotted *versus* the corresponding

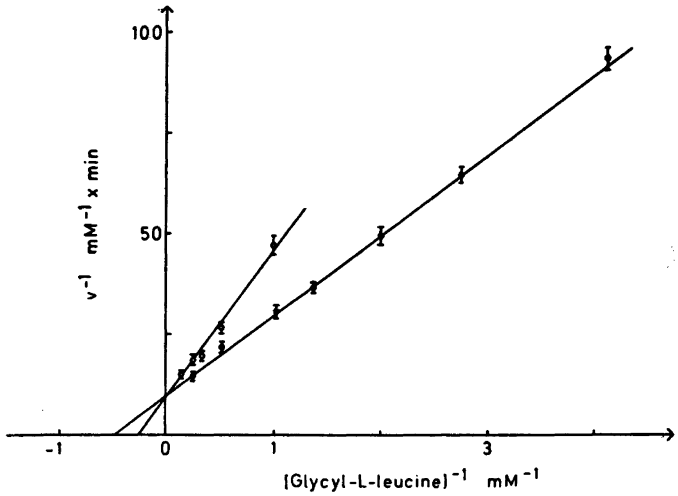

Fig. 3. Experimental data for the determination of the kinetic coefficients for the soluble dipeptidase without (●) and with (○) L-leucine (4 mM) using glycyl-L-leucine as substrate. ⊥ indicates 2 S. D. of the velocity measurements.

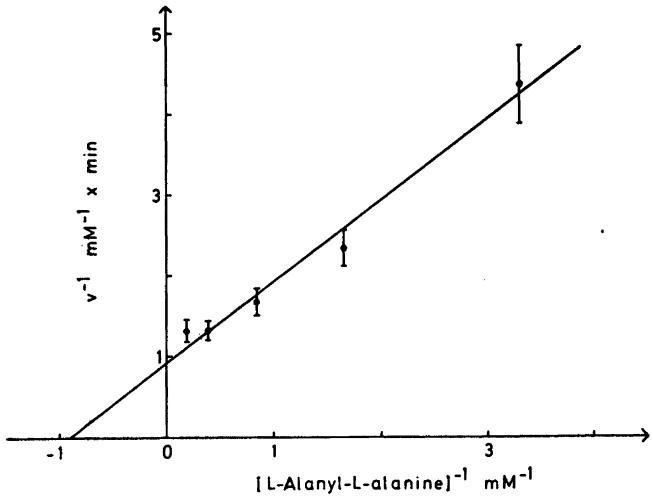

Fig. 4. Experimental data for the determination of the kinetic coefficients for the soluble dipeptidase using L-alanyl-L-alanine as substrate. A vertical bar indicates 2 S.D. of the velocity measurements.

substrate concentrations (Fig. 2). They were found to be located along a hyperbola and even at low substrate concentrations no sigmoid shape was observed. At high substrate concentrations the curve approached asymptotically a maximum value of velocity and substrate inhibition was not observed within the concentration range studied.

The determination of K_m- and V-values for the soluble dipeptidase were performed using the continuously recording procedure. To get a rapid and objective method for the calculation of the kinetic coefficients, with their standard errors, the statistical estimation method of Wilkinson [9] was used. The calculation procedure was translated into Univac-Algol and the ex-

perimental data were processed in a computer (Univac 1106, Recku, Copenhagen, Denmark). The experiments were performed in 0.05 M Tris-HCl buffer at pH 8.0 with glycyl-L-leucine and at pH 8.4 with L-alanyl-L-alanine, varying the peptide concentrations between 0.24 – 3.87 mM and 0.30 – 4.84 mM, respectively. The results obtained are presented in Fig. 3 and 4. Six different velocity measurements were made at each substrate concentration in the experiments with glycyl-L-leucine and the calculations resulted in a K_m of 2.1 (S.E. 0.13) mM and a V of 0.11 (S.E. 0.0034) mM per min. In the experiments with L-alanyl-L-alanine five separate velocity measurements were made at each substrate concentration and the calculations gave a K_m of 0.74 (S. E. 0.10) mM and a V of 0.94 (S. E. 0.039) mM per min.

Previous studies on soluble intestinal dipeptidase activities [10,11] have shown kinetic in accordance with the theory of Michaelis and Menten. This finding is also valid for studies performed on membrane bound intestinal peptidase activities using L-phenylalanyl-glycine and glycyl-L-phenylalanine as substrates [11] and for the renal dipeptidase.[12] K_m values have also been reported for the purified soluble monkey intestinal dipeptidase [8] without details on its kinetic behaviour. In the present study it has been found that for the two substrates studied the Michaelis-Menten kinetics may be applied. The found K_m values are of the same magnitude as those reported for the dipeptides with the enzymes mentioned above.

Using these kinetic data the molecular activity can be calculated to 2.4×10^5 min^{-1} and 6.4×10^5 min^{-1} for glycyl-L-leucine and L-alanyl-L-alanine, respectively, by using a molar extinction coefficient of 14.6×10^4 M^{-1} cm^{-1} for the soluble dipeptidase.[13]

Inhibitors of the enzyme

Amino acids. In the study of possible product inhibition of the dipeptidase reaction with glycyl-L-leucine it was observed, that L-leucine inhibited the reaction markedly, while glycine was only slightly inhibitory. Keeping the wide specificity of the soluble dipeptidase in mind [5] these observations prompted a more general study on the inhibitory effect of the amino acids.

Table 1. Influence of amino acids on the activity of the soluble dipeptidase.

Amino acid (L-forms) added (10 mM)	Dipeptidase activity (%)	Assay method[a]
–	100	A
Ala	95	A
Arg	80	A
Asp	95	A
Glu	99	A
Gly	96	A
His	37	B
Ile	59	A
Leu	55	A
Lys	94	A
Met	75	A
Phe	97	B
Pro	99	A
Ser	80	A
Thr	91	A
Trp	58	B
Val	73	A

[a] A. Spectrophotometric method.[7] B. TNBS-method.[5]

Each amino acid (Table 1) was mixed separately with glycyl-L-leucine and incubated with a solution of the dipeptidase. The activity was measured using the fixed time procedure. The results (Table 1) showed that L-histidine inhibited the reaction most strongly but also L-isoleucine, L-leucine, and L-tryptophan showed a marked inhibitory effect. L-Arginine, L-methionine, L-serine, and L-valine moderately slowed down the reaction rate, while the other amino acids tested had no or only a slight inhibitory effect on the dipeptidase reaction. This variation in the inhibitory effect of the amino acids, is, however, not parallel to our knowledge about the specificity of the dipeptidase,[5] and requires therefore a more detailed study to elucidate the inhibitory mechanism of the amino acids.

The type of amino acid inhibition was studied using L-leucine as inhibitor. L-Leucine (4 mM) was added separately to a series of glycyl-L-leucine solutions (0.99 – 5.94 mM). The reaction velocity of a dipeptidase solution was then assayed with the various substrate concentrations using the continuously recording procedure (0.05 M Tris-HCl buffer, pH 8.0). Five different velocity measurements were made at each substrate concentration. The experimental data

are presented in the double reciprocal form in Fig. 3. The apparent K_m and V were calculated to be 3.8 (S.E. 0.35) mM and 0.11 (S. E. 0.0054) mM per min, respectively, using the statistical estimation method described above. The K_m-values obtained for glycyl-L-leucine in the absence and presence of L-leucine were compared using the t-test [14] and were found to be significantly different (degrees of freedom 63, P = 99.9. As the V-values obtained showed no difference L-leucine can be regarded as a competitive inhibitor. From the determined kinetic coefficients K_i for L-leucine was calculated to be 5.0 mM.

Glycyl-D-leucine. Glycyl-D-leucine, not hydrolyzed by the enzyme,[5] was mixed with the L-form of the dipeptide in equal concentrations (12.3 mM) and incubated with an enzyme solution, using the fixed time procedure (0.1 M sodium phosphate buffer, pH 7.8). No change of the rate of hydrolysis was observed in this mixture when compared with a parallel incubation mixture containing the same amount of enzyme but glycyl-L-leucine only. Thus glycyl-D-leucine does not interfere with the substrate binding site on the enzyme.

SH-compounds. To ascertain stability of the dipeptidase during its purification, all solutions had to contain 2-mercaptoethanol (4 mM),[5] although this compound strongly inhibited the dipeptidase activity (Table 2). Other SH-compounds, when present in the same concentration, also inhibited the dipeptidase reaction with glycyl-L-leucine (Table 2). As a consequence of its presence in the enzyme stock solutions, the influence of 2-mercaptoethanol on the dipeptidase reaction was further investigated in concentrations valid for the assay mixtures. An enzyme stock solution was passed through a column of Sephadex G-25 Fine (0.07 M sodium phosphate buffer, pH 7.0, added 0.2 M NaCl) to remove the 2-mercaptoethanol and glycerol present. This enzyme solution was assayed according to the fixed time procedure against two series of glycyl-L-leucine solutions (0.25 mM and 12.3 mM), each series added 2-mercaptoethanol in concentration varying from 0 to 12 μM. No influence on the dipeptidase reaction was observed in the series with 12.3 mM substrate concentration, while a 2-mercaptoethanol concentration above 6 μM showed a slight inhibition of the activity in the series with 0.25 mM substrate concentration. To avoid inhibition in the kinetic experiments (see above), precautions were therefore taken not to allow the 2-mercaptoethanol concentration in the incubation mixtures to rise above 6 μM.

EDTA. The influence of EDTA (1 mM) on the dipeptidase reaction was measured using the fixed time procedure with glycyl-L-leucine as a substrate. EDTA was added to the substrate-buffer solution and no preincubation of the dipeptidase was undertaken. The inhibition observed (Table 3), suggests that the enzyme is dependent of a metal ion for its proper function.

SH-reagents. The influence of Hg^{2+} and PHMB on the dipeptidase reaction was studied. The reagents were added to the substrate-buffer solution and the dipeptidase activity was measured using the fixed time procedure with glycyl-L-leucine as substrate. In the concentrations used both reagents had strong inhibitory effect on the

Table 2. Influence of SH-compounds on the activity of the soluble dipeptidase. Assay conditions: Fixed time procedure (0.1 M sodium phosphate buffer, pH 7.8).

SH-compound added (4 mM)	Dipeptidase activity (%)
–	100
2-Mercaptoethanol	10
L-Cysteine	10
Thioglycolic acid	40
Cysteamine	30

Table 3. Influence of miscellaneous compounds on the activity of the soluble dipeptidase.

Compound added	Concentration mM	Dipeptidase activity (%)	Assay method[a]
–	–	100	A
EDTA	1.0	40	A
DFP	0.15	100	A
PCMB	0.1	44	B
Hg^{2+}	0.001	55	B
Co^{2+}	0.005	83	A
Mn^{2+}	0.005	84	A
Mg^{2+}	0.005	95	A
Zn^{2+}	0.005	90	A
Glycerol	50	95	A

[a] A. Spectrophotometric method.[7] B. TNBS-method.[5]

dipeptidase reaction (Table 3), suggesting that thiol group(s) of the soluble dipeptidase are necessary for its function.

Glycerol. During the purification procedure glycerol was used to achieve stable conditions for the dipeptidase.[5] Its influence on the dipeptidase reaction was therefore studied using the fixed time procedure with glycyl-L-leucine as substrate and with glycerol present in the substrate-buffer solution. In the concentration used only a slight inhibitory effect was recognized (Table 3).

DFP. The inhibitory effect of DFP was studied by preincubating a dipeptidase solution (0.8 units of activity per ml) for 20 min at 25 °C in an 0.1 M sodium phosphate buffer (pH 7.7), made 0.15 mM in respect to DFP. Parallel samples, pre-incubated as above, but without DFP added, served as a control. The dipeptidase activity was measured against glycyl-L-leucine using the fixed time procedure. No effect on the dipeptidase activity was observed under these conditions (Table 3). Chymotrypsin, included in the study and assayed against casein, showed a total inactivation under the same experimental conditions.[15]

Divalent metal ions. Co^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} were added directly to the glycyl-L-leucine solution. The activity of the soluble dipeptidase measured using the fixed time procedure, was only slightly influenced (Table 3), which is in accordance with observations earlier reported on the glycyl-L-leucine dipeptidase activity in crude extracts of pig intestinal mucosa.[7]

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Lignin Chromophores. Part I. Synthesis of Chromophores of the 2,4′- and 4,4′-Dihydroxystilbene Types

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Convenient syntheses of 2,4′- and 4,4′-dihydroxystilbenes (types C and E) and their hydroxymethyl derivatives (types D and F) are described.

The key step of the syntheses is a Knoevenagel condensation of appropriate aromatic aldehydes with suitably substituted arylacetic acids to give stilbenecarboxylic acids and/or their lactones. This step is followed by decarboxylation of the stilbenecarboxylic acids and removal of protecting (acetyl) groups (preparation of types C and E), or by esterification of the stilbenecarboxylic acids or lactones and reduction of the resulting esters with lithium aluminium hydride (preparation of types D and F).

The versatility of the method is shown. The analytical and spectral data for the various products and intermediates are summarised in tables.

During pulping, certain phenolic units in lignins are converted to hydroxystilbene structures. These may represent intermediates during the various pulping processes [1-3] or constitute potential chromophoric systems in the resulting pulps [1,4,5] and spent liquors.[6-8] Thus, phenolic units of the phenylcoumaran type (A, see Fig. 1) give 2,4′-dihydroxystilbene structures (C [9,10] and D [2]), and phenolic units of the 1,2-diarylpropane-1,3-diol type (B) yield 4,4′-dihydroxystilbene structures (E [11,12] and F). It has been suggested that these reactions in alkaline media proceed *via* intermediate methylene quinones (G and H)[1,4,9] and in acidic media *via* intermediate carbonium ions (I and J).[1,2,13] Elimination of the terminal hydroxymethyl group from these two types of intermediates gives stilbene structures which are unsubstituted at the olefinic carbon atoms (C and E).[1,4-13] Loss of their β-proton affords intermediate stilbene structures, substituted at one of the olefinic carbon atoms by a hydroxymethyl group (D and F).[2,13]

For studies of the reactions of the 2,4′- and 4,4′-dihydroxystilbene type structures (C–F) during pulping, bleaching and ageing representative models were needed.

Previously,[6,14] hydroxystilbenes have been synthesised by heating tris-hydroxyaryl-trithianes in the presence of heavy metals (Fe, Cu). The trithianes required can be prepared by treating the appropriate aromatic hydroxyaldehyde with an excess of hydrogen sulfide in acidic solution.[15] However, owing to various side reactions, in particular polymerisations, during the heat treatment of the trithianes, the yields obtainable by this desulfurisation method are usually low. Moreover, the desulfurisation method can only be used for the preparation of symmetrically substituted stilbenes.

The synthesis of asymmetrically substituted stilbenes entails the coupling of two different moieties by some type of condensation after protection of the phenolic hydroxyl groups. Grignard syntheses [16] and Wittig syntheses [16-22] have been used to achieve condensation between free,[18] benzylated,[16,21] acetylated,[16,22] methylated,[20] methoxymethylated,[21] or trimethylsilylated,[19] phenolic benzylhalogenides and aromatic aldehydes. These methods afford low overall yields of hydroxystilbenes, unsubstituted at the olefinic carbon atoms.

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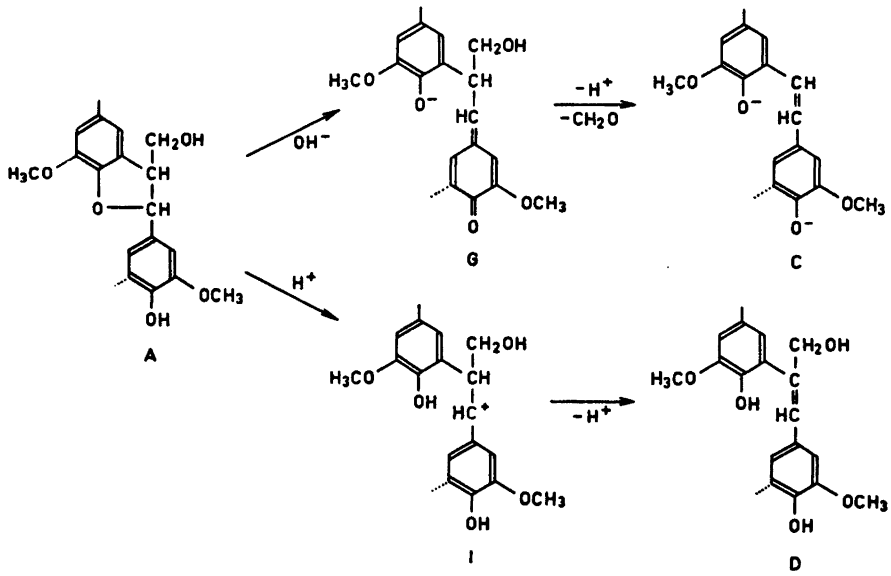
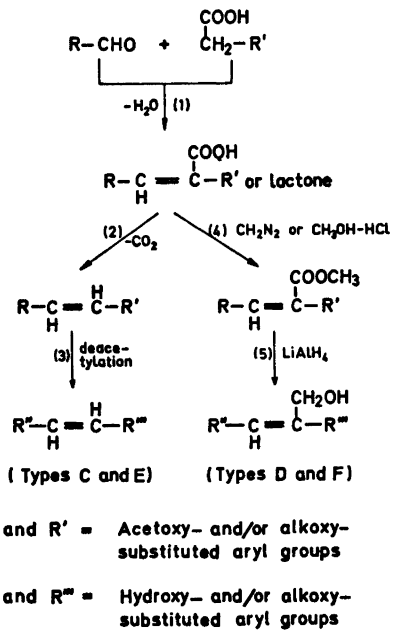


Fig. 1.

In the present communication, a convenient route of preparation for representatives of all four structural types (C – F) is described (see Scheme 1). The procedure involves:



R and R' = Acetoxy- and/or alkoxy-substituted aryl groups

R'' and R''' = Hydroxy- and/or alkoxy-substituted aryl groups

Scheme 1.

(1) Knoevenagel condensation of appropriate aromatic aldehydes with suitably substituted arylacetic acids to give stilbenecarboxylic acids or their lactones.[23–25]

(2) Decarboxylation of the stilbenecarboxylic acids [26,27] followed by

(3) Removal of protecting (acetyl) groups (preparation of types C and E)

(4) Alternative to (2): Esterification of the stilbenecarboxylic acids and conversion of lactones into methyl esters, followed by

(5) Lithium aluminium hydride reduction (preparation of types D and F).

By choosing appropriate reactants for the condensations and using the above reaction sequences, a wide variety of symmetrical and asymmetrical phenolic and non-phenolic stilbenes and hydroxymethyl-substituted stilbenes, differing in their patterns of aromatic substitution, could be synthesised. The various steps (1 – 5) in the preparation of some typical representatives (4,4'- and 2,4'-dihydroxystilbenes of types C – F) are described in the experimental section.

Yields, m.p. and elemental analyses of the intermediates and final products are given in Tables 1 – 6; the NMR-data are summarised in Tables 7 – 12. The final products were also characterised by mass spectra. Molecular ions

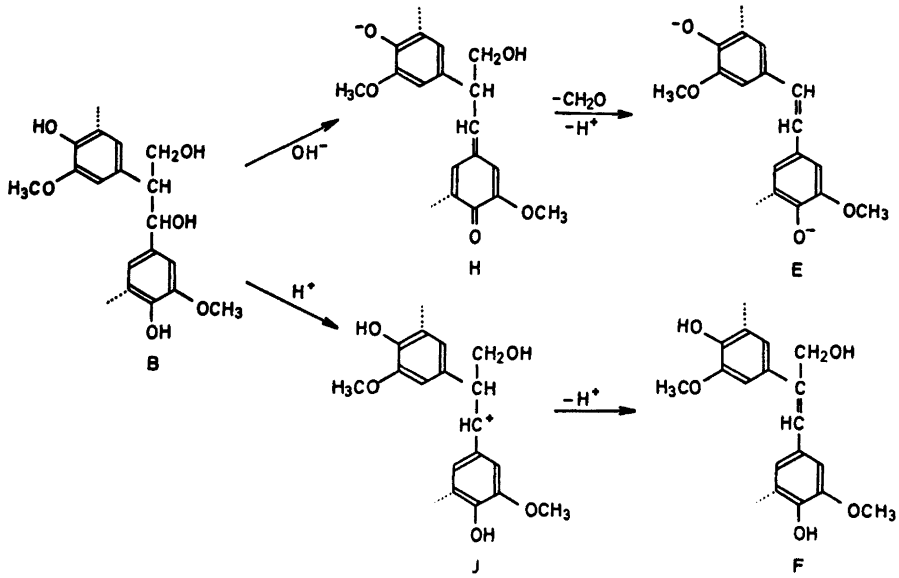


Fig. 2.

Table 1. Stilbenecarboxylic acids.

Compound	Yield %	M.p. °C	Elemental analyses C Calc.	C Found	H Calc.	H Found	O Calc.	O Found
Ia	59.8	205–207	69.23	69.07	5.13	5.18	25.64	25.53
IIa [44]	25.7	228–233	71.83	71.76	5.63	5.55	22.53	22.65
IIIa	65.7	216–218	63.00	63.03	5.00	5.10	32.00	31.92
IVa	49.8	178–180	64.51	64.70	5.38	5.55	30.11	30.00
Va	32.5	180–190	66.28	66.31	5.81	5.90	27.91	27.69
VIa	59.0	225–227	64.04	64.10	4.49	4.55	31.46	31.23
VIIa	39.4	232–238	66.67	66.54	5.26	5.4	28.07	27.93
VIIIa	42.2	217–220	62.69	62.58	5.47	5.62	31.84	31.78
IXa	53.2	228–229	69.23	69.25	5.13	5.12	25.64	25.68
Xa	51.3	179–182	63.00	63.02	5.00	5.08	32.00	31.89
XIa	51.0	188–191	64.51	64.70	5.38	5.55	30.11	30.00

Table 2. Lactones.

Compound	Yield %	M.p. °C	Elemental analyses C Calc.	C Found	H Calc.	H Found	O Calc.	O Found
XIV	15.4	157–158	76.19	76.19	4.77	4.81	19.05	19.15
XV	21.1	145–147	69.23	69.72	5.13	5.20	25.64	25.28
XVI	21.7	160–161	67.06	67.11	4.71	4.81	28.23	28.18
XVII	58.5	148–150	69.68	69.65	4.52	4.39	25.80	25.69
XVIII	71.8	149–153	67.80	67.70	5.08	5.10	27.12	27.24

Table 3. Hydroxystilbene acetates.

Compound	Yield %	M.p. °C	Elemental analyses C Calc.	C Found	H Calc.	H Found	O Calc.	O Found
Ib	48.8	141–147	76.12	76.22	5.79	6.01	17.91	17.85
IIIb [6]	58.5	227–229	67.42	67.38	5.62	5.68	26.96	27.01
IVb	53.6	130–132	69.52	70.02	6.09	6.10	24.37	23.81
IXb	62.8	109–110	76.13	76.44	5.96	5.62	17.89	17.35
Xb [22]	55.0	138–139	67.43	76.72	5.61	5.77	26.95	26.72
XIb	53.2	103–104	69.51	69.40	6.09	6.03	24.38	24.46

Table 4. Hydroxystilbenes.

Compound	Yield %	M.p. °C	Elemental analyses C Calc.	C Found	H Calc.	H Found	O Calc.	O Found
Ic [45]	96	130–131	79.65	79.57	6.19	6.27	14.16	14.12
IIIc [6,14]	98	215–216	70.59	70.45	5.88	5.83	23.53	23.63
Xc [21,22]	100	134–135	70.59	70.24	5.88	5.81	23.53	23.44

Table 5. Stilbenecarboxylic acid methyl esters.

Compound	Yield %	M.p. °C	Elemental analyses C Calc.	C Found	H Calc.	H Found	O Calc.	O Found
Id	100	124–126	69.94	69.59	5.52	5.86	24.54	24.75
IId	76.1	105–107	72.48	72.44	6.04	5.99	21.48	21.57
IIId	70.8	126–127	63.77	63.69	5.31	5.36	30.92	30.69
IVd	66.5	105–108	65.28	65.30	5.70	5.89	29.02	29.13
VId	64.5	94–96	64.86	64.69	4.86	4.83	30.28	30.42
VIId	77.2	161–164	67.42	67.70	5.62	5.71	26.97	26.92
VIIId	88.5	147–149	63.46	63.37	5.77	5.78	30.77	30.77
IXd	95.0	114–115	69.94	69.90	5.52	5.59	24.54	24.36
Xd	70.8	94–96	63.77	63.86	5.31	5.40	30.92	30.79
XId	78.5	92–94	65.30	65.48	5.69	5.62	29.00	28.91
XIId [a]	41.2	112–113	65.64	65.51	5.21	5.17	29.15	29.46
XIIId [a]	58.9	152–153	64.50	64.46	5.60	5.43	29.89	30.21

[a] Prepared by cleavage of the corresponding lactone with methanolic hydrogen chloride and subsequent acetylation.

and fragmentation patterns were in accordance with the expected data.

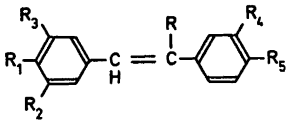
No attempts were made to increase the yields by systematic variation of the reaction conditions.

Some of the syntheses were not completed. Nevertheless, the data for the intermediates prepared are included in the tables.

In view of the versatility of this method, the small number of steps involved, the accessibility of the appropriate starting materials and the yields obtainable, the synthetic routes outlined above appear to compete well with those mentioned in the introduction.

The various compounds are assigned roman numerals according to their aromatic substitu-

tion pattern as follows:



I R_1 = OAc , R_2 = OCH_3 , R_3 = R_4 = R_5 = H

II R_1 = R_2 = OCH_3 , R_3 = R_4 = R_5 = H

III R_1 = R_5 = OAc , R_2 = R_4 = OCH_3 , R_3 = H

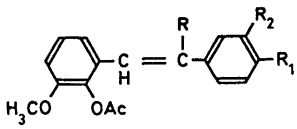
IV R_1 = OAc , R_2 = R_4 = R_5 = OCH_3 , R_3 = H

V R_1 = R_2 = R_4 = R_5 = OCH_3 , R_3 = H

VI R_1 = OAc , R_2 = OCH_3 , R_3 = H , R_4–R_5 = O–CH_2–O

VII R_1 = OAc , R_2 = R_3 = OCH_3 , R_4 = R_5 = H

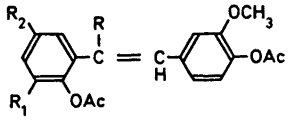
VIII R_1 = OAc , R_2 = R_3 = R_4 = R_5 = OCH_3



IX R_1 = R_2 = H

X R_1 = OAc , R_2 = OCH_3

XI R_1 = R_2 = OCH_3



XII R_1 = R_2 = H

XIII R_1 = OCH_3 , R_2 = CH_3

The indices used refer to:

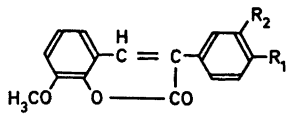
a, stilbenecarboxylic acids (R = COOH);

b, stilbenes unsubstituted at the olefinic carbon atoms (R = H);

c, deacetylation products of b (R = H, OH instead of OAc);

d, stilbenecarboxylic acid methyl esters (R = $COOCH_3$);

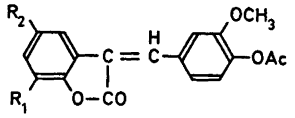
e, hydroxymethyl-substituted stilbenes (R = CH_2OH, OH instead of OAc).



XIV R_1 = R_2 = H

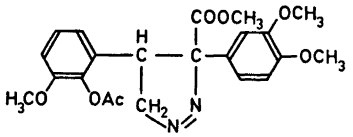
XV R_1 = OAc, R_2 = OCH_3

XVI R_1 = R_2 = OCH_3



XVII R_1 = R_2 = H

XVIII R_1 = OCH_3, R_2 = CH_3



XIX

EXPERIMENTAL

Melting points are corrected. Evaporations were carried out under reduced pressure.

Thin-layer chromatography (TLC) was performed using plates coated with a 0.25 mm thick layer of silica gel (Merck HF_{254}, type 60). The following solvent systems were used: chloroform, chloroform containing 5 % ethanol, chloroform containing 5 % ethyl acetate, toluene containing 5 % ethyl acetate, and diisopropyl ether:formic acid:water 90:7:3. Spots were visualised by exposure to iodine vapour or by spraying with a 3 % solution of vanillin in conc. sulfuric acid followed by heating at 110 °C for 5 – 10 min.

Column chromatography was accomplished using silica gel (Merck 0.05 – 0.5 mm, 70 – 325 mesh, ASTM) or silicic acid (Mallinckrodt, 100 mesh); solvent systems: chloroform containing 5 % ethyl acetate or toluene containing 5 % ethyl acetate. The separations were followed by TLC.

NMR-spectrometry. The NMR-spectra were run on a Perkin-Elmer R-12 spectrometer using $CDCl_3$, CD_3OD or $(CD_3)_2SO$ as solvents. Chemical shifts are given in δ (ppm downfield from tetramethylsilane, internal standard).

The mass spectra were recorded on a Perkin-Elmer 270 instrument at 70 eV using the direct inlet system. The temperature of the probe heater was 60 °C.

Table 6. Hydroxymethylstilbenes.

Compound	Yield %	M.p. °C	Elemental analyses C Calc.	C Found	H Calc.	H Found	O Calc.	O Found
Ie	49.5	98 – 100	75.00	75.03	6.25	6.31	18.75	18.90
IIe	53.2	83 – 85	75.53	75.38	6.71	6.54	17.76	17.81
IIIe	58.4	147 – 148	67.55	67.45	5.96	6.05	26.49	26.30
IVe	58.6	131 – 134	68.35	68.22	6.33	6.40	25.32	25.31
Ve	72.5	110 – 113	69.09	69.23	6.67	6.85	24.24	24.05
VIIIe diacetate [a]	74.2	amor-phous	64.17	64.26	6.09	6.18	29.73	29.79
IXe	64.6	139 – 141	75.00	75.15	6.25	5.90	18.75	18.98
Xe triacetate [a]	80.0	amor-phous	64.48	64.35	5.63	5.66	29.88	29.98
XIe	52.8	192 – 195	68.35	68.32	6.33	6.31	25.31	25.19
XIIe	73.8	125 – 126	70.60	70.36	5.87	5.97	23.51	23.56
XIIIe triacetate [a]	77.1	amor-phous	65.17	64.86	5.87	6.18	28.94	28.77

[a] Mixture of the *cis*- and *trans*-forms.

Table 7. Stilbenecarboxylic acids.

Compound	Chemical shifts (δ) [a,b] Aromatic and olefinic H	Methoxyl H	Acetyl H
Ia	7.52 – 6.63 (m, 9 H)	3.32 (s, 3 H)	2.18 (s, 3 H)
IIa[44]	7.40 – 6.70 (m, 9 H)	3.78 (s, 6 H)	
IIIa	7.12 – 6.55 (m, 7 H)	3.40 (s, 3 H)	2.23 (s, 3 H)
		3.72 (s, 3 H)	2.28 (s, 3 H)
IVa	7.10 – 6.67 (m, 7 H)	3.42 (s, 3 H)	2.20 (s, 3 H)
		3.67 (s, 3 H)	
		3.77 (s, 3 H)	
Va	7.05 – 6.35 (m, 7 H)	3.30 (s, 6 H)	
		3.70 (s, 6 H)	
VIa [c]	7.11 – 6.57 (m, 7 H)	3.50 (s, 3 H)	2.20 (s, 3 H)
VIIa	7.50 – 6.41 (m, 8 H)	3.40 (s, 6 H)	2.18 (s, 3 H)
VIIIa	7.14 – 6.33 (m, 6 H)	3.48 (s, 6 H)	2.20 (s, 3 H)
		3.65 (s, 3 H)	
		3.75 (s, 3 H)	
IXa	7.45 – 6.10 (m, 9 H)	3.82 (s, 3 H)	2.33 (s, 3 H)
Xa	7.00 – 6.15 (m, 7 H)	3.59 (s, 3 H)	2.22 (s, 3 H)
		3.75 (s, 3 H)	2.32 (s, 3 H)
XIa	7.00 – 6.20 (m, 7 H)	3.68 (s, 3 H)	2.34 (s, 3 H)
		3.77 (s, 3 H)	
		3.84 (s, 3 H)	

[a] Solvent: $(CD_3)_2SO$, except IXa: CD_3OD. [b] The signals of the carboxyl H appear between δ 7.70 and 7.90. [c] The signal of the methylene H appears at δ 6.02.

Table 8. Lactones.

Compound	Chemical shifts (δ) [a] Aromatic and olefinic H	Methoxyl H	Acetyl H	Others
XIV	7.70 – 7.05 (m, 9 H)	3.92 (s, 3 H)		
XV	7.72 – 6.95 (m, 7 H)	3.83 (s, 3 H) 3.92 (s, 3 H)	2.30 (s, 3 H)	
XVI	7.63 – 6.72 (m, 7 H)	3.81 (s, 3 H) 3.87 (s, 6 H)		
XVII	7.70 – 6.95 (m, 8 H)	3.80 (s, 3 H)	2.30 (s, 3 H)	
XVIII	7.75 – 6.85 (m, 6 H)	3.85 (s, 3 H) 3.92 (s, 3 H)	2.28 (s, 3 H)	2.33 (s, 3 H)

[a] Solvent: $CDCl_3$.

Table 9. Hydroxystilbene acetates.

Compound	Chemical shifts (δ) [a] Aromatic and olefinic H	Methoxyl H	Acetyl H
Ib	7.00 (s, 5 H) 7.35 (m, 5 H)	3.85 (s, 3 H)	2.30 (s, 3 H)
IIb [46,47]	7.30 – 6.85 (m, 10 H)	3.85 (s, 6 H)	
IIIb *cis*	6.83 (m, 6 H) 6.52 (s, 2 H)	3.55 (s, 6 H)	2.27 (s, 6 H)
IIIb *trans* [6]	7.00 (m, 8 H)	3.87 (s, 6 H)	2.30 (s, 6 H)
IXb	7.35 – 6.80 (m, 10 H)	3.78 (s, 3 H)	2.33 (s, 3 H)
Xb *cis*	6.80 (m, 6 H) 6.50 (d, 2 H)	3.73 (s, 3 H) 3.45 (s, 3 H)	2.18 (s, 3 H) 2.25 (s, 3 H)
Xb *trans* [22]	7.00 (s, 8 H)	3.78 (s, 3 H) 3.82 (s, 3 H)	2.28 (s, 3 H) 2.32 (s, 3 H)
XIb	7.02 – 6.35 (m, 8 H)	3.78 (s, 6 H) 3.52 (s, 3 H)	2.26 (s, 3 H)

[a] Solvent: $CDCl_3$.

Table 10. Hydroxystilbenes.

Compound	Chemical shifts (δ) [a] Phenolic OH	Aromatic and olefinic H	Methoxyl
Ic [45]	~9 (b, 1 H)	7.80 – 6.66 (m, 10 H)	3.85 (s, 3 H)
IIIc [6,14]	8.95 (s, 2 H)	7.28 – 6.62 (m, 8 H)	3.79 (s, 6 H)
Xc [21,22]	8.85 (s, 2 H)	7.50 – 6.50 (m, 8 H)	3.78 (s, 3 H) 3.80 (s, 3 H)

[a] Solvent: $(CD_3)_2SO$.

Table 11. Stilbenecarboxylic acid methyl esters.

Compound	Chemical shifts (δ) [a] Aromatic and olefinic H	Carbomethoxyl H	Methoxyl H	Acetyl H
Id	7.80 – 6.52 (m, 9 H)	3.78 (s, 3 H)	3.32 (s, 3 H)	2.22 (s, 3 H)
IId	7.70 – 6.40 (m, 9 H)	3.78 (s, 3 H)	3.35 (s, 3 H)	
			3.75 (s, 3 H)	
IIId	7.78 – 6.42 (m, 7 H)	3.80 (s, 3 H)	3.40 (s, 3 H)	2.21 (s, 3 H)
			3.70 (s, 3 H)	2.27 (s, 3 H)
IVd	7.70 – 6.45 (m, 7 H)	3.80 (s, 3 H)	3.40 (s, 3 H)	2.15 (s, 3 H)
			3.75 (s, 6 H)	
Vd	7.33 – 6.52 (m, 7 H)	3.85 (s, 3 H)	3.48 (s, 3 H)	
			3.80 (s, 9 H)	
VId [b]	7.72 – 6.65 (m, 7 H)	3.78 (s, 3 H)	3.50 (s, 3 H)	2.23 (s, 3 H)
VIId	7.72 – 6.70 (m, 8 H)	3.70 (s, 3 H)	3.70 (s, 6 H)	2.28 (s, 3 H)
VIIId	7.70 – 6.35 (m, 6 H)	3.85 (s, 3 H)	3.48 (s, 6 H)	2.25 (s, 3 H)
			3.75 (s, 6 H)	
IXd	7.80 – 6.27 (m, 9 H)	3.77 (s, 3 H)	3.77 (s, 3 H)	2.33 (s, 3 H)
Xd	7.78 – 6.67 (m, 7 H)	3.75 (s, 3 H)	3.59 (s, 3 H)	2.26 (s, 3 H)
			3.75 (s, 3 H)	2.33 (s, 3 H)
XId	7.73 – 6.22 (m, 7 H)	3.84 (s, 3 H)	3.66 (s, 3 H)	2.18 (s, 3 H)
			3.78 (s, 6 H)	
XIId	7.47 – 6.60 (m, 8 H)	3.72 (s, 3 H)	3.35 (s, 3 H)	2.09 (s, 3 H)
				2.20 (s, 3 H)
XIIId [c]	7.73 – 6.42 (m, 6 H)	3.76 (s, 3 H)	3.39 (s, 3 H)	2.19 (s, 3 H)
			3.66 (s, 3 H)	2.23 (s, 3 H)

[a] Solvent: $CDCl_3$. [b] Methylene H: 5.93 (s, 2 H). [c] Methyl H: 2.06 (s, 3 H).

Preparation of condensation reactants

o-*Vanillin acetate* by acetylation of *o*-vanillin (20 g) with acetic anhydride (30 ml) in pyridine (30 ml); yield 20 g, 78 %. Recrystallisation from isopropanol gave a product melting at 74 – 75 °C (lit.[28] 76 °C).

Syringaldehyde acetate from syringaldehyde (5 g) by acetylation with acetic anhydride (5 ml) in pyridine (5 ml); yield 5.9 g, 96 %. Recrystallisation from chloroform/hexane yielded the pure acetate, m.p. 115 – 117 °C (lit.[28] 114 °C).

4-Acetoxy-3-methoxyphenylacetic acid (homovanillic acid acetate) was prepared by oxidation of eugenol acetate (m.p. 23 – 28 °C, lit. [29] 27 °C) with potassium permanganate according to Ref. 30 with slight modifications. Total yield: 3.28 g, 94 %. After recrystallisation from glacial acetic acid, the acid melted at 137 – 138.5 °C, lit.[31] 139 – 140 °C.

3,4-Methylenedioxyphenylacetic acid (homopiperonalic acid) was analogously prepared from safrole. Yield 46 %, m.p. 125 – 127 °C (lit.[32] 127 – 128 °C).

6-Hydroxymethyl-4-methylguaiacol was obtained by reacting 4-methylguaiacol (creosol) with formaldehyde (300 ml, 3.7 mol) in a 5 % aqueous solution of sodium hydroxide according to Ref. 33. The colourless prisms (m.p. 50 – 51.5 °C) were recrystallised from ligroin; yield 24 %. Starting material (44 %) was recovered and *bis*-(2-hydroxy-3-methoxy-5-methylphenyl)-methane, m.p. 128 – 129 °C, was obtained in a low yield (4 %) from the fraction distilling at about 200 °C/5 mmHg.

2-Hydroxy-3-methoxy-5-methylphenylacetonitrile. 6-Hydroxymethyl-4-methylguaiacol (9.2 g, 0.055 mol) and sodium cyanide (5.5 g, 0.112 mol) were dissolved in anhydrous methanol and refluxed for 7 h (*cf.* Ref. 34). After removal of methanol the residue was dissolved in water and the solution neutralised with carbon dioxide. The nitrile precipitated in the form of brownish crystals (yield 7.45 g, 77 %) which were contaminated with a small amount of the above-mentioned diphenylmethane derivative and of a cyclic trimeric condensation product.[35] Recrystallisation from benzene-petroleum ether gave the pure nitrile; m.p. 62.5 – 63.5 °C). (Found: C 67.51; H 6.18; O 18.27; N 8.02. $C_{10}H_{11}O_2N$ (177.11) requires: C 67.80; H 6.21; O 18.08; N 7.91.) δ 6.80 – 6.53 (d, 2 H, arom. H); 6.58 (s, 1 H, phenol. H); 3.83 (s, 3 H, arom. OCH_3); 3.67 (s, 2 H, methylene H); 2.27 (s, 3 H, arom. CH_3).

Table 12. Hydroxymethylstilbenes.

Compound	Chemical shifts (δ) [a] Aromatic and olefinic H	Methylene H [e]	Methoxyl H	Solvent
Ie	7.25 – 6.53 (m) (9 H)	4.38 (s) (2 H)	3.38 (s) (3 H)	$CDCl_3$
IIe	7.22 – 6.40 (m) (9 H)	4.38 (s) (2 H)	3.40 (s) (3 H) 3.78 (s) (3 H)	$CDCl_3$
IIIc	6.75 – 6.25 (m) (7 H)	4.35 (s) (2 H)	3.38 (s) (3 H) 3.63 (s) (3 H)	$(CD_3)_2SO$
IVe	6.72 – 6.35 (m) (7 H)	4.20 (s) (2 H)	3.40 (s) (3 H) 3.65 (s) (3 H) 3.73 (s) (3 H)	$(CD_3)_2SO$
Ve	6.91 – 6.45 (m) (7 H)	4.45 (s) (2 H)	3.55 (s) (3 H) 3.80 (s) (3 H) 3.85 (s) (3 H) 3.92 (s) (3 H)	$CDCl_3$
VIe [b]	7.15 – 6.35 (m) (7 H)	4.32 (s) (2 H)	3.50 (s) (3 H)	$CDCl_3$
VIIIe diacetate [c]	7.09 – 6.20 (m) (6 H)	5.15, 4.87 (2s) (2 H)	3.98 – 3.43 (8s) (12 H)	$CDCl_3$
IXe	7.20 – 6.30 (m) (9 H)	4.45 (s) (2 H)	3.72 (s) (3 H)	$CDCl_3$
Xe triacetate [c]	7.13 – 6.28 (m) (7 H)	4.92, 4.83 (2s) (2 H)	3.57 (s) (3 H) 3.78 (s) (3 H)	$CDCl_3$
XIIe	7.25 – 6.45 (m) (8 H)	4.30 (s) (2 H)	3.37 (s) (3 H)	CD_3OD
XIIIe [d]	6.90 – 6.45 (m) (6 H)	4.34 (s) (2 H)	3.42 (s) (3 H) 3.80 (s) (3 H)	$CDCl_3$

[a] Acetates prepared from the hydroxymethylstilbenes, listed in this table, exhibit signals for the aliphatic acetyl H at δ 2.05 and for the aromatic acetyl H between δ 2.22 and 2.31. [b] The signal of the methylene H appears at δ 5.90. [c] Mixture of *cis*- and *trans*-forms. [d] Methyl H: 2.18 (s, 3 H). [e] The signals of the methylene protons were relatively broad indicating weak allylic coupling to the olefinic proton.

2-Hydroxy-3-methoxy-5-methylphenylacetic acid. The acetonitrile described above (4.6 g, 0.027 mol) was dissolved in conc. hydrochloric acid (1 l) and kept at room temperature for 2 days. The solution was extracted with chloroform and the combined chloroform extracts were extracted with water and dried. Evaporation gave colourless needles which were recrystallised from chloroform/hexane, m.p. 120 – 123 °C; yield 2.73 g, 56 %. [Found: C 61.66; H 5.80; O 32.62. $C_{10}H_{12}O_4$ (196.10) requires: C 61.22; H 6.12; O 32.65.] δ 8.05 (b, 1 H); 6.60 (s, 2 H); 3.85 (s, 3 H); 3.64 (s, 2 H); 2.25 (s, 3 H).

Heating the 2-hydroxyphenylacetic acid in glacial acetic acid afforded the corresponding lactone; m.p. 98 – 99 °C. [Found: C 67.33; H 5.52; O 27.03. $C_{10}H_{10}O_3$ (178.10) requires: C 67.44; H 5.61; O 26.96.] δ 6.66 (s, 2 H); 3.86 (s, 3 H); 3.62 (s, 2 H); 2.30 (s, 3 H).

Condensation (1)

trans-*4,4'-Diacetoxy-3,3'-dimethoxystilbene-α-carboxylic acid* (IIIa). A mixture of homovanillic acid acetate (6.82 g, 0.0304 mol), vanillin acetate (7.65 g, 0.0395 mol) and triethylamine (7.8 ml) in acetic anhydride (76 ml) was refluxed for 45 min. The bulk of acetic anhydride and of triethylamine was then removed by distillation under reduced pressure. The residue was repeatedly washed with water and brought to crystallisation by addition of ethanol. From the combined aqueous washing liquors an additional amount of stilbenecarboxylic acid crystallised and was added to the bulk of the material. Recrystallisation from ethanol gave the pure compound IIIa as yellowish crystals.

trans-*2,4'-Diacetoxy-3,3'-dimethoxystilbene-α'-carboxylic acid* (Xa) was prepared analogously by heating homovanillic acid acetate (6.82 g, 0.0395 mol) and *o*-vanillin acetate (7.65 g, 0.0395 mol) in acetic anhydride (76 ml) and triethylamine (7.8 ml) under reflux for 30 min. After removal of acetic anhydride and triethylamine by distillation under reduced pressure, the residue was dissolved in chloroform and the chloroform solution was extracted several times first with water and then with a saturated solution of sodium bicarbonate. From the combined bicarbonate extracts the stilbenecarboxylic acid (Xa) was obtained in the usual way. Recrystallisation from isopropanol gave the pure compound (6.25 g, 51.3 %, yellowish crystals).

3-(4-Acetoxy-3-methoxyphenyl)-8-methoxycoumarin (XV), formed by lactonisation from compound Xa, was isolated from the remaining chloroform solution. After extraction with sodium bisulfite (removal of the excess of *o*-vanillin acetate), extraction with water, drying (Na_2SO_4) and evaporation, a dark oil was obtained which crystallised on addition of isopropanol. Yield 1.0 g (8.2 %). Recrystallisation from isopropanol gave the pure lactone XV; colourless needles.

Prolongation of the reaction time to 2 h increased the yield of lactone to 22 %.

When 2-acetoxyphenylacetic acids and aromatic aldehydes were refluxed under similar conditions (preparation of type D stilbenes), lactonisation of the starting acids prevented condensation. Conversion of the 2-hydroxyphenylacetic acids into their piperidinium salts enabled condensation but the resulting 2-hydroxystilbene-α-carboxylic acid piperidides underwent extensive lactonisation yielding the corresponding five-membered stilbene lactones (*cf.* Ref. 36).

3-(4-Acetoxy-3-methoxy-benzylidene)-coumaran-2-one (XVII). *o*-Hydroxyphenylacetic acid (3.8 g, 0.025 mol) was dissolved in piperidine (30 ml) and the solvent was removed by distillation under reduced pressure. Large prisms of the *piperidinium salt of o-hydroxyphenylacetic acid* were obtained which after recrystallisation from benzene melted at 104–105 °C; yield 5.2 g (90.5 %) (Found: C 66.1; H 8.3; O 20.1; N 5.7. Calc. for $C_{13}H_{19}O_3N$: C 65.82; H 8.17; O 20.25; N 5.91).

The piperidinium salt (10.85 g, 0.457 mol) and vanillin acetate (11.45 g, 0.590 mol) were refluxed in acetic anhydride (50 ml) containing piperidine (1.6 ml) for 3 h. After removal of acetic anhydride by distillation, ice-water was added to the residue and the resulting oil was separated from the aqueous layer. Addition of ethanol brought about crystallisation. Repeated recrystallisation from the same solvent gave the pure coumaranone XVII; yield 8.3 g (58.5 %), yellowish crystals.

The reaction is likely to proceed as follows: piperidinium salt → piperidide → stilbenecarboxylic acid piperidide → coumaranone. This view is supported by the following results: Refluxing the piperidinium salt (0.5 g) in acetic anhydride (5 ml) for 1 h gave the piperidide; yield 0.4 g, m.p. 114–115 °C. Addition of vanillin acetate to a solution of the piperidide in acetic anhydride containing piperidine and refluxing the mixture for 2 h afforded coumaranone XVII. Finally, the piperidide of XIIa in the deacetylated form (Found: C 70.9; H 6.5; O 18.1; N 4.0. $C_{21}H_{23}O_4N$ requires: C 71.40; H 6.51; O 18.12; N 3.96) (250 mg) was converted into the coumaranone XVII by heating in acetic anhydride (3 ml) under reflux for 4 h. After removal of acetic anhydride and addition of ice to the residue, XVII was obtained as an oil which crystallised on standing; yield 216 mg (98.5 %); m.p. 148–150 °C (see Table 2).

The preparation of the piperidinium salt, its conversion into the piperidide and the condensation to the coumaranone XVII could be conveniently performed in one step with essentially the same result. Thus, addition of vanillin acetate to a solution of *o*-hydroxyphenylacetic acid and piperidine in acetic anhydride and refluxing the mixture for 2 h afforded XVII. The working-up was carried out as described above; yield 58 %.

3-(4-Acetoxy-3-methoxybenzylidene)-5-methyl-7-methoxycoumaran-2-one (XVIII) was similarly prepared from 2-hydroxy-3-methoxy-5-methylphenylacetic acid (see above) (0.795 g, 0.004 mol), piperidine (6.5 ml), vanillin acetate (0.84 g, 0.0043 mol) and acetic anhydride (9 ml). The product crystallised on addition of ethanol and was purified by recrystallisation from isopropanol; yellowish plates.

Decarboxylation (2)

trans-*4,4'-Diacetoxy-3,3'-dimethoxystilbene* (IIIb).[6] Stilbenecarboxylic acid IIIa (13.5 g, 0.034 mol) and hydroquinone (250 mg) were dissolved in freshly distilled quinoline (140 ml). Copper chromite [37] (250 mg) was added and the mixture was heated under reflux for 30 min in a steady stream of nitrogen passing through. After the decarboxylation, the quinoline solution was concentrated to about 25 ml by evaporation under reduced nitrogen pressure. The partially deacetylated product was reacetylated by addition of acetic anhydride (30 ml) and keeping the mixture overnight. After the usual working-up, an oil was obtained which crystallised on addition of isopropanol. The stilbene was purified by recrystallisation from toluene (yield 55.8 %). About the same yield of the *trans*-stilbene (58.3 %) and a minor amount of the amorphous *cis*-isomer (10.0 %) were obtained (see Table 3), when the acetylation mixture was separated on a silicic acid column.

trans-*2,4'-Diacetoxy-3,3'-dimethoxystilbene* (Xb)[22] was similarly prepared from stilbenecarboxylic acid Xa (6.0 g, 0.015 mol), hydroquinone (120 mg) and copper chromite (120 mg). After reacetylation and the usual working-up, an oil was obtained which was fractionated by chromatography on a silicic acid column. *trans*-Stilbene Xb (2.93 g, 54.9 %), the amorphous *cis*-isomer (about 100 mg, ~2 %), and lactone XV (0.6 g, 12 %) were isolated. *trans*-Stilbene Xb and lactone XV were further purified by recrystallisation from isopropanol.

Deacetylation (3)

trans-*4,4'-Dihydroxy-3,3'-dimethoxystilbene* (IIIc).[6,14] The *trans*-form of IIIb (0.59 g, 0.0015 mol) was dissolved in tetrahydrofuran in an atmosphere of nitrogen and the solution was

dropped to a cooled suspension of lithium aluminium hydride (0.47 g, 0.012 mol) in the same solvent (100 ml). After 15 min, the excess of lithium aluminium hydride was decomposed by addition of ethyl acetate saturated with water. Hydrochloric acid (2 M) was added until all the precipitate was dissolved and the solution was extracted repeatedly with ethyl acetate. The combined extracts were washed with water until neutral, dried and evaporated. The stilbene was recrystallised from ethanol.

trans-*2,4'-Dihydroxy-3,3'-dimethoxystilbene* (Xc)[21,22] was prepared from the corresponding diacetate (Xb, 182 mg) by addition of sodium (30 mg) to a solution in ethanol (10 ml). Neutralisation (CO_2) and evaporation of the solvent gave a residue which was dissolved in chloroform. The chloroform solution was extracted with water, dried (Na_2SO_4) and evaporated. The product was recrystallised from ethanol.

On exposure to air, the phenolic stilbenes undergo slow autoxidation with discoloration. The impurities can be removed by recrystallisation from ethanol containing small amounts of sodium borohydride.

In the preparation of phenolic hydroxymethyl-substituted stilbenes (types D and F), the reduction of the stilbenecarboxylic acid methyl ester group by lithium aluminium hydride (see below) is, of course, accompanied by the removal of acetyl groups. Thus, no extra deacetylation step is needed.

Esterification (4):

(a) starting from stilbenecarboxylic acids

4,4'-Diacetoxy-3,3'-dimethoxystilbene-α-carboxylic acid methyl ester (IIId). To a saturated solution of the stilbenecarboxylic acid IIIa (5.6 g, 0.014 mol) in dioxane (100 ml) an ethereal solution of diazomethane (about 0.02 mol) was dropped slowly. The mixture was kept at room temperature for 16 h. After removal of the solvents by distillation under reduced pressure, a yellowish oil was obtained which crystallised on standing. Recrystallisation from toluene gave the pure compound IIId.

The use of a larger excess of diazomethane lowered the yield owing to addition of the methylating reagent across the olefinic double bond. In one instance (esterification of XIa) the resulting 3,4-diarylpyrazoline derivative (XIX) was isolated, purified and identified by elemental analyses and NMR; m.p. 105–107 °C. (Found: C 61.7; H 5.6; O 25.8; N 6.6. $C_{22}H_{24}O_7N_2$ requires: C 61.68; H 5.60; O 26.16; N 6.54.) δ 6.97–6.55 (m, 6 H); 6.00–5.82 (1 q, 1 H); 5.05–4.57 (1 q, 1 H); 4.65–4.45 (1 q, 1 H (AMX spectrum) 3.75 (s, 3 H); 3.68 (s, 3 H); 3.62 (s, 6 H); 2.42 (s, 3 H).

2,4'-Diacetoxy-3,3'-dimethoxystilbene-α'-carboxylic acid methyl ester (Xd) was similarly prepared by treatment of stilbenecarboxylic acid Xa with diazomethane. The ester crystallised from the oily reaction product on prolonged standing in the refrigerator. Recrystallisation from toluene gave the pure compound. In addition to the ester, the oily reaction product contained the corresponding lactone (XV) and some other by-products not further characterised (TLC).

(b) starting from stilbene lactones

The yields of methyl esters were high except those of 2-acetoxystilbene-α-carboxylic acid methyl esters (XIId and XIIId) which had to be prepared be refluxing the corresponding stilbene lactones in an excess of methanolic hydrogen chloride.

2,4'-Diacetoxy-3'-methoxystilbene-α-carboxylic acid methyl ester (XIId). 3-(4-Acetoxy-3-methoxybenzylidene)-coumaran-2-one (XVII) (1.6 g) was dissolved in a 0.5 % solution of hydrogen chloride in anhydrous methanol (150 ml) and the solution was refluxed for 90 min.

After removal of the solvent and hydrogen chloride by evaporation, the residual oil was separated by preparative TLC and the fraction containing the deacetylated methyl ester was acetylated with acetic anhydride in pyridine. The resulting diacetyl compound XIId was purified by recrystallisation from ethanol.

2,4'-Diacetoxy-3,3'-dimethoxy-5-methylstilbene-α-carboxylic acid methyl ester (XIIId) was prepared analogously by refluxing a solution of 3-(4-acetoxy-3-methoxybenzylidene)-5-methyl-7-methoxycoumaran-2-one (XVIII, 0.818 g) in 1 % methanolic hydrogen chloride (75 ml) for 2.5 h. The product was worked up and purified as described for the preceding compound. Starting material (315 mg, 38.5 %) was also isolated.

Lithium aluminium hydride reduction (5)

The conversion of stilbenecarboxylic acid methyl esters into hydroxymethyl stilbenes was performed following the procedure described for the reduction of ferulic acid ethyl ester [38] and of acetylferulic acid methyl ester [39] to coniferyl alcohol with some modifications.

4,4'-Dihydroxy-3,3'-dimethoxy-α-hydroxymethylstilbene (*IIIe*). To a cooled suspension of lithium aluminium hydride (2.66 g, 0.07 mol) in anhydrous tetrahydrofurane (200 ml) a solution of 4,4'-diacetoxy-3,3'-dimethoxystilbene-α-carboxylic acid methyl ester (IIId, 4.1 g, 0.01 mol) in tetrahydrofurane (100 ml) was added at 0 °C in a nitrogen atmosphere during 20 min. The reduction was allowed to continue for 4 h at 0 °C with vigorous stirring and was followed by TLC.

Excess of lithium aluminium hydride was decomposed by addition of ethyl acetate satu-

rated with water. After dissolution of the precipitate by acidification with 2 M hydrochloric acid, the solution was extracted with ethyl acetate. Evaporation of the combined and dried (Na_2SO_4) extracts gave a colourless viscous oil which crystallised on standing. Recrystallisation from toluene yielded pure compound IIIe.

2,4'-Dihydroxy-3'-methoxy-α-hydroxymethyl stilbene (XIIe) was prepared similarly from 2,4'-diacetoxy-3'-methoxystilbene-α-carboxylic acid methyl ester (XIId). Recrystallisation from ether–petroleum ether gave the pure compound. An additional amount was isolated from the combined mother liquors by preparative column chromatography.

2,4'-Dihydroxy-3,3'-dimethoxy-5-methyl-α-hydroxymethylstilbene (XIIIe) was prepared in a similar manner from 2,4'-diacetoxy-3,3'-dimethoxy-5-methylstilbene-α-carboxylic acid methyl ester (XIIId).

The phenolic hydroxymethylstilbenes were stable, provided air (oxygen) and moisture were excluded.

Direct reductions of stilbene lactones with lithium aluminium hydride [40] (*cf.*, however, Ref. 41), aluminium hydride [42] and borohydride [43] resulted in complex mixtures consisting mainly of products formed by alkaline or reductive cleavage of the lactone ring with concomitant or subsequent reduction of the stilbene double bond (NMR).

Thus, compound XVII, when treated with sodium borohydride in ethanol-chloroform-water (7:4:1) at room temperature, afforded a mixture of products which was separated by chromatography on a column of silica gel yielding a main component, melting at 118–119 °C. This compound was acetylated with acetic anhydride in pyridine and identified as 2,4'-diacetoxy-3'-methoxy-α-acetoxymethylbibenzyl (XIIe, OAc instead of OH, $-CH_2-CH_2-$ instead of $-CH=CH-$). δ 7.43–6.43 (m, 7 H); 4.20 (d, 2 H); 3.60 (s, 3 H); 3.75–3.40 (m, 1 H); 2.89 (d, 2 H); 2.20 (s, 6 H); 1.89 (s, 3 H).

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Animal Carotenoids. 9.* On the Absolute Configuration of Astaxanthin and Actinioerythrin

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Dedicated to Professor Dr. techn. Nils Andreas Sørensen at the occasion of his 65th birthday December 8, 1974

The use of chiroptical methods for determination of the absolute configuration of astaxanthin (*1*) is discussed.

Chemical conversion of astaxanthin (*1*) diester into zeaxanthin (*7*) failed.

Consideration of the CD-spectrum of the tetrol *10*, obtained by $LiAlH_4$-reduction of astaxanthin (*1*) diester, led to the (3*S*,3′*S*)-assignment for astaxanthin (*1a*).

Chiroptical properties of actinioerythrin (*13*) are reported.

The constitution of astaxanthin (*1*) was elucidated by Kuhn and Sørensen [1] in 1938. Astaxanthin (*1*) is converted to astacene (*2*) under alkaline conditions in the presence of oxygen,[1] Scheme 1. The structure *1* of astaxanthin has subsequently been confirmed by Davis and Weedon [2] by partial synthesis of astacene (*2*) from canthaxanthin (*3*). However, the absolute configuration at 3,3′-positions has not yet been determined.

From feeding experiments with (^{14}C)-lutein it has been claimed that astaxanthin (*1*) in goldfish (*Carassius auratius*) is formed biogenetically from lutein.[3] Lutein, first considered to have (3*R*,3′*S*,6′*R*)-configuration,[4] was later demonstrated to possess (3*R*,3′*R*,6′*R*)-configuration (*4*).[5–7] If astaxanthin (*1*) were synthesized *in vivo* from lutein (*4*) without epimerization on the enzyme, astaxanthin should have 3*S*,3′*R*-configuration and be achiral. However, astaxanthin *ex Hommarus gammarus* (from crustacyanin) is optically active.[8a] Its biosynthesis in goldfish, therefore, calls for further attention.

From its chiroptical properties astaxanthin cannot be bound to positive centers of proteins

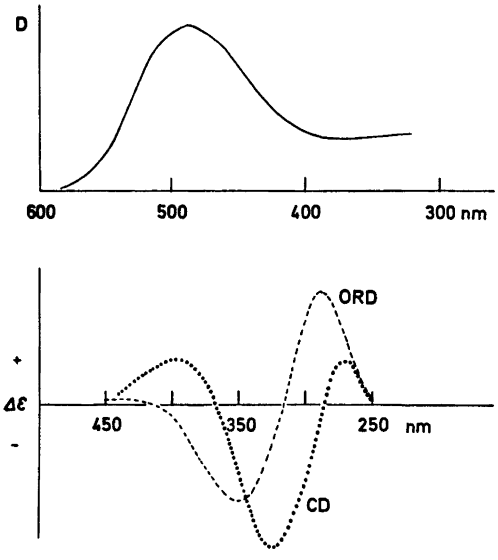

Fig. 1. Electronic absorption spectrum (—— top), CD-spectrum (· · ·, bottom) and ORD-spectrum (– – –, bottom; calculated from the CD-spectrum) of astaxanthin (*1*) *ex Hommarus gammarus* in chloroform solution.

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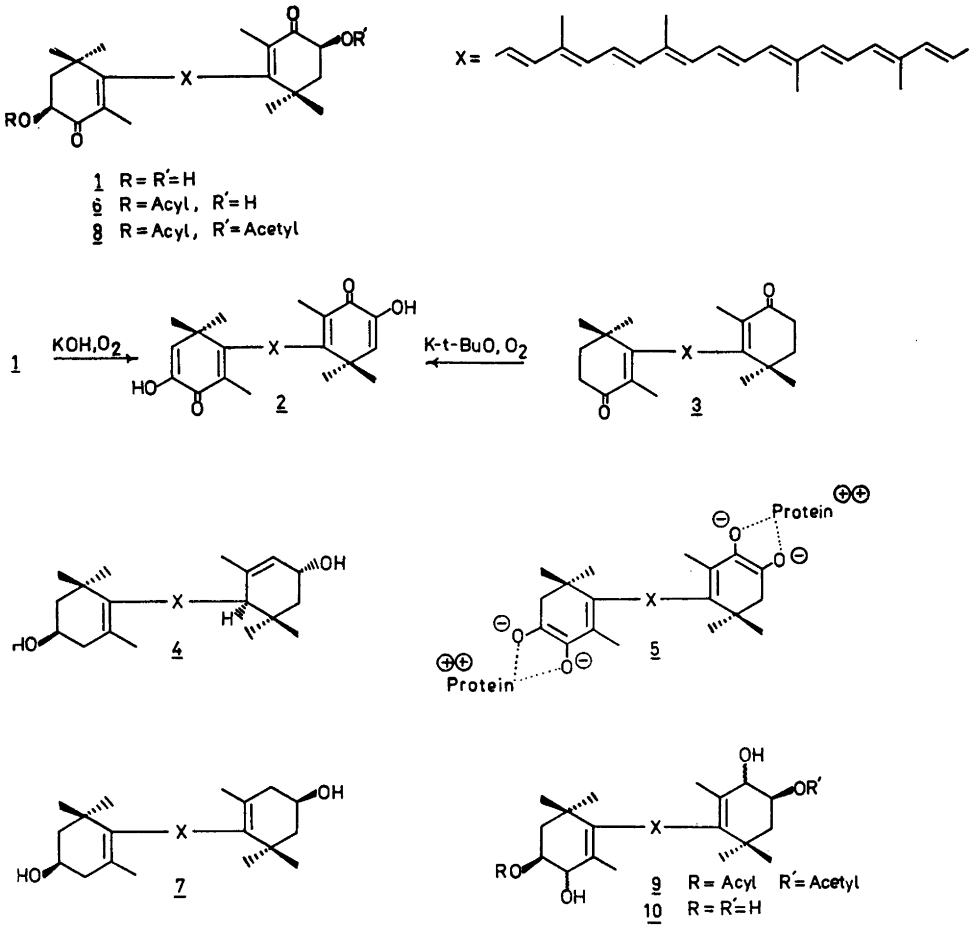


Scheme 1.

in lobster eggs as a bis-dianion (*5*),[1] because mere extraction with organic solvents cannot produce chirality of the carotenoid. The optical activity of astaxanthin *ex* ovoverdin is recently confirmed.[8b]

In order to establish the absolute configuration at the two chiral centers of astaxanthin (*1*) the use of chiroptical properties was considered. CD and ORD spectra of carotenoids have been investigated by several groups (*cf.*, *e.g.*, Refs. 8 – 10) but are theoretically not well understood.

Klyne and his colleagues [8a] have, quite successfully, developed an empirical rule which states that the two chiral end groups of a carotenoid contribute independently to the optical activity, so that contributions for these end groups are additive. This procedure is probably justified only for absorption bands connected with excitations of "partial chromophores" but not for the CD within the main band of all-*trans* carotenoids. However, due to the intense absorption of carotenoids in the latter region only few ORD/CD measurements are available for the visible region.[9,11]

Mills' rule [12,13] has also successfully been applied for determining the stereochemistry of 2- and 3-hydroxy-β-cyclogeranyl rings in carotenoids.[14,15] Recent work in our laboratories has further indicated that the preferred conformation of a chiral β-cyclogeneranyl ring is indeed of decisive importance for the CD spectra of carotenoids regardless of the type of substituents on the cyclohexene ring when dealing with half-chair conformations.[7]

RESULTS AND DISCUSSION

The ORD curve of a concentrated solution of astaxanthin (*1*) *ex* lobster has been reported by Buchwald and Jencks [16] for the 600 – 400 nm region and of astaxanthin *ex Hommarus gammarus* and of astaxanthin diacetate *ex Hommarus gammarus* and *ex Halocynthia papillosa* by Bartlett *et al.*[8a] in the 400 – 213 nm region. No stereochemical conclusions have been drawn from these data.

Fig. 1 shows the CD-spectrum of astaxanthin (*1*) *ex Hommarus gammarus* recorded in the 420 – 250 nm region. The ORD spectrum (450 – 250 nm) calculated [17] from this CD spectrum is consistent with data reported previously by Bartlett *et al.*[8a] In the 450 – 400 nm region their ORD curve deviates somewhat from that reported by Buchwald and Jencks.[16] In concentrated solution in the 600 – 400 nm region Buchwald and Jencks [16] probably measured some artefacts. The CD spectrum of astaxanthin monoester (*6*) *ex Haematococcus pluvialis*.[18] is shown in Fig. 2. In spite of the different solvents used, the CD spectra of *1* and *6* are very similar, thus supporting earlier reports [19] that individual chiral carotenoids have the same absolute configuration regardless of the biological source.

In general, the absolute configuration of a cyclohexenone can be derived from its CD spectrum in the range of the $n\rightarrow\pi^*$ band under the restrictions that this band can be unequivocally identified in the CD spectrum, and that a safe prediction can be made for the preferred conformation of the cyclohexenone ring.[20] Unfortunately, at least the first problem cannot be solved, because for astaxanthin (*1*) the CD band corresponding to the $n\rightarrow\pi^*$ transition cannot be identified. For simple conjugated oligo-enones, the $n\rightarrow\pi^*$ band appears at longest wavelengths corresponding to the transition of lowest energy. Calculations by Moore [21] have shown, however, that commencing with a polyene aldehyde of five carbon-carbon double bonds, the first $\pi\rightarrow\pi^*$ band should appear at longer wavelengths than the corresponding $n\rightarrow\pi^*$ band. In this connection recent work on a steriodal trienone has revealed that the $n\rightarrow\pi^*$ band occurs at *ca.* 360 – 380 nm and the $\pi\rightarrow\pi^*$ band at 345 nm.[22] With this short chromophore the separation of the two bands is rather small and distinctly visible only in unpolar solvents. In the case of astaxanthin (*1*), one would expect the $n\rightarrow\pi^*$ band to appear in the visible range where the anisotropy factor $g' = \Delta\varepsilon/\varepsilon$ is too small to give reliable CD data. Below 400 nm the CD spectrum of astaxanthin (*1*) resembles somewhat that of zeaxanthin (*7*), Scheme 1, which contains no carbonyl groups. A hypsochromic shift of the CD spectrum of *7* compared to that of *1*, is parallelled by a similar shift of the $\pi\rightarrow\pi^*$ band in the visible region of the corresponding isotropic electron absorption spectra. Such resemblance can, however, not be taken as proof for the same absolute configuration at the chiral centers C-3 and C-3′ without sufficient reference data.

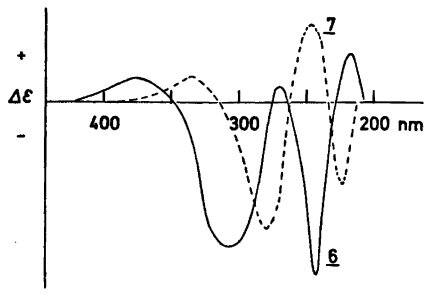


Fig. 2. CD-spectra of astaxanthin monoester (*6*, ——) and zeaxanthin (*7*, - - -) in EPA 5:5:2 solution.

For further work astaxanthin monoester (*6*) *ex Haematococcus pluvialis* [18] was used. All attempts to convert *6 via* its monoacetate *8* into zeaxanthin diester (or its enantiomer) failed.

These included the reduction of the two carbonyl groups of *8* to the tetrol-diester *9* with $NaBH_4$ followed by (i) SO_3 pyridine complex treatment and $LiAlH_4$-reduction,[23] (ii) formation of the *p*-toluene sulfonate [24] or methane sulfonate [25] derivative and subsequent $LiAlH_4$-reduction, (iii) replacement of hydroxyl by bromide followed by $LiAlH_4$-reduction.[26]

A second approach directed towards reduction of the keto groups of *6 via* N-derivatives, studied with canthaxanthin (*3*) as a model compound, was also unsuccessful. Thus, canthaxanthin (*3*) provided no hydrazone, phenylhydrazone or tosylhydrazone, but gave an oxime and a semicarbazone. Reduction of the semicarbazone under strongly alkaline conditions [24] failed. Previous attempts along the same lines have also been unsuccessful.[27]

Since a direct correlation with natural zea-

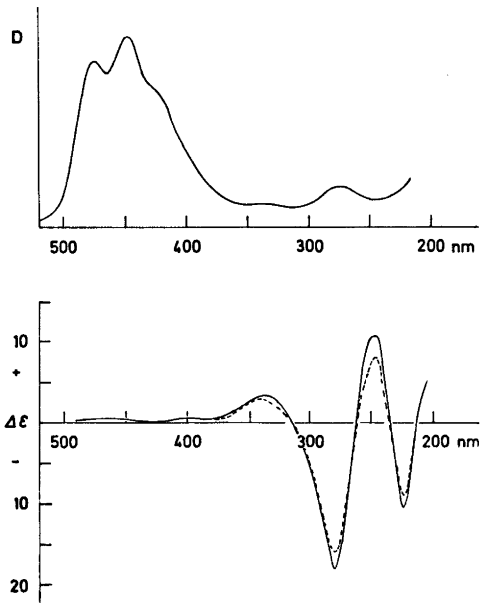


Fig. 3. Electronic absorption spectrum of the tetrol *10* (——, top), and CD-spectra of tetrol *10* (——, bottom) and zeaxanthin (*7*, – – –, bottom) *ex Flexithrix* strain QQ [30] in EPA 5:5:2 solution.

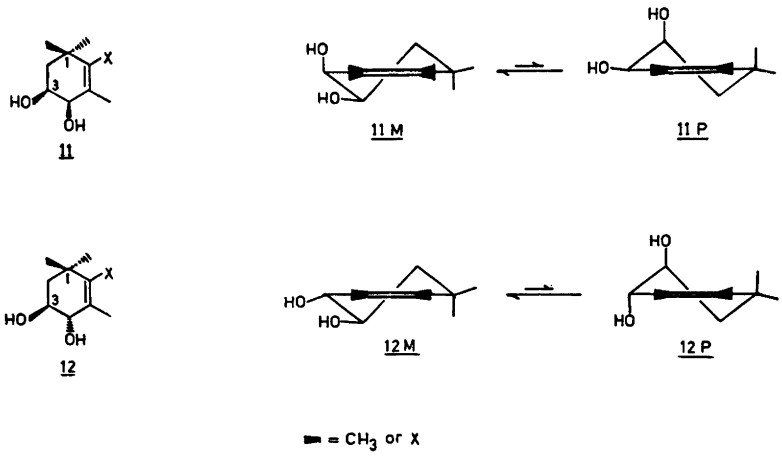
xanthin (*7*), known to have 3*R*,3′*R*-configuration [28,29] could not be achieved, chiroptical data of the tetrol *10* (obtained by $LiAlH_4$-reduction of *8*) was used to solve the stereochemistry of astaxanthin (*1*). The CD spectrum of the tetrol *10* together with that of zeaxanthin (*7*) *ex Flexithrix* sp.[30] with corrected [7] Δε-value is given in Fig. 3. The CD curves of *10* and *7* are very similar in shapes and signs of the individual Cotton effects; rotational strengths of the tetrol *10* being slightly higher than those reported for zeaxanthin (*7*).[7,8,31] By the following arguments this result is taken to prove the 3*S*,3′*S*-configuration of astaxanthin (*1*).

It is well known [32] that reduction by $LiAlH_4$ or $NaBH_4$ of a cyclohexanone preferentially leads to the equatorial alcohol if the ketone is unhindered, whereas, in the contrary case, the axial alcohol prevails. In the case of a cyclohexenone usually a mixture of epimers is formed,[33] especially if the ring is not rigid. Reduction of *8* was therefore expected to yield a mixture of three diastereomeric tetrols.

A series of attempts to isolate individual diastereomers of the tetrol *10* failed, thus also precluding the possible use of hydrogen bonding studies by IR. The systems investigated included that in which the diastereomeric tetrols derived from actinioerythrin (*13*) could be separated [34] and the one where Bodea and coworkers [35] claimed successful separation of $NaBH_4$-reduced astaxanthin.

However, separation of the tetrol *10* into individual diastereomers is not essential for our argumentation.

Assuming 3*S*,3′*S*-configuration for astaxanthin (*1*), the *cis* diol end group *11* will have 3*S*,4*R*-configuration and the *trans* diol end group *12* the 3*S*,4*S*-configuration, Scheme 2. Of the two half-chair conformations *12M* and *12P* associated with the *trans* diol *12*, *12M* will be



Scheme 2.

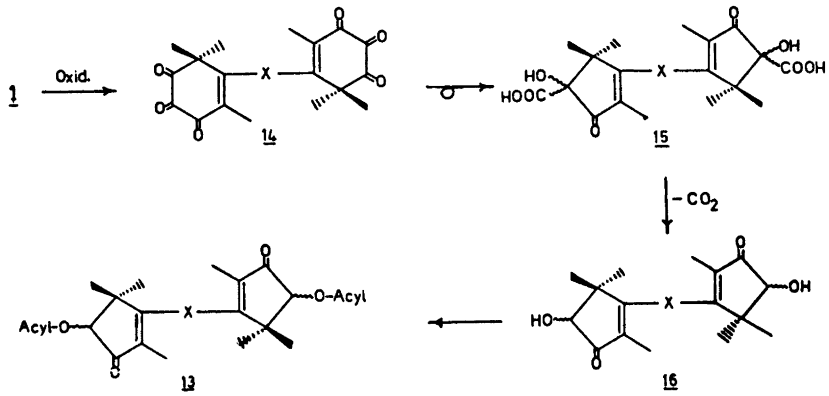
preferred because there is only one (1:3-Me:H) interaction as opposed to one (1:3-OH:Me) and one (1:3-OH:H) interaction present in the *12P*-conformation. In the case of the *cis* diol *11*, the *M*-helical conformation *11M* will also be preferred because the *P*-conformation contains a (1:3-OH:Me) interaction which is thought to be more severe than the sum of a (1:3-OH:H) and a (1:3-Me:H) interaction, *cf.* Ref. 36, especially in EPA solution where solvation will take place. Zeaxanthin (*7*), whose end group has the same configuration for the 3-OH-group, is also expected to have the ring predominantly in the *M*-conformation to avoid such a (1:3-OH:Me) interaction; this is consistent with ^{1}H NMR evidence.[37,38] The preponderance for the *M*-conformation of the cyclohexene half-chair of these end groups (*viz.* monohydroxylated end group of *7*, and *cis* and *trans* dihydroxylated *11* and *12* of *10*) will be greatest for the trans diol *12* end group and smallest for the *cis* diol end group *11*.

As already mentioned, there is reason to assume [10] that the signs of the CD bands are determined solely by the helicity of the (half-chair) conformation of the cyclohexene ring. This is consistent with another rule proposed [39] for the CD of conjugated dienes and enones, which states that the axial substituents (including hydrogen) in allylic position to the diene or α-position to the conjugated ketone determine the sign of the Cotton effects, *cf.* Ref. 40. On the basis of this hypothesis and the conformational analysis given above, the CD of *7*, *11*, and *12* should thus be mainly determined by the configuration at C-3 (or C-3′). As the signs and positions of the Cotton effects of *7* and the tetrol *10* are exactly the same, we conclude, therefore, that the configuration at C-3 and C-3′ of *10* and thus also of astaxanthin (*1*) is *S*.

Considering now the rotational strength observed for zeaxanthin (*7*) and the tetrol *10*, Fig. 3. From the arguments advanced above, complex hydride reduction resulting in a preponderance of products with the *cis* diol end group *11* would be expected to result in a $\Delta\varepsilon$-value for the tetrol *10* lower than for zeaxanthin (*7*). Preponderance of products with the *trans* diol end group *12* would, on the other hand, result in higher $\Delta\varepsilon$-values relative to zeaxanthin (*7*). The actual values observed ($\Delta\varepsilon = -14.8$ for *7*, *versus* $\Delta\varepsilon = -18.0$ for *10*) only allows the tentative conclusion that more of the *trans* diol end group *12* was formed than of the *cis* diol end group *11*.

Independent support for the stereochemical assignment of astaxanthin (*1*) may be possible through partial synthesis of the 3,3′-dimethyl ether of the tetrol *10* from optically active zeaxanthin (*7*) dimethyl ether for CD comparison with *10*. The route utilized by Surmatis and Thommen [41] for the synthesis of astaxanthin (*1*) dimethyl ether is one possible approach.

Another carotenoid with unknown stereochemistry is actinioerythrin (*13*, Scheme 3), which is a naturally occurring diester of a bis-cyclopentenone-α-ketol [34] with the unique 2,2′-bis-nor-carotenoid structure. It has been suggested that *13* is formed *in vivo* from astaxanthin (*1*) [42] by oxidation to *14* (Scheme 3), benzilic acid rearrangement to *15*, decarboxylation to the bis-α-ketol *16*, and finally esterification. As the



Scheme 3.

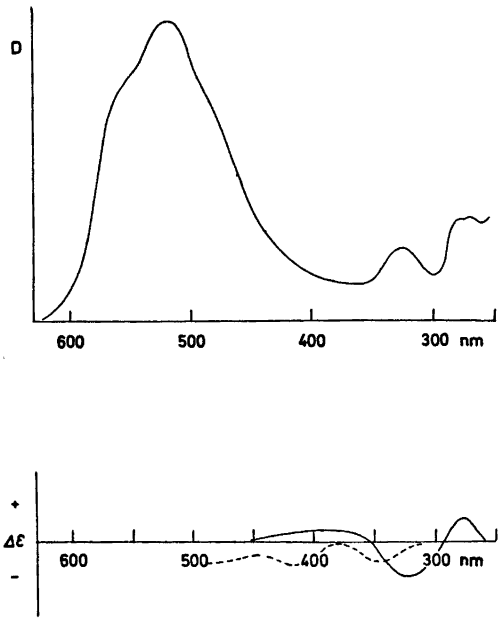


Fig. 4. Electronic absorption spectrum (——, top) of actinioerythrin (*13*), CD-spectrum (——, bottom) and ORD-spectrum (- - -, bottom; calculated from the CD-spectrum) of actinioerythrin (*13*) in chloroform solution.

prochiral intermediate *14* may be involved, the absolute configuration of the OR-group of *13* must not necessarily, from biosynthetic considerations, have the same streochemistry as that of astaxanthin (*1*). On the other hand, optical activity of *13* does not contradict such a biogenetic pathway since a chiral enzyme can stereospecifically react either with the *re*- or the *si*-side of the ring of *14*. Actinioerythrin *13* is indeed chiral as evidenced by its CD spectrum, Fig. 4. The ORD spectrum calculated [17] therefrom is also given in Fig. 4. In the case of *13*, a negative CD within the main absorption band in the visible region was observed. Because of high noise and a small anisotropy factor no quantitative values can be given, however. Due to the difficulties encountered in the interpretation of the CD of cyclopentenones [20] no conclusions about the absolute streochemistry of actinioerythrin (*13*) can be drawn at present, except that the two chiral centers of this molecule must have the same chirality.

EXPERIMENTAL

Materials. Astaxanthin *ex Hommarus gammarus* and astaxanthin monoester *ex Haematococcus pluvialis* were obtained from the collection of Professor N. A. Sørensen, and astaxanthin *ex* lobster eggs provided by Hoffmann-La Roche, Basel. Actinioerythrin was left from a previous investigation.[24]

Methods. Methods used were those commonly employed in the Trondheim laboratory.[24] CD spectra were recorded in Copenhagen in EPA (ether:isopentane:ethanol, 5:5:2) solution with a Roussel-Jouan Dichrographe II.

Acetylation of astaxanthin monoester (6). *6* (0.5 mg) in dry pyridine (2 ml) was treated with acetic anhydride (0.2 ml) for 6 h at 25 °C to yield quantitatively *8*; λ_{max} 485 nm ($CHCl_3$), 474 nm (MeOH); $R_F = 0.5$, silica gel G plate developed with 20 % APE (acetone in petroleum ether).

$LiAlH_4$ reduction of 8. *8* (0.5 mg) in dry ether (10 ml) was treated with $LiAlH_4$ at 25 °C for 3 min (representative experiment). After extractive isolation with $CHCl_3$, *10* was isolated and purified by TLC on silica gel G plates developed with APE. The tetrol *10* had the following properties: λ_{max} (430), 453, 480 nm (acetone), 451 nm (MeOH); *m/e* 600 (M), 582 (M − 18), 564 (M − 36), 546 (M − 54), 508 (M − 92) and 494 (M − 106); $R_F = 0.35$, silica gel G plates developed with 40 % APE.

Attempted separation of diastereomeric tetrols 10. Attempted separation of the diastereomeric tetrols *10* on kieselguhr paper, cellulose plates, cellulose columns, acetylated polyamide columns and MgO columns failed. On the latter column *10* failed to move with 10 % acetone in benzene; Nicoara *et al.*[25] reported successful separation of *10* in this system.

$NaBH_4$ reduction of 8. *8* (0.5 mg) in wet MeOH (10 ml) was treated with $NaBH_4$ at 0 °C for 5 min. After extractive isolation *9* was obtained in quantitative yield; λ_{max} (430), 453, 480 nm (acetone); 451 nm (MeOH); $R_F = 0.4$, silica gel G plates developed with 25 % APE.

Reactions of 9. *9* (0.1 mg) was treated for 20 h at 25 °C with SO_3-pyridine (10 mg) in dry THF (2 ml);[23] no sulfate ester was detected upon TLC analysis. Treatment of *9* (0.1 mg) with excess *p*-toluene sulfonyl chloride in ether or pyridine gave no ester. Treatment of *9* in dry pyridine with methane sulfonyl chloride resulted in degradation of the pigment. Attempted formation of the 4,4′-dibromo derivative of *9* by treatment with NBS-$(CH_3)_2S$ in CH_2Cl_2 [26] failed.

Reactions with canthaxanthin (3). *3* when treated with hydrazine hydrochloride, phenylhydrazine hydrochloride, or tosylhydrazine in MeOH or pyridine solvents failed to yield the corresponding hydrazones even at elevated temperatures. *3* smoothly formed the oxime and semicarbazone under standard conditions. The latter when treated with KOMe in dimethyl

sulfoxide [24] failed to yield the reduced product when treated at 100 °C in a sealed tube under N_2 for 30 min.

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Bacterial Carotenoids. XLIII.* C_{50}-Carotenoids. 13.** Synthesis of (2*R*,2′*R*)-2,2′-Dimethyl-β,β-carotene and Absolute Configuration of (2*R*,2′*R*)-2-(4-Hydroxy-3-hydroxymethyl-2-butenyl)-2′-(3-methyl-2-butenyl)-β,β-carotene

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This first assignment of absolute stereochemistry to a C_{50}-carotenoid, *C.p.* 450 = (2*R*,2′*R*)-2-(4-hydroxy-3-hydroxymethyl-2-butenyl)-2′-(3-methyl-2-butenyl)-β,β-carotene (*5*), followed from CD-correlation with natural (2*R*,2′*R*)-β,β-carotene-2,2′-diol (*4*) and (2*R*, 2′*R*)-2,2′-dimethyl-β,β-carotene (*12*) synthesized here.

All fourteen [1–4] known naturally occurring C_{45}- and C_{50}-carotenoids are distinguished by having additional C_5-isopentenyl units formally added to the 2- or 2,2′-positions on a C_{40}-skeleton; exemplified by bacterioruberin (*1*), bisanhydrobacterioruberin (*2*), and decaprenoxanthin (*3*), Scheme 1. Provided enzymatic control, this addition induces optical activity at C-2 (2′), indicated by asterisks in Scheme 1. From CD correlation bacterioruberin (*1*) and bisanhydrobacterioruberin (*2*) are known to possess the same absolute configurations.[5] Chiroptical properties of decaprenoxanthin (*3*) have also been reported.[5,6] However, despite rapid advances in the stereochemistry of C_{40}-carotenoids, no absolute configurations of C_{45}- or C_{50}-carotenoids have hitherto been reported.

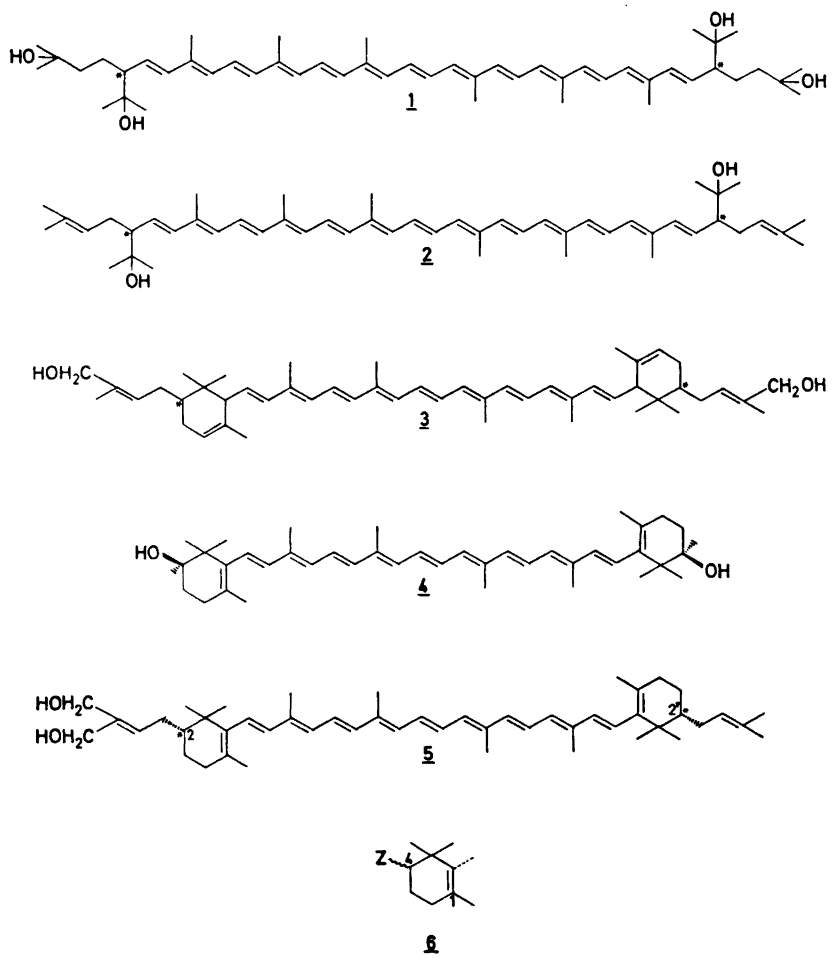
Recent reports on the absolute stereochemistry of (+)-(*R*)-β-irone (*7*, Scheme 2) [7] and tentative stereochemical assignment to (2*R*,2′*R*)-β,β-carotene-2,2′-diol (*4*) [8] prompted us to investigate the stereochemistry of some C_{50}-carotenoids; in particular that of 2-(4-hydroxy-3-hydroxymethyl-2-butenyl)-2′-(3-methyl-2-butenyl)-β,β-carotene (*5*; *C.p.* 450 [3]), Scheme 1.

(2*R*,2′*R*)-β,β-Carotene-2,2′-diol (*4*) and *C.p.* 450 (*5*, stereochemistry hitherto unknown) have common chromophores and differ only in the nature of the 2-substituent. Each have termini which may be regarded as 4-substituted cyclohexenes (see structure *6*, Scheme 1). From Mills' rule,[9,10] the optical rotation of cyclohexene systems with an asymmetric carbon at position 4 is caused by a preferred conformation of the cyclohexene ring itself and the nature of the substituent is not relevant. Application of Mills' rule may lead to erroneous results,[11] although its validity in the carotenoid series for the stereochemical correlation of (2*R*,2′*R*)-β,β-carotene-2,2′-diol (*4*) and zeaxanthin (3*R*,3′*R*)-β,β-carotene-3,3′-diol) has recently been confirmed.[12a] As a further model for CD-correlation (2*R*,2′*R*)-2,2′-dimethyl-β,β-carotene (12) was synthesized from (+)-(*R*)-β-irone (*7*). Optically inactive *12* has previously been synthesized by Eugster *et al.*[12b] by a different route.

RESULTS AND DISCUSSION

(+)-(6*R*)-β-Irone (*7*) was isolated by preparative GLC from a base-isomerized mixture of natural irones.[7] The synthesis of (2*R*,2′*R*)-2,2′-dimethyl-β,β-carotene (*12*) followed the route

* No. XLII. *Acta Chem. Scand. 27* (1973) 3040.
** No. 12. *Acta Chem. Scand. B 28* (1974) 244.



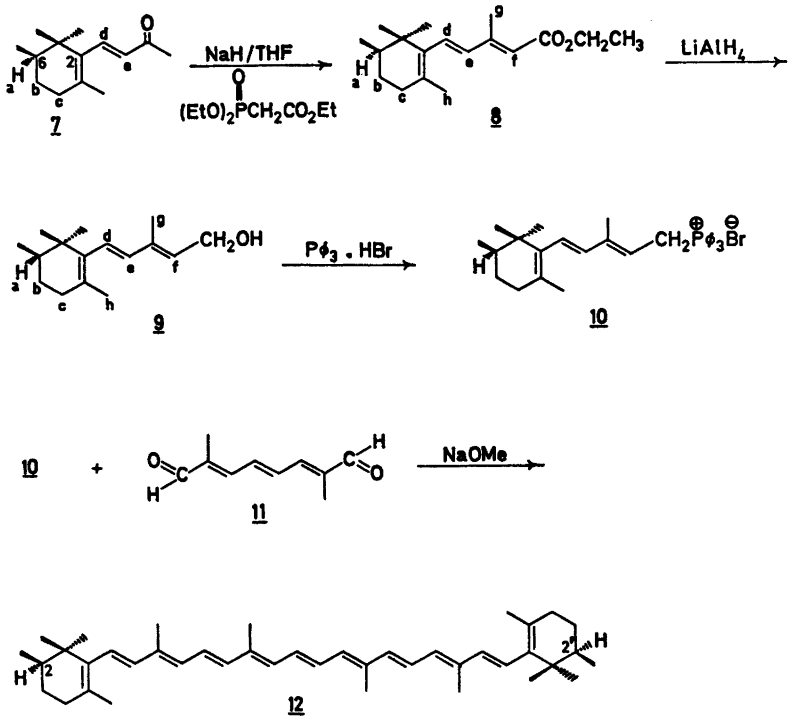
Scheme 1.

shown in Scheme 2. Treatment of *7* with ethyl diethylphosphonoacetate gave (*R*)-β-ironylidene acetate (*8*). Reduction with $LiAlH_4$ provided the allylic alcohol *9* which was converted to the corresponding phosphonium bromide *10*. Condensation of *10* with the trienedial *11* gave (2*R*,2′*R*)-2,2′-dimethyl-β,β-carotene (*12*).

The electronic absorption spectrum of *12* corresponded to that of β-carotene. The ^1H NMR spectrum, including signal assignments, is reproduced in Fig. 1. The upper mass region of the mass spectrum is shown in Fig. 2.

The CD spectra of *trans* (2*R*,2′*R*)-2,2′-dimethyl-β,β-carotene (*12*), (2*R*,2′*R*)-β,β-carotene-2,2′-diol (*4*) and *C.p.* 450 (*5*, available from a previous investigation [3]) are shown in Fig. 3. Immediately apparent is the similarity between the curves of *4* and *12*, possessing the same absolute stereochemistry, and differing only in the nature of the 2-substituent, hydroxyl *versus* methyl. This compatibility further confirms that the principle of Mills' rule is applicable to these carotenoid systems.

The CD spectrum of *C.p.* 450 (*5*) is virtually exactly opposite to those of *4* and *12*, revealing opposite absolute configuration in 2,2′-positions. *C.p.* 450 has therefore configuration *5*. Formally, *C.p.* 450 is (2*R*,2′*R*)-2-(4-hydroxy-3-hydroxymethyl-2-butenyl)-2′-(3-methyl-2-butenyl)-β,β-carotene.[13]



Scheme 2.

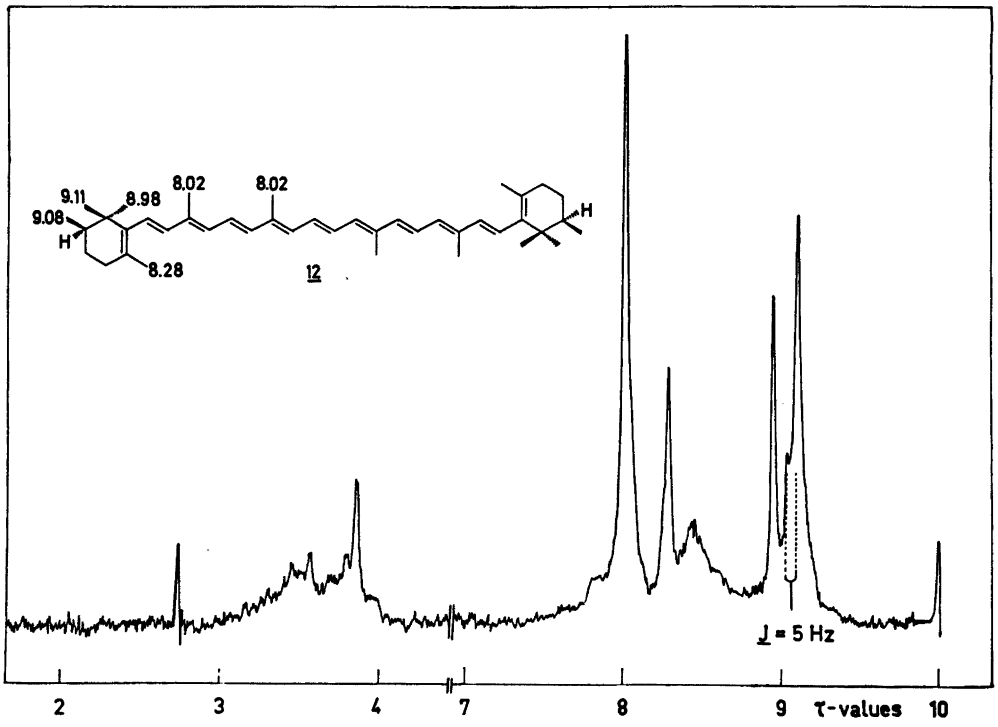


Fig. 1. ^{1}H NMR spectrum of (2*R*,2′*R*)-2,2′-dimethyl-β,β-carotene (*12*).

Fig. 2. Mass spectrum of (2*R*,2′*R*)-2,2′-dimethyl-β,β-carotene (*12*).

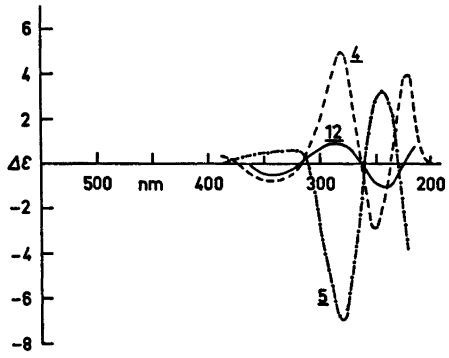


Fig. 3. CD-spectra of (2*R*,2′*R*)-2,2′-dimethyl-β,β-carotene (*12*, ———), (2*R*,2′*R*)-β,β-carotene-2,2′-diol (4, – – –) and *C.p.* 450 (*5*, - · - ·) in EPA.

Further studies on the absolute configurations of other C_{50}-carotenoids are in progress.

EXPERIMENTAL

Material and methods. All solvents were of analytical grade or distilled before use. Comparative TLC of carotenoid intermediates was on silica gel HF_{254} plates developed with petroleum ether-acetone solutions. Separation and purification of synthetic carotenoids were accomplished with alumina plates (1 mm) developed with petroleum ether-ethyl ether (97:3). Comparative chromatography of carotenoids was on Merck alumina HF_{254} Type E pre-coated plates (0.2 mm).

Instruments used were as specified elsewhere.[14] Specific rotations of carotenoid intermediates were obtained on a Perkin-Elmer 141 polarimeter and CD spectra recorded in EPA (ether-isopentane-ethanol, 5:5:2) on a Roussel-Jouane Dicrographe.

(+)-(*R*)-β-Irone (*7*) was isolated with an Aerograph Autoprep A-700 chromatograph using a 4.5 m × 12 mm metal column packed with Carbowax 20M (15 %) on Chromosorb W (60 – 80 mesh) at 190°C and 190 ml/min (helium). Carotenoid intermediates (*7*, *8*, *9* were examined for purity with a Perkin-Elmer F-11 gas chromatograph fitted with a 1.8 m glass column packed with OV-17 (5 %) on Chromosorb W;

temperature programmed from 110 °C at 2°/min.

C.p. 450 (5). 5 was available from an earlier reported study.[3] The CD spectrum is given in Fig. 3.

Composition of natural Iris oil. GLC analysis of natural Iris oil (Absolute Iris Essence) showed the following irone composition: (−)-(2*R*,6*R*)-*trans*-α-irone (numbering and nomenclature of irones follow that used by Rautenstrauch and Ohloff,[7] *cf.* *7*, Scheme 2), 20.7 % (of total irones); (+)-(2*S*,6*R*)-*cis*-α-irone (*cis* refers to the 2,6-substituents), 41.8 %; (+)-(2*S*,6*R*)-*cis*-γ-irone, 37.4 %.

Alkali-catalyzed isomerization of Iris oil. Iris oil (3 g) in a MeOH-H_2O (20 ml, 85:15) solution containing 10 % KOH was heated at 40 °C under N_2 for 24 h.[7] The ether extractable fraction was concentrated under vacuum and distilled at 0.3 Torr. A yellow oil (2 g) which distilled at 110 − 130 °C was collected and (+)-(6*R*)-β-irone (*7*, 0.85 g) isolated by preparative GLC. Irone composition of oil after isomerization: (+)-(6*R*)-β-irone (*7*), 61.6 %; (+)-(2*S*,6*R*)-*cis*-α-irone, 8.2 %; (−)-(2*R*,6*R*)-*trans*-α-irone, 30.2 %. Analytical GLC showed the β-irone (*7*) collected exceeded 98 % purity. The following physical properties for *7* were recorded: $[\alpha]^{20}$ (EtOH) 589 nm = 16°, 578 nm = 17°, 546 nm = 20° and 436 nm = 36°; λ_{max} 296 nm in MeOH; IR (liq.) 2980, 2920, 2860 (s, C−H), 1690, 1670 (s, C=O), 1605 (s, C=C), 1360 (s), 1250 (s, C=O), 980 cm^{-1} (m, *trans* −HC=CH−); τ($CDCl_3$; signal assignments with reference to *7*, Scheme 2) 9.083 d(3 H, *J* = 5 Hz, CH−CH_3), 9.080, 8.95 (6 H, *gem.* dimethyl), 8.8 − 8.4 (3 H, H-*a*,*b*), 8.27 (3 H, =C-CH_3), 8.1 − 7.8 (2 H, H-*c*), 7.75 (3 H, O=C−CH_3), 3.94 d(1 H, *J* = 16 Hz, H-*e*), 2.77 d(1 H, *J* = 16 Hz, H-*d*); *m/e* 206 (M), 191 (M − 15), 149 (M − 57), 135 (M − 71) and 122 (M − 84).

Ethyl (+)-(6R)-β-ironylideneacetate (8). Ethyl diethylphosphonoacetate [15a] (1.7 g) was added to a suspension of NaH (200 mg) in tetrahydrofuran (THF, 6 ml) and stirred at 20 °C for 1 h. The mixture was cooled to 0 °C and *7* (0.85 g) in THF (1 ml) added. The reaction was stirred for 3 h at 0 − 5 °C, then at 20 °C for an additional 15 h. Water was added and the mixture extracted with ether. The organic layer was dried with Na_2SO_4, concentrated under vacuum and chromatographed on a kieselgel column developed with benzene from which *8* was isolated (1.016 g, 89 %). GLC and ^1H NMR analysis showed *8* to consist of 85 % *trans*- and 15 % *cis*-isomers (around the new double bond). The product *8* had: $[\alpha]^{20}$ (EPA) 589 nm = 33°, 578 nm = 35°, 546 nm = 41°, and 436 nm = 81°; λ_{max} (MeOH) 260, 295 nm; ν_{max} (liq.) 2960, 2920, 2840 (s, C−H), 1715 (s, C=O), 1610 (s, C=C), 1450 (m), 1235 (s, C−O), 1153 (s, C−O), 1050 (s), 970 cm^{-1} (m, *trans* −CH=CH−); τ($CDCl_3$; signal assignments with reference to *8*, Scheme 2) 9.12, 8.97 (6 *H*, *gem.* dimethyl), 9.06 d(3 H, *J* = 2.5 Hz, −CH−CH_3), 8.72 t(3 H, *J* = 7 Hz, CH_2−CH_3), 8.7 − 8.3 (3 H, H-*a*,*b*), 8.32 (3 H, H-*h*) 8.1 − 7.8 (2 H, CH_3-*c*), 7.66 (3 H, CH_3-*g*), 5.77 q (2 H, *J* = 7 Hz, C$H_2$$CH_3$), 4.35 (1 H, *cis*-H-*f*, *cf.* Ref. 15b), 4.25 (1 H, *trans*-H-*f*), 3.97 d(1 H, *J* = 16 Hz, H-*e*), 3.40 d(1 H, *J* = 16 Hz, H-*d*); *m/e* 276 (M) 263 (M − 15), 231 (M − 45) and 203 (M − 73).

(+)-(6R)-β-Ironylidene-ethanol (9). *8*, (0.94 g) in THF (10 ml) was added to a stirred solution of $LiAlH_4$ (0.2 g) in THF (20 ml) at 0 °C during 30 min. Stirring was continued for 1 h and the reaction monitored by TLC (disappearance of starting material). The complex was decomposed by cautious addition of MeOH followed by a saturated solution of NH_4Cl. After ether extraction *9* (0.84 g, quantitative yield) was isolated; $[\alpha]^{20}$ (EPA) 589 nm = 30°, 578 nm = 31°, 546 nm = 37°, 436 nm = 68° and 365 nm = 327°; λ_{max} (MeOH) 237, 265 nm; ν_{max} (liq.) 3320 (s, OH), 2960, 2920, 2860 (s, C−H), 1000 (m, OH), 920 cm^{-1} (s, *trans* −CH=CH− τ($CDCl_3$; signal assignments with reference to *9*, Scheme 2) 9.13, 8.99 (6 H, *gem.* dimethyl), 9.09 d(3 H, *J* = *ca.* 5 Hz, −CH−CH_3), 8.7 − 8.3 (3 H, H-*a*,*b*), 8.32 (3 H, CH_3-*h*), 8.14 (3 H, CH_3-*g*), 8.25 − 7.85 (2 H, H-*c*), 5.17 d(2 H, *J* = 7 Hz, CH_2−OH), 4.38 broad t(1 H, *J* = 6.5 Hz, H-*f*), 3.95 (2 H, H-*d*,*e*; *m/e* 234 (M) and 219 (M − 15).

(6R)-β-Ironylidene-ethyltriphenylphosphonium bromide (10). A solution of *9* (0.8 g) and triphenylphosphonium bromide (1.5 g) in $CHCl_3$ (5 ml) was stirred at 20 °C for 48 h under N_2. The solvent was removed under vacuum and the oily residue dissolved in a minimum amount of acetone (5 ml) and chromatographed on a kieselgel column. Elution with acetone removed unreacted *9* and triphenylphosphine. *10* was eluted with MeOH and obtained as an amorphous powder after removal of solvent. Yield of *10* was 1.74 g (84 %).

(2R,2'R)-2,2'-Dimethyl-β,β-carotene (12). NaOMe (0.6 M) was slowly added to a stirred solution of *10* (200 mg) in dry MeOH (5 ml) until the phosphonium salt was completely converted to the deep red phosphorane. A solution of 2,7-dimethylocta-2,4,6-trienedial (*11*, 200 mg, prepared from the acetylenic analogue [16]) in CH_2Cl_2 (0.5 ml) was added and the mixture stirred. Progress of the reaction was monitored by TLC and additional phosphorane, generated externally, was added until *11* was completely converted to *12*. Water was added, the crude product extracted into ether, concentrated under vacuum and chromatographed on a kieselgel column developed with benzene. Yield of *12* (*cis-trans* mixture) was 42 mg (34 % based on dial *11*). *12* was purified on alumina plates from which the all *trans*-isomer was collected. Crystallized from MeOH-acetone solution *12* melted at 179 °C. Comparative TLC on precoated alumina plates developed with petroleum ether-ethyl ether (97:3) showed *12* to have R_F = 0.42; β,β-carotene in this system had R_F = 0.38. On Schleicher & Schüll paper No. 288 (Al_2O_3) developed with petroleum ether + ethyl ether (99 + 1) *12* had R_F = 0.33; β,β-carotene in the

same system had $R_F = 0.20$. *12* had λ_{max} (hexane) 265, (423), 449 [E (1 %, 1 cm) = 2428; $\varepsilon = 137\,000$ compared with $\varepsilon = 134\,000$ for β,β-carotene [17]], 476 nm [E(1 %, 1 cm) = 2072; $\varepsilon = 117\,000$], (acetone) (427), 451, 479 nm; $\tau(CDCl_3)$ see Fig. 1 with signal assignments; CD (EPA), see Fig. 3; *m/e* 564 (M), 549 (M − 15), 472 (M − 92), 458 (M − 106), 415 (M − 149), 406 (M − 158), 420 (M − 162), 373 (M − 191), 360 (M − 204), 347 (M − 217) and 282, see Fig. *2*.

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Mass Spectrometry of Onium Compounds. XXVI.[1] Ionisation Potentials in Structure Analysis of Gaseous Aminopyridine 1-Oxides

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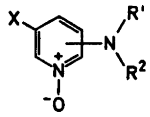
The isomeric aminopyridine 1-oxides, their methylamino and acetylamino analogues exist predominantly in the amine form in the gas phase in the mass spectrometer. The conclusion is based on ionisation potential data, and fragmentation characteristics, which have been compared with such data for *N*,*N*-dimethylamino and *N*,*N*-acetylmethylaminopyridine 1-oxides and 1-methoxypyridin-onimines.

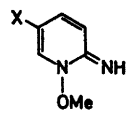
From ionisation potential measurements by electron impact in the mass spectrometer we have in recent reports [2,3] shown that the potentially tautomeric hydroxy-, mercapto-, and amino-pyridines in the gas phase exist predominantly in the enol and amine forms, respectively. The preference for the amino form in the gas phase has very recently been conclusively shown by a structural study of 2-aminopyridine by means of its microwave spectrum.[4] In this report we deal with the 1-oxides of the isomeric aminopyridines and their acylamino analogues. For comparative purposes in the interpretation of ionisation potential (IP) data corresponding *N*-methylamino analogues are included in the study. The IP data obtained are given in Table 1. The ionisation efficiency curves were recorded as previously described [5] and are interpreted by the semilog plot method.[6]

The influence of a substituent on the ease of ionisation depends on the nature of other substituents as well as on the ring system and relative position.[7] Generally an electron releasing substituent decreases the IP.[8] Thus the IP value for pyridine 1-oxide,[9] measured to 8.78

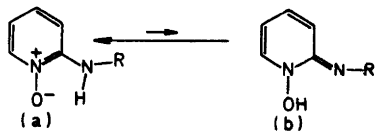
Table 1. Ionisation potentials for aminopyridine 1-oxides.



Comp.	Isomer	X	R^1	R^2	IP ± 0.05 eV
1	2	H	H	H	8.04
2	3	H	H	H	8.21
3	4	H	H	H	7.67
4	2	H	H	Me	7.67
5	3	H	H	Me	7.97
6	4	H	H	Me	7.45
7	2	H	Me	Me	7.62
8	3	H	Me	Me	7.85
9	4	H	Me	Me	7.32
10	2	H	H	Ac	8.05
11	3	H	H	Ac	8.40
12	4	H	H	Ac	7.76
13	2	H	Me	Ac	7.77
14	3	H	Me	Ac	8.18
15	4	H	Me	Ac	7.52
16	2	Cl	H	H	7.98
17	2	Cl	H	Me	7.61



(18) X = H, IP = 7.46 eV
(19) X = Cl, IP = 7.40 eV



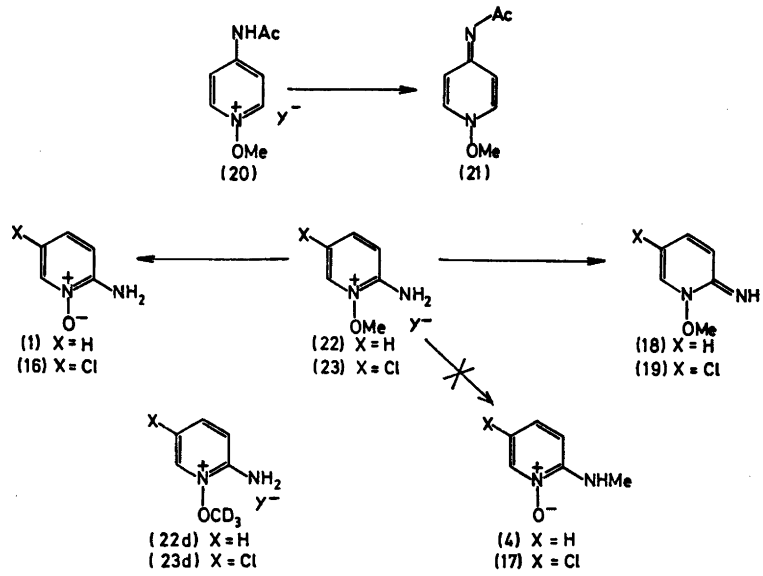
Scheme 1.

eV in the present work, is lowered on amine substitution (Table 1). Methylation of the amino group in aniline decreases the IP.[10] This effect was found to be very similar in all three aminopyridine isomers.[3]

The primary and secondary 2- and 4-aminopyridine 1-oxides can partially exist as the tautomeric 1-hydroxypyridin-2- and -4-onimines (Scheme 1). The *N*,*N*-dimethylaminopyridine 1-oxides, however, cannot tautomerise and must evaporate as such. The IP values (*7*–*9*) are increased by 0.2 eV from the 2- to the 3-isomer but decreased by 0.5 eV from the 3- to the 4-isomer. The same changes are seen for the primary amines (*1*–*3*) and for the secondary amines (*4*–*6*). The same pattern is evident also by comparison of the data for the 2-analogue series (*1*,*4*,*7*) with the 3- and 4-series; monomethylation has decreased IP with 0.2–0.3 eV relative to the parent amine and dimethylation has further reduced this value by 0.1 eV.

On prototropic shifts the 3-amines (*2*, *5*) can form the corresponding zwitterionic 1-hydroxy derivatives. The charge separation introduced will reduce the volatility compared to that of the non-charged amine form. On energetic grounds structures with charge separation should be disfavoured in the gas phase because at 10^{-6} Torr the charges cannot be dispersed by solvation or other intermolecular interactions. The 3-amines (*2*, *5*) must therefore be assumed present in the gas phase in the amine form. Consequently it follows from the systematic IP differences discussed above that the 2- and 4-isomers exist in the amine form in the gas phase. Furthermore the same IP pattern is seen for the acetyl derivatives (*10*–*12*) on comparison with the *N*-methyl analogues (*13*–*15*), and hence the same conclusion is reached. The amino form has also been found to be preferred by 2- and 4-aminopyridine 1-oxides and acylamino analogues in solid state and solution.[11,12]

Support for the above conclusion was to be sought in IP measurements of analogues locked in the onimine form by *O*-methylation. The 1-methoxy analogues, however, were only available as pyridinium salts with weak nucleophilic anions as *p*-toluenesulfonate or perchlorate anion. In the mass spectrometer these reactive molecules were largely decomposed, transmethylated on the nitrogen or demethylated. The cation from 4-acetamido-1-methoxypyridinium perchlorate (*20*, Scheme 2), however, appeared



Scheme 2.

to evaporate after proton loss as 1-methoxypyridin-4-*N*-acetylonimine (*21*). Unfortunately the relative molecular ion peak intensity was only of the order 1 % which with our present technique is too weak for obtaining reproducible IP data.

Similar studies for the *ortho* isomers also proved difficult. Thus the spectra from 1-methoxy-2-aminopyridinium toluene-*p*-sulfonate contain a signal for the mass number (*m/e* 124) of 1-methoxypyridin-2-onimine (*18*). Demethylation, however, is a competitive reaction to proton abstraction and evaporation of the onimine. Pyrolytic demethylation (*m/e* 110) was suggested by variable signal intensities. This deduction was confirmed by the appearance potential (AP) (8.02 eV) which was the same as IP for (*1*). The relative intensities for the signals corresponding to deprotonation and demethylation were increased from 1:8 to 1:2 on changing the anion from toluene-*p*-sulfonate to perchlorate. AP for the *m/e* 124 species was determined to 7.46 eV as compared to 7.67 eV for the isomeric *N*-methylpyridine 1-oxide (*4*). The experimental difficulties encountered and the closeness of these values made any conclusion uncertain. Support was sought in other derivatives. For this purpose the 5-chloro analogues (*17*) and (*23*) were prepared. A chlorine atom has little effect on the IP of an aromatic system.[8,9] This is also seen in the present work by comparison of the data (Table 1) for (*1*) and the 5-chloro analogue (*16*). IP for the *N*-oxide (*17*) was 7.61 eV as compared to 7.40 eV for the gaseous species from the 1-methoxy analogue (*23*). The difference is the same as between (*4*) and (*22*).

Any pyrolytic transmethylation in the salt (*22*) before evaporation (*22*→*4*) would affect AP for the *m/e* 124 species. Formation of (*4*) could occur by intra- or intermolecular reactions from (*22*). The latter possibility was investigated by deuteriation. The methyl group in (*22*) was perdeuteriated (*22d*) and mixed with equimolar quantities of the non-deuteriated 5-chloro analogue (*23*). The chlorine atom in (*23*) serves to mark the pyridine nucleus in the non-deuterated compound for identification after any transmethylation reaction. The chlorine atom is assumed not to significantly affect the activity. This assumption is supported by the very similar nature of the spectra from (*22*) and (*23*). A homogeneous mixture was strived at by evaporation in the instrument of a common methanol solution. In the case of intermolecular transmethylation four isotopically different molecular ion signals should be obtained.[18] The spectra, however, contained two such signals which exclude an intermolecular process. The conclusion was experimentally confirmed by mixing the deuteriated 5-chloro compound (*23d*) with (*22*). Comparative examination of the fragmentation spectra from the isomeric 2-methylaminopyridine 1-oxide (*4*) and 1-methoxypyrid-2-onimine (*18*) also excludes any significant intramolecular transmethylation. Thus $[M-OH]^+$ is the base peak in the spectrum of (*4*), other characteristic peaks being $[M-O]^+$ 18 % and $[M]^{+\cdot}$ 23 %. In the spectrum from (*22*) the molecular ion $[M]^{+\cdot}$ is the most intense peak, the relative intensities of the $[M-O]^+$ and $[M-OH]^+$ being 10 – 15 %. Similar differences were found in the spectra of the chlorine analogues (*16*, *23*). The gaseous molecules from (*22*) and (*23*) after proton loss are therefore assigned the respective onimine structures (*18*, *19*).

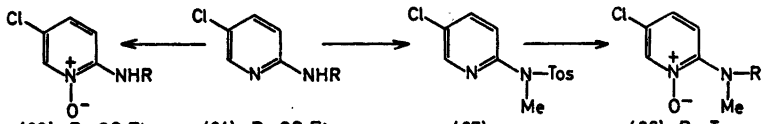
The IP effect of a methyl group relative to that of a hydrogen atom on a phenolic oxygen in simple systems is of the order 0.2 – 0.3 eV.[2,8] Using this value in conjunction with the IP's for (*18*) and (*19*) the IP's for the onimine forms of (*1*) and (*16*) are estimated to about 7.7 eV. The observed values are about 8.0 eV. The results are thus in agreement with the above concluded amino formulation.

From the larger dipole moments it is known that pyridine *N*-oxides are much more strongly polarised than corresponding pyridines.[14,15] High polarisation in the ground state and mesomeric stabilisation from the *N*-oxide group after electron removal are expected to be important factors in the observed decrease in IP for *N*-oxides relative to the corresponding pyridines.[3]

Characteristic primary fragments for the *N*-oxides and base peaks are recorded in Table 3. The mass spectra of heteroaromatic *N*-oxides are characterised by either the $[M-O]^+$ or $[M-OH]^+$ fragment, the latter being the more important when a hydrogen is readily extractable from an α-substituent.[16,17] All *N*-oxides show the $[M-O]^+$ fragment. The 2-isomers are of special interest for the study of the $[M-OH]^+$ fragment. In the 2-amino (*1*) the molecular ion is the base peak. The 2-

Table 2. Characteristic fragment intensities in the spectra of pyridine 1-oxides.

Substituents	$[M]^{+\cdot}$	% I	$[M-O]^{+}$	% I	$[M-OH]^{+}$	% I	$[M-Me]^{+}$	% I	$[M-CH_2CO]^{+\cdot}$	% I	$[M-Ac]^{+}$	% I	Base peak *m/e*
2-NH_2	110	*100*	94	26	93	11	–	–	–	–	–	–	
3-NH_2	»	88	»	27	»	10	–	–	–	–	–	–	54
4-NH_2	»	*100*	»	23	»	10	–	–	–	–	–	–	
2-NHMe	124	23	108	18	107	*100*	108	2	–	–	–	–	
3-NHMe	»	*100*	»	26	»	38	»	2	–	–	–	–	
4-NHMe	»	*100*	»	15	»	30	»	9	–	–	–	–	
2-NMe_2	138	8	122	20	121	*100*	123	7	–	–	–	–	
3-NMe_2	»	98	»	16	»	*100*	»	2	–	–	–	–	
4-NMe_2	»	*100*	»	33	»	38	»	5	–	–	–	–	
2-NHAc	152	25	136	9	135	–	137	5	110	*100*	109	–	110
3-NHAc	»	38	»	4	»	–	»	2	»	46	»	1	43
4-NHAc	»	9	»	45	»	–	»	4	»	24	»	2	94
2-NMeAc	166	7	150	5	149	–	151	–	124	33	123	1	107
3-NMeAc	»	*100*	»	16	»	–	»	2	»	36	»	61	
4-NMeAc	»	31	»	21	»	–	»	2	»	90	»	21	43
2-NH_2-5-Cl	144	*100*	128	69	127	7	–	–					
2-NHMe-5-Cl	158	30	142	45	141	*100*	143	3					



Scheme 3.

methylamino and 2-dimethylamino derivatives (*4,7*), however, have $[M-OH]^+$ as base peak, and the molecular ion has relatively low intensity. This suggests that $[M-OH]^+$ is preferentially formed by hydrogen abstraction from the carbon of the *N*-methyl group rather than from the electronegative amino nitrogen atom.

The primary fragmentation of the acetyl derivatives are characterised by the ready cleavage of the acetyl group as such or by expulsion as ketene preceeded or followed by *N*-oxide fragmentations.

The new compounds used in these studies were synthesised by modifications of established routes. The flow sheet for the 5-chloro derivatives is shown in Scheme 3. Amino groups must be deactivated by an acyl group for selective peroxide oxidation of the pyridine nitrogen atom such as in the carbamate (*24*). The acyl group is hydrolytically removed after oxidation. Methylation of the amino group in the carbamate (*24*) was unsatisfactory but proceeded readily in the sulfonamide (*25*) to form (*27*) which was oxidised and hydrolysed to (*17*).

EXPERIMENTAL

The mass spectra were recorded on an AEI MS902 mass spectrometer attached to an AEI DS30 data system. The electron energy was 70 eV and the ionising current 100 μA. During recording of the ionisation efficiency curves, repeller was at cage potential and the ionising current was 20 μA. Xenon was the reference compound. The IE-curves were interpreted by the semilog-plot method. The recorded IP values are the average of three determinations, the deviation being ± 0.05 eV. The compounds were introduced by the heated direct insertion probe at a source temperature of 220 °C.

Syntheses

2-Amino- (1) [18] and *3-aminopyridine 1-oxide (2)* [12,19] were synthesised according to literature.

4-Aminopyridine 1-oxide (3). 4-Nitropyridine 1-oxide (7.0 g, 0.05 mol) in ethanol (150 ml) was hydrogenated at 0.38 MPa* over 10 % palladium on charcoal for 1 – 2 h (until the uptake of hydrogen had almost ceased). The catalyst was then removed, the solution evaporated and the residual amine acetylated by refluxing for 1 h in a solution of acetic anhydride (3 ml) and ethyl methyl ketone (15 ml). The solid which crystallised from the cold solution was recrystallised from ethyl acetate-ethanol (1:1). 4-Acetamidopyridine 1-oxide (*12*) was thus obtained as needles, m.p. 264 – 265 °C (Lit. 260 – 261 °C).[12]

(*12*) thus obtained (1.5 g, 0.01 mol) was heated in 3 M HCl (12 ml) for 3 h, the reaction mixture evaporated and the residual title compound as HCl-salt crystallised from ethanol; yield 0.9 g (84 %), m.p. 181 – 183 °C.[20]

2-Methylamino- (4) [18] and *4-methylamino-pyridine 1-oxide (6)* [11] were synthesised according to literature.

3-Methylaminopyridine 1-oxides (5). 3-Acetylmethylaminopyridine 1-oxide (1.7 g, 0.01 mol) was dissolved in 3 M HCl (15 ml) and the solution refluxed for 4 h. Evaporation left the title compound as HCl-salt which was recrystallised twice from ethanol/ether; yield 1.2 g (74 %), m.p. 165 °C (Found: C 44.92; H 5.68. Calc. for $C_8H_{10}N_2O_2$: C 45.00; H 5.62).

2-Dimethylamino- (7) [18] and *4-dimethylaminopyridine 1-oxide (9)* [11] were prepared according to literature.

3-Dimethylaminopyridine 1-oxide (8). 3-Bromopyridine 1-oxide[21] (1.8 g, 0.01 mol) and 30 % aqueous dimethylamine (15 ml) were heated together at 155 °C for 48 h in a sealed glass tube. Potassium carbonate (1 g) was added to the solution before evaporation; the dried residue was extracted with ethanol, the ethanol solution heated with charcoal before evaporation of the filtrate. The residual material (1.1 g, 80 %) was crystallised from ethanol/ether in needles, m.p. 41 °C. (Found: C 60.85; H 7.51. Calc. for $C_7H_{10}N_2O$; C 60.86; H 7.24).

2-Acetamido- (10) [12] *3-acetamidopyridine 1-oxide (11)* [4,19] were prepared according to literature; the synthesis of *4-acetamidopyridine 1-oxide (12)* is described under (*3*) above.

2-Acetylmethylamino- (13) [18] and *4-acetylmethylaminopyridine 1-oxide (15)* [12] were prepared according to literature.

3-Acetylmethylaminopyridine 1-oxide (14). 3-Acetylmethylaminopyridine[22] (1.5 g, 0.01 mol) and 35 % hydrogen peroxide (1.8 ml) in acetic acid (6 ml) were heated at 70 – 80 °C for 24 h. The solution was then evaporated at reduced pressure, the residue dissolved in chloroform (20 ml) and treated with anhydrous potassium carbonate. After filtration and evaporation the

* 1 MPa ≈ 10.2 kg/cm².

residual material (1.5 g, 90 %) was recrystallised from ethanol/ethyl acetate, m.p. 163 – 165 °C. (Found: C 58.15; H 5.85. Calc for $C_8H_{10}N_2O_2$: C 57.83; H 6.02).

2-Amino-1-methoxypyridinium perchlorate (22) was prepared from its toluene-*p*-sulfonate.[18]

2-Amino-5-chloro-1-methoxypyridinium perchlorate (23). 2-Amino-5-chloropyridine 1-oxide (7.2 g, 0.05 mol) and methyl toluene-*p*-sulfonate (9.3 g, 0.05 mol) were heated together at 100 °C overnight to produce 1-methoxy-pyridinium toluene-*p*-sulfonate which after crystallisation from ethanol/ethyl acetate or ethanol/ether had m.p. 137 °C; yield 14.2 g (86 %).

The tosylate thus prepared (1.7 g, 0.005 mol) in ethanol (3 ml) was mixed with 60 % perchloric acid (0.8 ml) when the desired perchlorate was precipitated (1.1 g, 85 %); needles from ethanol, m.p. 100 °C (Found: C 28.12; H 3.10. Calc. for $C_6H_8Cl_2N_2O_6$: C 27.80; H 3.12). The 1-trideuteriomethoxypyridinium analogues (*22d*) and (*23d*) were prepared as above by *O*-methylation of the respective *N*-oxides using trideuteriomethyl toluene-*p*-sulfonate as the alkylating agent. Exchange of anion was again carried out in ethanolic solution by addition of perchloric acid.

4-Acetylamino-1-methoxypyridinium perchlorate (20) [12] was prepared by the above procedures.

2-Ethoxycarbonylamino-5-chloropyridine 1-oxide (24). Ethyl chloroformate (14 ml) was added dropwise to a stirred and cooled (0 °C) solution of 2-amino-5-chloropyridine (15.5 g) in pyridine (60 ml) and the solution left at room temperature. Water was added after 18 h and the precipitated material (68 %) crystallised from ethanol, m.p. 178 °C.

A solution of 2-ethoxycarbonylamino-5-chloropyridine thus obtained (20.1 g, 0.1 mol) and 30 % hydrogen peroxide (15 ml) in acetic acid (32 ml) was heated at 70 – 80 °C for 24 h. The solution was then evaporated at reduced pressure, the residue dissolved in chloroform (150 ml) and the solution heated and shaken with anhydrous potassium carbonate (10 g) for 10 min. Filtration and evaporation of the filtrate left the crude 1-oxide which crystallised in needles from ethanol; yield 21 g (97 %). (Found: C 44.48; H 4.18. Calc. for $C_8H_9ClN_2O_3$: C 44.34; H 4.15).

2-Amino-5-chloropyridine 1-oxide (16). 2-Ethoxycarbonylamino-5-chloropyridine 1-oxide (10.0 g, 0.05 mol) was heated under reflux in conc. HCl (20 ml) for 24 h. Evaporation and crystallisation of the residual material from ethanol furnished the title compound as HCl salt; yield 5.7 g (71 %), m.p. 163 °C. (Found: C 33.15; H 3.31. Calc. for $C_5H_5ClN_2O.HCl$: C 33.21; H 3.63).

The title compound was generated from its salt by addition of one equivalent of sodium ethoxide solution to a solution of the HCl-salt as above prepared. Removal of the salt, evaporation of the ethanol solution and recrystallisation of the residue from ethyl acetate/ethanol furnished the title compound, m.p. 193 °C. (Found: C 41.68; H 3.67. Calc. for $C_5H_5ClN_2O$: C 41.52; H 3.46).

5-Chloro-2-p-toluenesulfonamidopyridine (25). A solution of 2-amino-5-chloropyridine (12.9 g, 0.1 mol) and *p*-toluenesulfonyl chloride (22.5 g, 0.15 mol) in pyridine (25 ml) was heated on a steam-bath for 5 h. The precipitate formed on pouring the solution into water was recrystallised from benzene; m.p. 174 – 175 °C, yield 28.1 g (quantitative). (Found: C 51.07; H 4.29. Calc. for $C_{12}H_{11}ClN_2O_2S$: C 50.95; H 3.89).

5-Chloro-2-N-methyl-p-toluenesulfonamidopyridine (27). A solution of dimethyl sulfate (12.6 g, 0.1 mol) in acetone (50 ml) was added dropwise over 30 min to a refluxing mixture of 5-chloro-2-*p*-toluenesulfonamidopyridine (28.2 g, 0.1 mol) and anhydrous potassium carbonate (28.0 g, 0.2 mol) in acetone (500 ml). The heating was continued for another 3 h. The cold reaction mixture was then filtered and the filtrate evaporated. The oily product was next dissolved in chloroform (150 ml), the solution extracted with 10 % NaOH solution to remove any material not alkylated, and the organic solution washed and dried. Evaporation and recrystallisation from ethanol gave colourless prisms; m.p. 68 – 70 °C, yield 26.0 g (88 %). (Found: C 52.82; H 4.84. Calc. for $C_{13}H_{13}ClN_2O_2S$: C 52.61; H 4.38).

5-Chloro-2-methylaminopyridine 1-oxide (17). 5-Chloro-2-*N*-methyl-*p*-sulfonamidopyridine (14.8 g, 0.05 mol) and 30 % hydrogen peroxide (8 ml) in acetic acid (30 ml) were heated at 70 – 80 °C for 24 h. The solution was then evaporated and the residual oxide (*28*) subjected to hydrolysis by heating on a steam bath for 3 h in 80 % sulfuric acid (25 ml). The cold solution was diluted with water (20 ml) and made alkaline with 0.91 M ammonia before extraction with chloroform. The chloroform solution was washed, dried and evaporated and the residue crystallised from ethanol/ether; yield 6.4 g (81 %), m.p. 153 °C. (Found: C 45.64; H 4.47. Calc. for $C_6H_7ClN_2O$: C 45.42; H 4.41).

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The Crystal and Molecular Structure of 3-Hydroxyphenylalanine (*m*-Tyrosine)

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The crystal structure of 3-hydroxyphenylalanine has been determined by X-ray diffraction methods using 1690 observed reflections collected on a counter diffractometer. The crystals are monoclinic, space group $P2_1$, with cell dimensions $a = 5.95_6$ Å; $b = 5.29_9$ Å; $c = 13.86_6$ Å; $\beta = 104.3_6°$. The structure was refined to a conventional R-factor of 0.041, the standard deviations for bond lengths and angles involving non-hydrogen atoms are 0.002 Å and 0.1°, respectively.

The compound forms crystals each containing one enantiomer from solutions of the racemate. Both regarding molecular geometry and crystal packing of *m*-tyrosine the similarity to L-DOPA is pronounced.

3-Hydroxyphenylalanine (*m*-tyrosine) is a natural product occurring in certain plants [1] and also in mammals where the metabolic pathway from phenylalanine to L-DOPA partly may proceed through L-*m*-tyrosine.[2,3] The shift of the hydroxyl group from the 4-position in tyrosine to the 3-position in *m*-tyrosine gives rise to altered chemical as well as biological properties. L-*m*-Tyrosine behaves thus in some cases more like phenylalanine than like L-tyrosine [4] and it has been reported to act as a growth inhibitor.[5] Several of the biological activities of L-*m*-tyrosine have been shown to be analogous to those of L-DOPA [6,7] and the compound or its metabolically formed amines seem to interfere with the normal action of the neurotransmitters dopamine and noradrenaline.[8–10]

We have examined the crystal structure of 3-hydroxyphenylalanine as a part of a series of hydroxyphenyl- and pyridonalanines in order to study the variation in molecular conformation with varying crystal environment and to collect information useful for studies of relationships between structure and biological activity.

EXPERIMENTAL

A concentrated solution of oxalic acid in cold water was heated with an excess of 3-hydroxyphenylalanine and filtered. The solution was cooled down to room temperature and after a couple of weeks crystals of the pure compound appeared.

Oscillation and Weissenberg photographs showed the crystals to be monoclinic. The only condition for the presence of reflections was k even for ($0k0$). The number of molecules per unit cell being *two*, the only space group possible is $P2_1$ since $P2_1/m$ would require the molecule to exhibit mirror- or centrosymmetry.

Unit cell dimensions were calculated from diffractometer measurements of 28 reflections using CuKβ-radiation ($\lambda = 1.3922$ Å). The intensity data were recorded on an automatic Picker diffractometer using MoK-radiation monochromated by means of a graphite crystal. The specimen had approximate dimensions $0.2 \times 0.2 \times 0.5$ mm. Reflections with $\sin\theta/\lambda < 0.8$ were scanned in the $\theta - 2\theta$ mode at a rate of 1° min^{-1} in 2θ; the scan range was from 0.8° below $2\theta(\alpha_1)$ to 0.8° above $2\theta(\alpha_2)$. The background was measured for 30 s at each of the scan range limits. Three standard reflections were measured after every 100 reflections; they showed no significant change in intensity.

A total of 2010 independent reflections were measured of which 1690 were considered observed with intensities greater than 2.0 standard deviations. The standard deviations for the intensities were taken as $\sigma(I) = (c_T + (0.02\ c_N)^2)^{\frac{1}{2}}$ where c_T is the total number of counts and c_N the scan count minus background count. The data were corrected with Lorentz and polarization factors, but not for absorption ($\mu = 0.12$ mm^{-1}).

Atomic form factors used were those of Hanson *et al.*[11] for oxygen, nitrogen and carbon and of Stewart *et al.*[12] for hydrogen. Calculations during the structure investigation were performed by means of computer programs described in Ref. 13. The full-matrix least-squares refinement program minimizes $\sum w(\Delta F)^2$ where $w = \sigma^{-2}(F_o)$.

Table 1. Observed and calculated structure factors. The columns are h, k, l, $10 \times F_o$ and $10 \times F_c$.

Table 1. Continued.

Table 2. Fractional atomic coordinates and thermal parameters with estimated standard deviations ($\times 10^5$) The temperature factor is given by $\exp -(B_{11}h^2+B_{22}k^2+B_{33}l^2+B_{12}hk+B_{13}hl+B_{23}kl)$.

Atom	x	y	z	B_{11}	B_{22}	B_{33}	B_{12}	B_{31}	B_{23}
O1	−5262	−18235	33277	1845	2511	492	−1000	902	15
	20		9	32	43	7	60	24	29
O2	68186	44618	961	1943	2841	307	25	846	−45
	19	35	8	29	44	5	63	19	26
O3	91707	57078	15259	1279	4099	475	−1529	833	−793
	18	39	9	26	55	7	66	22	35
N1	30236	45820	81395	996	2616	280	141	464	154
	18	37	8	24	44	5	61	18	29
C1	41770	21794	29090	1338	1813	249	185	414	272
	22	35	9	32	43	7	67	25	31
C2	25070	3046	27666	1504	1828	282	12	490	−14
	24	35	10	32	47	6	64	23	27
C3	11133	191	34404	1311	1865	317	142	477	324
	21	38	10	31	47	7	66	23	31
C4	13811	16531	42402	1853	2565	299	189	666	201
	25	41	10	38	58	7	80	27	35
C5	30309	35378	43720	2380	2707	284	−234	602	−244
	30	43	11	47	61	7	90	29	36
C6	44357	38154	37161	1925	2118	310	−727	468	−42
	27	39	11	40	52	7	77	27	32
C7	57707	24282	22171	1368	1862	326	484	617	300
	22	37	10	32	43	7	67	25	31
C8	53179	47597	15462	1033	1525	259	−96	500	13
	19	36	9	26	38	6	59	20	28
C9	72549	50061	10005	1227	1533	337	66	710	57
	21	36	10	30	41	7	61	23	28

CRYSTAL DATA

3-Hydroxyphenylalanine (*m*-tyrosine), $C_9H_{11}NO_3$, monoclinic, $a=5.956(0.002)$ Å; $b=5.2992(0.0004)$ Å; $c=13.866(0.002)$ Å; $\beta=104.36(0.02)°$, ($t=18$ °C). $V=424.0$ Å^3; $M=181.19$; $F(000)=192$; $Z=2$; $D_{obs}=1.42$ g cm^{-3} (flotation); $D_{calc}=1.419$ g cm^{-3}. Absent reflections: ($0k0$) for k odd; space group $P2_1$.

STRUCTURE DETERMINATION

The positions of the phenyl ring atoms and the carbon and oxygen atoms directly attached to it were deduced from a sharpened Patterson map. The remaining non-hydrogen atoms were located from subsequent Fourier syntheses. Two cycles of full-matrix least-squares refinement with isotropic temperature factors reduced R to 0.15; further refinement with anisotropic temperature factors brought R down to 0.07. The hydrogen atoms could all be located from a difference Fourier map at this stage. The refinement of all positional parameters (except for one origin defining y-parameter), anisotropic

Table 3. Fractional atomic coordinates ($\times 10^4$) and B-values with estimated standard deviations for hydrogen atoms.

Atom	x	y	z	B
HO1	−505	−2660	2830	2.7
	29	42	12	0.4
HN1	2900	3141	495	2.3
	29	38	12	0.3
HN2	2825	5774	329	2.9
	33	47	15	0.4
HN3	2044	4784	1114	3.3
	37	49	15	0.4
H2	2306	−748	2203	2.3
	29	36	12	0.4
H4	470	1407	4711	3.6
	33	44	13	0.5
H5	3207	4797	4934	3.0
	35	46	13	0.4
H6	5470	4958	3783	4.1
	35	52	15	0.5
H71	7337	2511	2599	3.3
	33	47	13	0.4
H72	5519	987	1762	2.8
	30	43	13	0.4
H8	5329	6229	1934	1.3
	25	36	11	0.3

Table 4. Interatomic distances (Å) and bond angles (°).

Bond	Length	Corrected	Bond angles		Bond angles	
C1–C2	1.385	1.389	C1–C6–C5	119.7	C4–C5–H5	121
C2–C3	1.403	1.406	C6–C5–C4	121.0	C5–C4–H4	121
C3–C4	1.385	1.388	C5–C4–C3	119.7	C3–C4–H4	119
C4–C5	1.381	1.385	C4–C3–C2	119.7	C3–O1–HO1	79
C5–C6	1.388	1.388	C3–C2–C1	120.3	C3–C2–H2	121
C1–C6	1.394	1.399	C2–C1–C6	119.5	C1–C2–H2	119
C1–C7	1.513	1.515	C6–C1–C7	119.5	C1–C7–H71	109
C7–C8	1.530		C2–C1–C7	120.9	C1–C7–H72	109
C8–N1	1.490	1.498	C1–C7–C8	114.3	C8–C7–H71	108
C8–C9	1.534	1.538	C7–C8–C9	109.1	C8–C7–H72	105
C9–O2	1.250	1.257	C4–C3–O1	118.5	H71–C7–H72	111
C9–O3	1.248	1.255	O1–C3–C2	121.8	C7–C8–H8	110
C3–O1	1.362	1.363	C8–C9–O2	118.7	N1–C8–H8	108
C6–H6	0.85		C8–C9–O3	115.3	C9–C8–H8	108
C5–H3	1.01		O2–C9–O3	125.9	C8–N1–HN1	110
C4–H4	0.96		C7–C8–N1	110.9	C8–N1–HN2	113
C2–H2	0.94		N1–C8–C9	110.1	C8–N1–HN3	108
O1–HO1	0.82		C1–C6–H6	118	HN1–N1–HN2	105
N1–HN1	0.91		C5–C6–H6	123	HN1–N1–HN3	113
N1–HN2	0.88		C6–C5–H5	118	HN2–N1–HN3	108
N1–HN3	0.80					
C7–H71	0.95					
C7–H72	0.98					
C8–H8	0.95					

Hydrogen bonds	D···A	H···A	D–H	D–H···A
N1–HN1···O2 ($1-x,-\frac{1}{2}+y,-z$)	3.004	2.14	0.91	170
N1–HN2···O2 ($1-x,\frac{1}{2}+y,-z$)	2.889	2.07	0.88	150
N1–HN3···O3 ($-1+x,y,z$)	2.779	1.99	0.80	165
O1–HO1···O3 ($1-1+x,-1+y,z$)	2.786	1.97	0.82	171

thermal parameters for the non-hydrogen atoms and isotropic parameters for the hydrogen atoms was carried on until the shifts were negligible compared to the standard deviations. The final R-factor is 0.041 for 1690 reflections and R_w is 0.042. A difference Fourier synthesis showed electron densities between -0.2 eÅ^{-3} and $+0.3$ eÅ^{-3}.

The anisotropic thermal parameters were analysed in terms of rigid-body motion both for the whole molecule and for the hydroxyphenyl and alanine parts separately. The results indicated that the whole molecule does not behave as a rigid body whereas splitting the molecule into the two parts gave much better results. The latter description was adapted when correcting bond lengths for libration effects.

A comparison of observed and calculated structure factors is given in Table 1; final parameters for non-hydrogen atoms are listed in Table 2 and for hydrogen atoms in Table 3. Interatomic distances and bond angles are given in Table 4. Standard deviations were calculated from the correlation matrix; standard deviations in distances were found to be 0.002 Å and in angles 0.1° when hydrogen atoms were not involved.

DISCUSSION

Bond lengths and angles are shown in Fig. 1 in which the numbering of the atoms is indicated.

The molecular geometry is remarkably similar to that of 3,4-dihydroxyphenylalanine (DOPA).[14] Neglecting the extra hydroxyl group in DOPA, none of the bond lengths differs by more than 0.01 Å, nor the bond angles by more than 0.7° except for the external angles at C3. In the present structure the external angles are found to be 118.5° and 121.8° with the greater angle

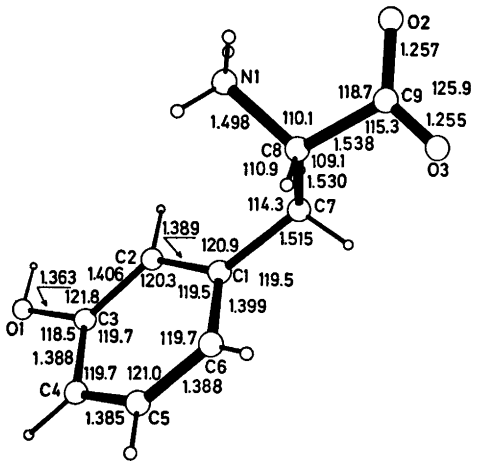

Fig. 1. Bond lengths (Å, corrected) and angles (°) in *m*-tyrosine.

cis to the phenol hydrogen atom which is situated in the phenyl ring plane. This feature is common for many phenol structures; the difference between the external angles in the present structure, 3,3° may be compared to that in L-tyrosine of 4.6°,[15] in DL-tyrosine of 4.4°,[16] and those in L-DOPA of 6.3 and 6.6°.

The similarity between *m*-tyrosine and DOPA applies also to the conformation of the molecules in the crystal. The following dihedral angles (°) were found:

	m-tyrosine	L-DOPA
C2 – C1 – C7 – C8	– 71.6	– 71.2
C1 – C7 – C8 – C9	171.6	175.2
N1 – C8 – C9 – O (*cis* to N)	– 16.0	– 14.4

The conformational angles are given as positive when progress along the atoms describes a clock-wise rotation.

All hydrogen atoms bonded to hetero atoms are involved in hydrogen bonds. Thus N1 and O1 act as hydrogen donors; the two carboxyl oxygen atoms are acceptors in four hydrogen bonds. In this way each molecule is connected to six neighbouring molecules through eight hydrogen bonds. The situation is illustrated in Fig. 2 which shows the crystal structure as seen along the *c*-axis. The pronounced similarity of the structures of *m*-tyrosine and L-DOPA may be seen from Fig. 3 where details of corresponding projections along *b* are visualized. Except for the *para* hydroxyl group in L-DOPA forming hydrogen bonds to corresponding groups in

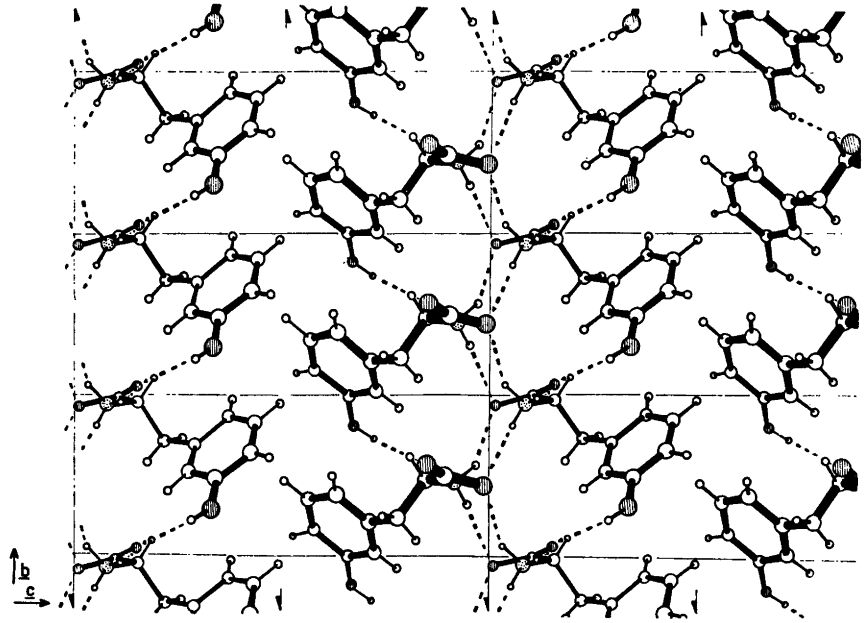

Fig. 2. The crystal structure as seen along the *a*-axis.

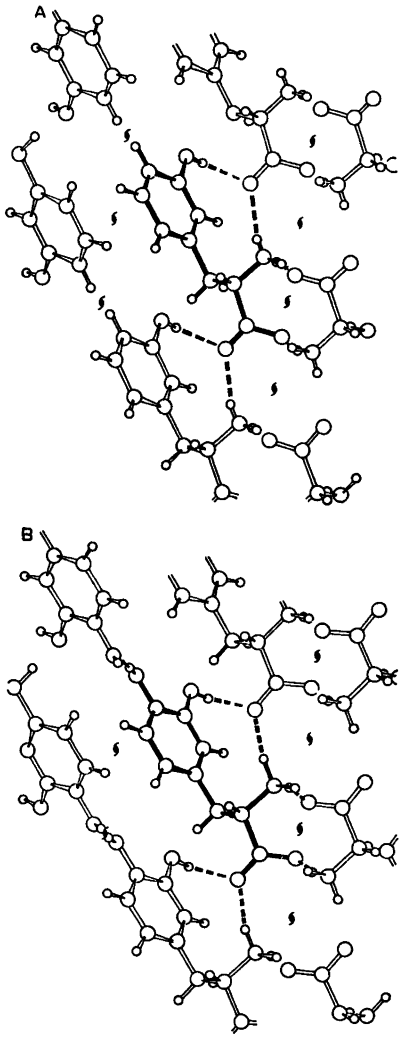

Fig. 3. The structure of *m*-tyrosine (A) and L-DOPA (B) as viewed along *b*.

molecules along a two-fold screw axis, the hydrogen system in the two structures is nearly identical. The N1–H···O3 bond of 2.78 Å (2.82 Å in L-DOPA) establishes chains of molecules along the *a*-axis. These chains are tied together through O1–H···O3 bonds of 2.79 Å (2.74 Å in L-DOPA) and through two N1–H···O2 bonds of 2.89 Å and 3.00 Å (2.87 Å and 3.02 Å in L-DOPA) to form double molecular layers normal to the (001) plane. Between the layers there are only weak van der Waals interactions.

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Aryl Cyanates. Reaction of Aryl Cyanates with Grignard Reagents. Kinetic Studies

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A kinetic investigation of the reactions of aryl cyanates with Grignard reagents and dibutylmagnesium in diethyl ether has been carried out. The reaction is best described as involving a concerted four-center mechanism in the rate-determining step, first order both in aryl cyanate and in the organomagnesium species: alkyl- or arylmagnesium halide and dialkyl- or diarylmagnesium, present in the Grignard reagents.*

For reactions of *m*-substituted phenyl cyanates with "butylmagnesium bromide" a linear correlation is obtained between the logarithm of the rate constants and the Hammett *sigma* values with a ϱ-value of $+0.97$. For reactions of *p*-substituted phenyl cyanates with "butylmagnesium bromide" it has not been possible to obtain a smooth curve when plotting the logarithm of the rate constants *versus* the Hammett *sigma* values.

The reaction constant for the reaction of phenyl cyanate with *p*-substituted "phenylmagnesium bromides" is calculated to be *ca.* -0.80.

"Arylmagnesium bromides" are found to be more reactive than dibutylmagnesium, which again is more reactive than "butylmagnesium bromide".

In another paper[1] we have described the product formation in the reaction between alkyl or aryl cyanates and Grignard reagents, and we now wish to report a kinetic investigation of the reaction between aryl cyanates and Grignard reagents.

The present work has been carried out in order to obtain information about the mechanism of the reaction (I) between aryl cyanates and Grignard reagents, which leads to the formation of aryloxymagnesium halides and nitriles:

$$\mathrm{ArOCN} + \mathrm{RMgX} \longrightarrow \mathrm{ArOMgX} + \mathrm{RCN} \qquad (\mathrm{I})$$

A thermographic method[2a,b] was used for the kinetic measurements, and this technique allowed rate measurements reproducible within ± 5 % by use of a concentration of 0.01 M of the reagent present in lesser amount. All measurements were carried out with one of the reagents in excess.

Fig. 1 shows three examples of plots of $\ln (\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T)$ * *versus* the reaction time, t, for the reaction of phenyl cyanate with "butylmagnesium bromide". From all kinetic measurements linear plots were obtained for at least 70 % transformation of the limiting reagent. The linearity of the plots means that the reaction order in phenyl cyanate is one. The pseudo first order rate constants (k_{obs}) are obtained from the slopes of the lines and found to be linearly dependent on the initial concentration of Grignard reagent (Fig. 2), and therefore the reaction order in "butylmagnesium bromide" is assumed to be one. Because of the association of most Grignard reagents at concentrations above 0.1 M[3] it is surprising that

* The Grignard reagents are in the following symbolized as "RMgX".

* $\mathrm{Ln}\, a/(a-x) = \ln (\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T) = k_{obs} \times t$, where ΔT_∞ is the total rise in temperature, ΔT_1 is the rise in temperature after 2 ms and ΔT is the rise in temperature at the time t. ΔT_1 is a correction for heat evolved from reaction between traces of impurities (water) and the Grignard reagent and from mixing.

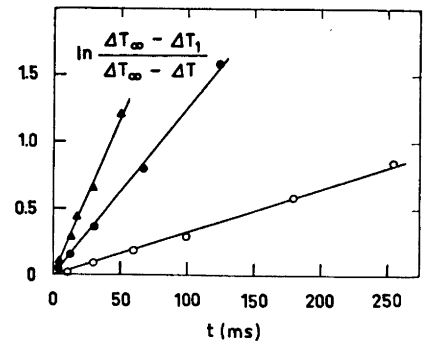


Fig. 1. Plot of $\ln (\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T)$ *versus* the reaction time, t, for the reaction of phenyl cyanate with excess "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{C_6H_5OCN} = 0.010$ M; $c^0_{\text{"}C_4H_9MgBr\text{"}} = 0.103$ M (○), 0.413 M (●) and 0.750 M (▲)).

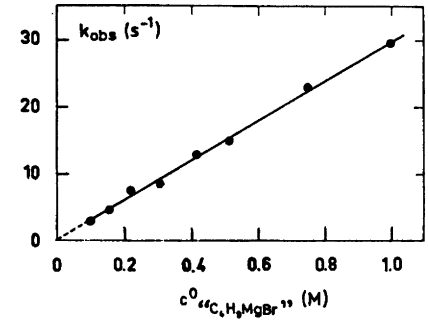


Fig. 2. Plot of the pseudo first order rate constant (k_{obs}) *versus* the initial concentration of the Grignard reagent for the reaction of phenyl cyanate with excess "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{C_6H_5OCN} = 0.010$ M).

the pseudo first order rate constant is linearly dependent on the initial concentration of "butylmagnesium bromide". However, the degree of association of "butylmagnesium bromide" has not been measured. This reagent could be associated to a lesser extent than the lower homologues because the degree of association decreases as the hydrocarbon chain is lengthened.[3] Another possibility is of course that the cyanate also reacts with the dimers (polymers) besides the organomagnesium species (butylmagnesium bromide and dibutylmagnesium) involved in the Schlenk equilibrium (reaction (II)).[4]

$$2\ C_4H_9MgBr \overset{K_s}{\rightleftharpoons} (C_4H_9)_2Mg + MgBr_2 \qquad \text{(II)}$$

In kinetic measurements with a constant excess of "butylmagnesium bromide" the pseudo first order rate constant was found to be increased by 9 % when the initial concentration of phenyl cyanate was doubled (Table 1). An increase is expected due to the temperature dependence of the rate constant. The doubling of the concentration of phenyl cyanate doubles the total rise in temperature. On this

Table 1. Pseudo first order rate constants for the reaction of phenyl cyanate with a constant excess of "butylmagnesium bromide" in diethyl ether at 25 °C.

$c^0_{C_6H_5OCN}$ (M)	$c^0_{\text{"}C_4H_9MgBr\text{"}}$ (M)[a]	k_{obs} (s^{-1})
0.010	0.220	7.6
0.020	0.220	8.3

[a] Sublimed magnesium ("Specpure", Johnson, Matthey Chemicals Ltd.) was used for preparation of the "butylmagnesium bromide" used in these experiments.

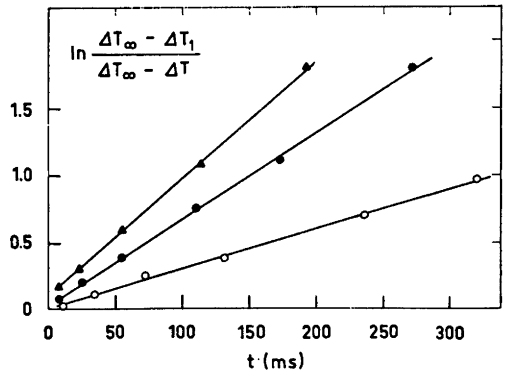


Fig. 3. Plot of $\ln (\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T)$ *versus* the reaction time, t, for the reaction of excess phenyl cyanate with "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{\text{"}C_4H_9MgBr\text{"}} = 0.010$ M; $c^0_{C_6H_5OCN} = 0.100$ M (○), 0.200 M (●) and 0.300 M (▲)).

basis it is difficult to say anything about catalysis by traces of transition metals present in the magnesium used for the preparation of the Grignard reagent.

Kinetic measurements with excess phenyl cyanate and 0.010 M "butylmagnesium bromide" likewise give linear pseudo first order plots (Fig. 3), and the pseudo first order rate constants obtained from the slopes of the curves are again linearly dependent on the initial concentration of the reagent in excess (phenyl cyanate). The k_{obs}-values found with excess phenyl cyanate are almost equal to the k_{obs}-values found in measurements with excess "butylmagnesium bromide" (Table 2).

Table 2. Pseudo first order rate constants for the reaction of phenyl cyanate with "butylmagnesium bromide" in diethyl ether at 25 °C.

$c^0_{C_6H_5OCN}$ (M)	c^0"C_4H_9MgBr" (M)	k_{obs} (s^{-1})
0.010	0.100	2.9
0.100	0.010	2.9
0.010	0.200	6.2
0.200	0.010	6.2
0.010	0.300	9.2
0.300	0.010	8.6

Because of the results reported above the rate of the reaction between phenyl cyanate and "butylmagnesium bromide" can be described by eqn. (1):

$$-d[C_6H_5OCN]/dt = k[C_6H_5OCN]\ ["C_4H_9MgBr"] \quad (1)$$

We have made kinetic measurements on the reaction of phenyl cyanate with excess dibutylmagnesium and found that the k_{obs}-value is a factor of 22 higher than the k_{obs}-value for the reaction between phenyl cyanate and "butylmagnesium bromide" (Tables 2 and 3). The pseudo first order plots are linear for up to 80 % transformation of phenyl cyanate (Fig. 4), and the pseudo first order rate constants are linearly dependent on the initial concentration of dibutylmagnesium (Table 3).

If only the dibutylmagnesium present in "butylmagnesium bromide" reacts with phenyl cyanate the k_{obs}-value for the reaction of 0.010 M phenyl cyanate with 0.100 M "butylmagnesium bromide" can be calculated to be 2.1 s^{-1},* which is 0.8 s^{-1} smaller than the observed value (Table 2). Therefore it seems reasonable to assume that both butylmagnesium bromide and dibutylmagnesium react when "butylmagnesium bromide" is reacting with phenyl cyanate.

Table 3. Pseudo first order rate constants for the reaction of phenyl cyanate with excess dibutylmagnesium in diethyl ether at 25 °C.

$c^0_{C_6H_5OCN}$ (M)	$c^0_{(C_4H_9)_2Mg}$ (M)	k_{obs} (s^{-1})
0.010	0.097	63
0.010	0.194	134

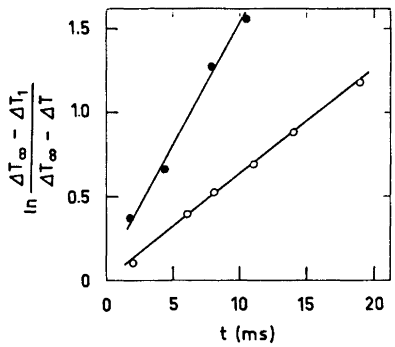


Fig. 4. Plot of $\ln (\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T)$ *versus* the reaction time, t, for the reaction of phenyl cyanate with excess dibutylmagnesium in diethyl ether at 25 °C ($c^0_{C_6H_5OCN} = 0.010$ M; $c^0_{(C_4H_9)_2Mg} = 0.097$ M (○) and 0.194 M (●)).

When magnesium bromide etherate is added to "butylmagnesium bromide" the k_{obs}-value becomes smaller (Table 4). The concentration of dibutylmagnesium decreases according to the Schlenk equilibrium when magnesium bromide is added, and therefore it is reasonable that the pseudo first order rate constant becomes smaller. However, the value is still high, which we suppose is due to the reactivity of butylmagnesium bromide.

* The concentration of dibutylmagnesium in 0.100 M "butylmagnesium bromide" is *ca.* 0.0032 M for $K_s = 1 \times 10^{-3}$ (Ref. 5). The calculation of k_{obs} is made with the assumption that k_{obs} is linearly dependent on the initial concentration of dibutylmagnesium in the interval 0–0.1 M, thus $k_{obs} = 63 \times 0.0032/0.097 = 2.1$ s^{-1}.

Table 4. Pseudo first order rate constants for the reaction of phenyl cyanate with excess "butylmagnesium bromide" in diethyl ether at 25 °C.

$c^0_{C_6H_5OCN}$ (M)	c^0"C_4H_9MgBr" (M)	$c^0_{MgBr_2}$ (M)	k_{obs} (s^{-1})
0.010	0.138	0.003	5.7
0.010	0.138	0.069	4.4

On basis of the results described above the rate expression (eqn. (1)) should consequently be:

$$-d[C_6H_5OCN]/dt = (k_2[C_4H_9MgBr] + k_2'[(C_4H_9)_2Mg])[C_6H_5OCN] \quad (2)$$

where k_2 and k_2' are the second order rate constants for the reaction of phenyl cyanate with butylmagnesium bromide and dibutylmagnesium, respectively. k_2' is calculated from the pseudo first order rate constant and the corresponding concentration of dibutylmagnesium (Table 3), $k_{obs} = k_2' \times c^0_{(C_4H_9)_2Mg}$, $k_2' = 650\ M^{-1}s^{-1}$. The second order rate constant (k_2) for the reaction of phenyl cyanate with butylmagnesium bromide can be calculated from the following equation:

$$k_{obs} = k_2 \times c^0_{C_4H_9MgBr} + k_2' \times c^0_{(C_4H_9)_2Mg} \quad (3)$$

For c^0"C_4H_9MgBr" = 0.100 M, $c^0_{C_4H_9MgBr} = 0.0936$ M, $c^0_{(C_4H_9)_2Mg} = 0.0032$ M, and $k_{obs} = 2.9\ s^{-1}$, which gives $k_2 = 8.6\ M^{-1}\ s^{-1}$.

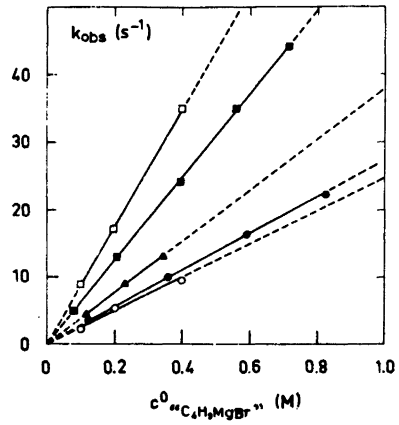


Fig. 5. Plot of pseudo first order rate constants *versus* the initial concentrations of the Grignard reagent for the reactions of *m*-substituted phenyl cyanates with excess "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{m\text{-}XC_6H_4OCN} = 0.010$ M; *m*-$(CH_3)_2NC_6H_4OCN$ (○); *m*-$CH_3C_6H_4OCN$ (●); *m*-$CH_3OC_6H_4OCN$ (▲); *m*-ClC_6H_4OCN (■); *m*-$CF_3C_6H_4OCN$ (□)).

Table 5. Pseudo first order rate constants for the reaction of substituted phenyl cyanates with "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{ArOCN} = 0.010$ M and c^0"C_4H_9MgBr" = 0.100 M).

X-C_6H_4OCN	k_{obs}(s^{-1})	log k/k_0	σ^0 [a]
C_6H_5OCN	2.9	0	0
m-$(CH_3)_2NC_6H_4OCN$	2.5	−0.07	−0.15[b]
m-$CH_3C_6H_4OCN$	2.8	−0.02	−0.07
m-$CH_3OC_6H_4OCN$	3.8	0.12	0.04
m-ClC_6H_4OCN	6.2	0.33	0.38
m-$CF_3C_6H_4OCN$	8.7	0.48	0.47
p-$CH_3C_6H_4OCN$	3.0	0.01	−0.125
p-$(CH_3)_2CHOC_6H_4OCN$	3.8	0.12	−0.45[c]
p-$CH_3OC_6H_4OCN$	4.8	0.22	−0.16
p-ClC_6H_4OCN	7.5	0.41	0.25
2,6-$(CH_3)_2C_6H_3OCN$	9.1	–	–

[a] σ^0-values from Ref. 6 unless another means is indicated. [b] This value is from Ref. 7. [c] This value is from Ref. 8.

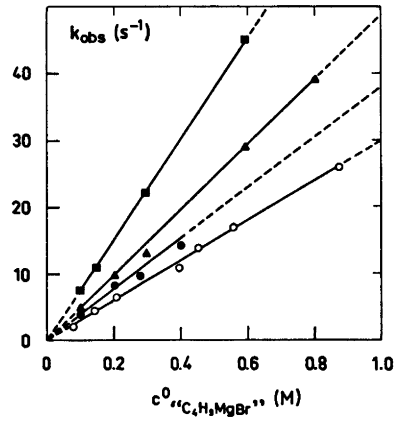


Fig. 6. Plot of pseudo first order rate constants *versus* the initial concentrations of the Grignard reagent for the reactions of *p*-substituted phenyl cyanates with excess "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{p\text{-}XC_6H_4OCN}$ = 0.010 M; *p*-$CH_3C_6H_4OCN$ (○); *p*-$(CH_3)_2CHOC_6H_4OCN$ (●); *p*-$CH_3OC_6H_4OCN$ (▲); *p*-ClC_6H_4OCN (■)).

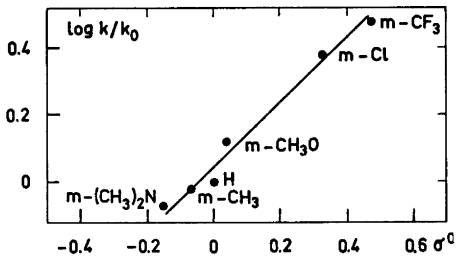


Fig. 7. Plot of log k/k_0 *versus* σ^0 for the reaction between *m*-substituted phenyl cyanates and "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{m\text{-}XC_6H_4OCN}$ = 0.010 M and c^0"C_4H_9MgBr" = 0.100 M).

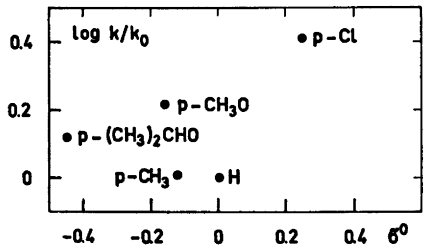


Fig. 8. Plot of log k/k_0 *versus* σ^0 for the reaction between *p*-substituted phenyl cyanates and "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{p\text{-}XC_6H_4OCN}$ = 0.010 M and c^0"C_4H_9MgBr" = 0.100 M).

Kinetic measurements have been carried out on reactions between *m*- and *p*-substituted phenyl cyanates and excess "butylmagnesium bromide". Pseudo first order plots were in all cases linear until 70 % transformation of the substituted cyanates, and the pseudo first order rate constants obtained from these plots were linearly dependent on the initial concentration of "butylmagnesium bromide" (Figs. 5 and 6).

The logarithm of the pseudo first order rate constants for the reactions of *m*-substituted phenyl cyanates with "butylmagnesium bromide" could be linearly correlated with the Hammett *sigma* values (Table 5 and Fig. 7). The slope of the line is positive (ϱ = +0.97). The logarithm of the pseudo first order rate constants found in reactions of *p*-substituted phenyl cyanates with "butylmagnesium bromide" could not be linearly correlated with the Hammett *sigma* values (Table 5 and Fig. 8). The rate constants are increased both when the substituent is electron-attracting (Cl) and when the substituent is electron-donating (CH_3, $(CH_3)_2CHO$ and CH_3O), and the points could not be connected by a smooth curve. It thus seems hazardous on this basis to draw any detailed mechanistic conclusions. However, the increased rate observed in reactions with *p*-substituted phenyl cyanates is in itself an indication of a change either in reaction mechanism or in the transition state.

The most reactive of the aromatic cyanates we have investigated is 2,6-dimethylphenyl cyanate (Table 5). The pseudo first order plots obtained in kinetic measurements with excess "butylmagnesium bromide" are linear up to 70 % transformation of 2,6-dimethylphenyl cyanate, and the pseudo first order rate constant is linearly dependent on the initial concentration of "butylmagnesium bromide" (Fig. 9). The apparent steric acceleration may be due to restricted rotation of the cyanate group with resulting decrease of conjugation with the phenyl group. Thus the inductive effect of the phenyl group becomes more pronounced, causing a reaction rate acceleration in accord with the observed positive sign of the reaction constant.

Aromatic Grignard reagents were found to be much more reactive towards phenyl cyanate than "butylmagnesium bromide" and more

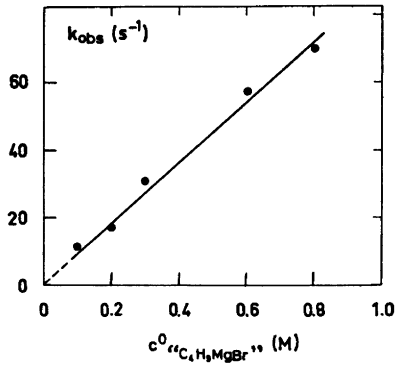
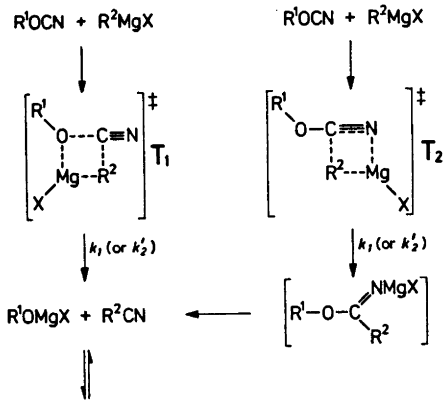


Fig. 9. Plot of pseudo first order rate constants *versus* the initial concentrations of the Grignard reagent for the reaction of 2,6-dimethylphenyl cyanate with "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{2,6\text{-}(CH_3)_2C_6H_3OCN} = 0.010$ M).

reactive even than dibutylmagnesium (Table 6). When the aromatic Grignard reagent is substituted in the *para*-position an electron-donating substituent (CH_3) makes the Grignard reagent more reactive towards phenyl cyanate and an electron-attracting substituent (Cl) reduces the reactivity of the Grignard reagent. From the k_{obs}-values and the Hammett *sigma* values a reaction constant (ϱ) of -0.8 can be calculated.



Scheme 1.

Table 6. Pseudo first order rate constants for the reaction of 0.010 M phenyl cyanate with different Grignard reagents (c^0"$_{RMgX}$" = 0.100 M) and dibutylmagnesium ($c^0_{R_2Mg} = 0.100$ M) in diethyl ether at 25 °C.

"RMgX" or R_2Mg	k_{obs} (s^{-1})
"*p*-$CH_3C_6H_4MgBr$"	248
"C_6H_5MgBr"	192
"*p*-ClC_6H_4MgBr"	85
$(C_4H_9)_2Mg$	65
"C_4H_9MgBr"	2.9
"*t*-C_4H_9MgCl"	<2.9

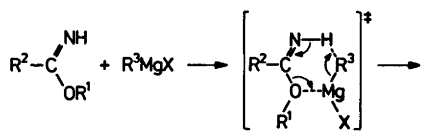
A radical mechanism for the reaction between aryl cyanates and Grignard reagents can probably be excluded. First of all the formation of only a few products in high yields does not indicate the presence of free radicals. In reactions of Grignard reagents where free radicals are formed several products are observed, as for example in the case of the reaction of "*t*-butylmagnesium chloride" with benzophenones.[9] In the reactions of benzophenone with different Grignard reagents "*t*-butylmagnesium chloride" also exhibited the highest measured reaction rate.[10] In the present case "*t*-butylmagnesium chloride" on the contrary has the smallest reaction rate. Furthermore, despite a number of experiments using flow technique we have not observed radicals in ESR measurements.

As shown above the reaction order for the reaction between aryl cyanates and Grignard reagents is two, first order in both reactants. For *m*-substituted aryl cyanates k_{obs}-values are linearly dependent on the initial concentration of the reagent in excess, signifying lack of complex formation prior to the rate-determining step. In the rate-determining step two alternative pathways have to be considered. *1.* Attachment of magnesium directly to the oxygen atom synchronously with nitrile formation. *2.* Attachment of magnesium to nitrogen followed by a further reaction, to give products (Scheme 1).

In case *1* the positive sign of the reaction constant, $\varrho_{ArOCN} = +0.97$, is in agreement with development of a negative charge on oxygen, as will be the case if phenolate is formed. Similarly the negative sign of the reaction

constant $\varrho_{ArMgX} = -0.80$ fulfills the electronic requirements for the formation of the bond between the nitrile group and the Grignard reagent. The size of the constants here observed will, however, normally be associated with a transition state of low polarity. Comparison with reaction constants for reactions of Grignard reagents where the mechanism is known has not been possible. The low polarity of the transition state seems to be evidence against reaction path *1*, but it is, however, hardly conclusive since a bond is formed to magnesium concurrently with breaking of the oxygen-carbon bond. This reduces the polarity of the transition state since the bond between oxygen and magnesium has some covalent character (27 %),[11] but the extent of this effect is uncertain.

The second pathway implies formation of an imido ester salt, for which no evidence has been brought to light despite carefull scrutiny.[1] Still this intermediate cannot be excluded on these grounds since it can be interpreted to mean that the rate constant for the decomposition of the imido ester salt is much bigger than the rate constant for the formation of the imido ester salt. Magnesium salts of simple imido esters are unknown in general, but a few examples of isolation of imido esters, where apparently the salts are intermediates, are reported when a strongly electron-attracting group is present in the molecule. Thus an imido ester can be obtained from the reaction between sodium malonodinitrile and phenyl cyanate,[12] and the reaction of ethyl cyanate with hydrogen cyanide in pyridine yields the corresponding imido ester.[13] The reaction of nitriles with an alcohol in the presence of alcoholate likewise yields imido esters.[14–16] These observations may be considered as an indication of the intermediacy of an imido ester also in the case of the reaction with Grignard reagents. On first consideration this gains support from the fact that in the reaction between imido esters and Grignard reagents, which similarly leads to alcoholates and nitriles, imido esters cannot be regenerated after mixing of the reactants even when using the flow technique.[17] Some caution, however, is necessary in the evaluation of this result, since it may well be that the abstraction of the proton from the imido ester by the Grignard reagent proceeds by a cyclic concerted reaction not leading to salt formation (III).



$$R^2CN + R^1OMgX + R^3H \qquad \text{(III)}$$

It is also in the case of pathway *2* difficult to evaluate what is to be expected with regard to the reaction constant. The aryl group in the cyanate is removed to a greater distance from the reaction center, and the screening effect of the oxygen atom could explain the low value of the reaction constant.

No conclusive evidence can be produced for either of the two alternatives, and evidently these reactions need more attention.

EXPERIMENTAL

All Grignard reagents were prepared in diethyl ether distilled from lithium aluminum hydride directly into the glass apparatus. This solvent was used in all experiments. The magnesium used (monosublimed, Dow Chemical Corp.) was washed with anhydrous diethyl ether. Every precaution was taken against oxygen and moisture. The halides used in the preparations of the Grignard reagents were distilled or recrystallized and their purity checked gas-chromatographically. In preparations of Grignard reagents an excess of magnesium was always used. The molarity of the Grignard reagents was determined by titration with standard acid and the content of halogen by titration with standard silver nitrate. The content of halogen was never more than 4 % higher than the content of Grignard reagent. The different concentrations were obtained by dilution of *ca.* 2 M standard solutions.

The aryl cyanates were prepared from phenols and cyanogen chloride in the presence of triethylamine.[18] The purity of the cyanates was checked by infrared and proton magnetic resonance spectroscopy and elemental analysis.

Dibutylmagnesium was prepared from "butylmagnesium chloride" and butyllithium.[19]

Magnesium bromide etherate was prepared from 1,2-dibromoethane.[20]

A detailed description of the apparatus used for the kinetic measurements is given in Ref. 2.

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Formation of β-Allenic Alcohols in Reactions of Acetylenes with Lithium Aluminium Hydride*

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β-Allenic alcohols can be prepared in low to good yields upon treatment of 5-alkoxy-, 5-tetrahydro-2-pyranyloxy-, and 5-trialkylamino-4-pentyn-1-ols (*1*—*10*) with $LiAlH_4$. The reactions proceed in some cases *via* detectable carbanionic intermediates (*21*).

The most convenient way to obtain β-allenic alcohols seems to be the $LiAlH_4$ reduction of alkenynols.[1-3] This reaction has also been applied to stereoselective synthesis.[4] Less used are the following methods: (*i*) the reaction of dihalocyclopropanes with alkyllithium, which was used for preparation of cyclic derivatives,[5] (*ii*) $LiAlH_4$ reduction of β-allenic aldehydes [4] and ketones,[6] (*iii*) addition of propadienyllithium derivatives to oxiranes,[7] and (*iv*) reduction of hydroxy propargylchlorides with a zinc-copper couple.[8]

α-Allenic alcohols are readily prepared by the reaction of 2-butyn-1-ol derivatives with $LiAlH_4$ (Scheme 1).[9-12] The reaction proceeds through an S_N2' mechanism, where X serves as a leaving group (Thp-oxy = tetrahydro-2-pyranyloxy).

Scheme 1.

In this paper we report on an extension of this reaction, in which β-allenic alcohols are formed from the homologous acetylenic derivatives *1*—*10* (Table 1) upon treatment with $LiAlH_4$ under suitable conditions.

RESULTS

The starting acetylenes *1*—*4*, *6*, and *7* were prepared using standard procedures, *i.e.* addition of lithiumalkynides to oxiranes in NH_3.[14] HMPA was used as co-solvent to increase nucleophilicity of the alkynides. *5* was made in analogy to similar compounds [13] and the ammonium salts *8* and *9* were prepared *via* the Mannich reaction [15] of the corresponding β-acetylenic alcohols followed by quaternization. Compound *10* originates from the addition of 3-piperidino-1-butyne to ethylene oxide.

Upon treatment of the acetylenic derivatives *1*—*7* with an excess of $LiAlH_4$ in tetrahydrofuran (THF) at 65 °C and the ammonium salts *8*—*10* at 35 °C the corresponding β-allenic alcohols *10*—*17* were formed in the indicated yields (*cf.* Table 1). No reaction occurs with compounds *1*—*7* when ether is used as a solvent. THF is used for compounds *8*—*10* because of its ability to dissolve the quaternary ammonium salts.[9] In all the reactions except that of *10* reduced allenes are formed as by-products (called "alkenols" in Table 1). In refluxing dioxane these alcohols are main products but so far they have not been further examined.

In some of the reactions one of the major products was the semi-reduced form of the starting acetylene, a fact that made possible a mechanistic interpretation of the formation

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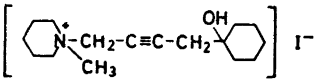
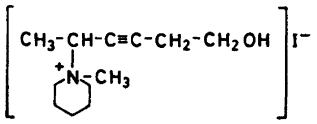
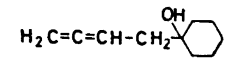
Table 1. $LiAlH_4$ reductions of acetylenic derivatives.

Starting acetylenes	B.p. °C/mmHg	Yield (%)	Reaction time (h)
Thp–O–CH_2C≡C–CH_2–CH_2OH *1*[24]	117/0.6	82	6
t-But–O–CH_2–C≡C–CH_2–CH_2OH *2*[a]	129/10	70	22
Thp–O | C_3H_7–CH–C≡C–CH_2–CH_2OH *3*[a]	114/0.3	73	22
t-But-O | C_3H_7–CH–C≡C–CH_2–CH_2OH *4*[a]	75/0.15	71	5
CH_3–O OH | | C_2H_5–CH–C≡C–CH_2–C–CH_3 | CH_3 *5*[a]	115/12	67	22
Thp–O–$C(CH_3)_2$–C≡C–CH_2–CH_2–OH *6*[a]	85/0.2	81	3
Thp–O OH | | C_2H_5–C–C≡C–CH_2–CH–CH_3 | CH_3 *7*[a]	108/0.4	90	6
$[(C_6H_5–CH_2)_2\overset{+}{N}–CH_2–C≡C–CH_2–CH_2–OH]\ I^-$ | CH_3 *8*		88[c]	2
 *9*		95[c]	2
 *10*		45[c]	2

[a] C and H analyses within ± 0.4 % of the calculated values. [b] Composition of the isolated yield. [c] Yield

Products	Yield GLC[d] (%)	B.p. °C/mmHg	Isolated yield (%)
$H_2C=C=CH-CH_2-CH_2-OH$ *11*[2](+ alkenols	96[b] 4[b])	80/45	55
11+	8		
t-But$-O-CH_2-CH=CH-CH_2-CH_2OH$ *18* (+ alkenols	84 7)		
$C_3H_7-CH=C=CH-CH_2-CH_2OH$ *12*[a] (+ alkenols	66[b] 34[b])	85/13	68
12+ *t*-But-O $C_3H_7-CH-CH=CH-CH_2-CH_2OH$ *19* (+ alkenols	10 40 5)		
$C_2H_5-CH=C=CH-CH_2-COH(CH_3)_2$ *13*+ CH_3-O $C_2H_5-CH-CH=CH-CH_2-COH(CH_3)_2$ *20* (+ alkenols	37 28 3)		
$(CH_3)_2C=C=CH-CH_2-CH_2OH$ *14*[a] (+ alkenols	92[b] 8[b])	75/20	62
$C_2H_5-C(CH_3)=C=CH-CH_2-CH(CH_3)-OH$ *15*[a] (+ alkenols	90[b] 10[b])	81/6	68
11+	97[b]	80/45	67
alkenols	3[b]		
 *16*[a] (+ alkenols	97[b] 3[b])	95/10	78
$CH_3-CH=C=CH-CH_2-CH_2OH$ *17*[4,5]	100[b]	60/11	70

of amino alcohol. Quaternization is quantitative. [d] Rel. areas of peaks. Not corrected for detector response.

of the β-allenic alcohols (*cf.* Discussion). Three of these compounds (*18*–*20*) were isolated by GLC and characterized (*cf.* Table 2).

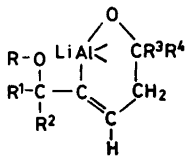
By use of GLC+MS it was easy to indicate a similar product, 5-Thp-oxy-3-octen-1-ol, from the reduction of *3* with $LiAlH_4$. In the mass spectrum of this compound there was no M^+ ion but the $M^+ - Thp\text{-}oxy$ peak was as predicted shifted two mass units compared with the corresponding peak in the spectrum of *3*.

In the reactions of *6* and *7*, where the Thp-oxy groups leave tertiary carbons, no products of this type were encountered.

5-*t*-Butoxy-3-octen-1-ol (*19*) obtained from the acetylenic derivative *4* has the *trans* configuration as indicated by GLC using the corresponding *cis*-alkenol for comparison. The latter originated from partial hydrogenation of *4* over a Lindlar catalyst. Compound *19* also shows strong absorption at 970 cm^{-1} in the IR spectrum. The finding is in accordance with the well known reduction of propargylic alcohols with $LiAlH_4$ to form *trans* allylic alcohols.[13]

DISCUSSION

After the initial alcoholate formation the reductions of *1*–*5* with $LiAlH_4$ proceed in two distinct steps (i) attack by $LiAlH_4$ on the triple bond to form *21* or a similar carbanion intermediate (*ii*) elimination of an alkoxy or Thp-oxy group.



21

Using D_2O to hydrolyze the $LiAlH_4$ reaction mixture from the acetylene *4* confirmed the presence of an intermediate like *21*. NMR analysis of the labeled *19* thus obtained proved that the deuterium occupied exclusively the 4-position; the proton at C-5 became a triplet and spin decoupling of the C-1 protons made the allylic protons at C-2 appear as a doublet ($J = 6$ Hz).

Some of the intermediates are surprisingly stable as can be seen in the reaction of *2*, where the intermediate constitutes 84 % after 22 h. It is interesting to note that the Grignard reagent from *t*-butyl-2-bromoallyl ether also shows remarkable stability, only giving rise to small amounts of allene through elimination of bromomagnesium *t*-butoxide.[17]

It has not been possible to determine whether the quaternary ammonium compounds *8*–*10* give β-allenic alcohols *via* similar long-lived intermediates.

The Thp-oxy group seems to be a better leaving group than *t*-butoxide (*cf.* the reactions of 1 and 2). This is in some contrast to two analogous reactions that we have examined. We have used the *t*-butoxy and Thp-oxy groups with approximately equal success in a modification[12] of Landor's preparation of α-allenic alcohols (*cf.* Scheme 1) and also in a S_N2' type reaction which gives *trans* homoallylic alcohols from $LiAlH_4$ reduction of 4-alkoxy-2-buten-1-ols.[15]

The β-allenic alcohols are susceptible to attack by hydride on the allenic group and further reduction to a mixture of unsaturated alcohols ("alkenols" in Table 1) occurs in most cases. The reaction temperature, which will minimize this reaction and yet get an acceptable reaction rate for compounds similar to *1*–*7*, seems to be 55–60 °C. The quaternary ammonium compounds react at a lower temperature(~35 °C) and only small amounts of alkenols (≤3 %) are formed as by-products.

Though β-allenic alcohols in a few cases are obtained in acceptable yields and purity by reaction of acetylenic Thp-oxy derivatives with $LiAlH_4$ (Table 1) it can be concluded that quaternary ammonium compounds like *8*–*10* seem to be the most suitable substrates for their preparation by this method.

EXPERIMENTAL

General. IR spectra were run on a Perkin-Elmer Infracord 157 G spectrophotometer using liquid films between NaCl discs. NMR spectra were obtained in $CDCl_3$ with tetramethylsilane as internal standard, using a Perkin-Elmer R-12 B spectrometer. These spectra were routinely recorded and are in full agreement with the proposed structures. Mass spectra were obtained at 70 eV with an AEI MS-30 mass spectrometer connected to a Pye 104 gas chromatograph. Columns; 1.5 m glass columns packed with 5 % Carbowax 20 M or 3 % OV-17 on Gas-Chrom Q. Correct mass spectral data were obtained for all products in Table 1.

Table 2. Spectral data of compounds *11*–*20*.

Compound	IR[a] (cm^{-1})	NMR (δ)
11[2]	1955	5.40–4.87 (m, 1 H)
	860	4.80–4.54 (m, 2 H)
	842	3.90–3.48 (m, 2 H)
		2.73 (s, 1 H)
		2.50–1.98 (m, 2 H)
12	1958	5.34–4.89 (m, 2 H)
	875	4.86–4.54 (t, 2 H)
		2.50–1.74 (m, 4 H)
		2.00 (s, 1 H)
		1.74–1.13 (m, 2 H)
		1.08–0.73 (t, 3 H)
13	1958	5.38–4.98 (m, 2 H)
	874	2.35–1.92 (m, 4 H)
		1.82 (s, 1 H)
		1.27 (s, 6 H)
		1.19–0.88 (t, 3 H)
14	1963	5.12–4.65 (m, 1 H)
		3.77–3.49 (t, 2 H)
		2.36–1.97 (q, 2 H)
		1.73 (s, 1 H)
		1.69–1.63 (d, 6 H)
15	1961	5.27–4.80 (m, 1 H)
		4.16–3.53 (m, 1 H)
		2.25–1.95 (t, 2 H)
		2.15–1.78 (m, 2 H)
		2.04 (s, 1 H)
		1.74–1.60 (d, 3 H)
		1.32–1.11 (d, 3 H)
		1.11–0.77 (t, 3 H)
16	1951	5.49–4.90 (m, 1 H)
	877	4.81–4.53 (m, 2 H)
	837	2.36–2.05 (m, 2 H)
		1.87–1.15 (m, 11H)
17[1,3]	1960	5.33–4.80 (m, 2 H)
	870	3.87–3.47 (t, 2 H)
		3.25 (s, 1 H)
		2.50–1.94 (m, 2 H)
		1.84–1.43 (t, 3 H)
18	1360	5.84–5.57 (m, 2 H)
	968	4.00–3.78 (m, 2 H)
		3.78–3.51 (t, 2 H)
		2.60–2.18 (m, 2 H)
		2.03 (s, 1 H)
		1.30 (s, 9 H)
19	1363	5.65–5.42 (m, 2 H)
	970	4.04–3.77 (m, 1 H)
		3.77–3.48 (t, 2 H)
		2.45–2.03 (m, 2 H)
		2.14 (s, 1 H)
		1.58–1.09 (m, 4 H)
		1.17 (s, 9 H)
		1.07–0.76 (t, 3 H)
20	972	5.98–5.10 (m, 2 H)
		3.68–3.17 (m, 1 H)
		3.26 (s, 3 H)
		2.39–2.17 (d, 2 H)
		1.98 (s, 1 H)
		1.77–1.15 (m, 2 H)
		1.23 (s, 6 H)
		1.04–0.82 (t, 3 H)

[a] Only bands of diagnostic value are listed.

For GLC analyses a 2.7 m glass column containing 3 % OV-25 was used. Individual compounds were isolated on a 300 × 0.94 cm aluminium column packed with 20 % Carbowax 20 M on Chromosorb W (60–80).

All reactions with $LiAlH_4$ and Grignard reagents were performed under nitrogen.

Propargylic ethers used in the subsequent preparations were 3-(tetrahydro-2-pyranyloxy)-propyne,[18] *t*-butyl propargyl ether,[19] 3-(tetrahydro-2-pyranyloxy)-1-hexyne,[10,11] 3-*t*-butoxy-1-hexyne,[15] 3-methyl-3-(tetrahydro-2-pyranyloxy)-1-butyne,[20] and 3-methyl-3-(tetrahydro-2-pyranyloxy)-1-pentyne.[20]

Preparation of 1–4, 6, and 7. To 1.14 mol of lithium amide in 1000 ml of liquid ammonia the appropriate propargylic ether (1 mol, see below) dissolved in 100 ml of THF was added during 30 min. 2.4 mol of ethylene oxide or propylene oxide was added and then 100 ml of HMPA. The mixture was stirred overnight and the ammonia allowed to evaporate. Water was added and the products taken up in ether, which was washed with saturated NH_4Cl and several times with 0.01 M hydrochloric acid to remove all traces of HMPA, dried over Na_2SO_4–K_2CO_3 and distilled. For boiling points and yields see Table 1.

2-Methyl-6-methoxy-4-octyne-2-ol (5). Addition[20] of dihydropyran to 2-methyl-4-pentyne-2-ol[21] gave the tetrahydropyranyl protected alcohol (b.p. 96 °C/14 mmHg) in 85 % yield. This (25.0 g; 0.137 mol) in 50 ml of THF was added dropwise during 0.5 h at room temperature to ethylmagnesium bromide [prepared in ether-THF (1:4) from ethyl bromide (19.0 g; 0.174 mol) and magnesium (4.25 g; 0.174 mol)] and stirring was continued for 1 h to give the corresponding acetylenic Grignard derivative. To this reagent propionaldehyde (7.25 g; 0.25 mol) in 25 ml of THF was added dropwise during 0.5 h and the mixture was stirred at room temperature for 2 h. Dimethyl sulfate (23.6 g; 0.187 mol) was added during 0.5 h and then 50 ml of HMPA. The mixture was refluxed for 10 h. After cooling, water was added and the product was taken up in light petroleum, which was then evaporated. The residue was methanolyzed by stirring with 0.5 g of *p*-toluenesulfonic acid in 400 ml of methanol at room temperature for 1 h. 10 g of K_2CO_3 was added and the mixture diluted with 400 ml of ether. Filtering and distillation yielded *5*.

Reactions of 1–7 with $LiAlH_4$. 0.02 mol of the acetylenes 1–7 in 20 ml of THF was slowly dropped to an ice-cooled, stirred suspension of $LiAlH_4$ (0.03 mol for Thp-oxy derivatives and 0.022 for the others). The mixture was then kept at an oil bath temperature of 70 °C and the reactions were followed by GLC (*cf.* Table 1). Work-up using NaOH-water[22] yielded the products in Table 1.

3,4-Pentadien-1-ol[2] *(11) from 8.* Dibenzylamin (11.8 g; 0.06 mol), 3-butyn-1-ol (4.0 g; 0.057 mol), paraformaldehyde (3 g; 0.1 mol)

and 0.2 g of Cu(I)Br were mixed in 25 ml of dry dioxane and refluxed for 1.5 h. The mixture was poured into 100 ml of water, acidified to pH ~ 2 and extracted twice with ether. The aqueous phase was made alkaline with conc. ammonia and extracted three times with ether (3 × 100 ml). Drying over K_2CO_3 and evaporation of solvents yielded 5-dibenzylamino-3-pentyn-1-ol (14 g; 88 %). This crude amino alcohol was treated with an 100 % excess of methyl iodide in refluxing acetone for 1 h. Evaporation yielded the ammonium iodide *8* to which 75 ml of THF was added. $LiAlH_4$ (3.02 g; 0.08 mol) was added in small portions with ice-cooling and swirling. The mixture was then kept in an oil bath of 35 °C during 2 h. Work-up using the NaOH method [22] and distillation yielded the title compound.

1-(2,3-Butadienyl)cyclohexanol (16) was similarly prepared from 1-(2-propynyl)cyclohexanol [21] and piperidine.

3,4-Hexadien-1-ol [4] *(17)*. 3-Piperidino-1-butyne [23] (from the propargylic tosylate and piperidine) was added to ethylene oxide *via* its lithium derivative as described for *1–4*. HMPA was omitted. 5-Piperidino-3-hexyn-1-ol was obtained in 45 % yield. (B.p. 110 °C/0.1 mmHg). This alcohol was treated as described in the preparation of *11* to give the title compound.

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Palladium(II) Catalyzed Aromatic Acetoxylation. V. Mixed Amine Acetatonitratopalladium(II) Complexes as Catalysts in the Acetoxylation of Chlorobenzene

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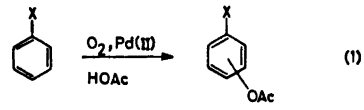
The palladium(II) acetate catalyzed acetoxylation of aromatic compounds has been further investigated, using chlorobenzene as the substrate. Addition of cooxidants, such as dichromate or nitrate, speeds up the reaction by a factor of about 50 but decreases the *meta* selectivity somewhat. In the presence of nitrate (or nitric acid or nitrogen dioxide) Pd metal never precipitates during the reaction. The nitrate catalyzed reaction could be run with a high degree of *meta* selectivity (up to 65 % of the product mixture) if an amine was added in an amount equivalent to the formation of a bisaminepalladium(II) complex. When 2,2′-bipyridine was used for complexation, the compound acetato(2,2′-bipyridine)nitratopalladium(II) monohydrate could be isolated from the reaction mixture. This complex, synthesized separately, possessed good catalytic activity for acetoxylation of chlorobenzene.

Previous work in this series [1,2] and by others [3–14] has established that direct acetoxylation of aromatics — in the ring and/or in the α position of an alkylsubstituted aromatic compound — can be achieved *via* a palladium acetate catalyzed process in boiling acetic acid in an oxygen atmosphere, as shown for nuclear acetoxylation of a monosubstituted benzene derivative in eqn. 1. The isomer distribution turned out to be anomalous under these or similar conditions,[2,10,14] the *meta* acetoxy derivative being the predominant isomer from a number of monosubstituted benzenes with otherwise *ortho*, *para*-directing substituents (*i.e.* in electrophilic substitution). These findings were rationalized in terms of an addition-elimination mechanism, in which electrophilic attack by a palladium(II) species is the initial step.[15]



(1)

Under the conditions described above, the reaction is, however, too slow to be of any practical value,[1] and we have therefore continued our studies of Pd(II) catalyzed aromatic acetoxylation with the aim of finding ways of speeding up the process while retaining the *meta* selectivity. This latter feature is of obvious interest from the synthetic point of view, since it gives a one-step route to phenol derivatives that are otherwise only accessible by multi-step procedures.

Some clues — apart from the well known effect of adding strong acid [3,6] — as to which directions one should look for additional catalytic effects in aromatic acetoxylation were to be found in the literature. Thus, Henry [10] had shown that the addition of a co-oxidant, such as potassium dichromate, to the $Pd(OAc)_2$/HOAc system favors nuclear acetoxylation in a substrate (toluene) that otherwise would give predominantly α acetoxylation.[7–9] Also here a selectivity for *meta* substitution was noticeable (*ortho*:*meta*:*para* equal to 19:62:19 in the presence of methanesulfonic acid). In the analogous vapor phase reaction, acetoxylation over a palladium based catalyst,[16] a similar facilitation of nuclear acetoxylation, coupled to a certain degree of *meta* selectivity, was

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noticed when the catalyst contained a co-oxidant. Moreover, Henry [10] as well as other investigators [6,12,14] has found that the addition of nitrate ion, nitric acid, or oxides of nitrogen has an accelerating effect upon aromatic acetoxylation, yet that nitration then becomes a significant side reaction.

We have applied these and similar ideas to the Pd(II) catalyzed nuclear acetoxylation of chlorobenzene, a substrate that after some preliminary experimentation was found to be a good model compound, since both nitration and biaryl coupling were only minor side reactions. The results indicate that it is indeed possible to speed up the reaction by a factor of about 50 while still retaining the *meta* selectivity to a high degree. Mixed amine acetatonitratopalladium(II) complexes were found to be best in this respect. The 2,2′-bipyridine-acetatonitratopalladium complex was isolated and characterized and found to possess good catalytic activity.

RESULT

A first approach to increase the rate of a reaction is to change the solvent. Since DMF has turned out to be a superior solvent for the palladium acetate catalyzed oxidation of terminal alkenes to ketones,[17] we first tried a mixture of acetic acid and DMF (1:1 v/v) as a solvent for the acetoxylation of chlorobenzene under oxygen. However, DMF acted as a reductant for Pd(II) acetate at the temperature employed and hence this solvent cannot be used. The main product from this reaction was, somewhat curiously, *N*,*N*-dimethylacetamide.

N,*N*-Dimethylacetamide in a mixture with acetic acid was then tried but with similar negative results; at 90 °C Pd(II) is reduced by the solvent mixture and acetoxylation runs with chlorobenzene at 65 and 85 °C did not yield any acetoxylation products. Dimethyl sulfoxide as a co-solvent also underwent oxidation by Pd(II), giving the corresponding sulfone. Thus, none of the dipolar aprotic solvents tried is inert towards Pd(II).

Next we added a strong acid to the reaction mixture (Table 1, experiments 2 and 3). As expected, this changed the reaction path from acetoxylation to predominant biaryl coupling, a mixture of dichlorobiphenyls (at least five of the six possible ones) being obtained. With methanesulfonic acid this process was essentially instantaneous and should be of pre-

Table 1. Acetoxylation of chlorobenzene by $Pd(OAc)_2$ in acetic acid after 4 h at 115 °C[a].

Experiment No.	Atmosphere	Added component	Acetoxychlorobenzenes Yield,[b] %	Isomer distribution *o*	*m*	*p*
1	O_2	None[c]	2.9	3	71	26
2	O_2	CF_3COOH (5 ml)	2.0	16		84[d,e]
3	O_2	CH_3SO_3H (2 ml)	Trace[e]			
4	O_2	$K_2Cr_2O_7$ (15 mmol)	56	15	45	39
5	N_2	$K_2Cr_2O_7$ (15 mmol)	56	15	46	38
6	O_2	$K_2Cr_2O_7$ (15 mmol), CF_3COOH (5 ml)	146	40	32	28
7	O_2	$K_2Cr_2O_7$ (15 mmol), CH_3SO_3H (2 ml)	22	59	19	22
8	O_2	$K_2Cr_2O_7$ (15 mmol), CCl_3COOH (7 ml)	16	70	14	16
9	O_2	$Cu(OAc)_2.H_2O$ (1 mmol)	Trace[f]			
10	O_2	$Sn(OAc)_2$ (1 mmol)	None			
11	O_2	$K_2S_2O_8$ (1 mmol)	9	8	56	36
12	O_2	$(NH_4)_2S_2O_8$ (1 mmol)	3.6	18	46	36
13	O_2	H_2O_2 (12 mmol)	Trace[g]			
14	O_2	NO_2 (10 mmol)	61	27	33	40

[a] All experiments were performed with 1.0 mmol of $Pd(OAc)_2$ and 9.9 mmol of chlorobenzene. The total amount of acetic acid and added liquid component was 50 ml. [b] Based on $Pd(OAc)_2$. [c] Reported [2] *o*:*m*:*p* = 3:88:9. [d] Difficult to analyze due to the small amount formed. [e] The main product was a mixture of isomeric dichlorobiphenyls. [f] Traces of phenyl acetate were detected. [g] H_2O_2 decomposed as soon as Pd(0) precipitated.

parative interest if the isomer distribution can be controlled. Trifluoroacetic acid also caused the formation of predominantly biaryl products, although a low yield of acetoxychlorobenzenes was secured.

Following Henry's observations,[10] we performed experiments in which potassium dichromate was added as a co-oxidant (Table 1, experiments 4 and 5). Both reactions, which differed from each other by being run under oxygen and nitrogen, respectively, gave acetoxylation products in a reasonably fast process (about 20 times faster than in the absence of potassium dichromate), showing that the finding that toluene is predominantly acetoxylated in the ring under these conditions is due to a rate increase of the nuclear substitution reaction. The reaction became even faster when trifluoroacetic acid was present together with potassium dichromate (146 % yield after 4 h), whereas under otherwise identical conditions methanesulfonic acid and trichloroacetic acid decreased the rate (Table 1, experiments 6 – 8). However, these rate increases are accompanied by changes in the isomer distribution, so that the desired *meta* selectivity has disappeared. Instead, the *ortho* isomer is slightly favored. No coupling was noticed in the experiments with dichromate present.

Some other oxidants were tried (experiments 9 – 13 in Table 1) but with no apparent success with respect to the aims of this investigation. In these cases acetoxylation did occur, but the reactions were either too slow or complicated by side reactions. Moreover, these reactions as well as all mentioned above suffered from the disadvantage of Pd metal precipitation with a concomitant sluggishness of the reoxidation of Pd(0) to Pd(II). Apart from this practical drawback, Pd(0) can catalyze unwanted reactions.

However, the last experiment (No. 14 in Table 1) gave very promising results from several points of view. Addition of NO_2 to the reaction solution caused a fairly rapid acetoxylation process to occur while at the same time the solution remained homogeneous during the run. Only a trace of chloronitrobenzenes was formed as a by-product, and the isomer distribution, although not directly in favor of the *meta* isomer, was only little affected.

A number of runs (Table 2) in which NO_2 was used as a co-oxidant were performed. Since water is formed during the reaction it was

Table 2. Acetoxylation of chlorobenzene in acetic acid after 4 h at 115 °C in the presence of NO_2.[a]

Experiment No.	Added component	Acetoxychlorobenzenes Yield,[b] %	Isomer distribution *o*	*m*	*p*	Nitration, %[b]
14	None	61	27	33	40	Trace
15	H_2O (2 mmol)	59	26	34	40	Trace[c]
16	CF_3COOH (5 ml)	49	17	49	34	20
17	CF_3COOH (10 ml)[d]	11	5	51	44	Trace[e]
18	NaOAc (10 mmol)	29	24	42	34	5
19	HNO_3 (0.2 ml)	92	25	34	41	Trace[f]
20	HNO_3 (1.0 ml)	110	26	33	41	Trace[f]
21	Pyridine (1.0 mmol)	75	13	57	30	5
22	Pyridine (1.3 mmol)	88	6	61	33	4
23	Pyridine (2.0 mmol)	45	6	61	33	5
24	Pyridine (4.0 mmol)	3.6	3	68	29	Trace
25	2,2′-Bipyridine (1.0 mmol)	79	5	48	47	2
26	2,2′-Bipyridine (1.0 mmol), HNO_3 (1.0 ml)	0				
27	2,2′-Bipyridine (1.0 mmol)	0[g]				

[a] All experiments were performed with 1.0 mmol of $Pd(OAc)_2$, 10.0 mmol of NO_2, and 9.9 mmol of chlorobenzene in an oxygen atmosphere. The total amount of acetic acid and added liquid component was 50 ml. [b] Based on $Pd(OAc)_2$. [c] The reaction was followed to 140 % yield; at this stage the solution was still catalytically active. [d] At 80 °C. [e] Chlorophenols (5 %) were also formed. [f] Bisacetoxylation (4 %) also occurred. [g] No NO_2 added.

Table 3. Acetoxylation of chlorobenzene by $Pd(OAc)_2$ in the presence of complexing agents and NO_2 in acetic acid at 115 °C [a].

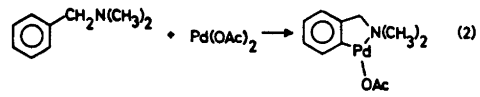
Experiment No.	Complexing agent	Reaction period, h	Acetoxychlorobenzenes Yield,[b] %	Isomer distrib. *o*	*m*	*p*	Nitration, %[b]
23	Pyridine (2 mmol)	4	45	6	61	33	5
25	2,2′-Bipyridine (1 mmol)	4	79	5	48	47	Trace
26	Isoquinoline (2 mmol)	4	88	6	65	30	Trace
27	Ethylenediamine (1 mmol)	4	81[c]	15	40	45	Trace
28	1,4-Diazabicyclo[2,2,2]octane (1 mmol)	1	20	21	44	45	1
29	Benzalaniline (1 mmol)	1	Trace				
30	*N*,*N*-Dimethylbenzylamine (2 mmol)	1	34	11	47	42	Trace
31	Acetylacetone (1 mmol)	4	65	23	37	40	Trace
32	EDTA (1 mmol)	1	18	20	45	35	1

[a] All experiments were performed with 1.0 mmol of $Pd(OAc)_2$, 10.0 mmol of NO_2, and 9.9 mmol of chlorobenzene in an oxygen atmosphere. The total amount of acetic acid and added liquid component was 50 ml. [b] Based on $Pd(OAc)_2$. [c] Yield after 1 h 52 %.

first established that small amounts of water had no effect on the yield and product distribution (nor had acetic anhydride), and that additives like trifluoroacetic acid, sodium acetate, or nitric acid did not have any deleterious effect upon the yield but on the other hand did not increase the *meta* selectivity to the desired level. Complexing bases of the pyridine type (Tables 2 and 3) were better in this respect and did not decrease the rate if not added in excessive amounts (*cf.* Table 2, experiments 23 and 24). All factors considered, isoquinoline appears to be the best complexing base hitherto found (experiment 26), the percentage of the *meta* isomer being 61 %, the yield 88 % after 4 h, and the occurrence of nitration almost negligible.

In the experiment (No. 15) with water present, the reaction was run for 27 h, after which time the yield was 250 %, based on the Pd(II) acetate used. The reaction mixture was still catalytically active and no Pd metal had precipitated. The isomer distribution changed slightly with time, being 17:37:46 at the discontinuation of the experiment, presumably due to different reactivity of the three isomers toward further acetoxylation. A second acetoxylation process does take place, as shown by the detection of at least three isomers of diacetoxychlorobenzene (22 % yield). Bisacetoxylation was noticed in two other cases as well (experiments 19 and 20).

Ethylenediamine was the most strongly rate accelerating base tried, the yield after 1 h being 52 % and after 4 h 71 %. A similar high initial rate was noticed with benzyldimethylamine (34 % after 1 h) but this reaction later slowed down appreciably (7 % during the second hour). In both cases the reaction mixture turned dark, probably due to oxidative degradation of the amine; it is also possible that benzyldimethylamine is removed in a palladation reaction, such as that depicted in eqn. 2:



(2)

This type of compound is known from the literature.[18]

When NO_2 in excess was added to a solution of palladium acetate and 2,2′-bipyridine in acetic acid, a yellow precipitate was formed. The same precipitate was also produced when the reaction mixture from experiment No. 25 was allowed to cool. A solution of the filtered and dried complex in acetic acid has the same catalytical effect as the initial solution, without any extra NO_2 being added. Elemental analyses and spectral data (IR, NMR) showed that the salt must be acetato(2,2′-bipyridine)nitratopalladium(II) monohydrate. This complex reacted

Table 4. Acetoxylation of different substrates by some catalysts in acetic acid at 115 °C.[a]

Exp. No.	Substrate	Reaction period, h	Catalyst	Acetoxy derivatives Yield[b]	Isomer distribution *o*	*m*	*p*
33	Anisole	2	2,2′-Bipy Pd(OAc) (NO_3)	Low[c]	10	41	49
34	Phenyl acetate	2	2,2′-Bipy Pd(OAc) (NO_3)	Low	20	60	20
35	Chlorobenzene	4	2,2′-Bipy Pd(OAc) (NO_3)	70	2	60	38
36	Benzene	4	(Isoquin)$_2$Pd(OAc)$_2$+NO_2[e]	610[d]			
37	Methyl benzoate	4	(Isoquin)$_2$Pd(OAc)$_2$+NO_2[e]	0			

[a] All experiments were performed with 1 mmol of the complex, 9.9 mmol of the substrate, and 50 ml of HOAc in an O_2 atmosphere. [b] Based on the amount of complex. [c] A mixture of nitroanisoles was the main product. [d] Yield of nitrobenzene, 305 %. [e] 5.5 mmol.

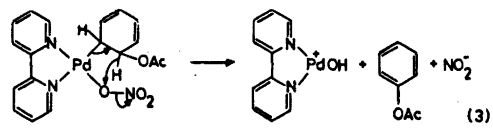
with excess nitric acid in HOAc and gave a solution of (2,2′-bipyridine)dinitratopalladium(II), which was catalytically inactive.

The mixed acetatonitrate complex was then used as a catalyst for the acetoxylation of a number of substrates (Table 4). Chlorobenzene (experiment 35) gave a higher *meta* selectivity than and almost the same yield of acetoxychlorobenzenes as in experiment 25 (Table 3) in which the complex was generated in solution. Compounds with activating groups gave acetoxylation products but in low yield; as expected, nitration is the major process in the case of anisole (experiment 33) and, in spite of much experimentation, it has not yet been possible to suppress this reaction. Even benzene is nitrated to a fairly high degree (experiment 36) under similar conditions. Methyl benzoate did not react at all. Phenyl acetate gave a mixture of diacetoxybenzenes as the main product but at a surprisingly low rate, which might explain why earlier attempts to acetoxylate phenyl acetate under slightly different conditions have failed.[10]

DISCUSSION

The primary aim of this investigation has been to increase the rate of the Pd(II) catalyzed nuclear acetoxylation of aromatic compounds while retaining the anomalous *meta* selectivity noticed earlier. At the same time it was desirable to find a better method of reoxidizing the Pd(0) state, so that no Pd metal would precipitate. Otherwise, there is a risk that the heterogeneous reoxidation reaction will be rate-limiting and/or that Pd metal might cause unwanted reactions.

The system palladium(II) acetate/NO_2 or HNO_3 in combination with oxygen represents a first step toward the realization of these goals, even if the *meta* selectivity is not so pronounced in these cases and in some cases published in the patent literature[14] (toluene, *o*:*m*:*p* = 16:41:43; isopropylbenzene, *o*:*m*:*p* = 0:59:41, *t*-butylbenzene, *o*:*m*:*p* = 0:43:57). However, modification of the acetatonitrato catalyst by adding a pyridine base increases the *meta* selectivity, 2,2′-bipyridine and isoquinoline being the best ligands so far studied. The isolation of a defined complex with good catalytic activity indicates that it is essential that palladium has both nitrate and acetate as a ligand (the corresponding complexes with two nitrate or acetate groups, respectively, were catalytically inactive). This is in accordance with the mechanism discussed earlier,[15] in which the first step is the reversible formation of a π complex between the palladium species, PdL_4, and the aromatic compound which then undergoes attack by a nucleophile (in acetoxylation, acetate ion) to form an adduct. Loss of $PdHL_3$ completes the reaction sequence.



(3)

It is the last step that is affected favorably by the addition of a co-oxidant, nitrate ion being especially effective since it can be present within

the coordination sphere of the adduct (eqn. 3) and regenerate a Pd(II) species directly.

The improvements of the acetoxylation process described in this paper have made the reaction feasible from the practical point of view, at least for laboratory use. A remaining drawback is the nitration reaction which becomes especially pronounced in compounds with activating substituents. Attempts to find other oxidants with properties similar to those of nitrate ion have so far failed.

EXPERIMENTAL

Materials. All chemicals used were purchased in the highest commercial quality available or prepared according to literature methods (see also Parts I and II of this series [1,2]). Solutions of nitrogen dioxide were prepared by heating a known amount of lead nitrate and dissolving it in glacial acetic acid. Identification of compounds was based on mass spectral/gas chromatographic (LKB 9000 system) comparison with authentic samples. Analyses [1,2] were performed with a Varian 1700 gas chromatograph in conjunction with a disc integrator. Biphenyl was used as an internal standard.

Analytical oxidation experiments. These were performed according to method B in Part I [1] with the modifications described in the tables.

Preparation of acetato(2,2'-bipyridine)nitratopalladium(II) monohydrate. 2,2'-Bipyridinepalladium(II) acetate [19] was dissolved in acetic acid and an excess of nitrogen dioxide was dissolved in the solution. The yellow-red precipitate was collected and recrystallized from acetic acid or DMF, m.p. 202 °C (dec.). IR (KBr): 3600 – 2950 (broad); 1600(w); 1385(s) cm^{-1}. NMR (DMSO-d_6): δ 2.10(s, 3); 3.35(s, 2); 7.7 – 8.6(m,8). (Found: C 35.8; H 3.24; N 10.2. Calc. for $C_{12}H_{13}N_3O_6Pd$: C 35.8; H 3.24; N 10.5).

Diacetoxychlorobenzenes. The retention times of the three isomers were 12, 13, and 14 min, respectively, on a 2 m × 0.3 cm 5 % neopentylglycol succinate on Chromosorb W column (120 – 200 °C at 10 °C/min). The mass spectra of the three isomers were very similar with a small parent peak at *m/e* 228 (2 – 3 %) and major peaks at *m/e* 186 (15 – 17), 146 (20 – 30), and 144 (100).

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Radiation Induced Effects on Exchangeable Hydrogens in Crystalline Carbohydrates

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Crystalline mono- and disaccharides, unlabeled and labeled with deuterium or tritium in the exchangeable positions, were irradiated with ^{60}Co γ-rays. Hydrogen gas, H_2, is formed with G-values of 3.0 – 3.8 in unlabeled carbohydrates. Mass spectrometry of the hydrogen gas formed in the deuterium labeled carbohydrates showed that only 2 – 4 % is in the form of HD and that no D_2 is detectable. Irradiation of the tritium labeled carbohydrates transfers tritium to non-exchangeable positions and a large part of this tritium is present in the original carbohydrate. G-Values of 1 – 2 can be calculated for the transfer process assuming that no isotope effect is present. The results indicate that the radiation induced transfer from exchangeable positions to non-exchangeable positions in crystalline carbohydrates occurs at an early stage following the absorption of the radiation.

Radiation induced hydrogen transfer from exchangeable sites to non-exchangeable sites has been detected in several types of compounds in the solid state.[1,2] In amino acids, labeled with deuterium in the exchangeable positions, transfer of exchangeable hydrogen to carbon-bound hydrogen in the radiation induced free radicals has been investigated by ESR (Ref. 1 and quoted refs.). Smitherman *et al.*[2] have studied transfer from exchangeable to non-exchangeable sites in lyophilized samples of proteins, nucleic acid and D-glucose by means of tritiated compounds and have suggested that destruction of secondary radicals may be responsible for the radiation induced hydrogen transfer.

In the present investigation the extent of the radiation induced hydrogen transfer to positions at carbon atoms has been determined in some crystalline carbohydrates labeled with tritium in the exchangeable positions. In parallel studies with non-labeled samples and samples labeled with deuterium in the exchangeable positions quantitative determinations of the radiation induced formation of hydrogen gas, H_2, HD, and D_2, have been performed in order to evaluate the involvement of the exchangeable hydrogens in the formation of hydrogen gas.

EXPERIMENTAL

The ^{60}Co γ-irradiation source and the non-labeled carbohydrates have previously been described.[3]

Labeling of the exchangeable hydrogens in the carbohydrates was made by crystallization in aqueous solvents containing tritiated or deuterated water. The specific activities of the tritiated samples were in the range 22 – 76 mCi/mol. The theoretical exchange of deuterium for hydrogen, as calculated from the ratio of the number of exchangeable deuterium atoms to the number of exchangeable hydrogen plus deuterium atoms in the crystallization solution, ranged from 55 % (in sucrose) to 87 % (in α-D-glucose).

The tritiated carbohydrates were irradiated in the presence of air and then dissolved in water bubbled with nitrogen. The water was distilled off at reduced pressure and temperatures not exceeding 35 °C. Dissolution of the residue in water and distillation were repeated until all exchangeable tritium had been removed and the solid residue had a constant specific activity. This took about eight cycles. Syntheses of di-*O*-isopropylidene-D-fructose and di-*O*-isopropylidene-D-glucose were made directly on the unirradiated and irradiated samples whereas syntheses of the octa-*O*-acetyl derivatives of sucrose and trehalose were made on

samples from which the exchangeable tritium had been removed. Di-*O*-isopropylidene-D-glucose was prepared according to Glen *et al.*[4] and the other derivatives were synthesized and purified as described earlier.[3] The determination of radioactivity was mainly done with aqueous scintillation with Instagel (Packard Inc.).

For the determination of hydrogen gas the samples were irradiated in sealed glass ampoules. After irradiation the ampoule was opened when attached to a small flask having a second side arm closed with a rubber membrane through which water was added for dissolution of the sample and head space gas withdrawn for analysis. Hydrogen gas was determined by gas chromatography with a 2.5 m × 2.1 mm Molecular Sieve 5A 60 – 80 mesh column at 50 °C and with hot wire detection at 100 °C. The determination of HD and D_2 relative to H_2 was made on a CH-4 Atlas M-A-T mass spectrometer at the Mass Spectrometry Laboratory, Karolinska Institute, Stockholm, on the head space gas of samples irradiated *in vacuo* and on the head space gas of samples irradiated in the presence of air and then dissolved in water *in vacuo*.

RESULTS

Labeling of the carbohydrates with tritium in the exchangeable positions occurred to the extent expected from the amount of added tritium and the number of exchangeable positions in the carbohydrates and the crystallization solvent. This indicates that no measureable isotope effect is present in the recrystallization labeling procedure.

Upon irradiation, followed by removal of the exchangeable tritium by repeated distillation, a dose related increase of non-exchangeable tritium was present in the samples (*cf.* Figs. 1 and 2). Except for trehalose dihydrate the tritium incorporation is linearly dependent on the dose up to at least about 10^{21} eV/g. With the assumption that no isotope effect is operating in the transfer process the following

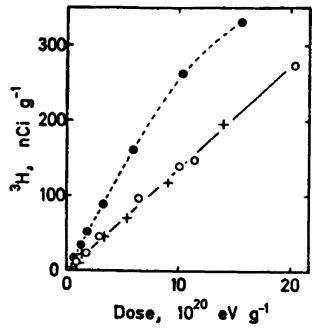

Fig. 1. The radiation induced transfer of ^3H from exchangeable to non-exchangeable positions in crystalline carbohydrates labeled with ^3H in the exchangeable positions (mCi/mol carbohydrate); dose *vs.* amount of non-exchangeable ^3H per g irradiated carbohydrate; ● D-fructose 35 mCi/mol, O α-D-glucose 22 mCi/mol, and + α-lactose.H_2O 66 mCi/mol.

Table 1. Radiation induced transfer of tritium in crystalline carbohydrates from exchangeable positions to non-exchangeable positions determined after (a) repeated evaporation of water and (b) derivatization and purification with respect to the original carbohydrate.

Compound and sp. activity	Dose 10^{20} eV/g	nCi non-exchangeable ^3H per g[a] (a) of irradiated sample	(b) of original carbohydrate
D-Fructose 35 mCi/mol	5.9	160	110
	10.4	264	165
	16.0	331	249
α-D-Glucose 22 mCi/mol	10.1	139	108
Sucrose 61 mCi/mol	11.4	113	63
Trehalose·$2H_2O$ 76 mCi/mol	15.8	214	72

[a] After correction for tritium incorporation in non-exchangeable positions in unirradiated samples.

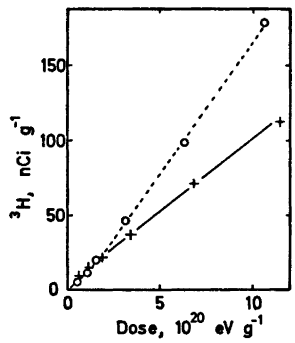

Fig. 2. As for Fig. 1 with O trehalose.$2H_2O$ 76 mCi/mol and + sucrose 61 mCi/mol.

G-values are obtained: D-Fructose 2.3, α-D-glucose 1.8, α-lactose monohydrate 1.2, and sucrose 0.8. In the case of trehalose dihydrate there is an exponentially increasing dose response up to about 10^{21} eV/g (Fig. 2) with an initial G-value of about 0.9 and a slope in the dose region $0.5 \times 10^{21} - 10^{21}$ eV/g corresponding to a G-value of about 1.7. A G-value of 0.95 for transfer of exchangeable hydrogens to non-exchangeable positions has been reported for lyophilized D-glucose samples, the modification of which was not reported.[2] Tritium is also present in non-exchangeable positions in unirradiated samples; these incorporations were generally small (*i.e.* 0.3 – 5 nCi/g) compared with the radiation induced incorporations and they have been corrected for in the calculations.

By preparation of the di-*O*-isopropylidene derivative directly from unirradiated and irradiated D-fructose it was found that most of the radiation induced tritium incorporation (about 70 %) is present in D-fructose itself (Table 1). A similar investigation on the other carbohydrates by means of the suitable acetyl derivatives resulted in a high tritium incorporation into the acetoxy methyl group. Derivatization after removal of the exchangeable tritium was, however, feasible and analyses of sucrose and trehalose dihydrate showed that a large part of the radiation induced incorporation is in the original carbohydrate (Table 1). The same seems to hold also for α-D-glucose as determined by analyses of its di-*O*-isopropylidene derivative prepared directly from the crystalline samples; in this case, in contrast to D-fructose, an appreciable tritium incorporation (178 nCi/g) occurred in the unirradiated sample. The difference may be that the D-fructose derivative is prepared with only anhydrous acetone and zinc chloride whereas also the presence of another acid, *e.g.* phosphoric acid, is necessary for D-glucose.

Analyses of hydrogen gas formation in the dose range $10^{19} - 4 \times 10^{20}$ eV/g showed that the total yield, obtained after dissolution of the samples, is linearly dependent on the dose; $G(H_2)$ was found to be 3.8 for α-D-glucose, lactose monohydrate, and maltose monohydrate 3.1 for sucrose and 3.0 for D-fructose and trehalose dihydrate. Depending on the type of carbohydrate, various portions of the hydrogen gas are released from the crystals in the solid state; the per cent released was 15 – 25 for α-D-glucose, D-fructose and sucrose, 50 – 60 for lactose monohydrate, and 70 – 80 for maltose monohydrate and trehalose dihydrate. The present result on hydrogen gas formation in α-D-glucose is the same as that reported by Phillips and Baugh.[5]

Mass spectrometric analyses of the relative amount of HD to H_2 performed on α-D-glucose, D-fructose, sucrose, and trehalose dihydrate deuterated in the exchangeable positions and irradiated with doses of about 1.5×10^{20} eV/g showed that only 2 – 4 % of the hydrogen gas was in the form of HD. Analyses of D_2 was usually not possible, probably due to contamination from helium, except for one analysis of each of α-D-glucose, sucrose, and trehalose dihydrate; in these analyses no peak was detected at mass number 4 while the HD was detected; this result indicates that D_2 at most is only one fifth to one tenth of HD. Control experiments with non-labeled samples dissolved in D_2O after irradiation showed that no or negligible amounts of HD were formed.

DISCUSSION

Several mechanisms have been discussed for the radiation induced hydrogen transfer from exchangeable to non-exchangeable sites.[2] Among these are reactions involving hydrogen atoms and molecular hydrogen. The present data show that no more than a very small part of the radiation induced hydrogen gas is derived from the exchangeable hydrogens in the carbohydrates studied. This infers that neither

hydrogen gas nor such hydrogen atoms which are precursors to hydrogen gas play a significant role in the radiation induced hydrogen transfer from exchangeable to non-exchangeable positions.

Exchange reactions of secondary radicals, as observed in amino acids,[1] are not likely mechanisms, as irradiated polycrystalline carbohydrates,[6] and single crystals of sucrose [7] and trehalose [8] deuterated in exchangeable positions show the same ESR spectra as non-labeled samples.

The radiation induced transfer of hydrogen to non-exchangeable sites, as measured by tritium incorporation, is, except for trehalose dihydrate, linearly dependent on the dose over a very large dose range. In the same dose range, formation of stable, secondary free radicals occurs with decreasing efficiency with increasing doses; this also occurs at doses below 2×10^{19} eV/g for sucrose.[9] It is thus unlikely that removal of secondary radicals is related to the tritium incorporation. The fact that a large part of the tritium incorporated into non-exchangeable positions is present in the original carbohydrate favours an interpretation that the transfer occurs at an early stage following the absorption of the radiation.

Further studies, particularly to investigate into which position(s) the exchangeable hydrogens are incorporated, may give additional insight into the transfer mechanism and may also lead to methods to synthesize labeled carbohydrates.

Acknowledgement. This investigation has been supported by grants from the Swedish Atomic Research Council.

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Alkyl Cyanates. XVI. Reaction of Alkyl Cyanates with Grignard Reagents. Kinetic Studies

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A kinetic investigation of the reactions of alkyl cyanates with Grignard reagents* and dibutylmagnesium in diethyl ether has been carried out by means of thermographic measurements. In the presence of excess organomagnesium reagent first order plots are obtained for reaction of the cyanate, but the kinetic order in organomagnesium reagent is less than one and decreasing at high concentrations. In the presence of excess cyanate the same kinetics are observed, the order in organomagnesium reagent being one and the order in cyanate decreasing at high concentrations, which corresponds to a mechanism involving complex formation between the reactants. Addition of $MgBr_2$ to the organomagnesium reagent depresses the observed first-order rate constants. The depression is consistent with both R_2Mg and RMgX contributing to the reactions.

The reactivity of aromatic Grignard reagents in reactions with alkyl cyanates is higher than the reactivity of dibutylmagnesium, which again is higher than the reactivities of aliphatic Grignard reagents. The Hammett ϱ-value for the reactions of isobutyl cyanate with *p*-substituted "phenylmagnesium bromides" is found to be *ca.* -1.5. On the basis of the kinetic data and the ϱ-value the reactions are best described as involving a concerted four-center mechanism in the rate-determining step.

In another paper we have reported on the product formation in the reaction between alkyl or aryl cyanates and Grignard reagents,[1] and now we wish to report on a kinetic investigation of the reaction between alkyl cyanates and Grignard reagents.

The present work has been carried out with the aim of obtaining deeper insight into the reactions between alkyl cyanates and Grignard reagents which lead to the formation of alkoxymagnesium halides and nitriles (I):

$$R^1OCN + R^2MgX \longrightarrow R^1OMgX + R^2CN \qquad (I)$$

A thermographic method was used for the kinetic measurements.[2] This technique allowed reproducible rate measurements within ± 5 % when the limiting reagent was present in a concentration of 0.01 M. At lower concentrations the accuracy of measurements becomes unsatisfactory. At higher concentrations the error of the k_{obs}-values becomes too high due to the temperature dependence. All measurements were carried out with one of the reactants in excess.

Fig. 1 shows three examples of plots of $\ln (\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T)$* *versus* the reaction time, *t*, for the reaction of ethyl cyanate with „butylmagnesium bromide". From all kinetic experiments linear plots were obtained up to at least 70 % reaction of the limiting reagent. The linearity of the plots means that the reaction order in ethyl cyanate is one. The pseudo first order rate constants (k_{obs}) are obtained from the slopes of the lines. In Fig. 2 is shown how the pseudo first order rate constants vary with the initial concentrations of "butylmagnesium

* The Grignard reagents are in the following symbolized as "RMgX".

* $\text{Ln}\, a/(a-x) = \ln (\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T) = k_{obs} \times t$, where ΔT_∞ is the total rise in temperature, ΔT_1 is the rise in temperature after 2 ms and ΔT is the rise in temperature at the time *t*. ΔT_1 is a correction for heat evolved from reaction between traces of impurities (water) and the Grignard reagent and for heat of mixing.

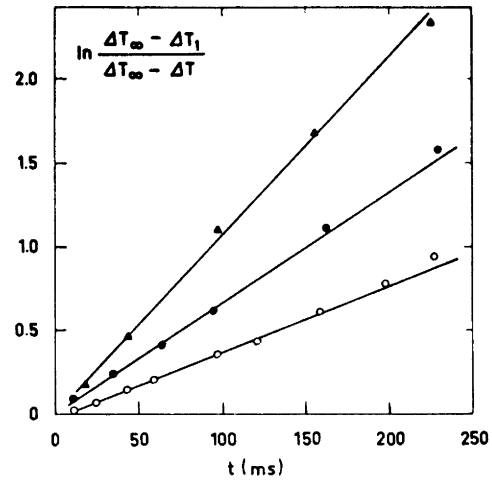


Fig. 1. Plots of ln $(\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T)$ *versus* the reaction time, t, for the reaction of ethyl cyanate with excess "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{C_2H_5OCN}$ = 0.010 M; c^0"C_4H_9MgBr" = 0.100 M (○), 0.200 M (●) and 0.600 M (▲)).

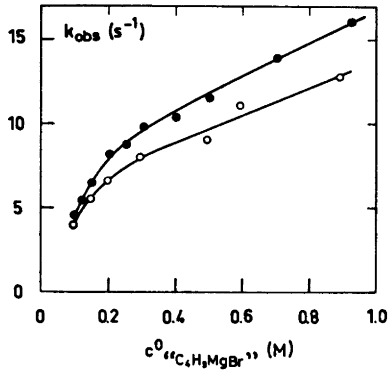


Fig. 2. Plots of the pseudo first order rate constants *versus* the initial concentrations of "butylmagnesium bromide" for reactions of this Grignard reagent with ethyl cyanate (○) and isobutyl cyanate (●) in diethyl ether at 25 °C (c^0_{ROCN} = 0.010 M).

bromide" for the reactions of this Grignard reagent with ethyl and isobutyl cyanate. Because of the curvature of the plots shown it is concluded that the reaction order in Grignard reagent is less than one.

Kinetic experiments with excess isobutyl cyanate and 0.010 M "butylmagnesium bromide" likewise give linear pseudo first order

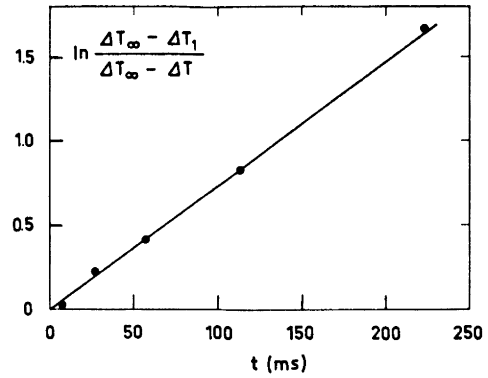


Fig. 3. Plot of ln $(\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T)$ *versus* the reaction time, t, for the reaction of 0.138 M isobutyl cyanate with 0.010 M "butylmagnesium bromide" in diethyl ether at 25 °C.

plots (Fig. 3), and a plot of the pseudo first order rate constant *versus* the initial concentration of isobutyl cyanate is very similar to the plot obtained with excess "butylmagnesium bromide" (Fig. 4). The deviation appears roughly to be within the limits of the accuracy of measurements of this method.

Kinetic measurements on the reaction of isobutyl cyanate with dibutylmagnesium in diethyl ether have been carried out. The pseudo first order plot is linear up to 80 % reaction of isobutyl cyanate (Fig. 5). From the slope of the

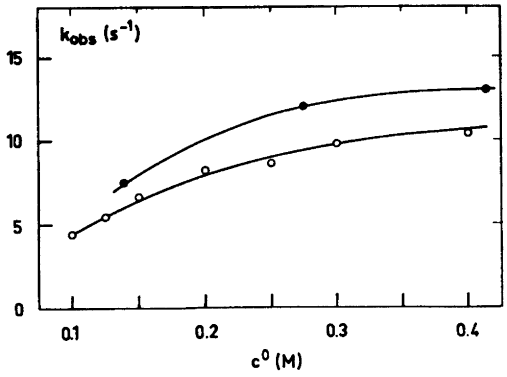


Fig. 4. Plots of the pseudo first order rate constant *versus* the initial concentrations of the reactant in excess for the reaction of isobutyl cyanate with "butylmagnesium bromide" in diethyl ether at 25 °C (○: 0.010 M i-C_4H_9OCN and excess "C_4H_9MgBr"; ●: 0.010 M "C_4H_9MgBr" and excess i-C_4H_9OCN).

Table 1. The influence of the magnesium bromide concentration on the pseudo first order rate constant for the reaction of isobutyl cyanate with "butylmagnesium bromide" in diethyl ether at 25 °C.

$c^0_{i\text{-}C_4H_9OCN}$ M	$c^0_{"C_4H_9MgBr"}$ M	$c^0_{MgBr_2}$ M	k_{obs} (s^{-1})
0.010	0.138	0.003	6.8
0.010	0.138	0.069	4.0
0.010	0.138	0.138	1.5

line a pseudo first order rate constant of 47 s^{-1} is obtained for $c^0_{i\text{-}C_4H_9OCN} = 0.010$ M and $c^0_{(C_4H_9)_2Mg} = 0.100$ M.

When a large amount of magnesium bromide etherate is added to "butylmagnesium bromide" the pseudo first order rate constant is decreased, but the value of the rate constant is still considerable (Table 1). Therefore it is concluded that both butylmagnesium bromide and dibutylmagnesium present according to the Schlenk equilibrium [3] (II) react with the cyanate

$$2\ C_4H_9MgBr \overset{K_s}{\rightleftharpoons} (C_4H_9)_2Mg + MgBr_2 \qquad (II)$$

when "butylmagnesium bromide" reacts with isobutyl cyanate. A decrease of k_{obs} on addition of magnesium bromide is expected because of a lowered content of the most reactive species, dibutylmagnesium. However, it cannot be excluded that magnesium bromide also forms a complex with the cyanate because the pseudo first order rate constant is decreased more than expected.* However, we have not been able to show the presence of a complex between a cyanate and magnesium bromide. The only products obtained when the reactants are mixed are magnesium cyanate and alkyl bromide. This result is due to nucleophilic attack of the halide ion on the cyanate, a wellknown process,[5,6] but of course primary complex formation is still possible.

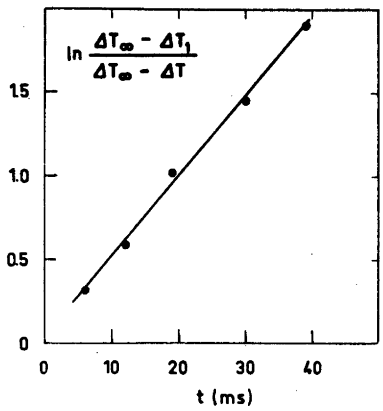


Fig. 5. Plot of ln $(\Delta T_\infty - \Delta T_1)/(\Delta T_\infty - \Delta T)$ *versus* the reaction time, t, for the reaction of isobutyl cyanate with excess dibutylmagnesium in diethyl ether at 25 °C ($c^0_{i\text{-}C_4H_9OCN} = 0.010$ M and $c^0_{(C_4H_9)_2Mg} = 0.100$ M).

As shown above (Figs. 2 and 4) the pseudo first order rate constant is not linearly dependent on the initial concentration of the reactant in excess, regardless of whether it is the Grignard reagent or the alkyl cyanate. We therefore conclude that complexes between the cyanate

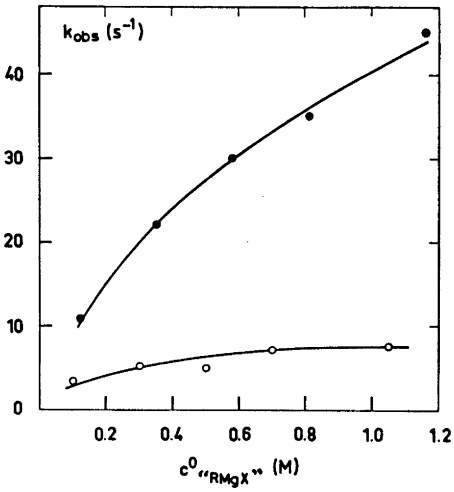


Fig. 6. Plot of the pseudo 1st order rate constant *versus* the initial concentration of the Grignard reagent for the reactions of isobutyl cyanate with "methylmagnesium bromide" (○) and "ethylmagnesium bromide" (●) in diethyl ether at 25 °C ($c^0_{i\text{-}C_4H_9OCN} = 0.010$ M).

* The contribution to the k_{obs}-value from the reaction between isobutyl cyanate and dibutylmagnesium present in 0.100 M "butylmagnesium bromide" can be calculated to be *ca.* 1.5 s^{-1}. The concentration of dibutylmagnesium in 0.100 M "butylmagnesium bromide" is *ca.* 0.0032 M for $K_s = 1 \times 10^{-3}$ (Ref. 4). The calculation of the contribution to the k_{obs}-value is made with the assumption that $k_{obs}(R_2Mg)$ is linearly dependent on the initial concentration of dibutylmagnesium in the interval 0–0.1 M; thus $k_{obs} = 47 \times 0.0032/0.100 = 1.5$ s^{-1}.

Table 2. Pseudo first order rate constants for the reaction of 0.010 M isobutyl cyanate with Grignard reagents and dibutylmagnesium in diethyl ether at 25°C ($c^0_{\text{"RMgX"}} = c^0_{R_2Mg} = 0.100$ M).

"RMgX" or R_2Mg	k_{obs} (s^{-1})
"*p*-$CH_3C_6H_4MgBr$"	619
"C_6H_5MgBr"	244
"*p*-ClC_6H_4MgBr"	85
$(C_4H_9)_2Mg$	47
"C_2H_5MgBr"	10
"C_4H_9MgBr"	4.4
"CH_3MgBr"	3.5
"*t*-C_4H_9MgCl"	< 3.5

and the magnesium species in the Grignard reagent have been formed.

In Fig. 6 are shown plots of the pseudo first order rate constant *versus* the initial concentration of the Grignard reagent for the reactions of isobutyl cyanate with "methylmagnesium bromide" and "ethylmagnesium bromide". The pseudo first order rate constant is not linearly dependent on the initial concentration of the Grignard reagent in these cases either. Therefore the mechanism of the reactions of isobutyl cyanate with these Grignard reagents is assumed to be the same as for the reaction with "butylmagnesium bromide".

Alkyl cyanates are found to react faster with aromatic Grignard reagents than with dibutylmagnesium and with aliphatic Grignard reagents (Table 2). An electron-donating substituent (CH_3) in the *p*-position of a "phenylmagnesium bromide" increases the reactivity, and on *p*-substitution with an electron-attracting group (Cl) the reactivity decreases (Table 2). From the k_{obs}-values and the Hammett *sigma* values a reaction constant of *ca.* −1.5 is calculated signifying electron deficiency at the magnesium carbon atom in the transition state. This effect of substitution is only slightly more pronounced than what we have found in reactions of phenyl cyanate with *p*-substituted "phenylmagnesium bromides". In this case the constant is *ca.* −0.8.[7]

A free radical mechanism for the reaction between alkyl cyanates and Grignard reagents can be excluded for the same reasons discussed in another paper [7] concerning the reaction between aryl cyanates and Grignard reagents.

Since complex formation takes place in these reactions two alternatives are possible which are kinetically indistinguishable. *1.* A complex is formed in equilibrium with the starting materials. Rate-determining decomposition of the complex leads to products. *2.* The complex formed is in equilibrium with the reactants but it does not lead to products but represents a "blind alley".

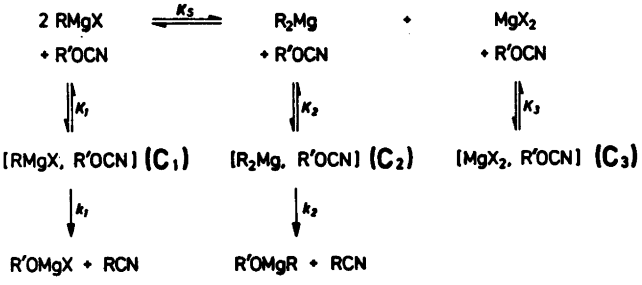
From the rate expression $v_{obs} = k_{obs} \times [R'OCN]_{total} = k_{obs}([R'OCN] + [C_1] + [C_2] + [C_3])$ together with $v = k_1[C_1] + k_2[C_2]$ and $v = k_1 \times [RMgX][R'OCN] + k_2[R_2Mg][R'OCN]$, respectively, for Scheme 1 and 2 the following equation is derived:

$$\frac{1}{k_{obs}} = \frac{1}{k'} \times \frac{1}{[RMgX]} + \frac{k''}{k'} \qquad (1)$$

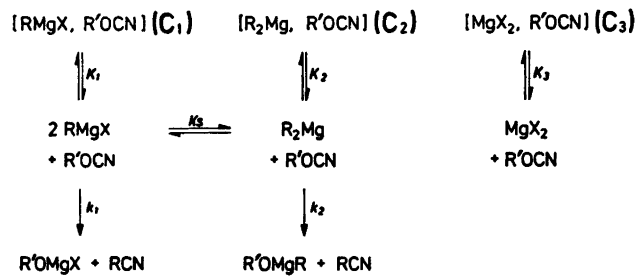
In case *1*, $k' = k_1K_1 + k_2K_2K_s^{\frac{1}{2}}$ and $k'' = K_1 + (K_2 + K_3)K_s^{\frac{1}{2}}$. In case *2*, $k' = k_1 + k_2K_s^{\frac{1}{2}}$ and $k'' = K_1 + (K_2 + K_3)K_s^{\frac{1}{2}}$.

As seen from Fig. 7 good agreement with eqn. (1) is obtained when $1/k_{obs}$ is plotted against 1/[RMgX], supporting the assumption of reversible complex formation.

The reactions between alkyl cyanates and Grignard reagents leading to nitriles and alco-

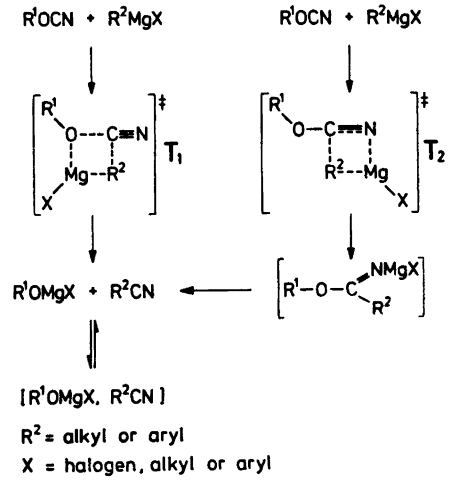


Scheme 1.



Scheme 2.

holates are strongly exothermic whereas complex formation between MgX_2 and cyanate does not give rise to any noticeable evolution of heat. According to the Hammond principle [8] the transition states leading to products should therefore be more similar to the reactants than to the products and likewise more similar to the complexes. Two transition states T_1 and T_2 in accordance with this formulation leading to the observed products have been visualized in Scheme 3. It is clearly not possible to distinguish between those two alternatives from the kinetics or from the final products. However, the presence of an intermediate such as the imido-ester salt shown in Scheme 3 certainly would point to the likelihood of T_2. Despite numerous



Scheme 3.

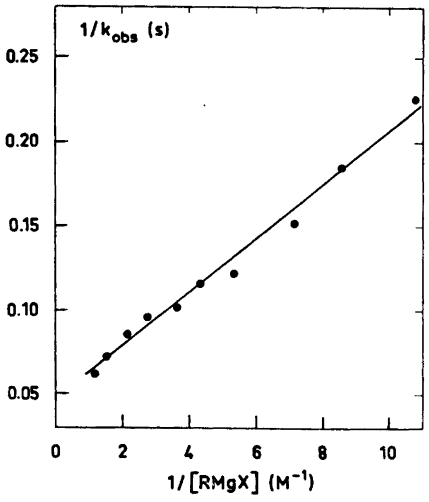


Fig. 7. Plot of $1/k_{obs}$ *versus* 1/[RMgX] for the reaction of isobutyl cyanate with excess "butylmagnesium bromide" in diethyl ether at 25 °C ($c^0_{i\text{-}C_4H_9OCN} = 0.010$ M).

attempts [1] it has, however, not been possible to obtain any evidence for this substance and no distinction between T_1 and T_2 can thus be made.

EXPERIMENTAL

All Grignard reagents were prepared in diethyl ether distilled from lithium aluminium hydride directly into the glass apparatus. This solvent was used in all experiments. The magnesium used (monosublimed, Dow Chemical Corp.) was washed with anhydrous diethyl ether. Every precaution was taken against oxygen and moisture. The halides used in the preparation of the Grignard reagents were distilled or recrystallized and their purity checked gas-chromatographically. Grignard reagents were always prepared with an excess of magnesium. The molarity of the Grignard reagents was determined by titration with standard acid and the content of halogen by titration with standard silver nitrate. The content of halogen was never more than 4 % higher than the content of Grignard reagent.

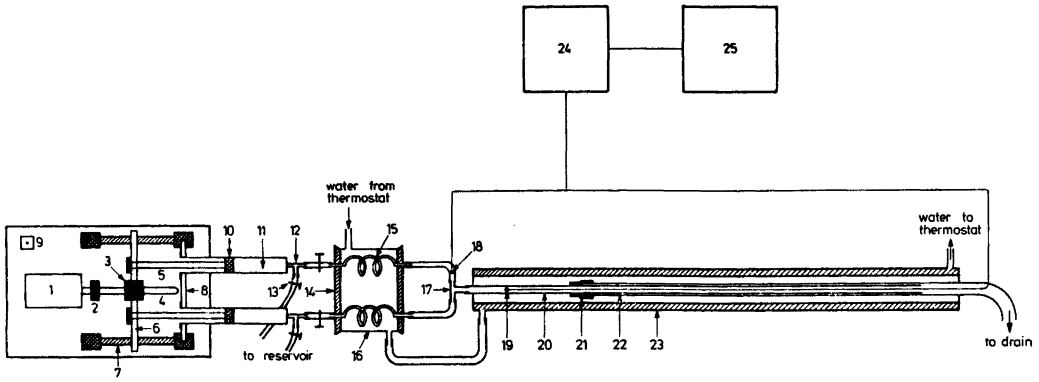


Fig. 8. Schematic drawing of the apparatus used for the kinetic measurements.

The different concentrations were obtained by dilution of *ca.* 2 M standard solutions.

The alkyl cyanates were prepared from 5-alkoxy-1,2,3,4-thiatriazoles.[9,10] The purity of the cyanates was checked by infrared and nuclear magnetic resonance spectroscopy and elemental analysis.

Dibutylmagnesium was prepared from "butylmagnesium chloride" and butyllithium.[11]

Magnesium bromide etherate was prepared from 1,2-dibromoethane and magnesium.[12]

Explanation of Fig. 8: 1. Berger synchronous motor, type RSM 65 NG, 220 V, geared down to 60 rpm; *2.* Ball bearing. *3.* Brass bushing with 14 mm thread, 1 mm pitch; *4.* Shaft with 14 mm thread, 1 mm pitch; *5.* Piston rod; *6.* Crossbar driving the pistons back and forth; *7.* Guide rod; *8.* Stand for piston cylinders; *9.* Switch; *10.* Teflon piston; *11.* Metrohm piston cylinder; *12.* Stainless steel T-tube, diameter 0.6 mm; *13.* Clamp; *14.* Rubber stopper; *15.* Stainless steel tubing, diameter 0.6 mm, length 1.5 m; *16.* Glass cylinder with circulating water from a thermostat; *17.* Stainless steel T-tube, diameter 0.6 mm; *18.* Reference thermocouple of copper-constantan; *19.* Measuring thermocouple of copper-constantan; *20.* Reaction tube, diameter 0.75 mm (Polystan polyethene tubing); *21.* Rubber tubing for fastening the outlet tubing to which the measuring thermocouple is fastened; *22.* Outlet tubing; *23.* Thermostated glass cylinder (air bath); *24.* Philips DC-microvoltmeter PM 2436; *25.* Philips recorder PM 8100. The thermocouples were soldered with tin. With 50 ml Metrohm piston cylinders a flow rate of 2788 mm/s is obtained; with 20 ml cylinders, the rate is 1152 mm/s.

Acknowledgement. The authors are very grateful to Professor Martin Ettlinger for his interest in and valuable discussions of this work.

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Studies on a Soluble Dipeptidase from Pig Intestinal Mucosa. Structural Properties

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A soluble dipeptidase (glycyl-L-leucine dipeptidase, EC 3.4.13.2) purified from pig intestinal mucosa was investigated for some of its structural properties. A molecular weight of 104 000 was determined under native conditions by gelfiltration but under denaturing conditions the molecular weight was 52 000 as determined by gelfiltration and polyacrylamide gel electrophoresis. These findings might suggest the native dipeptidase to be composed of two polypeptide chains of uniform molecular weight. The two polypeptide chains may be identical as only one N-terminal amino acid, proline, was found. The amino acid composition and the molar extinction coefficient (14.6×10^4 M^{-1} cm^{-1}) were determined. The soluble dipeptidase contained carbohydrate (about 0.3 %). The number of free SH-groups of the soluble dipeptidase was found to be about 5 when determined with *p*-hydroxymercuribenzoate but was higher (8 – 17) when determined with Ellman's reagent.

Little is known about the characteristics of the exopeptidases of the small intestine, a fact which is in contrast to the information available for the proteolytic enzymes of pancreas and ventricle. The purification and the specificity of a soluble dipeptidase (glycyl-L-leucine dipeptidase, EC 3.4.13.2) from the pig intestinal mucosa have earlier been reported.[1] The kinetics of the enzyme and the influence of some common enzyme inhibitors on its activity have been described.[2] In this paper the molecular weight and the amino acid composition are reported together with some other data on its structural properties.

MATERIALS AND METHODS

Enzyme. The soluble dipeptidase was prepared and tested for homogeneity as described earlier.[1] The enzyme preparation used had a specific activity of 1400 units [2] of activity per mg protein. It was stored (– 20°C) in 0.07 M sodium phosphate buffer containing 0.2 M NaCl, 4 mM 2-mercaptoethanol, and 12.5 % (w/v) glycerol and had a concentration of 0.4 mg per ml.

Chemicals. Bovine serum albumin was bought from Armour Pharmaceuphtical Co., Ltd., Eastbourne, England. Dansyl chloride, dansyl amino acids, acrylamide and *N,N'*-methylenebisacrylamide were purchased from BDH Chemicals Ltd., Poole, England. ε-Dansyl-lysine was a gift from Dr. Johan Stenflo, Malmö General Hospital, Malmö, Sweden. Yeast alcohol dehydrogenase was obtained from Calbiochem, San Diego, U.S.A. and bovine chymotrypsinogen from Mann Res. Labs., New York, U.S.A. HCl (suprapure), HBr (suprapure), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) and Schiff's reagent were manufactured by Merck, Darmstadt, Germany. Blue Dextran 2000, Sephadex G-200, Sephadex G-25 Fine, and Sepharose 6B were bought from Pharmacia, Uppsala, Sweden, and sodium dodecyl sulfate (sequanal grade) from Pierce Chemical Co., Illinois, U.S.A. *E. coli* alkaline phosphatase (type III), sperm whale myoglobin, egg albumin, sodium *p*-hydroxymercuribenzoate (PHMB), milk xanthine oxidase (grade II), human transferrin, *nor*-leucine, cysteic acid, and methionine sulfone were bought from Sigma Chem. Co., St. Louis, U.S.A. Nitrogen (oxygen free) was a product of Norsk Hydro A/S Oslo, Norway. Other chemicals used were of analytical grade and the water used was distilled and de-ionized.

Determination of molecular weight

Native conditions. For the estimation of the molecular weight of the soluble dipeptidase under native conditions the method of Andrews [3] was used. A Sephadex G-200 column was loaded with a sample containing xanthine oxidase, alcohol dehydrogenase, alkaline phos-

phatase, egg albumin, and the soluble dipeptidase. The sample applied was 12.5 % (w/v) in respect to glycerol and was then applied to the column underneath the eluent. Fractions of about 2.5 ml were collected and their precise volumes determined by weight. The concentrations of alcohol dehydrogenase and egg albumin in the fractions were analyzed by measuring the absorbance at 230 nm (PMQ II Spectrophotometer, Zeiss, Oberkochen, Germany). The concentration of dipeptidase (substrate: glycyl-L-leucine), alkaline phosphatase (substrate: *p*-nitrophenyl phosphate) and xanthine oxidase (substrate: hypoxanthine) in the fractions were measured using established methods.[4-6]

Denaturing conditions. The molecular weight of the soluble dipeptidase was also estimated under denaturing conditions using polyacrylamide gel electrophoresis and gelfiltration in dodecyl sulfate. Before the experiments the soluble dipeptidase and the molecular weight standards (serum albumin, egg albumin, chymotrypsinogen, myoglobin) were treated in 1 % (w/v) dodecyl sulfate and 1 % (v/v) 2-mercaptoethanol for 2 min at 100 °C.[7] In some experiments the soluble dipeptidase was pretreated with dodecyl sulfate only under the same conditions.

The polyacrylamide gel electrophoresis in dodecyl sulfate was performed essentially as described by Weber *et al.*[7] using a total monomer concentration in the gel of 10 % (w/v). The *N,N'*-methylenebisacrylamide was 1 % (w/v) of the total monomer concentration. About 20 μg of each protein were applied and the gels were stained with Coomassie Brilliant Blue. Three parallel gels were run in each experiment, one containing the molecular weight standards, a second the soluble dipeptidase, and a third the molecular weight standards and the soluble dipeptidase together.

Gelfiltration in dodecyl sulfate [8] was performed using Sepharose 6B. The column was eluted using a peristaltic pump (Varioperpex, LKB Produkter AB, Bromma, Sweden). Fractions of equal volume were collected each 15 min and their absorbance measured at 230 nm. The void volume in every experiment was determined by Blue Dextran. Before and after the experiments with the enzyme the column was calibrated with the molecular weight standards (100 μg of each applied).

Determination of amino acid composition and E(1 %, 1 cm) value

A solution of the dipeptidase (1 ml) was thoroughly dialyzed, (dialysis tubes, Visking Co., Chicago, U.S.A.) against 3 × 500 ml 0.05 M *N*-ethylmorpholine-acetate buffer (pH 8.5). The absorbance at 280 nm of the dialyzed sample was measured with the used dialysis buffer as a reference and the sample was then transferred to three different ampoules (300 μl in each). After lyophilization of the samples, 1 ml 6 M HCl and a crystal of phenol were added.[9] The ampoules were flushed with nitrogen and after a thorough evacuation [10] they were closed and hydrolyzed for different times (24, 48, 72 h) at 110 °C. After performed hydrolysis the HCl was removed, using a rotoevaporator (40 °C). The amino acid analysis was performed on an amino acid analyzer (Multichrom, Model 4255, Beckman, Munich, Germany) using the dual column system.[10]

The E(1 %, 1 cm) value was calculated from the absorbance and the amino acid analysis data using the finally calculated amino acid composition.

The amount of the sulfur containing amino acids of the soluble dipeptidase was determined using the oxidation with performic acid.[11] A solution of dipeptidase solution (300 μl) was dialyzed as described above. The sample was lyophilized, dissolved in 100 μl formic acid and 2 ml performic acid was added. As no precipitates were observed during the oxidation the process was interrupted after 4 h by 300 μl HBr (47 %) and then 15 ml of water was added and the sample was lyophilized. The hydrolysis (24 h) was performed as described above. The amount of cysteic acid and methionine sulfone formed was determined using the amino acid analyzer and their content was related to the leucine content. The yield of cystine (20 nmol) in the procedure was determined in separate experiments.

The tryptophan content was determined spectrophotometrically using the method of Edelhoch.[12] A dipeptidase solution (300 μl) was first dialyzed against 2 l 0.02 M sodium phosphate buffer (pH 6.5) and then against 200 ml 6 M guanidine chloride (Norit treated) in the same buffer. The absorbance of the sample was measured using the last dialysis buffer as a reference and afterwards given norleucine (50 nmol). It was then hydrolyzed (24 h) and analyzed for its amino acid content. The protein content was calculated both from the absorbance data and from the amino acid analysis.

Determination of N-terminal amino acid

The end-group analysis of the soluble dipeptidase was performed using the dansyl chloride method.[13] Prior to the reaction with dansyl chloride, the dipeptidase solution (300 μl) was made 1 % (w/v) in respect to dodecyl sulfate and 2-mercaptoethanol according to Weiner *et al.*[14] The dipeptidase solution was then dialyzed overnight against a 0.2 M *N*-ethylmorpholine-acetate buffer (pH 8.5) containing 0.16 % dodecyl sulfate and lyophilized. The lyophilized sample was dissolved in water and *N*-ethylmorpholine (50 μl of each) and then 75 μl of a dansyl chloride solution (25 mg/ml dimethylformamide) was added. The reaction

was interrupted after 3 h by 500 μl ice-cold trichloroacetic acid (20 % w/v) added drop by drop. The formed precipitate was spun down and washed four times with 500 μl 1 M HCl each time to get rid of the dansylic acid. To the dried precipitate 50 μl 6 M HCl was added and the sample was hydrolyzed for 16 h. After performed hydrolysis the HCl was removed in a rotoevaporator. The sample was then dissolved in 10 μl acetone-acetic acid (3:2, by vol.) and subjected to chromatography on polyamide sheets (Cheng Chin Trading Co., Taipei, Taiwan). The chromatographic systems used were water-90 % formic acid (200:3, by vol.) and benzene-acetic acid (9:1, by vol.).[15] In order to separate dansyl-arginine, dansyl-histidine, and ε-dansyl-lysine the additional solvent systems ethyl acetate-methanol-acetic acid (20:1:1, by vol.) and 0.05 M trisodium phosphate-ethanol (3:1, by vol.)[16] were run.

Determination of number of free SH-groups

The number of free SH-groups of the soluble dipeptidase was determined using Ellman's reagent [17] and PHMB.[18]

Prior to the reaction with Ellman's reagent 2-mercaptoethanol was removed from the dipeptidase solution using either dialysis against 3 × 1 l 0.05 M Tris-HCl buffer (pH 8.1), containing 10 mM EDTA or gelfiltration on a Sephadex G-25 column (0.9 cm × 11.7 cm) equilibrated in the same buffer. The absorbance of the sample at 280 nm was measured (0.15–0.30). To 300 μl of the sample were then added 10 % (w/v) dodecyl sulphate solution (15 μl) and Ellman's reagent (25 μl, 5 mg/ml methanol). The course of the reaction was followed by measuring the absorbance at 412 nm during a 30 min period and the concentration of free SH-groups was calculated using a molar extinction coefficient of 13 600 M^{-1} cm^{-1} for the 3-carboxylato-4-nitro-thiophenolate ion.[17]

Before the reaction with PHMB the dipeptidase solution (150 μl) was dialyzed overnight against 0.1 M sodium phosphate buffer (pH 7.5). The solution was measured for its absorbance at 280 nm, made 0.5 % (w/v) in respect to dodecyl sulfate and then added 75 nmol PHMB. The reaction was allowed to proceed overnight at room temperature. The mercury content of the dipeptidase was measured by a method developed in our laboratory using an atomic absorption spectrophotometer (Model 103, Perkin-Elmer, Norwalk, Connecticut, U.S.A.), equipped with a flameless mercury analysis system (Carlsen, J. B. *Anal. Biochem. In press*).

Determination of carbohydrate content

The carbohydrate content of the soluble dipeptidase was analyzed by polyacrylamide gel electrophoresis in combination with Schiff's reagent.[19] About 75 μg of the soluble dipeptidase was run on two parallel gels each. After completion of the electrophoresis, one gel was stained with Coomassie Brilliant Blue and the other one with Schiff's reagent. A dilution series of transferrin was run in parallel in order to get a semiquantitative estimation of the carbohydrate content of the dipeptidase.

RESULTS AND DISCUSSION

The molecular weight of the soluble dipeptidase was estimated to be 104 000 under native conditions (Fig. 1), assuming it to be globular and of the same grade of hydration as the molecular weight standards. In comparison to earlier reported molecular weight data on dipeptidases this value is close to that found for the purified intestinal monkey dipeptidase [20] but somewhat higher than observed for the mouse ascites tumor dipeptidase [21] and much higher than that reported for the renal dipeptidase.[22]

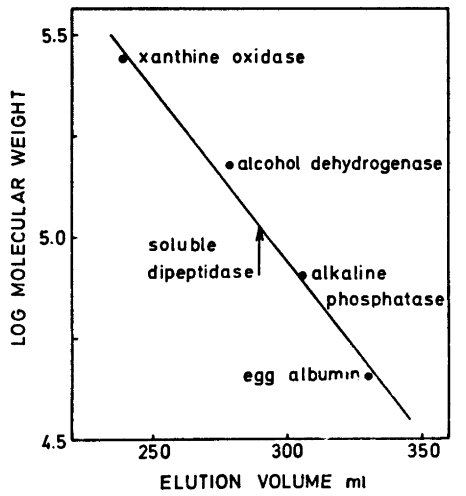

Fig. 1. Determination of the molecular weight of the soluble dipeptidase on Sephadex G-200. The column (2.5 cm × 93 cm) was equilibrated in and eluted with 0.1 M sodium phosphate buffer (pH 7.0), containing 4 mM 2-mercaptoethanol, at a constant flow rate of 5 ml/h. The sample (2.7 ml) applied contained 150 μg soluble dipeptidase.

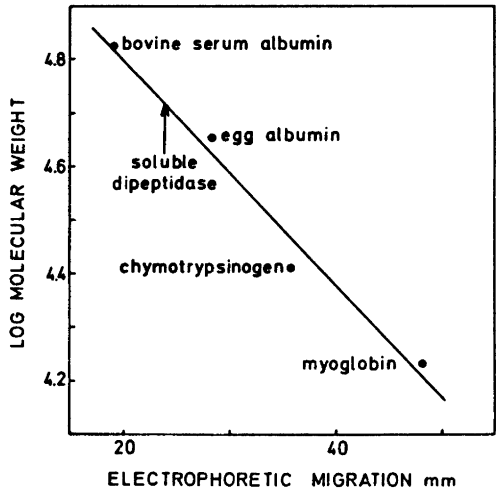

Fig. 2. Determination of the molecular weight of the soluble dipeptidase under denaturing conditions using polyacrylamide gel electrophoresis. The experiment was performed applying 8 mA per gel for 4 h.

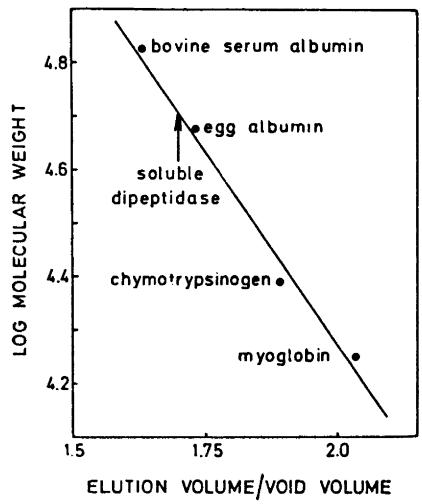

Fig. 3. Determination of the molecular weight of the soluble dipeptidase under denaturing conditions using gelfiltration on Sepharose 6B (room temperature). The column (0.9 cm × 57 cm) was equilibrated in and eluted with 0.07 M sodium phosphate buffer (pH 7.2), containing 1 % dodecyl sulfate, at a constant flow rate of 1.75 ml/h.

The molecular weight estimated for the reduced and dodecyl sulfate treated enzyme when determined by gel electrophoresis was 52 000 (Fig. 2). Irrespective of the enzyme being reduced and dodecyl sulfate treated or only dodecyl sulfate treated, the gel electrophoresis resulted in only one dominating band with a constant position, when the soluble dipeptidase was run alone. Likewise one peak corresponding to a molecular weight of 51 000 (Fig. 3) was obtained in the experiments on Sepharose 6B, irrespective of which of the two pre-treatments that had been used.

These molecular weight data may suggest the soluble dipeptidase to be composed of two polypeptide chains with a uniform molecular weight and associated with non-covalent bonds. Since N-terminal amino acid analysis revealed proline only, the two polypeptide chains might be identical.

The amino acid composition of the soluble dipeptidase is given in Table 1. Figures are given for mol % and for the number of amino acids per molecule of enzyme using a molecular weight of 104 000. The yield of cystine in the oxidation experiments with performic acid was determined to 83 % and this figure was used in the calculations. The cysteic acid and methionine sulfone content obtained in the amino acid analysis was related to the other amino acids by means of the leucine content. The figure for the tryptophan content was obtained by relating it to the leucine content and to the absorbance at 280 nm. In both cases a value of 18.4 mol of tryptophan per mol of dipeptidase was obtained.

A value of 14.0 for E(1 %, 1 cm) was found. Using a molecular weight of 104 000 the molar extinction coefficient was calculated to be 14.6×10^4 M^{-1} cm^{-1}.

The Ellman reaction with the soluble dipeptidase was rapid and complete within 10 min but the results obtained varied. When the 2-mercaptoethanol was removed by gelfiltration the reaction gave 12 – 17 mol SH-groups per mol dipeptidase while removal of the 2-mercaptoethanol by dialysis resulted in 8 – 11 mol SH-groups per mol dipeptidase. When the dipeptidase was analyzed with the PHMB-reagent only 4.8 – 5.1 mol SH-groups per mol enzyme were found showing that this reagent is less reactive to the soluble dipeptidase than Ellman's reagent. The number of free SH-groups obtained indicates that the soluble dipeptidase contains only few disulfide bonds, if any.

Table 1. Amino acid composition of the soluble dipeptidase. The values of the stable and quantitatively liberated amino acids are given as the mean of the results from the three different hydrolysis times with the coefficient of variation. The number of residues per molecule dipeptidase is calculated on the basis of a molecular weight of 104 000.

Amino acid	Mol %	Coefficient of variation (%)	Number of residues per molecule dipeptidase
Lys	7.05	3.9	66
His	2.56	5.3	24
Arg	3.76	3.5	35
Asx	10.11	0.77	94
Thr [a]	4.32	–	40
Ser [a]	6.06	–	57
Glx	11.03	1.3	103
Pro	4.84	2.2	45
Gly	8.87	4.7	83
Ala	6.89	0.78	64
Cys [b]	1.79	–	17
Val [c]	6.87	–	64
Met [b]	1.95	–	18
Ile [c]	5.47	–	51
Leu	9.40	1.4	88
Tyr [a]	3.60	–	34
Phe	3.55	5.6	33
Trp [d]	1.97	–	18
Total number of residues			934

[a] Extrapolated to zero time by assuming destruction according to a first order reaction.[10] [b] Values obtained after performic acid oxidation.[11] [c] Values obtained from 72 h hydrolysis. [d] Determined spectrophotometrically.[12]

By comparing the intensity of the color obtained with the Schiff reagent on the band of the soluble dipeptidase with the intensity of the bands obtained for transferrin, the carbohydrate content of the enzyme was estimated to be 0.3 %. Recently, the carbohydrate content of another pig intestinal peptidase has also been reported.[23]

A comparison with the prolidase recently reported purified from pig intestinal mucosa [24] and now investigated for its structural properties [25] shows the same molecular weight and subunit structure. It has a similar carbohydrate content. There are, however, some differences in the amino acid composition of the two enzymes. The content of tyrosine and tryptophan of the soluble dipeptidase is higher than that found for the prolidase. This finding is consistent with the differences found in the $E(1\ \%, 1\ \text{cm})$ values for the two enzymes. The two enzymes also display differences in their covalent structure, which may explain the differences in stability of the enzymes and in their reactivity to Ellman's reagent.

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Triterpenes. The Synthesis of Novel 18βH,19β-Substituted Lupane Derivatives

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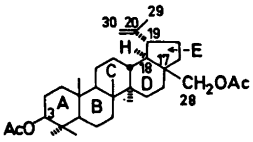
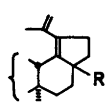
Catalytic hydrogenation of the conjugated diene (II), obtained from betulin diacetate (I) by mercuric acetate dehydrogenation, has been shown to proceed *via* 1,2- and 1,4-addition to compounds (IVb) and (VIIa). The latter is capable of further hydrogenation to (VIb). Acid catalysed isomerisation of (VIIIa) gives a novel isomer (XII) of betulin diacetate (I).

Betulin diacetate (I) gives on dehydrogenation with mercuric acetate the conjugated diene (II).[1,2] Analogously [3] to the corresponding lupane derivative (III), the hydrogenation of (II) with PtO_2-catalyst gives a mixture of the dihydro diacetate (IVb) with an endo double bond in ring E, and a tetrahydro diacetate of unknown stereochemistry at C-18 and C-19, differing from dihydrobetulin diacetate (V). For this compound we now present formula (VIb), which follows from the results outlined below.



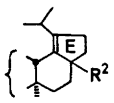
I



3ß-OAc

II R = $-CH_2OAc$

III R = $-CH_3$

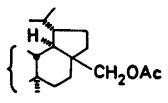


3ß-OR^1

IVa R^1 = -H, R^2 = $-CH_2OH$

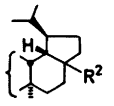
IVb R^1 = -Ac, R^2 = $-CH_2OAc$

XIV R^1 = -Ac, R^2 = $-CH_3$



3ß-OAc

V

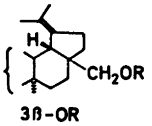


3ß-OR^1

VIa R^1 = -H, R^2 = $-CH_2OH$

VIb R^1 = -Ac, R^2 = $-CH_2OAc$

XVII R^1 = -Ac, R^2 = $-CH_3$



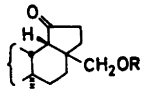
3ß-OR

VIIa R = -Ac

VIIb R = -H

The hydrogenation of diene (II) in the presence of Pd/$BaSO_4$-catalyst gives quantitatively the 1,4-addition product with the double bond exo to ring E. The stereochemistry at C-18 is determined from the reactions presented below and is found to be 18β H as in formula (VIIa). Ozonisation of the exo double bond in compound (VIIa) and reduction of the ozonide with $(MeO)_3P$ under neutral conditions gives a five membered ring ketone, which has a large positive value of molecular ellipticity $[\theta]_{313}$ +6270° indicating [4] a 18βH-structure for the ketone (VIIIa). The m.p., $[\theta]$, and $[\alpha]_D$ are in agreement with values reported for the compound (VIIIa).[5-7]

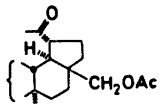
Acid catalysed isomerisation of the exo double bond in the diacetate (VIIa) gives another diacetate having an isopropenyl side chain and being different from betulin diacetate (I). Ozonolysis of the terminal methylene group gives a 19-acetyl derivative, which does not epimerise even under alkaline hydrolysis. It is known [5] that the 18αH,19αAc-isomer (IX) [5,8] is stable as such and this side chain will be base epimerised into the unknown 18αH,19βAc-isomer only under special circumstances involving further reaction with the 17β-CH_2OH group to give the hemiacetal (X).[5] As the new 19-acetyl compound is different from the stable



3ß-OR

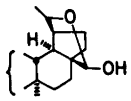
VIIIa R = -Ac

VIIIb R = -H



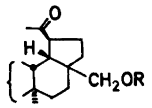
3ß-OAc

IX



3ß-OH

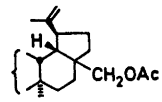
X



3ß-OR

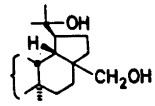
XIa R = -Ac

XIb R = -H



3ß-OAc

XII



3ß-OH

XIII

18αH,19αAc-isomer and *tert*-BuO⊖ only hydrolyses it, and does not give the hemiacetal (X), which should be formed from the 18αH,19βAc-isomer, the stereochemistry at C-18 must be 18βH. This stereochemistry also applies to the preceding isopropenyl compound. The CD curve of the new 19-acetyl derivative ($[\theta]_{281} - 3990°$) gives the 19β-acetyl stereochemistry for the compound, when the octant rule projection is drawn with the least hindered conformation as in Fig. 1.[4] The large [θ] value indicates hindered rotation about the C-19/C-20 axis and is caused mainly by the hydrogens at C-12. According to Dreiding models the corresponding 18βH,19αAc-derivative would be highly crowded and hardly

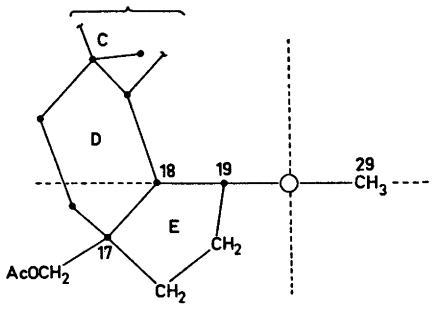
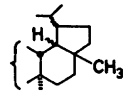


Fig. 1.

capable of resisting epimerisation. For the reasons given above we present the 18βH,19βAc-structure (XIa) for the new acetyl compound and correspondingly the 19β-isopropenyl side chain structure (XII) for its precursor, an isomer of betulin diacetate (I).

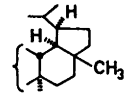
The treatment of the 19β-acetyl compound (XIa) with methyllithium gave the triol (XIII), which on reacetylation lost a molecule of water and gave mainly the terminal methylene compound (XII) and a small amount of the tetrasubstituted exo double bond compound (VIIa). This reaction and the ozonolysis of (VIIa) to the ketone (VIIIa) indicates that C-18 does not become involved in the acid catalysed isomerisation of the tetrasubstituted exo double bond compound (VIIa) and thus the 18βH-structure is correct for the compound (VIIa).

Hydrogenation of the terminal methylene in the 19β-isopropenyl compound (XII) gave a dihydro derivative, identical with the tetrahydro compound from the hydrogenation of the diene (II) (IR, ^{1}H NMR, mixed m.p., $[\alpha]_D$, mass spectra, TLC). These hydrogenation products therefore have the 18βH,19β-isopropyl structure (VIb). Hydrogenation of the lupane derivative (III) with PtO_2-catalyst has been reported [3] to give a mixture of the dihydro compound (XIV) and tetrahydro compound (XV). Because the amount of the tetrahydro compound (XV) does not increase with a prolonged reaction time, it was assumed [3] that (XIV) and (XV) must be



3ß-OAc

XV



3ß-OAc

XVI

formed *via* different routes. However, it has been suggested [3] that the tetrahydro compound (XV) is formed by addition of hydrogen from the α-face to the dihydro compound (XIV), a mechanism which leads to 18αH,19β-isopropyl structure (XVI). The hydrogenation of the betulin derived diene diacetate (II) under the same reaction conditions with PtO_2-catalyst gives similary two compounds (IVb) and (VIb) and the amount of the tetrahydro derivative (VIb) does not increase during extended reaction time. Because the tetrahydro compound (VIb) has *trans*-hydrogens at C-18 and C-19 it

cannot be formed *via cis*-addition of hydrogen to dihydro compound (IVb). Instead its formation by the addition of hydrogen from α-face to the 1,4-addition product (VIIa) is possible, and, indeed, the hydrogenation of the exo double bond compound (VIIa) with PtO_2-catalyst gave the same tetrahydro compound (VIb) as obtained from the diene (II) under the same conditions. Thus the hydrogenation of diene (II) with PtO_2-catalyst in EtOAc-AcOH solution gives first both the dihydro compound (IVb) and dihydro compound (VIIa). The former resists further hydrogenation while the latter gives the tetrahydro compound (VIb).

Finally, we note that the *cis*-structure (XVI) assigned,[2] without proof, to the tetrahydro derivative from the diene (III), should probably be replaced by the *trans*-structure (XVII), in view of our results in the betulin series (*i.e.*, II→VIb).

EXPERIMENTAL

Melting points are uncorrected. ^{1}H NMR spectra were recorded on a Varian A-60 spectrometer in $CDCl_3$ (unless stated otherwise) and related to internal TMS, the IR spectra on a Perkin-Elmer 125 spectrometer using KBr pellets, mass spectra on a Perkin-Elmer 270 B mass spectrometer, CD curves in dioxane solution on a Cary 61 spectrorotameter, specific rotations in $CHCl_3$ solution (unless stated otherwise) on a Perkin-Elmer 141 polarimeter, and elemental analyses with a F&M 185 CHN-analyser.

The typical values for the hydroxy- or acetoxy-, 3αH, and 17β-CH_2-groups, present in all the synthetised compounds, are omitted from the spectral data.

Hydrogenation of 3β,28-diacetoxy-lupa-18,20-(30)-diene (II).(a) *With PtO_2-catalyst.* 3β,28-Diacetoxy-lupa-18,20(30)-diene (II) [1,2] (3 g) and PtO_2-catalyst (0.6 g) in EtOAc-AcOH (1:1, 170 ml) were shaken for 20 h under hydrogen at room temperature and normal pressure. After that, the reaction mixture contained, according to TLC, two compounds at ratio of about 3:7, which does not change on prolonged reaction time. The catalyst was filtered off and the solvent removed. For better resolution of the two compounds the reaction mixture was hydrolysed by refluxing in KOH/EtOH for 1 h, worked up, and chromatographed on silica plates impregnated with 10 % $AgNO_3$.

The less polar 3β,28-dihydroxy-18β(H),19α-(H)-lupane (VIa) (0.4 g), recrystallised from EtOH, had m.p. 255 °C, $[\alpha]_D$ +11° (*c* 1.45), ^{1}H NMR ($CDCl_3$-CS_2) δ 0.68 – 0.95 (7 Me groups), (Found: C 81.29; H 11.87. Calc. for $C_{30}H_{52}O_2$: C 81.02; H 11.79). Diacetate (VIb), from (VIa) by refluxing 10 min in Ac_2O, had m.p. 210 °C (Ac_2O), $[\alpha]_D$ +20° (*c* 1.5), M+ 528.

The more polar 3β,28-dihydroxy-lup-18-ene (IVa) (1.6 g), recrystallised from EtOH, had m.p. 220 °C, $[\alpha]_D$ −24° (*c* 1.1), δ 0.75 – 1.05 (7 Me groups), 2.05 – 2.5 (3H, m), 3.0 – 3.8 (4H, m). (Found: C 81.33; H 11.60. Calc. for $C_{30}H_{50}O_2$: C 81.39; H 11.38). Diacetate (IVb) m.p. 211 °C, $[\alpha]_D$ +15° (c 1.5), δ 0.82 – 1.05 (7 Me groups).

(b) *With Pd/$BaSO_4$-catalyst.* 3β,28-Diacetoxy-lupa-18,20(30)-diene (II) [1,2] (2 g) and 10 % Pd/$BaSO_4$-catalyst (0.4 g) in EtOAc (140 ml) were shaken for 17 h under hydrogen at room temperature and normal pressure. Only one compound resulted according to TLC. Catalyst was filtered off and the solvent evaporated. Crystallisation from EtOH yielded 3β,28-diacetoxy-18β(H)-lup-19(20)-ene (VIIa), (1.8 g), m.p. 197 °C, $[\alpha]_D$ −19° (*c* 1.15), M+ 526, δ 0.83 – 0.95 (5 Me groups), 1.68 (2 Me, s), 2.2 – 2.6 (3H, m). (Found: C 77.69; H 10.58. Calc. for $C_{34}H_{54}O_4$: C 77.52; H 10.33). Hydrolysis with KOH/EtOH gave the diol (VIIb), m.p. 177 °C, $[\alpha]_D$ −36° (*c* 1.21), δ 0.75 – 0.95 (5 Me groups), 1.70 (2 Me, s), 2.15 – 2.6 (3 H, m).

Ozonolysis of 3β,28-diacetoxy-18β(H)-lup-19-(20)-ene (VIIa). 3β,28-Diacetoxy-18β(H)-lup-19(20)-ene (VIIa) (1 g) in EtOAc-CH_2Cl_2 (1:1, 75 ml) was ozonised at −75 °C until the solution remained slightly blue. Excess ozone was driven off with a nitrogen stream and the ozonide reduced with $(MeO)_3P$. The mixture contained (TLC) one major and several minor components. The major component was separated by chromatography on silica plates with $CHCl_3$ eluent. Recrystallisation from EtOH gave 3β,28-diacetoxy-20,29,30-trisnor-18β(H)-lupan-19-one (VIIIa) (0.35 g), m.p. 250 °C, $[\alpha]_D$ +31° (*c* 1.12) (Ref. 5, m.p. 250 – 253 °C, $[\alpha]_D$ +35.5°), $[\theta]_{313}$ +6270°. ν_{max} 1730, δ 0.70 – 0.98 (5 Me groups), 4.05 – 4.35 (2 H, m). Hydrolysis in KOH/EtOH gave the diol (VIIIb) m.p. 263 °C (EtOH), $[\alpha]_D$ +31° (THF, *c* 1.13) (Ref. 7, m.p. 254 – 6 °C, $[\alpha]_D$ +40.5° $[\theta]_{313}$ +7260°), ν_{max} 1730. Reacetylation of VIIIb) with Ac_2O gave (VIIIa).

Isomerisation of 3β,28-diacetoxy-18β(H)-lup-19(20)-ene (VIIa). 3β,28-Diacetoxy-18β(H)-lup-19(20)-ene (VIIa) (1.5 g) and *p*-toluenesulfonic acid (0.2 g) in AcOH (150 ml) were refluxed for 1.5 h. Reaction mixture contained, according to TLC, starting material and a less polar compound. Chromatography on silica impregnated with 10 % $AgNO_3$ with light petroleum (b.p. 60 – 80 °C)-benzene (3:1) eluent gave 3β,28-diacetoxy-18β(H),19α(H)-lup-20(30)-ene (XII). Recrystallisation from EtOH gave (0.6 g) m.p. 174 °C, $[\alpha]_D$ +6° (*c* 1.04), ν_{max} 3080, 1640, 885, δ 0.85 (5 Me groups), 0.99, 1.07, 1.70 (à 1 Me, s), 2.9 (1 H, m), 4.7 (2 H, br.d. 6 Hz). (Found: C 77.96; H 10.33. Calc. for $C_{34}H_{54}O_4$: C 77.52; H 10.33).

Ozonolysis of 3β,28-diacetoxy-18β(H),19α(H)-lup-20(30)-ene (XII). 3β,28-Diacetoxy-18β-(H),19α(H)-lup-20(30)-ene (XII) (0.35 g) in

$CHCl_3$-CH_2Cl_2 solution (1:1, 50 ml) was ozonised at −75 °C until slightly blue. Unreacted ozone was driven off with nitrogen and the ozonide reduced with $(MeO)_3P$. Chromatography and recrystallisation from EtOH gave 3β,28-diacetoxy-30-nor-18β(H),19α(H)-lupan-20-one (XIa) (0.15 g), m.p. 207 °C, $[\alpha]_D$ +27° (*c* 1.25), $[\theta]_{281}$ −3990°, ν_{max} 1725, δ 0.83 (3 Me groups), 0.79 (2 Me groups), 2.17 (1 Me, s), 3.1 (1 H, m). (Found: C 74.95; H 9.94. Calc. for $C_{33}H_{52}O_6$: C 74.96; H 9.91). Hydrolysis with KOH/EtOH gave the diol (XIb) m.p. 234 °C (EtOH), $[\alpha]_D$ +22° (*c* 1.0), ν_{max} 1695, 1355, δ 0.75−0.97 (5 Me groups), 2.17 (1 Me, s). Compound (XIa) does not react on standing for 3 h in AcOH-TsOH solution, and refluxing (XIa) in *tert*-BuOH with *tert*-BuO^-K^+ only hydrolyses (XIa) to (XIb).

Methylation of 3β,28-diacetoxy-30-nor-18β-(H),19α(H)-lupan-20-one (XIa). 3β,28-Diacetoxy-30-nor-18β(H), 19α(H)-lupan-20-one (XIa) (0.25 g) in abs. ether (50 ml) was purged with a nitrogen stream, cooled to −75 °C and 1 ml of methyllithium (2 M-solution in ether) was added. The reaction mixture was allowed to warm slowly to room temperature, washed with water, dil. H_2SO_4, $NaHCO_3$, and dried. Chromatography on silica plates gave two compounds. The less polar compound was found to be hydrolysed starting material, 3β,28-dihydroxy-30-nor-18β(H), 19α(H)-lupan-20-one (XIb), and the major component was 3β,20,28-trihydroxy-18β(H), 19α(H)-lupane (XIII), crystallised from EtOH (0.13 g), m.p. 220 °C, $[\alpha]_D$ −4° (*c* 0.87), δ 0.75−1.20 (7 Me groups). Acetylation of (XIII) by refluxing in Ac_2O for 10 min gave approximately 1:9 mixture of diacetates (VIIa) and (XII) resulting from dehydration and acetylation reactions, respectively.

Hydrogenation of 3β,28-diacetoxy-18β(H), 19α-(H)-lup-20(30)-ene (XII) and 3β,28-diacetoxy-18β(H)-lup-19(20)-ene (VIIa). Both diacetates (XII) and (VIIa) were hydrogenated as the diene diacetate (II) above with PtO_2-catalyst. Both reactions gave only one compound on TLC and both were identical (m.p., mixed m.p., [α]d, IR, ^1H NMR, TLC, mass spectra) with the tetrahydro compound (VIb) from the hydrogenation of the diene (II).

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A Comparison of Three Phenol Methods for Extraction of Rat Liver RNA

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Three commonly used phenol methods for extraction of rat liver total RNA, requiring the use of 4-aminosalicylate, naphthalene-1,5-disulfonate, and heat, respectively, have been compared.

Different amounts of giant heterogeneous nucleoplasmic RNA were isolated by the three methods. The amounts isolated, being dependent upon the ionic strength of the extraction liquid, were inversely related to the amounts of rapidly labeled RNA present in the 4 – 28 S region. No method was able to extract additional rapidly labeled RNA after extraction by any of the other methods, suggesting that the differences observed in the patterns were caused by aggregation/disaggregation during extraction.

A previously undescribed, extremely rapidly labeled, small molecular fraction was isolated, more abundantly by the 4-aminosalicylate method than by the others. It was tentatively identified as growing polynucleotide chains. The amounts isolated were partly dependent upon the ionic strength of the aqueous phase used for extraction.

Isolated rat liver total RNA may give different sedimentation and electrophoretic patterns according to the method of extraction (*e.g.*, the methods of Parish and Kirby,[1] Attardi *et al.*,[2] Warner *et al.*[3]). The differences are found both for rapidly labeled RNA (rlRNA) including giant heterogeneous nucleoplasmic RNA (giant hnRNA), and for ribosomal RNA (rRNA). This may be explained by differential extraction, configurational changes, aggregation/disaggregation phenomenons, or by splitting of covalent bonds during the extraction.

The present comparison of the above-mentioned methods was performed as part of an attempt to determine which of the isolated RNA structures represented *in vivo* existing molecules, and which were caused by the extraction procedures.

MATERIALS AND METHODS

Animals. Male 250-g CD*F inbred rats (Charles River Breeding Laboratories, Wilmington, Mass.), fed *ad libitum*, were killed at intervals varying from 1 min to 48 h after intraperitoneal injection of radioactive precursors.

Chemicals. Orotic acid-5-^{3}H (22.3 Ci/mmol) and orotic acid-6-^{14}C (60.8 mCi/mmol), The Radiochemical Centre, Amersham, United Kingdom. Pronase (B-grade), Calbiochem. Ribonuclease II-A and Deoxyribonuclease (DN-C), Sigma Chemical Company. The latter was freed of RNAase by isoelectric focusing in a 4 – 6 pH-gradient, and stored frozen. (Both Worthington RNAase-free DNAase and Sigma electrophoretically purified DNAase were found to contain traces of RNAase.) Purified human salivary α-amylase, gift from Dr. A. Hensten Pettersen, Department of Microbiology, Dental Faculty, University of Oslo.

Extraction procedures. The extraction procedures require the use of 4-aminosalicylate, naphthalene-1,5-disulfonate, and heat (60 °C), respectively, for the release of rlRNA. To minimize the possibility of degradation by RNAase, of aggregation due to salting-out procedures [4] or to high temperature,[5] DNA and glycogen were removed by specially purified enzymes, and heat extraction was performed at 55 °C. Bentonite (treated according to Fraenkel-Conrat *et al.*[6]), 1 mg/ml, and dextran sulfate, 0.1 mg/ml, were added to all aqueous solutions except to the TKM-buffer (Tris 0.05 M, KCl 0.025 M, $MgCl_2$ 2.5 mM; pH 7.0) used for incubation with DNAase and amylase, where

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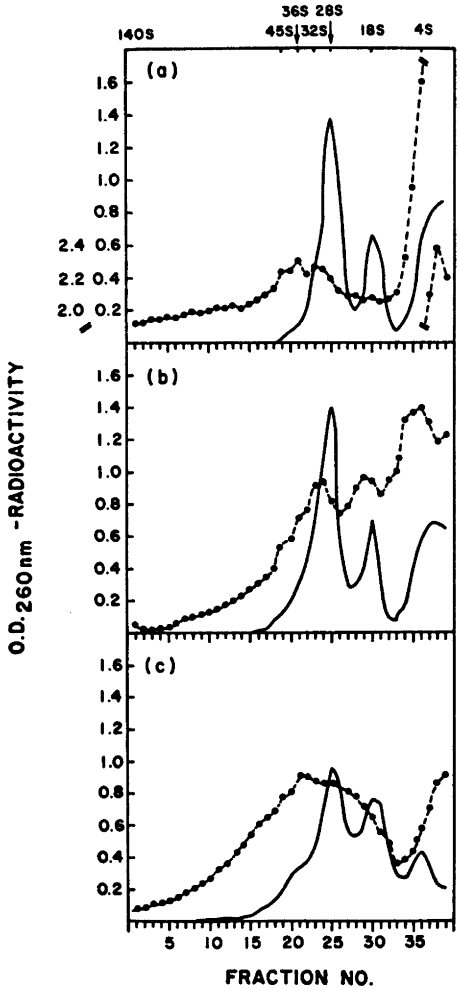
bentonite was omitted. All glassware was cleaned with chromic-sulfuric acid, 0.1 M sodium hydroxide, or 2 % w/v SDS prior to rinsing in doubly distilled water. To avoid selective loss of low-molecular-weight RNA, all collections of precipitates were performed by centrifugation of ethanolic solutions at not less than 10 000 *g* for 1½ h. Phenol and *m*-cresol were distilled under reduced pressure before use.

Method 1 was based upon the 4-aminosalicylate method of Parish and Kirby.[1] However, the extraction was performed at 4 °C, and DNA and glycogen were removed by exposure to 250 μg DNAase and 500 μg amylase in 25 ml TKM-buffer at 25 °C for 1 h. The enzymic treatment was terminated by shaking with 2 % w/v SDS and ½ volume phenol reagent at 4 °C for 15 min, and the nucleic acids recovered from the aqueous phase by ethanol precipitation.

Method 2 (modified from Attardi *et al.*[2]). The aqueous phase, used for homogenization and extraction, consisted of 2 % w/v SDS and 0.5 % w/v sodium naphthalene-1,5-disulfonate in TKM-buffer. Otherwise, the procedure was identical to Method 1.

Method 3 (from Warner *et al.*[3]). This method was also identical to Method 1, except for the



METHOD 1

GRADIENT	38400	dpm
PELLET	19700	"
TUBE	1400	"
TOTAL	59500	dpm

METHOD 2

GRADIENT	38300	dpm
PELLET	1100	"
TUBE	0	"
TOTAL	39400	dpm

METHOD 3

GRADIENT	37100	dpm
PELLET	2750	"
TUBE	0	"
TOTAL	39850	dpm

Fig. 1a–c. Ultracentrifugation of rat liver total RNA isolated by the three methods. 1 mg RNA, extracted 20 min after administration of 50 μCi ^{14}C-orotic acid, was dissolved in 0.1 M sodium acetate pH 6.0 and applied directly on 30 ml 10–40 % w/w linear sucrose gradients made up in the same buffer. (a) RNA_1; (b) RNA_2; (c) RNA_3. Spinco SW 25.1 rotor, 24 000 rpm, 5 °C, 14 h. ——— OD_{260}; – – – Radioactivity (dpm × 5 × 10^{-4}).

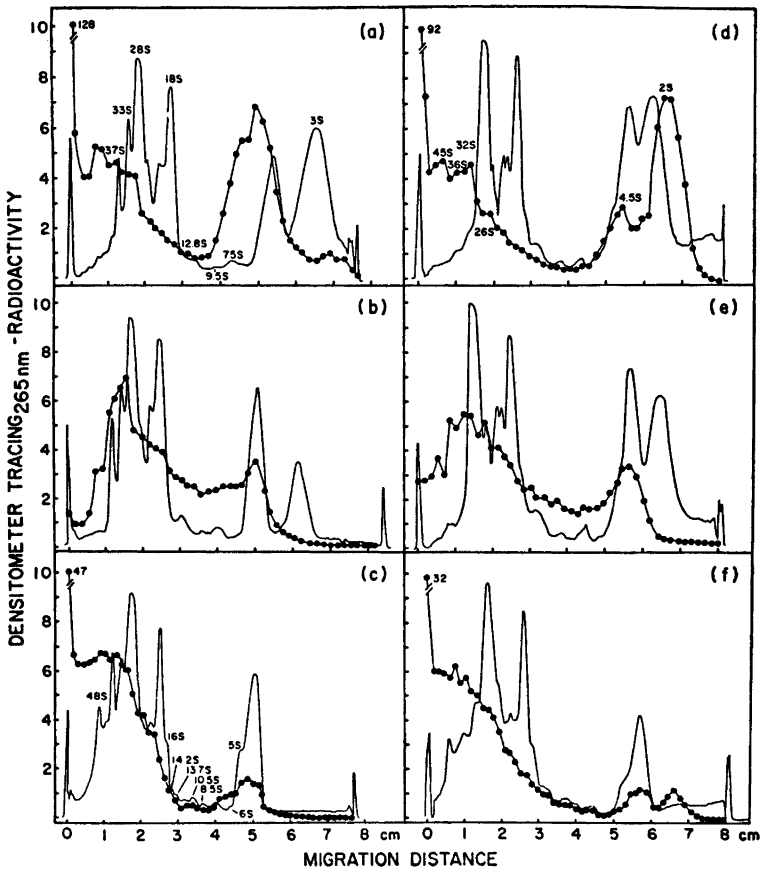


Fig. 2a–f. Gel electrophoresis of 300 μg rat liver total RNA labeled for 20 min, in magnesium-buffer (a–c), and in EDTA-buffer (d–f). (a,d) RNA_1; (b,e) RNA_2; (c,f) RNA_3. 2.6 % w/v acrylamide, 12 mA/tube, 20 °C, 2 ¾ h. ——— Densitometer tracing (265 nm); ● Radioactivity (dpm/mg gel).

following: The aqueous phase consisted of 1 % w/v SDS and 10 mM EDTA in 0.05 M sodium acetate pH 5.1. Extraction was performed by heating at 55 °C (true temperature of the solution) for 3 min before chilling. After re-extraction at 55 °C, the combined aqueous phases were treated with ½ volume phenol at 4 °C.

In the exhaustive extractions, temperatures gradually increasing up to 100 °C were used for all methods.

Ultracentrifugation. Routinely 1 mg RNA, dissolved in 1 ml gradient buffer, was applied on top of linear 30 ml 10–40 % w/w sucrose gradients containing 0.1 M sodium acetate pH 6.0, and run at 5 °C and 24 000 rpm for 14 h in a Spinco SW 25.1 rotor. Fractions of 0.8 ml were collected, diluted to 2 ml, and read spectrophotometrically. Liquid scintillation counting was performed as described previously.[7] *s* values were approximated by the method of Martin and Ames.[8]

Gel electrophoresis. Homogeneous 2.6 % acrylamide gels with a diameter of 10 mm and a length of 8 cm were run and scanned as described.[9] As the electrophoretic conditions were found to be disaggregating, electrophoresis was performed both with magnesium in the buffer for stabilization, and in EDTA-buffer. The migration of the individual fractions was affected to a slightly different degree. The buffers consisted of 2 % v/v glycerol in 0.05 M sodium phosphate with 1 mM EDTA (EDTA-buffer) or with 5 mM magnesium acetate (magnesium-buffer) pH 6.25. After scanning, the gels were frozen, and cut into 1.5 mm slices. These were weighed, hydrolyzed,[10] and counted.

RESULTS AND DISCUSSION

*Characteristics of RNA_1, RNA_2 and RNA_3.** By all methods 40 – 50 mg RNA was extracted per liver. The specific activity of RNA_1 was some 50 % higher than that of the others (Fig. 1a – c).

UV-patterns. All materials had UV-absorbing peaks in the region > 28 S, representing aggregates of rRNA (Fig. 2a – c). The aggregates of RNA_1 and RNA_2 were to a large extent dissociated by electrophoresis in EDTA-buffer at room temperature (Fig. 2d,e), while the more abundant aggregates of the hot-phenol-extracted RNA_3 were harder to disaggregate (Fig. 2f).

When run in magnesium-buffer, more peaks were visible between the ribosomal peaks and in the 4 – 18 S region in RNA_3 than in the cold-extracted RNAs (Fig. 2a – c). These extra peaks were quite reproducible on careful electrophoretic examination. Traces of several of them could be seen when RNA_1 and RNA_2 were run under more dissociating conditions (Fig. 2d,e). Ultracentrifugation demonstrated the same differences between the materials as gel electrophoresis, although the resolution was less (Fig. 1a – c). Furthermore, the 28 S RNA_3 peak then appeared lower and broader than the other 28 S peaks, and it often sedimented more slowly.

Dissociation of 5 S RNA from nuclear 28 S RNA,[11] and formation of rRNA aggregates during heating are probably responsible for part of the reduction of the 28 S RNA_3 peak. Its broadening and reduction in sedimentation rate indicate that conformational changes may be caused by the heating. The increased amount of UV-absorbing material between the ribosomal peaks and in the 4 – 18 S region might partly be derived from ribosomal RNA. This is supported by the release of corresponding material in RNA_1 and RNA_2 upon heating.[12]

The 3 S peak was not labeled using orotic acid as radioactive precursor, and probably represents DNA breakdown products from the DNAase treatment.

Radioactivity patterns. Varying amounts of rapid label remained confined to the top slices of the gels (> 60 – 70 S) (Fig. 2a – c). After labeling for 20 min, only about 50 % of $rlRNA_1$ entered the gels. Almost all $rlRNA_2$ entered, while 10 – 15 % of $rlRNA_3$ was excluded. Except for the material in the 1 – 3 S rlRNA peak (see below), the excluded material was the most rapidly labeled. Upon ultracentrifugation, all RNA sedimenting faster than 65 S had the same labeling kinetics, suggesting that these molecules all belong to the same group. $rlRNA_1$ tended to adhere to the nitro-cellulose tube walls when centrifuged. Because of the great differences in the patterns, at least two – if not all three methods – must give distorted pictures of the *in vivo* state of rlRNA.

The radioactivity in the 4 – 28 S region was quantitatively inversely related to the amount of the appearent giant rlRNA. In doubly labeled RNA, the short label: long label ratio in the 4 – 28 S region was slightly lower than in the rapidly sedimenting RNA. This could indicate that the label in the 4 – 28 S region represents the same molecular groups as the giant structures, in addition to some other, more slowly labeled molecules.

In RNA_1 an additional peak of radioactivity, 25 – 30 % of the total, migrated in the 6 – 8 S region or in the 1 – 3 S region, depending on the electrophoresis being performed in magnesium- or EDTA-buffer. It always sedimented as a 1 – 3 S peak. It was inconstantly present in RNA_2 and RNA_3. Only traces were regained after ethanol precipitation from EDTA-buffer. After thorough DNAase degradation during extraction, so that no 3 S UV-absorbing material was left, it was absent. It was the first fraction to become labeled, with significant amounts of label already after 2 min, and with maximal labeling after 10 min. For all labeling times it retained a strikingly solitary character, never merging with the RNA of higher molecular weight.

Chromatin associated 3 S RNA has been described.[13–16] The existence of this RNA as a separate entity has recently been questioned.[17–19] The smaller size of our 1 – 3 S rlRNA and its extremely rapid labeling do not indicate similarity between these fractions. According to these parameters our fraction may represent growing polynucleotide chains. During the isolation, it is possibly precipitated bound to DNA or to other macromolecular structures. The electrophoretic migration at 6 – 8 S in

* Subscript 1, 2, or 3: That which pertains to Extraction method 1, 2, or 3.

magnesium-buffer could be caused by charge differences, as it sedimented at 1 – 3 S, irrespective of EDTA or magnesium being present in the buffer.

Exhaustive extraction. To investigate whether the differences in the rapid label patterns were caused by selective extraction of rlRNAs, the extraction according to each of the methods was repeated until no more radioactivity was released into the aqueous phase. No additional radioactivity could then be extracted by any of the methods, even if the temperatures were gradually increased to 100 °C. Any residual interphase contained only negligible amounts of radioactivity when solubilized and counted directly. These studies, as well as the labeling kinetics, and the conversion studies performed by heating RNA,[12] indicate that the extra amount of label obtained with Method 1 is due to 1 – 3 S rlRNA not being precipitated by the other methods. The other major differences in the radioactivity patterns could be due to different aggregational or conformational states of the isolated RNAs.

Effects of the composition of the aqueous phase. The presence of the large amounts of rapidly sedimenting $rlRNA_1$ and the rapidly labeled 1 – 3 S peak was investigated based on the difference in composition between the aqueous solutions of Methods 1 and 2. The patterns were unaffected by an exchange of SDS for tri-isopropylnaphthalene sulfonate, by addition of 6 % v/v butan-2-ol to extraction $buffer_2$, by addition of EDTA to the extraction buffers, or by omission of *m*-cresol from the phenol reagent. Addition of potassium chloride to increase the ionic strength of extraction $buffer_2$ up to that of aqueous $phase_1$ resulted in a radioactive recovery of about 80 % of the usual. A strong increase in radioactivity was seen in the 1 – 3 S region, while the distribution in the rest of the gradient was intermediate between RNA_1 and RNA_2 (Fig. 3). The ionic strength of the extraction liquid therefore seems to be essential. A specific effect of the extracting agents on the droplet size in the water-phenol emulsion could possibly also influence the patterns.

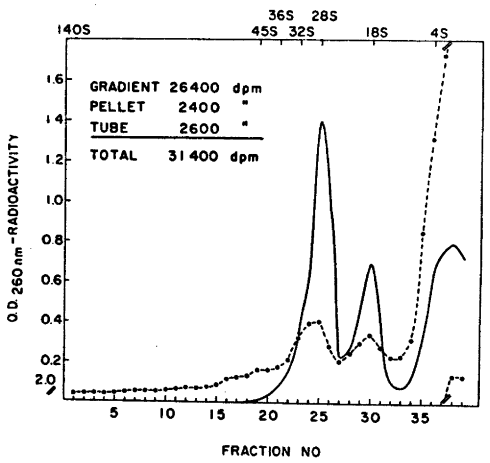


Fig. 3. Ultracentrifugation of 1 mg rat liver total RNA, isolated by Method 2 with 0.1 M KCl added to the extraction buffer, 30 min after administration of 50 μCi ^{14}C-orotic acid. Legend otherwise as in Fig. 1.

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Enzymatic Properties of Pig Intestinal Proline Dipeptidase

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The basic enzymatic properties of pig intestinal proline dipeptidase (aminoacyl-L-proline hydrolase, EC 3.4.13.9) were studied. The enzyme was inhibited both by substrate and product. pH-optima and K_m of the reactions with L-alanyl-L-proline (6.8 and 0.29 mM) and glycyl-L-proline (7.4 and 0.13 mM) as also K_i of the proline inhibition (0.21 mM at pH 7.4 and 0.49 mM at pH 6.8) were determined. The values were used for the calculation of theoretical progression curves, which were in good accordance with the experimental ones. 2-Mercaptoethanol, used as a stabilizer of the enzyme preparation showed inhibitory properties. The prolidase activity was not affected by DFP whereas thiol-reagents inhibited the enzyme. Bivalent metal ions showed no influence on the activity. Inhibition by 1,10-phenantroline, partially reversible by Zn^{2+}, suggested this metal to be involved in the catalysis.

Proline dipeptidase (also known as prolidase, aminoacyl-L-proline hydrolase, EC 3.4.13.9) is a dipeptide hydrolase which almost exclusively cleaves dipeptides of the aminoacyl-L-proline or aminoacyl-L-hydroxyproline type. It was first recognized by Bergmann and Fruton [1] and since then several reports on the enzyme from various animal sources have appeared.[2-8] The basal enzymatic characteristics of the pure enzyme, necessary for further enzyme kinetic work, have now been investigated on the pig intestinal prolidase, recently obtained as a homogeneous preparation.[9]

The present report settles the optimal assay conditions for the enzyme and investigates the possibility of product inhibition. A theoretical progression curve, based on the experimental data, is calculated and compared to the one obtained experimentally. In addition, the prolidase is characterized in respect to some common enzyme inhibitors and activators.

EXPERIMENTAL PROCEDURES

Enzyme preparation. The enzyme was prepared according to final purification Procedure II as described elsewhere.[9] It was stored frozen at −20 °C in small samples in 0.05 M Tris-HCl buffer (pH 7.5), made 4 mM in respect to 2-mercaptoethanol. The concentration of the enzyme was about 50 units of activity per ml. Under these conditions the enzyme was found to be stable for several months. 2-Mercaptoethanol was present to prevent aggregation of the enzyme. For each experiment one of the stored samples was used. After thawing it was generally diluted with 0.1 M potassium phosphate buffer (pH 6.8) at 4 °C to give a concentration of about 0.5 units of activity per ml, a concentration suitable for adequate substrate hydrolysis during a 10 min time period using the ordinary substrate concentration. Although the inhibitory effect observed for 2-mercaptoethanol at higher concentrations became negligible by this dilution, samples used for experiments involving low substrate concentrations were dialyzed (dialysis tubes, Visking Co., Chicago, U.S.A.) over-night against the buffer to further dilute the 2-mercaptoethanol.

Chemicals. L-Alanyl-L-proline was purchased from Fluka AG Chem. Fabrik, Buchs, Switzerland, and glycyl-L-proline was obtained from Sigma Chem. Comp., St. Louis, U.S.A. They were found chromatographically pure (thin layer chromatography, Merck DC Alufolien Cellulose 0.1 mm; butanol – acetic acid – water 4:1:1 by vol., ninhydrin reagent). In all experiments involving the continuously recording procedure the substrate concentration was calculated on the basis of the determined molar extinction coefficient of the peptide bond. Both substrates were dried as described by Bergmann *et al.*[10] until constant weight was obtained. Di-isopropylfluorophosphate (DFP) and *p*-hydroxymercuribenzoate (crystalline sodium salt, PHMB) were products of Sigma, St. Louis, U.S.A. 1,10-phenantroline was obtained from Merck, Darmstadt, Germany. De-ionized and glass distilled water was used

throughout. All other reagents used were of analytical grade.

Enzyme assay. Prolidase activity was assayed according to the method of Josefsson and Lindberg[11] with the following modifications. The dipeptide and amino acid solutions were prepared in their suitable concentrations using 0.1 M KH_2PO_4 buffer solution adjusted to proper pH with 0.1 M KOH (pH-meter 28, Radiometer, Copenhagen, Denmark). To get a higher precision of the method, 100 µl of the enzyme solution were mixed with 750 µl of the buffered substrate (2 mM) solution. Two samples were used for each digestion time. They were incubated at 25 °C for a suitable time period (5 – 60 min). Because of the small amounts of protein in the samples the ethanol precipitation procedure was omitted and instead the hydrolysis was interrupted by the addition of 750 µl 0.5 M H_3PO_4. The samples were directly read in a Zeiss spectrophotometer PMQ II at 226 nm (L-alanyl-L-proline) and 224 nm (glycyl-L-proline). In certain experiments the sensitivity of the method was increased by mixing a greater volume of enzyme solution into a smaller volume of substrate solution. The total volume of the incubation solution was always the same as in ordinary assays, as was the final substrate concentration.

In experiments where the substrate concentration was varied, alternative wavelengths from 205 nm and upwards were used to compensate for the absorbance differences. To get an approximation of the initial velocity a continuously recording procedure was chosen. The spectrophotometer was provided with a logarithm recorder (Servoger R. E. 514.9) and a thermostated cuvetteholder for cuvettes with 1 cm light path. The enzyme solution (20 µl) was mixed with pre-temperated, buffered substrate solution (1000 µl), and subsequently transferred to the cuvette. The mixing and transfer process took about 15 s. The enzyme concentration was chosen to allow approximation of the initial velocity and the reading wavelength was chosen to give a starting absorbance between 0.7 and 0.8. The spectrophotometer was set to zero before each experiment using a mixture of the enzyme solution and a buffered solution of the corresponding amino acids at appropriate concentrations.

Unit of prolidase activity. One unit of prolidase activity was defined as the amount of enzyme hydrolyzing 1 µmol L-alanyl-L-proline (1.8 mM) per min at 25 °C and pH 6.8.

RESULTS

Factors affecting stable assay conditions

Stability as a function of pH. The buffer prepared for these studies was made from a stock solution containing equimolar concentrations of H_3PO_4, citric acid, and H_3BO_3. After adjusting the solution to appropriate pH with 0.1 M KOH, water was added to give a final concentration of 0.1 M for each of the constituents. Samples of the enzyme stock solution were diluted ten times with the described buffer solution, preadjusted to pH-values varying from 2.9 to 10.3. The samples, containing 3 units of activity per ml, were stored for 30 min at 25 °C and subsequently diluted another ten times with 0.1 M potassium phosphate buffer (pH 7.0) to accomplish a nearly neutral solution before the samples were assayed for their activity. All pH-values were controlled during the experiments in parallel experiments, where the enzyme stock solution was substituted with 0.05 M Tris-HCl buffer (pH 7.5). As shown in Fig. 1 there is no difference in enzyme stability in the pH-range between 5.5. and 8.5.

Stability as a function of time, temperature, and enzyme concentration. The stability of the prolidase at 0 °C (melting ice) was studied in 0.1 M potassium phosphate buffer (pH 6.8), using an enzyme concentration of 0.5 units of activity per ml. Assays (1.8 mM L-alanyl-L-proline) were performed at suitable time intervals during a time period of 3 h from the time of preparing the diluted enzyme solutions. The results showed a small decrease of the activity during the first hour and then a more stable activity. Thus practically constant assay values were obtained during a 2 h period if the enzyme solution first was left for 1 h at 0 °C.

The stability of the prolidase after being preincubated as above was also studied at 25 °C at the enzyme concentrations 0.03, 0.06,

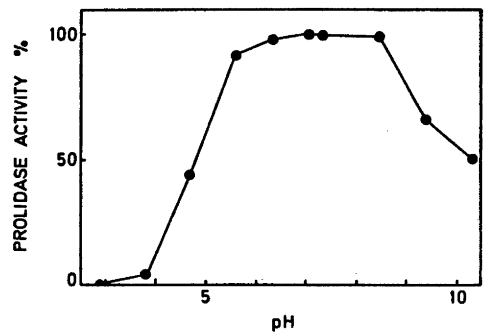

Fig. 1. Stability of prolidase as a function of pH. Substrate: L-alanyl-L-proline.

and 0.15 units of activity per ml. The same experimental procedure as above was used and the time period was 1 h. A continuously proceeding slow loss of activity was observed in all incubations, although the loss was most pronounced during the first 20 min period.

This slow loss of the enzyme activity was, however, without influence in the assay procedure, as it was found that progression curves (1.8 mM L-alanyl-L-proline) when followed to 80 % hydrolysis at the three different enzyme concentrations showed identical profiles, *i.e.* the enzyme concentrations of 0.03 units of activity per ml resulted in the same hydrolysis figure, when incubated for 100 min as did the one containing 0.15 units of activity per ml, when incubated for 20 min. This finding thus suggests a substrate stabilisation of prolidase.

Factors affecting optimal assay conditions

Reaction velocity as a function of pH. The influence of pH on the reaction velocity was investigated in the pH-range from 6.0 to 7.9 using 0.1 M potassium phosphate buffer. Measurements of the pH at the start and at the end of the incubation period, made in parallel experiments, showed a change of less than 0.04 pH-units. The results, shown in Fig. 2, gave a velocity maximum for the enzyme reaction at pH 6.8 when L-alanyl-L-proline was the substrate. A similar experiment displayed a pH-optimum of 7.4 for the enzyme reaction when glycyl-L-proline was the substrate.

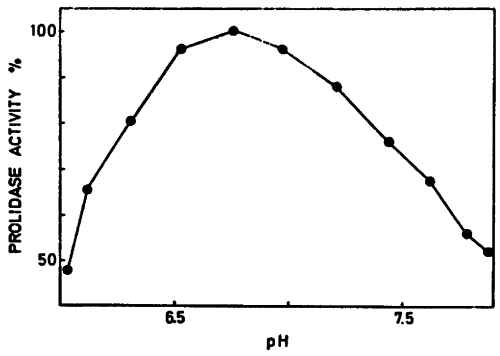

Fig. 2. Prolidase activity as a function of pH. Substrate: L-alanyl-L-proline.

Reaction velocity as a function of substrate concentration. The dependence of reaction velocity on substrate concentration was studied at five different substrate concentrations using L-alanyl-L-proline and glycyl-L-proline as substrates. Each experiment consisted of a double analysis at each substrate concentration and was run at the pH-optima proper to each of the reactions. The enzyme solution, dialyzed against 4000 vol. of 0.05 M Tris-HCl buffer (pH 7.5) over-night and subsequently frozen in portions suitable for each experiment, was appropriately diluted and kept at 0 °C for 1 h before the start of each experiment. Five experiments of this type were run for each substrate. A reference assay made at the beginning and end of each experiment showed neither an alteration of the activity during the test period nor a difference between the five separate experiments. Therefore the data could be statistically analyzed as one experiment with ten measurements at each substrate concentration. Assuming a classical Michaelis-Menten kinetics, the method of Wilkinson [12] could be used. The calculation including 4 of the 5 concentrations was performed on a Univac 1106 computer and resulted in a K_m of 0.29 (S.E. 0.025) mM and a V of 0.077 (S.E. 0.0033) mM/min for the reaction with L-alanyl-L-proline and a K_m of 0.13 (S.E. 0.015) mM and a V of 0.062 (S.E. 0.0011) mM/min for the reaction with glycyl-L-proline. Fig. 3a – b gives the data plotted as suggested by Lineweaver and Burk.

Product inhibition

Most of the naturally occurring amino acids were separately added to the buffered L-alanyl-L-proline solution in a concentration of 2 mM to investigate their possible role as inhibitors for the prolidase reaction. L-Tyrosine, L-phenylalanine and L-tryptophan were excluded because they disturbed the assay method. Of the various amino acids L-proline in consistency with earlier findings [3] was found to be a rather strong inhibitor, whereas the other amino acids besides cysteine, caused no or only slight inhibition. The inhibition induced by cysteine is probably explained by its thiol-group (*cf.* influence on the activity with 2-mercaptoethanol).

The extent and kind of inhibition with L-proline were further investigated by adding

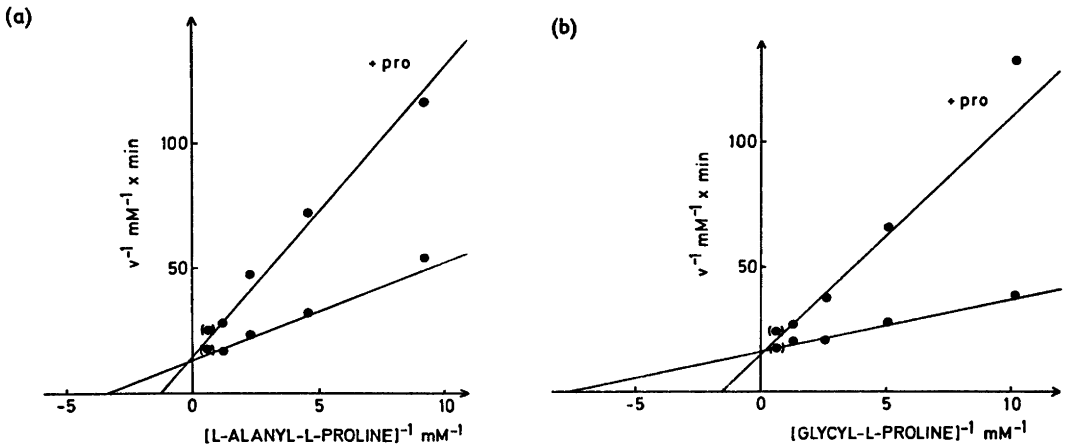

Fig. 3. Lineweaver-Burk plots of prolidase hydrolysis of (a) L-alanyl-L-proline and (b) glycyl-L-proline and the effect of L-proline (0.8 mM) on the reactions. ● Mean value of 10 measurements. Enzyme concentration during incubation (a) 0.03 units of activity per ml; (b) 0.1 units of activity per ml.

the amino acid (0.8 mM) to incubations with varying substrate concentrations. The experiment was run in the same way as described for the determination of the kinetic coefficients. In fact, the experiments described above and the inhibitor experiments were mixed with each other for making comparisons of the V-values more justified. Using the same statistical analysis procedure as above the apparent K_m was found to be 0.76 (S.E. 0.10) mM in the experiments with L-alanyl-L-proline and 0.62 (S.E. 0.039) mM in the experiments with glycyl-L-proline. The corresponding values for V were 0.069 (S.E. 0.0063) mM/min and 0.068 (S.E. 0.0038) mM/min, respectively. Fig. 3a – b gives the experimental data plotted as suggested by Lineweaver and Burk.

Other inhibitors

Influence of DFP on the activity. 150 µl of an 0.2 mM solution of DFP in 0.1 M potassium phosphate buffer (pH 7.7) were added to 50 µl of the enzyme solution, corresponding to a thousand-fold excess of the reagent. The samples in parallel with controls, in which the DFP solution was replaced by the buffer, were incubated at 25 °C for 20 min and then assayed (1.8 mM L-alanyl-L-proline) for their enzyme activity. No decrease of activity was observed in the DFP-containing samples when compared to the controls. Using the same experimental conditions the DFP solution was found to cause a complete inactivation of chymotrypsin,[13] when assayed against casein.

Influence of thiol-reagents on the activity. The essentiality of free SH-groups for the activity of prolidase was demonstrated in experiments with PHMB, Ag^+, Cu^{2+}, and Hg^{2+}, all known to react with thiol-groups of proteins. The enzyme was incubated at 25 °C for 10 min with the compounds added separately to a buffered L-alanyl-L-proline solution (pH 6.8) at concentrations of 1, 10, and 100 µM (Table 1). Preincubations of prolidase with a hundred-fold excess of PHMB (molar basis) for 16 h in 0.05 M Tris-HCl buffer (pH 7.5, 4 °C) resulted in a complete inactivation, which remained also after the excess of PHMB had been removed by dialysis.

Table 1. Effect of thiol-reagents on the prolidase activity.

Concentration µM	Activity % Ag^+	Cu^{2+}	Hg^{2+}	PHMB
0	100	100	100	100
1	0	100	100	50
10	0	60	10	40
100	0	30	0	20

Influence of 2-mercaptoethanol on the activity. An enzyme solution, free from 2-mercaptoethanol by dialysis, was assayed according to the general procedure but using a series of buffered L-alanyl-L-proline solutions, containing varying concentrations (0 – 4 mM) of 2-mercaptoethanol. The results revealed a 50 % inhibition at a 2-mercaptoethanol concentration of 0.7 mM, while very low 2-mercaptoethanol concentrations (10 μM) did not affect the prolidase activity.

Influence of certain metal ions on the activity. $CoCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$ from aqueous stock solutions (0.02 M) were separately added to buffered L-alanyl-L-proline and glycyl-L-proline solutions at concentrations of 10 and 100 μM. The activity of a prolidase solution was assayed according to the general assay procedure using the various substrate solutions. No obvious effect on the enzyme activity was observed with any of the metal ions when compared to an incubation with no metal added. The influence of the metal ions on the prolidase activity was also investigated after the enzyme was treated with 1,10-phenantroline. An enzyme solution (0.05 M Tris-HCl buffer pH 7.5) was stored during a fortnight at 4 °C with and without 1,10-phenantroline (1 mM) and then assayed against L-alanyl-L-proline. The results showed a 90 % decrease of the activity in the 1,10-phenantroline containing solution compared to only a 20 % decrease in the control solution. 1,10-phenantroline was then removed by dialysis and the enzyme solution was given the four metal ions separately at concentrations as above. Repeated assays of the prolidase activity then showed partial reactivation in the samples containing Zn^{2+}, while no effect was observed in the other samples.

DISCUSSION

The omission of the centrifugation step in the original assay procedure [22] increased the precision of the method as did the increase of the volumes, thereby making it more suitable for these studies. At a substrate concentration of 1.8 mM the coefficient of variation of the assay was found to be 4.5 % ($n = 20$). The sensitivity of the method can be increased by decreasing the substrate concentration, as the inadequacy caused by not measuring the reaction at maximal velocity can be compensated for by the use of the general formula given in this report. Systematic errors due to the hygroscopic nature of the substrates, *i.e.* varying water content, can be overcome by using the molar extinction coefficient of the peptide bond. The applicability of Lambert-Beers law was verified within the whole wavelength region used.

The pH-optima for the prolidase reaction with L-alanyl-L-proline and glycyl-L-proline agree well with the values earlier reported for crude intestinal extracts of various species.[5–7,14] Peters,[15] however, reported a pH-optimum of 8.0 for the glycyl-L-proline hydrolysis by a quinea-pig intestinal extract. This pH-optimum for the glycyl-L-proline reaction was also given for the purified pig kidney prolidase.[4]

As separate experiments with substrate concentrations higher than 2 mM demonstrated substrate inhibition, the data for obtaining the kinetic coefficients were processed both with and without the values of 1.6 mM substrate concentration (Fig. 3). The kinetic coefficients obtained with a four point analysis fitted best in the progression curve (see below) thus making these values more probable in spite of the lower statistical precision.

Using the t-test according to Cleeland,[16] it was found that the two values of K_m and the two apparent K_m values obtained from the inhibition experiments with proline differed ($P = 99.9$ %). No differences between the corresponding V values could be demonstrated (L-alanyl-L-proline, $P = 90$ %, glycyl-L-proline, $P = 90$ %). These findings suggest L-proline as a competitive inhibitor and result in a K_i for the inhibition of 0.49 mM at pH 6.8 and 0.21 mM at pH 7.4.

Since variation of buffer (0.1 M Tris-HCl, pH 7.5, and 0.1 M potassium phosphate, pH 7.5) did not affect the prolidase activity and the enzyme activity was stable during the incubation period, the experimental progression curve could be completely explained by product inhibition and by the decrease of enzyme saturation with substrate.

Jennings and Niemann [17] have described an integrated rate equation (1) for a product inhibited enzyme model. This equation, using a K_m of 0.29 mM and a K_i of 0.49 mM for the

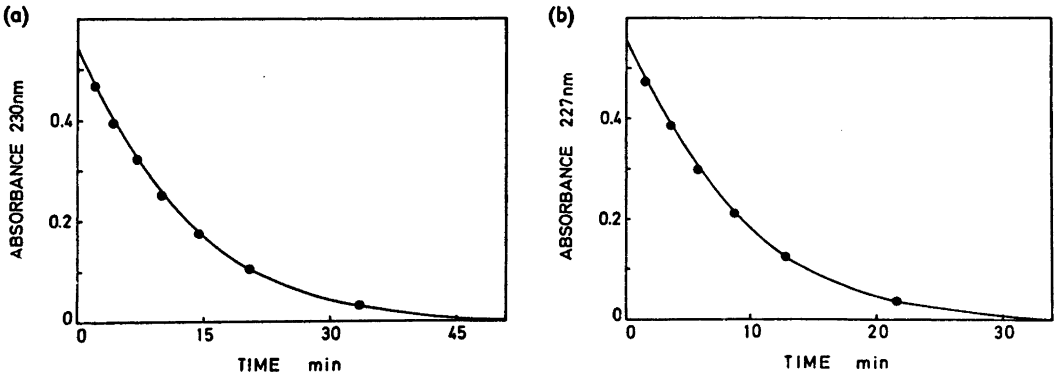

Fig. 4. Progression curves of prolidase with (a) 0.71 mM L-alanyl-L-proline and (b) 0.64 mM glycyl-L-proline as substrates. The theoretical values were calculated according to eqn. (1) at a constant interval of substrate concentration. The fitting of the equation to the enzymatic activity of the experiments were made as described in the text. ——— Experimental curve; ● calculated values.

prolidase reaction with L-alanyl-L-proline and a K_m of 0.13 mM and a K_i of 0.21 mM for the reaction with glycyl-L-proline is plotted in Fig. 4. In each of the curves, one point (substrate concentration, S_t; time, t) around 50 % hydrolysis was first taken from the experimental data to obtain $k_3 \times E$, and thereby fit the equation to the experimental curves.

$$k_3 E t = K_m(1 + S_0/K_i) \ln S_0/S_t + (1 - K_m/K_i)(S_0 - S_t) \quad (1)$$

E enzyme concentration
S_0 substrate concentration to time zero
S_t substrate concentration to time t
k_3 rate constant of the enzyme-substrate complex dissociation

This equation permits evaluation of the kinetic coefficients from a few progression curves instead of their estimation from many separate determinations of initial velocities.

The equation was also used to calculate the molecular activity (k_3) for the enzyme at 25 °C, using a molar extinction coefficient of 11.2×10^4 M^{-1} cm^{-1} for prolidase (280 nm)[18]. The molecular activity was found to be 3×10^4 and 8×10^3 min^{-1} for L-alanyl-L-proline and glycyl-L-proline, respectively. The value of the glycyl-L-proline hydrolysis is low as compared to that reported by Davis and Smith.[4] The discrepance is, however, partlyexplained by the higher experimental temperature used by them.

The experiments with DFP and thiol-reagents suggest prolidase as an enzyme dependent on free SH-group(s) for its activity, which is in agreement with earlier observations on pig kidney prolidase.[4]

The inhibition observed in this study with the thiol-compounds, earlier also reported with glutathione,[4] could be explained by the binding of the sulfur to an essential metal of the enzyme. This metal may be zinc, as suggested from the experiments including 1,10-phenantroline. As the enzyme activity in our studies, in contrast to earlier findings with higher metal concentrations,[2-4,6,7,15] was found to be unaffected with Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} and also stable in the presence of 1 mM EDTA,[9] the metal is proposed to be strongly bound to the enzyme.

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Mechanism of the Grignard Addition Reaction. XI. Electrode Kinetics and Chemical Reactivity of Grignard Reagents

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Electrolysis of alkylmagnesium bromides using platinum electrodes lead to Tafel plots of slope 0.13 for allyl-, benzyl-, and *t*-butylmagnesium bromide, but 0.32 for methylmagnesium bromide. Two different mechanisms for the discharge process are therefore indicated. Ethyl-, butyl-, and isopropylmagnesium bromide behave like methyl at low current densities, but change over toward the *t*-butyl slope at high current densities. Values of the anodic polarisation at high current densities are correlated to the reactivity of the reagent toward benzophenone.

By electrolysis of alkylmagnesium bromides in ether solution using platinum electrodes a polarization is observed which varies with the nature of the alkyl group. These "decomposition potentials" were measured by Evans *et al.* in 1935.[1] Since magnesium is produced at the cathode and hydrocarbons attributable to the free radical form at the anode, the difference in decomposition potentials was assumed by Evans to reflect a characteristic difference in the anodic discharge potential of each alkyl anion.

In the study of the reactivity of the Grignard reagent toward benzophenone an initial transfer of a single electron from Grignard reagent to ketone has been assumed to be rate determining[2,3] and since this SET process might be related to the electrode process it was found of interest to try to correlate the chemical reactivity of the Grignard reagents with the electrochemical reactivity.

No experimental details were given by Evans concerning electrodes or current densities and serious objections may be raised against the oversimplified interpretation. More recently Psarras and Dessy[4] made an investigation of the polarographic behaviour of Grignard reagents, but failed to determine characteristic anodic potentials. Martinot made an electrokinetic investigation of several Grignard reagents[5] and found decreasing electrochemical reactivity in the order *t*-butyl > i-butyl > ethyl > butyl. Martinot found identical Tafel slopes of 0.20 for the four reagents in the anodic process, while the cathodic process for the four reagents had a Tafel slope of -0.008.

During the present work a simple procedure was used which usually gave reproducible values for the polarisation of the anode and allowed the determination of Tafel slopes as well as relative values of the electrochemical reactivity for several alkylmagnesium bromides.

A cell with two platinum electrodes was charged with *ca.* 0.8 M alkylmagnesium bromide in ether. A potential was set up by means of a constant current generator. The equilibrium value of the potential was measured exactly by means of a digital voltmeter for values of the current density I_D varying from 10^{-5} to 10^{-1} A/cm^2. For each reading of the potential E, a correction was made by increasing the current to two times the original value and noting the following increase in electrode potential E_{inc}. The apparent back electromotoric force was then taken as:

$$\text{bEMF} = E - E_{inc} \qquad (1)$$

The values of bEMF were plotted against the logarithm of the current density; Fig. 1.

The potential between the electrodes is considered the sum of ohmic resistance potentials, concentration potentials, and overvoltage for both electrode processes. It was assumed that

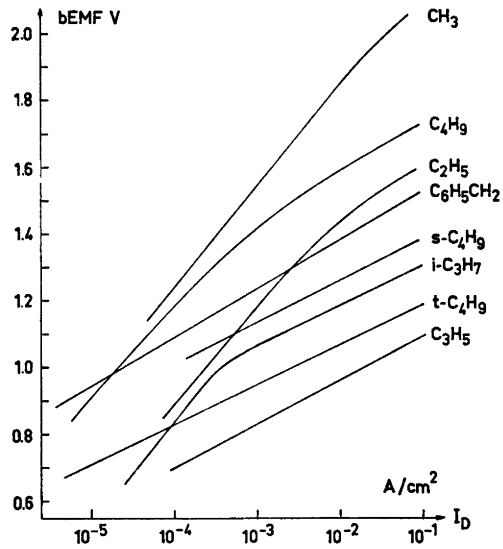

Fig. 1. Back electromotoric force (see text) *versus* log current density during electrolysis of alkylmagnesium bromides between platinum electrodes in diethyl ether at 20 °C.

the current densities applied were so small that the concentration polarisation was proportional to I_D just like the ohmic potential drop. Variations in the calculated values of bEMF was therefore considered representative for variations in overvoltage, and since for reasons which will be explained later the cathodic potential could be considered almost constant, the variations were ascribed specifically to anodic overvoltage.

Overvoltage usually follows the Tafel equation

$$\eta = a + b \log I_D \qquad (2)$$

From the Tafel plots obtained with the various Grignard reagents it seems that the values of the slope b in eqn. (2) may vary between two extremes. A low value, *ca.* 0.13, is observed with allyl, benzyl, and *t*-butylmagnesium bromide, while a high value, *ca.* 0.32, is obtained for methylmagnesium bromide. With reagents like isopropyl-, ethyl-, butylmagnesium bromide, *etc.* a change in slope is observed from the high value, 0.32, at low current densities to the low value 0.13, at high current densities. A simple interpretation would be the operation of two mechanisms for discharge of alkylmagnesium species at the anode: (1) reaction at platinum, and (2) reaction at "radical saturated" platinum. The simple discharge of alkylmagnesium species at the platinum surface to form alkyl radical and magnesium ion would be in operation at low current densities or with extremely shortlived radicals like methyl. A more complex discharge mechanism would apply if a sufficient concentration of surface bound radicals are present on the anode. The electron transfer may in this case occur with a lower activation than necessary with pure platinum, as if the surface bound radicals served as a catalyst for the electron transfer. With reagents forming stable radicals like allyl, benzyl, and *t*-butyl, the saturation is obtained even with the lowest current densities and with methyl it is not obtained even with the highest, but with isopropyl and ethyl saturation may be obtained of the current density is high enough.

For identical values of b the value of a in (2) would represent a measure of the electrochemical reactivity of the reagent. Since at the highest I_D values the slopes of the plots tended toward a common value (0.13), bEMF for the various Grignard reagents are given in Table 1 for a current density of 0.06 A/cm^2. The Tafel slope problems of course make the values for especially methyl and ethyl rather dubious.

It has been implicated in the interpretation that the overvoltage may be ascribed to the anode process. This conclusion seemed permissible from experiments in which a $Mg/MgBr_2$ reference electrode was included in the system. At low current densities the potential between the reference electrode and the cathode was near zero and at increasing I_D the potential increased linearly with I_D indicating that overvoltage is of much less importance at the magnesium cathode than at the anode. In the work of Martinot the cathodic overvoltage for six alkylmagnesium bromides varied ±5 mV for 0.6 M solutions, and the Tafel slope for the cathodic processes was smaller than 0.01.

In order to see if the electrode process is related to the rate determining step in the reaction of Grignard reagents with benzophenone, pseudo first order rate constants were obtained by means of the thermographic method [6] for the reaction of 0.02 M Grignard reagent with 0.25 M benzophenone in ether as shown in Table 1.

Table 1. Pseudo first order constants, k_{obs}, in s^{-1} for the reaction of 0.02 M alkylmagnesium bromide with 0.25 M benzophenone in diethyl ether at 20 °C. Excess bromide relative to the base titration is given as % Br^-. Anodic overvoltage $\eta_{0.06}$ (see text) is given in Volts, molarity of electrolysed solution given as [RMgBr].

R	% Br^-	k_{obs}	$\eta_{0.06}$	[RMgBr]
CH_3	0.8	0.3	1.98	1.00
C_3H_5	2.8	5.0	1.57	0.87
i-C_3H_7	13.0	133	1.28	0.99
C_4H_9	4.4	2.6	1.70	1.09
i-C_4H_9	9.6	0.90	1.60	0.98
s-C_4H_9	19.5	64	1.36	1.00
t-C_4H_9	30.0	400	1.16	0.69
C_3H_5	19.7	16 000	1.07	0.68
$C_6H_5CH_2$	15.0	21	1.50	1.02
c-C_5H_{10}	9.9	214	1.35	0.98

The logarithm of the rate constants was plotted against the anodic overvoltage for $I_D = 0.06$ A/cm² relative to the magnesium cathode; see Fig. 2. An approximate linear correlation is obtained for methyl-, ethyl-, isopropyl-, *t*-butyl, and benzylmagnesium bromide, which might indicate that the SET mechanism which was suggested for *t*-butylmagnesium bromide [3] may be a relevant model for many simply alkylmagnesium bromides. This would not mean that free radicals are actual intermediates, but only that the alkyl assumes a significant amount of radical character in the transition state. The stability of the radical type transition state may be increased by pairing between benzophenone ketyl radical and the alkyl radical.

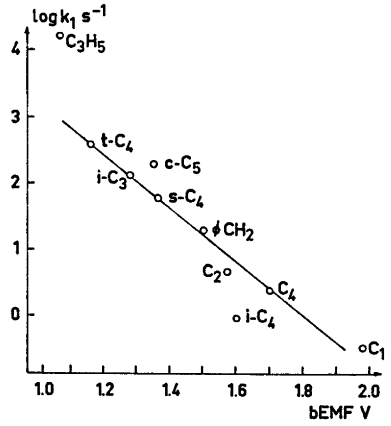

Fig. 2. Pseudo first order rate constants for the reaction at 20 °C of alkylmagnesium bromide (0.02 M) in diethyl ether with 0.25 M benzophenone. C_1, C_2, C_3, C_4, *etc.* means methyl-, ethyl-, propyl-, butylmagnesium bromide, *etc.*

The reactivity of some Grignard reagents is not very well correlated with the discharge potential. It seems that β-branching as in isobutyl represents more steric hindrance for the chemical reaction than for the electrode process. For allyl- and cyclopentylmagnesium bromide the reaction is faster than would be predicted from the discharge potentials. The mechanism of reaction for these reagents may be different or it may be a hybrid between the stepwise SET mechanism and appropriate polar or concerted mechanisms.

A certain amount of radical character or at least electron deficiency at the α-carbon in the transition state is always indicated in reactions in which the ethyl reagent is more reactive that the methyl reagent which applies to most of the reactions of Grignard reagents which have been investigated kinetically. A correlation of rate data for the metalation of 1-hexyne with Grignard reagents with Evans decomposition potentials was attempted by Dessy in 1955.[7] If this correlation is established it would suggest the operation of a SET mechanism even in this type of reaction.*

EXPERIMENTAL

The Grignard reagents were prepared from sublimed magnesium ("Specpure", Johnson, Matthiey & Co.) as reported.[8] The electrolysis cell consisted of a 10 × 75 mm Pyrex test tube. The electrodes were smooth platinum wires (0.6 mm) which were placed 6 mm apart in a rubber stopper with 65 mm extending into the tube. The electrodes were cleaned before each experiment by washing with dilute acid and with acetone. They were then heated in a bunsen burner. The cell was assembled and copper leads were connected to a Fluke 382 A current calibrator and to a Takeda-Riken digital volt-

* *Note added in proof.* A recent report [9] demonstrates a linear correlation of log rate for reaction of RMgBr with di-*t*-butyl peroxide in ether with Evans decomposition potentials. The correlation is improved if $\eta_{0.06}$ is used. Reaction of Grignard reagents are shown to have a rate determining SET step. The reactivity of benzophenone is 3.3×10^5 times greater than the reactivity of di-*t*-butyl peroxide toward the same Grignard reagent.

meter. The cell was evacuated to 0.1 mmHg through a hypodermic needle and 3 ml of the Grignard reagent were injected through the rubber stopper. The tube was placed in water at 20 °C and the current calibrator was adjusted to 1.000 mA. After 10 min the potential between the electrodes was read with two decimals and the current was increased to 2.000 mA. The new value of the potential was read exactly and from the measurements obtained the back electromotoric force was calculated according to eqn. (1). The procedure described was repeated every 10 min until a constant value for the bEMF was obtained, which usually occurred within 30 min. With methyl- and ethylmagnesium bromide up to 1 – 2 h were required to obtain a constant value. After the measurement at 1.000 mA the bEMF was measured according to the same principle at 0.4, 0.2, 0.1, 0.04, 0.02, 0.01, 0.004, 0.002, 0.001, 0.0004, 0.0002, and 0.0001 mA. The results are shown as plots of bEMF *versus* log I_D in Fig. 1.

For measurements using an external reference electrode two cells were connected through a Pyrex G 4 fritte. The reference electrode was a piece of sublimed magnesium connected to a platinum wire and suspended in a saturated solution of magnesium bromide in diethyl ether (2.5 M). During measurements using isopropyl- and allylmagnesium bromide the reference electrode was only 35 – 40 mV anodic to the cathode of the electrolysis cell at low current densities. Based on this observation and on the observation of Martinot [5] the magnesium-coated platinum cathode was considered useful as an intrinsic reference for comparison of anodic potentials.

The values of the back electromotoric force at a current density of 60 mA/cm² was taken as representative for the anodic discharge potential of the specific Grignard reagent relative to the $Mg/MgBr_2$ reference and are tabulated in Table 1.

The kinetic measurements were performed using a thermographic method.[6] The standard concentrations were 0.02 M Grignard reagent and 0.25 M benzophenone. The liquid speed in the reaction tube was usually 3 000 mm/s, but for measurement of allylmagnesium bromide the speed was 13 800 mm/s in a glass capillary of 0.24 mm inside diameter. The high speed allowed temperature measurement *ca.* 0.15 ms after mixing. From the time/temperature readings pseudo first order plots were prepared according to the expression

$$\ln [(T_\infty - T_0)/(T_\infty - T_t)] = kt$$

The plots were linear usually for more than 50 % of the reaction and values of k_1 were obtained from this linear part of the plot.

The logarithm of the rate constants were plotted *versus* the anodic discharge potentials of the respective reagents as shown in Fig. 2.

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Short Communications

Partial Purification of Cysteine Synthase (*O*-Acetylserine Sulfhydrylase) from Onion (*Allium cepa*)

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In a previous investigation on the sulfur metabolism of *Allium* plant tissues it was found that cysteine derivatives can be formed enzymatically by condensation of various thiols with serine.[1] It was proposed that this reaction is mediated by the second member in a two-enzyme system which forms cysteine from serine. Most workers in this field now agree that cysteine is formed by the following reactions:
Acetyl-CoA + serine ⟶ *O*-acetylserine + CoA
O-Acetylserine + sulfide ⟶ L-cysteine + acetate.

The first step is catalyzed by serine acetyl transferase (EN 1972 2.3.1.30). The enzyme of the second step is known in the literature as *O*-acetylserine sulfhydrylase. However, according to the 1972 Enzyme Nomenclature it should be named cysteine synthase (EN 1972 4.2.99.8), not to be confused with cysteine synthase (EN 1964 4.2.1.22). In this paper the 1972 Nomenclature will be followed. The enzyme reacts not only with sulfide but also with methyl mercaptan and ethyl mercaptan, forming *S*-methylcysteine and *S*-ethylcysteine, respectively (for literature, see Ref. 1). In order to study further the mechanism of the biosynthesis of cysteine derivatives in onion, an attempt has now been made to purify the cysteine synthase of onion. Becker and coworkers have purified the cysteine synthase of *Salmonella typhimurium*,[2] and their procedure has been applied to the onion enzyme. However, the much lower enzyme content in onion and the presence of large amounts of polysaccharides necessitated the design of other initial purification steps. Also, it was found beneficial to raise the pH throughout the purification procedure.

Experimental. Materials. The onions used were different batches of freshly harvested or stored bulbs obtained from the local market. *O*-Acetyl-L-serine was synthesized by acetylation of L-serine in perchloric acid/acetic anhydride/acetic acid solution.[3] Other reagents were of commercial origin.

Enzyme assay. Cysteine formation was measured by the assay of Becker *et al.*[2]

Purification of cysteine synthase. The initial purification steps were performed at temperatures between 0 and +4 °C. The peeled and sliced onion (3 kg) was homogenized for 30 s at medium speed in a 5 l Waring Blendor with 1500 ml extraction buffer (0.1 M Tris-HCl, pH 8.2, containing 0.01 M 2-mercaptoethanol). A certain amount of solid Tris (about 14.5 g) was added immediately before the homogenization to establish a pH of 8.2 when the homogenization process was finished. The required amount of Tris varied from batch to batch of onion and was estimated from a small scale experiment. The slurry was strained through cheesecloth and centrifuged in a refrigerated centrifuge for 20 min at 10 400 *g*. One gram activated charcoal (BDH, for decolorizing purposes) was added per 100 ml supernatant, and the mixture was stirred for 5 min in an ice-bath and immediately centrifuged as above. Two grams moist Ca-phosphate gel was added per 100 ml supernatant, and the mixture was stirred for 10 min and centrifuged as above.[4] After this step all operations were carried out at room temperature. The pH of the supernatant was re-adjusted to 8.2 with 1 M ammonia, and solid ammonium sulfate was added to 40 % saturation. After stirring for 45 min and centrifugation, the precipitate was discarded and the supernatant was made 60 % saturated with ammonium sulfate. The solution was stirred for one hour and allowed to stand for another hour and centrifuged. The precipitate was dissolved in water and dialyzed overnight against 0.1 M Tris-HCl containing 0.01 M 2-mercaptoethanol.

First Sephadex filtration. The dialyzed enzyme was applied to a Sephadex G-100 column (52 cm × 2.7 cm ⌀), equilibrated with 0.1 M Tris-HCl – 0.01 M 2-mercaptoethanol, and eluted with the same buffer. Fractions of 5 ml were collected and the active fractions were pooled.

DEAE-Cellulose chromatography. The pooled enzyme was applied to a DEAE-cellulose column (14 × 1 cm ⌀), equilibrated with 0.1 M Tris-HCl – 0.01 M 2-mercaptoethanol, and was eluted with a 1 liter linear gradient of 0 to 0.35 M NaCl in the same buffer. Fractions of 5 ml were collected, and the fractions with the highest activities were pooled.

Second Sephadex filtration. The pooled enzyme from the previous step was dialyzed against polyethylene glycol 6000 (Shell) until the volume was reduced to about 2 ml, and then applied to

a Sephadex G-100 column (55 × 1.7 cm ⌀), equilibrated with 0.1 M Tris-HCl, pH 7.4. The column was eluted with the same buffer. The enzyme peak was pooled and stored at −18 °C.

Results and discussion. The initial adsorption steps clear the enzyme extract from many interfering substances, and gel filtration is a powerful means of further purification. In a Sephadex separation the shape of the UV profile varied with the batch of onion, but the enzyme activity always was eluted between two major UV peaks. In further purification on DEAE-cellulose the enzyme was eluted approximately in the same way as the *Salmonella* enzyme when purified on a DEAE-Sephadex column.[2] Chromatography on Sephadex G-100 also indicates similarity of enzyme from both sources. Like the *Salmonella* enzyme the purified onion enzyme is fairly stable at room temperature.

The enzyme activity varied much between different batches of onion, investigated at different times of the year, the reason for this remaining unknown. In a favourable case *ca.* 900 enzyme units were obtained from 3 kg onion (measured from the pooled enzyme after the first Sephadex fractionation). The result of the purification could not be presented in terms of specific activity since protein determination was unreliable in the crude extract and too low after the chromatographic steps. Protein determination as well as further characterization of the enzyme would have required a considerable scale-up of the whole isolation procedure. Onion is a poor source of the enzyme compared with microorganisms such as *Salmonella* in which the synthesis of this enzyme can be derepressed by growth in a medium containing L-djenkolic acid as the sole sulfur source.[5]

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Synthesis of *S*-Substituted Cysteine Derivatives by the Cysteine Synthase (*O*-Acetylserine Sulfhydrylase) of Onion (*Allium cepa*) and *Escherichia coli*

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Previous investigations showed that *Allium* plants can form a wide variety of cysteine derivatives from externally supplied thiols and serine.[1] It was proposed that this reaction is mediated by the non-specific action of cysteine synthase (EN 1972 4.2.99.8). This enzyme was previously known as *O*-acetylserine sulfhydrylase. It is the second member of the two-enzyme system which normally forms cysteine from serine. The cysteine synthase of onion has now been partially purified.[2] In this investigation we have found that cysteine synthase is non-specific with regard to a wide range of thiols, and there is no difference in this respect between enzyme of plant and bacterial origin.

Materials and methods. Substrate. O-Acetyl-L-serine was synthesized by acetylation of L-serine in perchloric acid/acetic anhydride/acetic acid solution.[3] Labeled substrate was prepared on a micro scale from L-serine-C14(U) by the same method. The labeled substrate (specific activity 10 μCi/μmol) was divided in aliquots, vacuum dried and stored dry at −18 °C until use.

Enzyme. The partial purification of onion cysteine synthase is described elsewhere.[2] The enzyme was partially purified from *E. coli* using essentially a small-scale modification of the procedure of Becker *et al.*[4] The starting material was 1 g frozen cells of *E. coli* ATCC 4157, grown in a glucose/inorganic salt medium, and kindly provided by Mr. Seppo Vilkki, Phil. Lic. The cells were mixed with 9 ml buffer (0.05 M Tris-HCl pH 7.8 and 0.01 M 2-mercaptoethanol) and 13 ml ballotini glass beads and disintegrated by continuous circulation for 2 min at 0 °C in a silicone tube agitated by a fast peristaltic pump. The enzyme was purified by streptomycin precipitation and ammonium sulfate fractionation as described by Becker *et al.*[4] After this step the enzyme was dialyzed against 0.05 M Tris-HCl buffer pH 7.6. The specific activity was 1.46 units per mg protein.

Reaction. O-Acetyl-L-serine labeled with L-serine-C14(U) was incubated with the enzyme for 30 min in small, conical, stoppered test tubes under conditions similar to those described in the assay for cysteine synthase activity,[4] with the sulfide ion replaced by one of the following thiols: methyl, ethyl, propyl, allyl, butyl, or benzyl mercaptan. One μl of each thiol was

a Sephadex G-100 column (55 × 1.7 cm ⌀), equilibrated with 0.1 M Tris-HCl, pH 7.4. The column was eluted with the same buffer. The enzyme peak was pooled and stored at −18 °C.

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Synthesis of *S*-Substituted Cysteine Derivatives by the Cysteine Synthase (*O*-Acetylserine Sulfhydrylase) of Onion (*Allium cepa*) and *Escherichia coli*

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Previous investigations showed that *Allium* plants can form a wide variety of cysteine derivatives from externally supplied thiols and serine.[1] It was proposed that this reaction is mediated by the non-specific action of cysteine synthase (EN 1972 4.2.99.8). This enzyme was previously known as *O*-acetylserine sulfhydrylase. It is the second member of the two-enzyme system which normally forms cysteine from serine. The cysteine synthase of onion has now been partially purified.[2] In this investigation we have found that cysteine synthase is non-specific with regard to a wide range of thiols, and there is no difference in this respect between enzyme of plant and bacterial origin.

Materials and methods. Substrate. O-Acetyl-L-serine was synthesized by acetylation of L-serine in perchloric acid/acetic anhydride/acetic acid solution.[3] Labeled substrate was prepared on a micro scale from L-serine-C14(U) by the same method. The labeled substrate (specific activity 10 μCi/μmol) was divided in aliquots, vacuum dried and stored dry at −18 °C until use.

Enzyme. The partial purification of onion cysteine synthase is described elsewhere.[2] The enzyme was partially purified from *E. coli* using essentially a small-scale modification of the procedure of Becker *et al.*[4] The starting material was 1 g frozen cells of *E. coli* ATCC 4157, grown in a glucose/inorganic salt medium, and kindly provided by Mr. Seppo Vilkki, Phil. Lic. The cells were mixed with 9 ml buffer (0.05 M Tris-HCl pH 7.8 and 0.01 M 2-mercaptoethanol) and 13 ml ballotini glass beads and disintegrated by continuous circulation for 2 min at 0 °C in a silicone tube agitated by a fast peristaltic pump. The enzyme was purified by streptomycin precipitation and ammonium sulfate fractionation as described by Becker *et al.*[4] After this step the enzyme was dialyzed against 0.05 M Tris-HCl buffer pH 7.6. The specific activity was 1.46 units per mg protein.

Reaction. O-Acetyl-L-serine labeled with L-serine-C14(U) was incubated with the enzyme for 30 min in small, conical, stoppered test tubes under conditions similar to those described in the assay for cysteine synthase activity,[4] with the sulfide ion replaced by one of the following thiols: methyl, ethyl, propyl, allyl, butyl, or benzyl mercaptan. One μl of each thiol was

supplied giving an unknown actual concentration in the aqueous phase because of the wide variation in water solubility and volatility of the thiols used. After 30 min acetic acid was added to stop the reaction, and the mixture was dried in a vacuum desiccator. The reaction products were dissolved in water and subjected to thin layer chromatography with methyl ethyl ketone/pyridine/water/acetic acid (70 + 15 + 15 + 2 v/v) followed by autoradiography.[1,5]

Results. When the enzymes from onion and *E. coli* were incubated with labeled *O*-acetylserine and one of the thiols methyl, ethyl, propyl, allyl, or benzyl mercaptan, distinct spots of *S*-methylcysteine, *S*-ethylcysteine, *S*-propylcysteine, *S*-allylcysteine, and *S*-benzylcysteine, respectively, appeared on the autoradiograms. When butyl mercaptan was tested the reaction was only weak, which may reflect the low water solubility of this compound.

Discussion. Although the biosynthesis of *trans*-(+)-*S*-(propen-1-yl)cysteine sulfoxide from *S*-(2-carboxypropyl)cysteine in onion has been elucidated the origin of the side chain in other *S*-substituted cysteine derivatives has remained unknown.[1] At present, there is no evidence about the origin of the allyl side chain in *S*-allylcysteine sulfoxide in garlic. The side chain of *S*-propylcysteine sulfoxide, a minor component in onion, may be formed by hydrogenation of propenylcysteine sulfoxide, although this has not yet been experimentally proved. The metabolism of *S*-methylcysteine and its sulfoxide has been studied in several higher plants (for literature, see the reviews of Thompson [6] and Granroth [1] and some more recent publications [7–11]). This compound can be formed either by methylation of cysteine or by thiomethylation of serine, but the former reaction seems to be the normal one.

The demonstration in this investigation of the non-specific action of cysteine synthase from two different sources suggests that the ability to form cysteine derivatives from thiols and serine is wide-spread and may be an important detoxification mechanism. In onion sulfur metabolism it explains the side-chain recycling mechanism previously observed.[1] This is a reaction in which the thioalkyl moiety of an *S*-substituted cysteine derivative is combined with serine to form a new molecule with the same structure as the original. This reaction is sometimes confusing and makes the investigation of onion sulfur metabolism more intricate.

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The N-Terminal Amino Acid Sequence of *Bacillus subtilis* α-Amylase

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While it has been reported that native α-amylase isolated from *Bacillus subtilis* has a weight of 48 000 daltons, this weight is reduced to approximately 24 000 in the presence of 6 M guanidine.HCl, *i.e.* two subunits of equal weight are obtained.[1] Unequivocal proof that the primary structure of these two subunits is identical will have to come from sequence studies. Preliminary results, which are reported here, suggest that the enzyme molecule contains only one kind of polypeptide chain.

Two commercial preparations of the α-amylase, one manufactured by Novo Industries,** the other procured from Sigma Chemical Company and labeled Type II-A were examined. Both products exhibited identical elution volumes upon chromatography on Sephadex G-100. (Employing 0.025 M sodium acetate buffer, pH 6.0, as elution medium, a major fraction and a minor-slower migrating one were obtained. Utilizing 0.025 M ethylenediaminetetraacetate buf-

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In our studies with the sequencer, the two preparations yielded identical results.

For the automatic Edman degradation the Beckman sequencer (Model 890B) and the slow protein-Quadrol program was utilized.[3] Phenylthiohydantoins were identified by gas chromatography.[4]

When the intact enzyme was analyzed, the sole sequence at the amino end was found to be:

1 2 3 4 5 6 7 8
Val – Asn – Gly – Thr – Leu – Met – Gln – Tyr –
9 10 11 12
Phe – Glu – Trp – Tyr

As expected, after exposure of the enzyme to CNBr a component (A) was isolated that began as follows:

Gln – Tyr – Phe – Glu – Trp – Tyr

(The treatment with CNBr was performed by allowing a solution of 100 mg enzyme in 25 ml 70 % formic acid to stand for 17 h at room temperature followed by dilution with water and lyophilization. Chromatography on Sephadex G-100 utilizing 0.2 M ammonium hydroxide and monitored at 280 nm yielded two peaks. The first one to emerge was the fraction designated above as component A. This material seemed homogeneous upon polyacrylamide gel electrophoresis while the second fraction appeared heterogeneous).

Further studies of the primary structure of the α-amylase are in progress.

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The Effect of *gem*-Dimethyl Groups in the Cyclization of Diynes

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Since the smallest ring obtainable by oxidative coupling of a *gem*-dimethyl substituted diyne has been shown [1] to be the 18-membered, and the possibility of making cyclic compounds with *gem*-dimethyl groups in different positions is also limited in this method, other cyclization methods had to be considered in order to prepare certain *gem*-dimethyl-substituted rings required for conformational studies.

Cyclization of terminal diynes with dibromides in liquid ammonia to give unsubstituted cyclic diynes has been described earlier [2,3] and this method has now been tried on *gem*-dimethyl substituted diynes.

The cyclization reactions are specified in Table 1 where the corresponding yields and melting points of the cyclic acetylenes formed are also given.

No cyclization to the corresponding 16-membered ring was obtained using 5,5-dimethylnona-1,8-diyne and dibromoheptane. With the same diyne and dibromononane the yield was only 6 %. The longer diyne chain, 6,6-dimethylundeca-1,10-diyne, however, reacted with the shorter dibromide, dibromopentane, to give 15 % yield of cyclic product, and with the *gem*-dimethyl substituted dibromoheptane to give a cyclic, crystalline reaction product in 59 % yield.

The results can be explained by considering the possible conformations of the reactants. Bends on the carbon chain, caused by *gauche* bonds, will most easily occur at the carbon alpha to the acetylene bond or at the *gem*-dimethyl substituted carbon.[3,4] As pointed out earlier,[1] this effect and the additional effect of the steric requirements of the *gem*-dimethyl groups, reduces the number of probable conformers for 5,5-dimethylnona-1,8-diyne to only one, shown in Fig. 1A, having the *gem*-dimethyl group at the "corner" of the chain. The terminal acetylene groups are, however, quite distant in this conformer, and they point in directions unfavourable for cyclization. This explains why no cyclization was obtained with 5,5-dimethylnona-1,8-diyne in its reaction with 1,7-dibromoheptane, as well as the low yield obtained with the same diyne and 1,9-dibromononane. With a *gem*-dimethyl group in the 4-position of dibromoheptane the dibromide chain should more easily become bent and the chances for cyclization should increase; in another work [5] the corresponding 16-membered ring was indeed obtained.

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Table 1. Yields and melting points in cyclization of *gem*-dimethyl substituted diynes and dibromides.

Reactants		Cyclic product	Yield	M.p.
Diyne	Dibromide		%	Cyclic diyne °C
5,5-Dimethylnona-1,8-diyne	1,7-dibromo heptane	[5,5-dimethylcyclohexadeca-1,8-diyne]	0	
»	1,9-dibromo-nonane	5,5-dimethylcyclo-octadeca-1,8-diyne	6	Liquid
6,6-Dimethylundeca-1,10-diyne	1,5-dibromo-pentane	6,6-dimethylcyclo-hexadeca-1,10-diyne	15	26
»	4,4-dimethyl-1,7-dibromo-heptane	6,6,15,15-tetramethyl-cyclo-octadeca-1,10-diyne	21 – 59	91

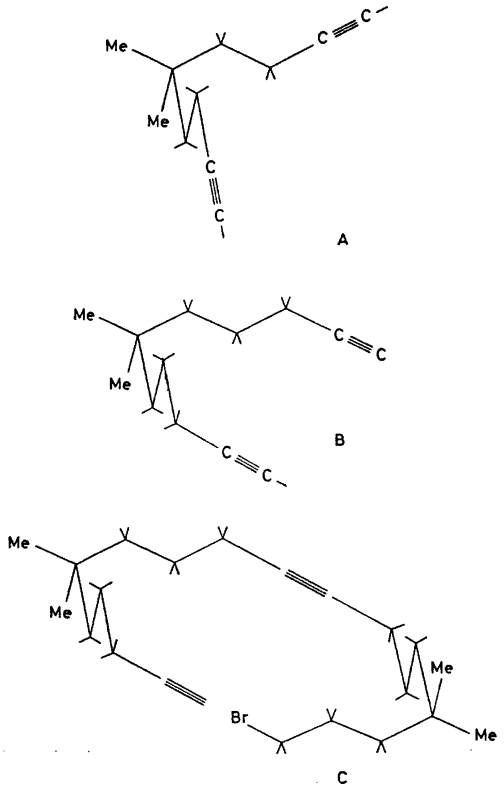

Fig. 1. Conformations before cyclization of:
A, 5,5-dimethylnona-1,8-diyne.
B, 6,6-dimethylundeca-1,10-diyne.
C, 6,6-dimethylundeca-1,10-diyne in reaction with 4,4-dimethyl-1,7-dibromoheptane.

side is shown in Fig. 1B. A conformation with the *gem*-dimethyl groups on a "corner" and with parallel terminal acetylene bonds well fitted for cyclization is then possible. This explains why this diyne gave with 1,5-dibromopentane a cyclic product in 15 % yield. In Fig. 1C the same diyne is shown to fit perfectly together with a conformationally more favourable dibromide. Here the two reactants can take similar conformations and their reacting end groups thereby come in very close positions before the final cyclization. This explains the good yield of cyclic diacetylene obtained in the reaction, as well as the stability of the conformation of the crystalline cyclic compound. It melts as high as 91 °C and shows the same infrared absorptions in the solid phase as in carbon disulfide solution.

Experimental. 5,5-Dimethylcyclo-octadeca-1,8-diyne. To a solution of sodamide, made by dissolving sodium (2.2 g = 0.094 mol) in liquid ammonia (600 ml) was added 5,5-dimethylnona-1,8-diyne [1] (7.0 g = 0.047 mol). After 1 h of stirring 1,9-dibromononane (13.5 g = 0.047 mol) was added. Stirring was continued under refluxing ammonia for 5 d. A condenser with solid carbon dioxide was used. Dry diethyl ether was added (400 ml) and stirring continued for 2 d. Thereafter water was added (500 ml), the ether phase thoroughly washed with water and dried with magnesium sulfate. After filtration and evaporation of ether the residue (9.0 g) was purified on an alumina column, eluted with a solution of benzene/pentane 1/3 whereby *5,5-dimethylcyclo-octadeca-1,8-diyne* (0.8 g = 6 %) was isolated. The compound did not crystallize above – 100 °C. Mol. w. 272 by mass spectrometry. Calc. M = 272. Further identified by the hydrogenated product.[8]

6,6-Dimethylundeca-1,10-diyne. Monosodium acetylide was prepared [6] by passing acetylene into a solution of sodium amide, prepared from sodium (5 g) in liquid ammonia (300 ml). 1,7-Dibromo-4,4-dimethylheptane, prepared in 8 steps from β,β-dimethylglutaric acid [7] was added during 20 min and the reaction mixture stirred for 40 h. The ammonia was allowed to evaporate,

water and diethyl ether added, the ether extract washed with water and dried over magnesium sulfate. Evaporation of the ether gave the crude *6,6-dimethylundeca-1,10-diyne* (7.6 g = 65 %) which was found by IR spectroscopy to contain no dibromide and was used in the next step without further purification.

13,13-Dimethylcyclohexadeca-1,8-diyne. To a stirred solution of sodamide, made from sodium (2.02 g = 0.088 mol) in liquid ammonia (600 ml), was added 6,6-dimethylundeca-1,10-diyne (7.5 g = 0.044 mol). After 1 h 1,5-dibromopentane (12 g) was added and the stirring continued for 8 d under refluxing ammonia. The ammonia was slowly evaporated, the residue dissolved in water and the water solution extracted with ether. The ether extracts were dried with magnesium sulfate, and the ether evaporated. The crude reaction product (7.3 g) was purified on an alumina column, and by elution with a benzene/pentane 1/3 solution was obtained the 13,13-dimethylcyclohexadeca-1,8-diyne (1.5 g = 15 %), m.p. 26 °C. (Found: C 88.33; H 11.45. Mol. w. 244 (by mass spectrometry). Calc. for $C_{18}H_{28}$: C 88.45; H 11.55. Mol. w. 244).

6,6,15,15-Tetramethylcyclo-octadeca-1,10-diyne. 6,6-Dimethylundeca-1,10-diyne (8.5 g = 0.05 mol) was added to a solution of sodamide, made from sodium (2.3 g = 0.1 mol) in liquid ammonia (600 ml) and stirred for 1.5 h.

4,4-Dimethyl-1,7-dibromoheptane [7] (14.3 g = 0.05 mol) was added and the reaction mixture was stirred and refluxed for 5 d. Dry diethyl ether (500 ml) was added, the ammonia evaporated and the ether solution stirred at room temperature for 2 d. Water was added, the ether solution washed with more water, dried with magnesium sulfate. After evaporation of ether the crystalline residue (8.5 g) was dissolved in petane and purified through a short alumina column. After recrystallization with ethanol was obtained: *6,6,15,15-tetramethylcyclo-octadeca-1,10-diyne* (3.0 g = 21 %, 8.5 g = 59 % yield as crystalline reaction product), m.p. 91 °C. (Found: C 87.79; H 12.04. Mol. w. 300 (by mass spectrometry. Calc. for $C_{22}H_{36}$: C 87.92; H 12.08. Mol. w. 300).

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Base-promoted Non-stereospecific 1,2-and 1,4-Elimination Reactions

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The stereochemistry of elimination reactions from the two diastereomeric 1-(1-acetoxyethyl)-indenes (A_1 and A_2) and from 3-(1-acetoxyethyl)indene (B) have been studied in MeO^-/MeOH. The mechanisms of the elimination reactions are discussed.

When a methanol solution 0.03 M in substrate was reacted with KOMe or NaOMe (0.06 M) at 30 °C, *trans-* and *cis-*1-ethylideneindene (C_1 and C_2) were obtained (Scheme 1). The proportions varied considerably with the substrate structure (A_1, A_2, and B). The thermodynamic equilibria exclusively favour C_1 and C_2, no trace of A_1, A_2, or B being observed after long reaction times. Neither any epimerization of A_1 or A_2 nor any 1,3-proton transfer to B could be observed when a deficiency of base was used. The reactions were studied using a quench – – extraction – NMR procedure. The results are given in Table 1.

Table 1. Product compositions obtained with A_1, A_2, and B in MeO^-/MeOH at 29.96 ± 0.07 °C.

Initial substrate conc. 0.03 M	Initial [NaOMe] M	Initial [KOMe] M	$\frac{100\ C_2}{C_1+C_2}$
A_1	0.06		37.1 ± 2.0
A_1		0.06	37.0 ± 2.0
A_2	0.06		8.1 ± 2.0
A_2		0.06	8.2 ± 2.0
B	0.06		22.6 ± 2.0
B		0.06	22.5 ± 2.0
A_1[a]	0.02		38 ± 3
A_2		0.01	7.4 ± 2.0

[a] 0.05 M.

A preliminary correlation between diastereomers and structures (A_1 and A_2) has been made assuming *anti* elimination from substrate A_2 to be favoured over *syn* elimination. The same structure assignments were obtained using Cram's rule. In NMR-spectra of mixtures of C_1 and C_2 the methyl group of C_2 appears at 0.18 ppm lower field. The proximity of the methyl group of C_2 to the deshielding region of the benzene ring is assumed to yield proton resonance at lower field than the methyl protons of C_1.

water and diethyl ether added, the ether extract washed with water and dried over magnesium sulfate. Evaporation of the ether gave the crude *6,6-dimethylundeca-1,10-diyne* (7.6 g = 65 %) which was found by IR spectroscopy to contain no dibromide and was used in the next step without further purification.

13,13-Dimethylcyclohexadeca-1,8-diyne. To a stirred solution of sodamide, made from sodium (2.02 g = 0.088 mol) in liquid ammonia (600 ml), was added 6,6-dimethylundeca-1,10-diyne (7.5 g = 0.044 mol). After 1 h 1,5-dibromopentane (12 g) was added and the stirring continued for 8 d under refluxing ammonia. The ammonia was slowly evaporated, the residue dissolved in water and the water solution extracted with ether. The ether extracts were dried with magnesium sulfate, and the ether evaporated. The crude reaction product (7.3 g) was purified on an alumina column, and by elution with a benzene/pentane 1/3 solution was obtained the 13,13-dimethylcyclohexadeca-1,8-diyne (1.5 g = 15 %), m.p. 26 °C. (Found: C 88.33; H 11.45. Mol. w. 244 (by mass spectrometry). Calc. for $C_{18}H_{28}$: C 88.45; H 11.55. Mol. w. 244).

6,6,15,15-Tetramethylcyclo-octadeca-1,10-diyne. 6,6-Dimethylundeca-1,10-diyne (8.5 g = 0.05 mol) was added to a solution of sodamide, made from sodium (2.3 g = 0.1 mol) in liquid ammonia (600 ml) and stirred for 1.5 h.

4,4-Dimethyl-1,7-dibromoheptane [7] (14.3 g = 0.05 mol) was added and the reaction mixture was stirred and refluxed for 5 d. Dry diethyl ether (500 ml) was added, the ammonia evaporated and the ether solution stirred at room temperature for 2 d. Water was added, the ether solution washed with more water, dried with magnesium sulfate. After evaporation of ether the crystalline residue (8.5 g) was dissolved in petane and purified through a short alumina column. After recrystallization with ethanol was obtained: *6,6,15,15-tetramethylcyclo-octadeca-1,10-diyne* (3.0 g = 21 %, 8.5 g = 59 % yield as crystalline reaction product), m.p. 91 °C. (Found: C 87.79; H 12.04. Mol. w. 300 (by mass spectrometry. Calc. for $C_{22}H_{36}$: C 87.92; H 12.08. Mol. w. 300).

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Base-promoted Non-stereospecific 1,2-and 1,4-Elimination Reactions

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The stereochemistry of elimination reactions from the two diastereomeric 1-(1-acetoxyethyl)-indenes (A_1 and A_2) and from 3-(1-acetoxyethyl)indene (B) have been studied in MeO^-/MeOH. The mechanisms of the elimination reactions are discussed.

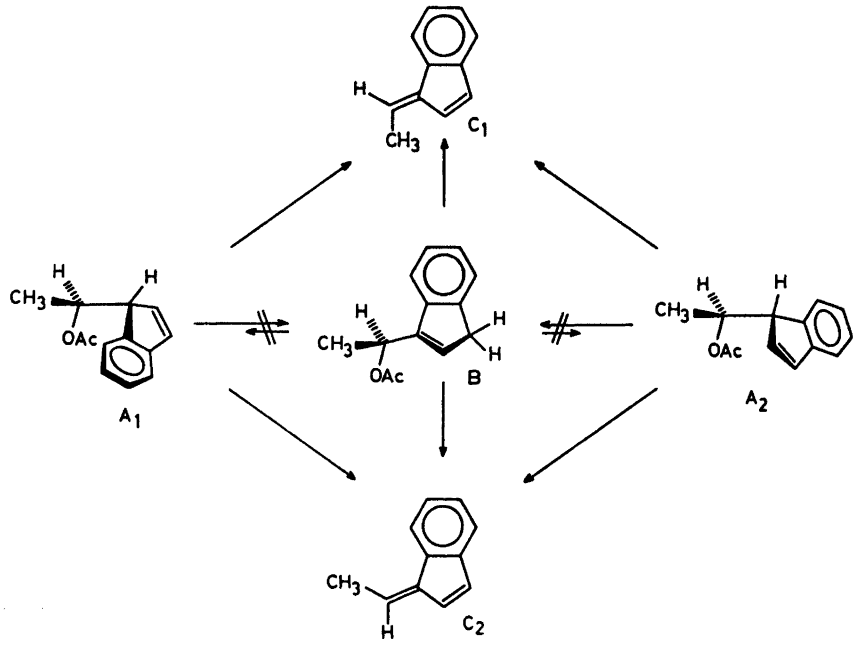
When a methanol solution 0.03 M in substrate was reacted with KOMe or NaOMe (0.06 M) at 30 °C, *trans-* and *cis-*1-ethylideneindene (C_1 and C_2) were obtained (Scheme 1). The proportions varied considerably with the substrate structure (A_1, A_2, and B). The thermodynamic equilibria exclusively favour C_1 and C_2, no trace of A_1, A_2, or B being observed after long reaction times. Neither any epimerization of A_1 or A_2 nor any 1,3-proton transfer to B could be observed when a deficiency of base was used. The reactions were studied using a quench – – extraction – NMR procedure. The results are given in Table 1.

Table 1. Product compositions obtained with A_1, A_2, and B in MeO^-/MeOH at 29.96 ± 0.07 °C.

Initial substrate conc. 0.03 M	Initial [NaOMe] M	Initial [KOMe] M	$\frac{100\ C_2}{C_1+C_2}$
A_1	0.06		37.1 ± 2.0
A_1		0.06	37.0 ± 2.0
A_2	0.06		8.1 ± 2.0
A_2		0.06	8.2 ± 2.0
B	0.06		22.6 ± 2.0
B		0.06	22.5 ± 2.0
A_1[a]	0.02		38 ± 3
A_2		0.01	7.4 ± 2.0

[a] 0.05 M.

A preliminary correlation between diastereomers and structures (A_1 and A_2) has been made assuming *anti* elimination from substrate A_2 to be favoured over *syn* elimination. The same structure assignments were obtained using Cram's rule. In NMR-spectra of mixtures of C_1 and C_2 the methyl group of C_2 appears at 0.18 ppm lower field. The proximity of the methyl group of C_2 to the deshielding region of the benzene ring is assumed to yield proton resonance at lower field than the methyl protons of C_1.



Scheme 1.

The equilibrium constants for interconversion of the substrates were determined in an experiment using pyridine as catalyst. Pyridine was used because its catalytic efficiency in the 1,3-proton transfer reactions is much larger than the rate with which it effects elimination. Only trace amounts of elimination products were obtained. The equilibrium constants were $[B]_{eq}/[A_1]_{eq} \approx 22$, $[B]_{eq}/[A_2]_{eq} \approx 26$, and $[A_1]_{eq}/[A_2]_{eq} \approx 1.2$.

As shown in Table 1 the olefin C_1 is the predominant product in the reactions with all substrates. The same product compositions were obtained with KOMe and NaOMe as bases. Our analytical method did not allow accurate determinations of the absolute second order rate constants because the reactions were too fast ($k_{A1} \approx 0.07$ $M^{-1}s^{-1}$). However, it has been possible to measure relative rates of elimination reactions from the three substrates. Thus, mixtures of A_1 and A_2 or A_1 and B have been reacted in competion experiments with a deficiency of base. The ratios of elimination rates $k_{A1}:k_{A2}:k_B$ were found to be 1:5.2:1.6.

The results show that A_1 and A_2 exclusively give 1,2-elimination reactions and B exclusively 1,4-elimination reactions.

The studied reactions are expected to proceed by mechanisms in the E1cB–E2 part of the mechanistic spectrum. The results give indications of the operation of irreversible carbanion mechanisms, but they do not exclude concerted mechanisms. Carbanion mechanisms are supported by the fact that the elimination reactions from the three substrates converge towards olefin C_1. If the reactions are non-concerted, they all could have a common carbanion as intermediate. Rotation around the $C_\alpha - C_\beta$ bond interconverts different conformations of the carbanion. Thus, the substrates either could give fixed compositions of carbanion conformers, or the intermediate(s) formed have time to interconvert. The experimental results clearly show that an equilibrium mixture of the carbanion conformers is not formed.

The absence of competing epimerization and 1,3-proton transfer reactions is evidence against pre-equilibrium E1cB-mechanisms. In a similar reaction system Ahlberg and Bengtsson[1,2] have found that ion-pairs are intermediates in elimination reactions from 1-(2-acetoxy-2-propyl)indene and 3-(2-acetoxy-2-propyl)indene in methanol with tertiary amines as bases.

The stereochemistry of elimination reactions near the borderline E1cB-E2 has been studied by Cristol and Pappas,[3] who have reported stereoconvergent elimination reactions from *erythro-* and *threo*-2-*p*-toluenesulfonyl-1,2-diphenyl-1-chloroethane in NaOH/EtOH and explained the convergency with a duality of mechanism (E2/E1cB). Cram, Greene and

Depuy [4] have made an extensive study of elimination reactions from diastereomeric substrates. 1,2-Diphenyl-1-propyl-X (X = Br, Cl or NMe_3^+) was reacted in primary, secondary, and tertiary alkoxide/alcohol media. Furthermore the chloride was studied in potassium 2-octyloxide/benzene. All the elimination reactions were *anti* eliminations with one exception, the *erythro* isomer with NMe_3^+ as leaving group gave in *t*-BuOK/BuOH *syn* elimination. Later studies [5] have shown that no exchange is involved, *i.e.* a pre-equilibrium E1cB-mechanism is excluded. Alunni and Baciocchi [6] have also examined the stereochemistry of elimination reactions. They found that 1-phenyl-2-X-propanes (X = halogen) in EtONa/EtOH eliminate with a *trans/cis* ratio ranging from about 25 for the bromo and chloro compounds to 112 for the flouro compound. Sufficient activation of a substrate destroys the stereospecificity and presumably changes the mechanism from E2 to E1cB.[5]

Examples of 1,4-elimination reactions, promoted by base are not common in the literature. Cristol and coworkers [7,8] have studied 1,4-elimination reactions from *cis*- and *trans*-9,10-di-X-dihydroanthracene derivatives (X = halogen, OH, OAc, and OCOPh) with sodium hydroxide in ethanolic dioxane. *syn*-1,4-Elimination was found to be preferred. The diols react presumably with carbanion mechanisms, but the mechanisms of the other reactions are not settled. Naphthalene tetrachlorides with sodium methoxide in methanol/acetone gave 1,2- and 1,4-elimination reactions.[9] Other substrates which have been found to give 1,4-elimination reactions are 1-chloro-2-alkylperfluorocyclobutene and -pentene in KOH/EtOH or MeOH. Contrary to other studies of 1,4-eliminations these two substrates have free rotation around the $C_\gamma - C_\delta$ bond. Mixtures of *cis*- and *trans*-olefins were obtained as products.[10]

Ahlberg and Bengtsson [1,3] have recently reported the first true 1,4-elimination reactions, *i.e.* it was demonstrated that the reactions were not mixtures of 1,3-proton transfer and 1,2-elimination. The substrate, 3-(2-acetoxy-2-propyl) indene, was reacted with tertiary amines as bases in methanol.

The mechanisms of the above reported elimination reactions are under further study in these laboratories.

Experimental. The substrates were prepared by addition of acetaldehyde to indenylmagnesium bromide.[11,12] Chromatographic separation of the alcohols formed followed by zinc dichloride catalysed esterification with acetic anhydride gave diastereomerically pure *threo*- and *erythro*-1-(1-acetoxyethyl)indenes. Rearrangement of the diastereomeric acetate mixture in pyridine produced 3-(1-acetoxyethyl)indene.

Stock solutions of NaOMe and KOMe were prepared by dissolving pure-cut pieces of the metals in methanol (Fluka spectrograde quality dried over molecular sieves) under nitrogen atmosphere. The reactions were carried out in a V-formed flask; 5 ml substrate solution was placed in one of the shanks and 5 ml base solution in the other. The flask was placed in a thermostat at a temperature of 29.96 ± 0.07 °C. After 10 min in the thermostat the reaction was started by shaking the flask. 90 min later the reaction mixture was quenched according to a method previously described by Ahlberg,[1] who has also reported the procedure for determining the equilibrium constants. The analysis were made with NMR-technique.

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Syntheses and Conformations of *gem*-Dimethyl Substituted Cyclo-octadecanes. The 1,1-Dimethyl and the 1,1,4,4-Tetramethyl Derivatives

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Two *gem*-dimethyl substituted cyclo-octadecanes have earlier been synthesized; a tetramethyl derivative diametrically substituted in positions 1 and 10 and an octamethyl derivative symmetrically substituted in the four positions 1, 4, 10, and 13.[1-3] Increased substitution in cyclo-octadecane was shown to raise the melting point, to lower the melting entropy and to increase the conformational stability. The octa-methyl substituted derivative was found to be conformationally homogeneous and to take the same compact conformation in solution as in the crystal.

We have now extended this investigation to cyclo-octadecanes substituted with *gem*-dimethyl groups in only one position and in positions 1 and 4, giving molecules with lower constitutional symmetry than those studied earlier.

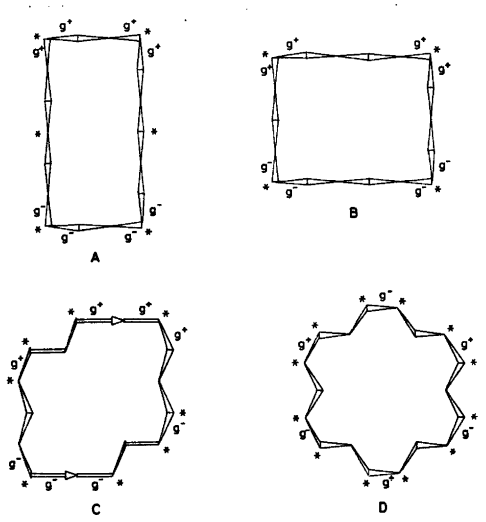

Fig. 1. Possible diamond-lattice conformations of cyclo-octadecane. g = gauche bonds. * = allowed positions for *gem*-dimethyl groups.

1,1-Dimethylcyclo-octadecane and 1,1,4,4-tetramethylcyclo-octadecane were synthesized by hydrogenation of the corresponding cyclic diynes.[4,5] In Table 1 are shown the melting points and the enthalpies and entropies of melting of these compounds together with the results of those earlier determined.

The infrared spectra were taken of the compounds as crystals in potassium bromide and dissolved in carbon disulphide. The spectra of the two phases were not identical.

In Fig. 1 are shown[6] the four possible diamond lattice conformations of cyclo-octadecane and the "allowed" positions for the space-demanding *gem*-dimethyl group. Conformations of lowest energy will, however, be obtained when those of the allowed ring positions which are situated between two *gauche* bonds carry the *gem*-dimethyl groups because thereby a minimum of new gauche-butane interactions are introduced. Dimethyl substitution in the relative positions 1, 4, 10, and 13 between two gauche bonds is only possible in conformation A and this is in accordance with the observation that 1,1,4,4,10,10,13,13-octamethylcyclo-octadecane is conformationally homogeneous. A similar rectangular 3-bond bridge conformation as Fig. 1A is also found by X-ray analyses in an unsubstituted cyclic hydrocarbon, cyclotetratriacontane.[7]

For dimethyl substitution in the relative positions 1 and 10 low-energy and, at the same time, sterically allowed positions exist both in conformation A and C, also in accordance with the observation that more conformers than the crystal conformer exist in solution.

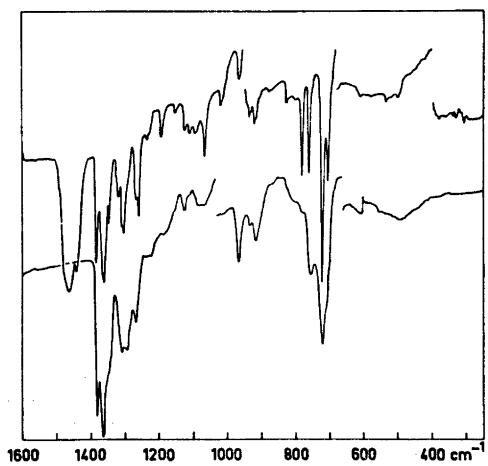

Fig. 2. Infrared spectra of 1,1,4,4-tetramethylcyclo-octadecane as KBr-disc at −50 °C. (upper curve) and in CS_2-solution (lower curve).

Conformational inhomogeneity in 1,1-dimethylcyclo-octadecane is as expected. For the 1,1,4,4-tetramethylcyclo-octadecane the above reasoning should lead to conformational homogeneity. The IR-spectrum in Fig. 2 shows, however, that although the crystal conforma-

Table 1. Melting points, enthalpies, and entropies of melting and conformational homogeneity of cyclo-octadecanes.

	M.p. °C	ΔH kcal/mol	ΔS e.u.	Conformational homogeneity in solution
Cyclo-octadecane	73	9.4[a]	30.1[a]	Inhomogeneous
1,1-Dimethylcyclo-octadecane	10	5.7	20.1	»
1,1,4,4-Tetramethylcyclo-octadecane	37	7.8	25.1	»
1,1,10,10-Tetramethylcyclo-octadecane	86	9.5	26.1	»
1,1,4,4,10,10,13,13-Octamethyl-cyclo-octadecane	165	6.4[a]	14.9[a]	Homogeneous

[a] The compounds have transition points [1] and the values represent the total of transition and melting.

tion is obviously present in solution, the broadening of the bands seems too pronounced to be due to vibrational scattering and could be caused by the existence of other conformers in solution.

In our earlier investigation of *gem*-dimethyl substituted cyclo-octadecanes a raise in melting point and a drop in melting entropy was found with increased degree of substitution.[1] The two new *gem*-dimethyl substituted derivatives of cyclo-octadecane did not follow this regularity. Although the changes in entropies when going from the crystal to the melt are of the expected magnitude, the melting points and melting enthalpies for both compounds are surprisingly much lower than for cyclo-octadecane itself. We think that this is explained by the fact that the first two *gem*-dimethyl substituted cyclo-octadecanes investigated both had a centre of symmetry while the two new compounds, 1,1-dimethyl- and 1,1,4,4-tetramethylcyclo-octadecane both lack this high degree of symmetry. As the crystal packing of the molecules may not be so tight in unsymmetrical molecules as in symmetrical and the melting enthalpy thereby lowered [2] the result is a low melting point in unsymmetrical molecules that is not necessarily due to lower conformational stability.

Experimental. 1,1-Dimethylcyclo-octadecane. To 5,5-dimethylcyclo-octadeca-1,8-diyne [4] (0.2 g) dissolved in cyclohexane (50 ml) was added palladium on charcoal (0.1 g) and the solution fully hydrogenated at 344 kPa (40 h). The catalyst was filtered off, the solvent evaporated, the residue dissolved in pentane and filtered through alumina. Evaporation of pentane gave: *1,1-dimethylcyclo-octadecane.* M.p. 10 °C. Mol.w. 280. (Mass spectrometry). (Found: C 85.72; H 14.35. Calc. for $C_{20}H_{40}$: C 85.63; H 14.37).

1,1,4,4-Tetramethylcyclo-octadecane. 12,12,15-15-Tetramethylcyclo-octadeca-1,7-diyne [5] (0.47 g) was hydrogenated as described above. Recrystallization from ethanol gave: *1,1,4,4-tetramethylcyclo-octadecane.* Mol.w. 308 (Mass spectrometry). (Found: C 85.69; H 14.39. Calc. for $C_{22}H_{44}$: C 85.63; H 14.37).

The infrared spectra were recorded in a Perkin-Elmer Grating Infrared Spectrophotometer 457. For the calorimetric measurements a Perkin-Elmer Differential Scanning Calorimeter IB was used.

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Frontal Gel Chromatography of a Humic Acid

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Gel chromatography has been widely applied in the study of humic acids to obtain discrete fractions or at least reduce their polydispersity. The inherent difficulties of the method, when applied to humic acids, have unfortunately not been considered in detail in most cases. Swift and Posner have recently given the first careful treatment of the subject.[1] They point out that a meaningsful separation requires that "(A) the elution volume of a substance is largely independent of sample concentration and flow rate and (B) the whole of the applied sample (*i.e.* the final peak) is eluted within the total column volume". The last point has been completely neglected by most investigators although the adsorption effects were strongly emphasized already in 1967.[2] Swift and Posner showed that only elution with alkaline buffers (pH of the order of 9) can possible fulfil the condition of negligible adsorption. Tentative molecular weight calibrations were later made using appropriate buffers[3] (one calibration necessary for each buffer).

In the study of humic acids one often has large amounts of samples of low concentrations. Therefore, frontal analysis will give a clearer insight of the complications of the fractionation procedure than zonal analysis. An example of such a frontal analysis will be given in this paper. It partly confirms the conclusions of Swift and Posner and gives at the same time some additional information about the system studied.

Experimental. The humic acid was obtained from a peat soil by pyrophosphate extraction at pH 7. The acid was precipitated with hydrochloric acid, dissolved to pH 7, dialysed and lyophilized. A 0.2 % solution in boric acid-borax buffer (0.033 M − 0.0082 M + 0.02 % NaN_3) was prepared at pH 8.5.

80 ml of this acid was fractionated by gel chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). Two columns (2.54 cm × 33 cm), equilibrated with buffer, were used (void volume 70 ml, total volume 150 ml); in each run 4 ml humic acid were eluted with the buffer. 10 ml fractions were collected at an elution speed of 25 ml/h. A typical elution pattern is shown in Fig. 1. Four fractions were taken out, roughly where indicated, and collected from all the twenty runs. These collected fractions are numbered 1 − 4 in the following. Small but measurable amounts of humic acid are obtained after the total volume, indicating some adsorption even at pH 8.5.

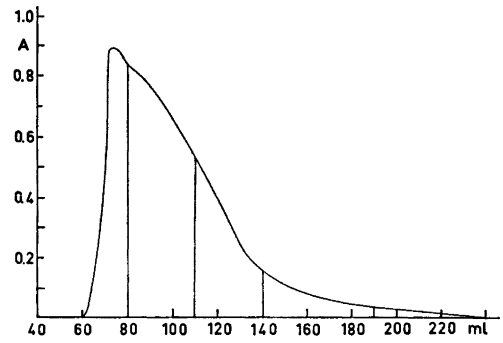

Fig. 1. Example of a zonal gel chromatographic analysis on G-100 of the humic acid. The collection of four main fractions 1 − 4 is indicated. Absorbances at 400 nm as function of elution volume.

Frontal analysis was made of each fraction on the same Sephadex G-100 column. The samples applied were large enough to give rise to a plateau level in each experiment (165 ml of fraction 1 was used, 207 ml of fraction 2, 275 ml of fraction 3 and 273 ml of fraction 4). 15 ml fractions were collected and the absorbance values at 400 nm and 500 nm measured in a 10 mm cuvette on a Zeiss PMQ II Spectrophotometer. In Fig. 2 the results obtained at 400 nm are given as histograms with tentatively drawn elution profiles. The shifts from sample to buffer are indicated by vertical lines.

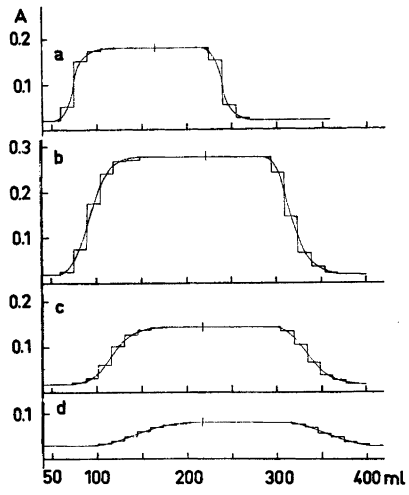

Fig. 2. Frontal gel chromatographic analysis on G-100 of the humic acid at pH 8.5. Absorbances at 400 nm as function of elution volumes. (a) Fraction 1, (b) Fraction 2, (c) Fraction 3, (d) Fraction 4.

The fractions were then recollected and dialysed against a phosphate buffer (Radiometer Type S1001) so that fractions 1–4 at pH 6.5 were obtained with the same concentrations as at pH 8.5. Frontal analyses were made in the same way as above (using 259 ml of fraction 1, 350 ml of fraction 2, 380 ml of fraction 3 and 395 ml of fraction 4). The results are given in Figure. 3.

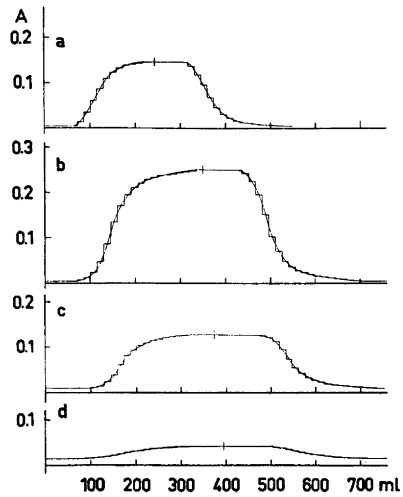

Fig. 3. Frontal gel chromatographic analysis on G-100 of the humic acid at pH 6.5. Absorbances at 400 nm as function of elution volumes. (a) Fraction 1, (b) Fraction 2, (c) Fraction 3, (d) Fraction 4.

Discussion. 1. The histograms at pH 8.5 are enantiographic within the accuracy of the experiment; the front and the back elutions do not differ appreciably. The situations of the half-values of the front and back also agree reasonably well. This means that no complicating equilibria exist in the system and that any existing adsorption must be linear. (The fact that no humic acid appears after the total volume does not itself prove the absence of adsorption; the last fractions still can be retarded.) The skewness of every histogram is in agreement with the shape of the corresponding fraction in the zonal analysis. Some of the fractions were rerun after dilution and similar patterns obtained, further confirming that a true fractionation existed.

2. At pH 6.5 the results of the frontal analyses are quite different. All fractions are appreciably retarded. The histograms are, however, still enantiographic within the limits of experimental accurary. Only two main explanations seem to be possible: the molecules have decreased in size or the adsorption (admittedly linear adsorption) is greater at the lower pH. The mere existence of a retardation cannot definitely distinguish between the two possibilities. The results show the correctness and importance of the statement by Swift and Posner that size calibrations must be made for each type of buffer.

3. The resolution achieved can easily be evaluated from the frontal analyses. When fraction 1 at pH 8.5 has passed to 90 %, fraction 2 has already passed to 37 %; and for 90 % of fraction 2, 50 % of fraction 3 has been obtained. At pH 6.5 the situation is slightly better for the resolution between fraction 1 (containing the excluded part) and fraction 2 (90 % of fraction 1 corresponds to 30 % of fraction 2), while the following resolution is much worse (90 % of fraction 2 corresponds to 80 % of fraction 3). It seems natural to explain these effects as due to a larger adsorption at pH 6.5 than at pH 8.5. Even at the higher pH the resolution is not as good as would be expected for only size separation. Some adsorption would lead to a trailing of the high-molecular fractions to overlap with the subsequent less high-molecular fractions, and would be increasingly disturbing for the last fractions as is observed. As long as the adsorption is linear it does not affect the size calibration for a given buffer but it makes the resolution of the fractionation worse.

4. The ratios A_{400}/A_{500} found for the three first fractions (the low concentrations of the fourth made the determinations very uncertain) are 2.60, 2.54, and 2.36 at pH 8.5 and 2.75, 2.62, and 2.36 at pH 6.5. The fractionation is thus reflected in the absorption spectra indicating larger similarity with the "core structure" (*cf.* Ref. 4) for the low-molecular fractions.

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Friedel-Crafts Reactions. II.* Amidoalkylation by *N*-Acetoxymethyl-*N*-methylformamide in the Presence of Trifluoroacetic Acid

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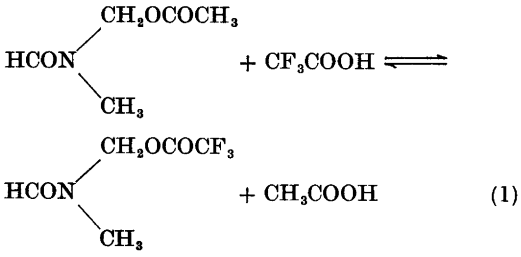
Amidoalkylation of aromatic compounds has received considerable attention, since it represents a convenient method for the synthesis of derivatives of benzylamines.[2] The reaction is generally carried out using *N*-hydroxymethylamides or *N*-hydroxymethylimides in the presence of a catalyst (proton or Lewis acids). The major limitation of the reaction often is the preparation of the *N*-hydroxymethylamide and its instability towards isolation, and it would therefore be an advantage to use the corresponding esters as starting materials.[2]

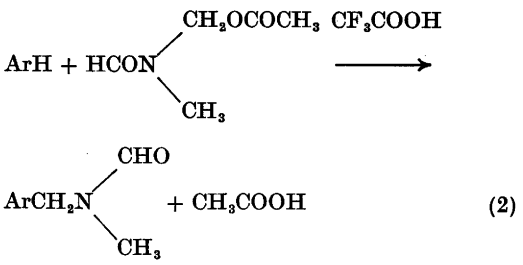
A direct synthesis of *N*-acetoxymethyl-*N*-methylformamide and *N*-formyloxymethyl-*N*-methylformamide has been achieved by anodic oxidation of *N*,*N*-dimethylformamide in acetic acid and formic acid, respectively.[3] The formyloxy derivative is capable of amidomethylating aromatic compounds in the presence of hydrochloric or sulfuric acid.[4]

When *N*-acetoxymethyl-*N*-methylformamide is dissolved in trifluoroacetic acid, an equilibrium is established, as can be shown by NMR, according to eqn. 1.



(1)

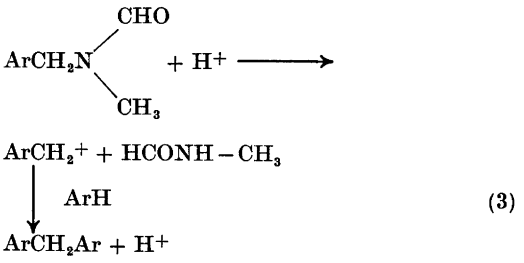
Upon addition of an aromatic compound a reaction takes place, leading to the formation of an *N*-benzyl-*N*-methylformamide derivative. The overall reaction is represented by eqn. 2. The rate of the reaction is determined by the concentration of trifluoroacetic acid as well as the nucleophilicity of the aromatic compound. Some examples of the synthetic scope of the reaction is shown in Table 1. The reactions with mesitylene, pentamethylbenzene and 1,3,5-trimethoxybenzene are very efficient under mild reaction conditions. Benzene, however, is a



(2)

weak nucleophile in this reaction and requires a considerable reaction period to give a reasonable yield of the product even in the presence of an additional catalyst (CF_3SO_3H).

The only side-reaction of any importance is the formation of diphenylmethanes. This probably takes place in an acid-catalyzed reaction according to eqn. 3.[1] However, the formation of



(3)

diphenylmethanes can be almost totally suppressed by selecting the reaction conditions properly (using equivalent amounts of acetate and aromatic compound, minimum amounts of catalyst).

Experimental. *N*-Acetoxymethyl-*N*-methylformamide was prepared in the following way using a concentric capillary gap cell.[5] A solution of *N*,*N*-dimethylformamide (5.0 mol), acetic acid (1.5 l) and Bu_4NBF_4 (0.023 mol) was electrolyzed at a current of 50 A and an applied voltage of 40 – 60 V with a temperature of 60 °C. When 2 F/mol of *N*,*N*-dimethylformamide had been passed, the electrolysis was interrupted. Acetic acid and *N*,*N*-dimethylformamide were removed by distillation up to 70 °C/20 mmHg. The product was then collected at 70 – 75 °C/1.5 mmHg (601 g; 92 % yield). The acetate has also been prepared on a 50 mol scale using a large electrolysis cell operating at 350 A.[6]

General procedure. Equivalent amounts of *N*-acetoxymethyl-*N*-methylformamide and the aromatic compound were dissolved in 25 ml of the solvent. Trifluoroacetic acid was added and the mixture was stirred (see Table 1 for details of the reaction conditions). When the reaction was over, trifluoroacetic acid and the solvent were removed by evaporation *in vacuo*. The residue was dissolved in ether, washed with sodium bicarbonate solution until neutral, washed with

* Part I, *cf.* Ref. 1.

Table 1. Yields of *N*-benzyl-*N*-methylformamides in the reaction between aromatic compounds (0.1 mol) and *N*-acetoxymethyl-*N*-methylformamide (0.1 mol) in the presence of trifluoroacetic acid.

ArH	CF_3COOH (mol)	Solvent	Reaction time (h)	Yield (%)
Benzene	0.5[a]	$CHCl_3$[b]	96	40
Mesitylene	0.25	CH_2Cl_2[c]	16	93
Pentamethyl benzene	0.25	$CHCl_3$[b]	5	85
1,3,5-Trimethoxy-benzene	0.1	CH_2Cl_2[c]	20	81

[a] CF_3SO_3H (0.01 mol) was used as an additional catalyst. [b] At reflux temperature. [c] At room temperature.

water, and finally dried over anhydrous sodium sulfate. After filtration and removal of the ether by evaporation *in vacuo* the product was isolated as shown below. The mixture from the reaction with 1,3,5-trimethoxybenzene was diluted with methylene chloride and worked up as above (the product was poorly soluble in ether).

N-*Benzyl-N-methylformamide.* The product was isolated by distillation, b.p. 148–150 °C/20 mmHg (6.0 g; 40 % yield). The NMR spectrum was in agreement with published data,[7] MS: *m/e* 149 (100 % abundance), 148 (18), 134 (11), 120 (7), 106 (18), 92 (10), 91 (73), 79 (15), 65 (14), 42 (18).

N-*(2,4,6-Trimethylbenzyl)*-N-*methylformamide.* The residue was dissolved in 50 ml of hexane. Crystallization took place at −20 °C giving the product, m.p. 59–61 °C (17.8 g; 93 % yield). NMR (in $CDCl_3$): δ 2.27 and 2.28 (9 H, $ArCH_3$), 2.72 and 2.80 (3 H, $N-CH_3$), 4.50 and 4.67 (2 H, $N-CH_2$), 6.90 (2 H, ArH), 8.20 (1 H, CHO). MS: *m/e* 191 (15), 133 (20), 132 (100), 117 (11).

N-*(2,3,4,5,6-Pentamethylbenzyl)*-N-*methylformamide.* The residue was dissolved in 50 ml of boiling hexane. After cooling, the precipitate was filtered off, giving the product, m.p. 87–89 °C (18.7 g; 85 % yield). NMR (in $CDCl_3$): δ 2.22 (15 H, $ArCH_3$), 2.58 and 2.75 (3 H, $N-CH_3$), 4.45 and 4.67 (2 H, $N-CH_2$), 8.00 and 8.08 (1 H, CHO). MS: *m/e* 219 (10), 161 (11), 160 (100), 145 (9).

N-*(2,4,6-Trimethoxybenzyl)*-N-*methylformamide.* The residue was dissolved in a mixture of 100 ml of cyclohexane and 25 ml of benzene at boiling. After cooling, the precipitate was filtered off, giving the product, m.p. 103–105 °C (19.3 g; 81 % yield). NMR (in $CDCl_3$): δ 2.72 (3 H, $N-CH_3$), 3.80 (9 H, CH_3O), 4.35 (2 H, $N-CH_2$), 6.12 (2 H, ArH), 8.25 (1 H, CHO). MS: *m/e* 239 (47), 210 (17), 182 (10), 181 (100), 180 (13), 168 (17), 136 (16), 121 (22).

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Electrolytic Generation of Nucleophiles. IV.* Reductive Alkylation and Acylation of Disulfides

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Mayer and coworkers [3] have briefly reported on the electrolytic reductive methylation of various disulfides with dimethyl sulfate in aqueous isopropanol on a rather small scale. We have extended the reaction to aprotic medium with a variety of electrophiles to present a general alternative way of preparing sulfide derivatives and demonstrate another example of *in situ* reaction of electrochemically generated nucleophilic species.

The electrolytic reduction of a disulfide bond leads to a thiolate anion which could either be protonated to give the corresponding mercapto derivative [3] or react with an appropriate electrophile present, thus creating a new C–S linkage:

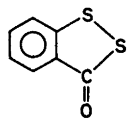
$$R-S-S-R \xrightarrow{2e^-} 2RS^- \xrightarrow{H^+} RSH$$
$$\qquad\qquad\qquad\qquad\quad \xrightarrow{R'X} R-S-R'+X^-$$

In the present investigation have been employed readily available (mostly symmetric) disulfides which yielded products of relatively low volatility (Tables 1 and 2). One advantage of the electrochemical method was already pointed out by Mayer and coworkers:[3] Derivatives can be made from disulfide precursors where the corresponding thiols are unstable or not known as in the case of 3*H*-1,2-benzodithiol-3-one and -3-thione. Another feature is that controlled potential electrolysis can work selectively on substrates which contain other reducible functions as exemplified by bis(2-nitrophenyl)disulfide. This is of course also possible by normal chemical procedures, if a suitable reducing agent can be found.

For a given disulfide the choice of the electrophile is restricted by the fact that the S–S bond must be easier reducible than the electrophile itself. Methyl chloride in an aprotic polar solvent, such as *N*,*N*-dimethylformamide (DMF), was found to be a convenient and efficient methylating reagent for the reductive methylation of most disulfides in DMF (Table 1). This has also recently been used by Simonet and coworkers [4] in electrolytic reductive alkylation of sulfonyl chlorides. Results from experiments with some other alkylating and acylating agents are given in Table 2 showing that the alkylations mostly give better yields than the acylations. In the experiment with *N*,*N*-dimethylcarbamoyl chloride, the electrophile was reduced at the potential applied, so that a good yield was not to be expected. However, the fact that our attempts to prepare the wanted dimethylthiocarbamoyl dimethylcarbamoyl sulfide by conventional methods [5] were unsuccessful, makes the relatively low yield (44 % crude) more acceptable. The addition of pyridine did not affect the product yield significantly

* *Cf.* Refs. 1–2.

Table 1. Results from reductive methylation of disulfides (5.0 g or 5.0 ml) with CH_3Cl in DMF/0.5 M LiCl.



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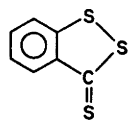

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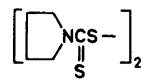

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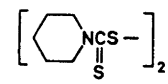

4

Starting material	$-E$ (V) *vs.* Ag/AgCl/0.5 M Cl^-	Product yield (%) Crude	Isolated	B.p./mmHg or M.p. (°C)	n_D^{25}
$[C_6H_5CH_2S-]_2$	1.2	95	82	84–87/10	1.5608
$[C_6H_5S-]_2$	0.85	95	83.5	70–71/9	1.5835
$[p\text{-}CH_3C_6H_4S-]_2$	1.0	95	85.5	85–88/10	1.5730
$[C_6H_5COS-]_2$	0.8	90	70	112–120/10	1.5820
$[o\text{-}NO_2C_6H_4S-]_2$	0.3	94	91	67–68	
$[o\text{-}C_2H_5O-CO-C_6H_5S-]_2$	0.6	95	89	106–109/0.15	1.5754
1	0.15	98	85.5	53–54	
2	0.35	84	78	59–60	
$[(CH_3)_2NCSS-]_2$	1.0	98	81	46–47	
3	0.8	94	88	90–91	
4	1.2	86	78	36–37	

Table 2. Results from reductions of disulfides (5.0 g or 5.0 ml) with other electrophiles in DMF/0.5 M LiCl.

Starting material	$-E$ (V) *vs.* Ag/AgCl/0.5 M Cl^-	Electrophile (ml)	Product yield (%) Crude	Isolated	B.p./mmHg or M.p. (°C)	n_D^{25}
$[CH_3S-]_2$	1.2	$C_6H_5CH_2Cl$ (18)	–	63	82–86/9	1.5604
$[CH_3COS-]_2$	0.8	» (5)	87	76.5	65–75/0.25	1.5564
$[C_6H_5S-]_2$	0.8	» (5.5)	98	92	41–42	
$[C_6H_5CH_2S-]_2$	1.2	$CH_3CHClCH_3$ (20)	90	75.5	100–104/10	1.5362
»	1.2	$(CH_3CO)_2O$[a] (15)	95	89.5	68–72/0.25	1.5564
$[o\text{-}NO_2C_6H_4S-]_2$	0.2	» [a] (15)	87	68.5	118–125/0.25	1.5910
»	0.2	CH_3COCl (10)	–	39	110–120/0.15	1.5912
»	0.3	CH_3I (2.2)	89	84	68–69	
»	0.3	$(CH_3O)_2SO_2$ (10)	98	91	66–68	
»	0.3	$(CH_3CH_2O)_2SO_2$ (20)	82	76.5	115–125/0.25	1.6234
$[(CH_3)_2NCSS-]_2$	0.4[b]	$(CH_3)_2NCOCl$[c] (5)	44	37	76–77	
$[C_6H_5COS-]_2$	0.4	CH_2Br_2 (2)	98	66[d]	118–119	
»	0.4	CH_2Cl_2 (1.2)	57	34[d]	108–110	

[a] 5 ml of pyridine added. [b] Solvent acetonitrile/0.8 M $NaClO_4$, sat. LiCl in the reference electrode. [c] 2 ml of pyridine added. [d] The product was di-*S*-thiobenzoylmethane.

in this particular case, but it proved to be advantageous for the reductions with acetic anhydride.

In most experiments DMF has been employed as the solvent, but acetonitrile may also be useful for reductive alkylations and acylations. In some cases, *e.g.* dibenzyldisulfide, the yield of methylated product was considerably lower in acetonitrile (82 %) than in DMF (95 %), whereas with *N*,*N*-dimethylcarbamoyl chloride as the electrophile the yield was higher in acetonitrile than in DMF. Ethanol and presumably other protic solvents are inferior to the aprotic solvents and a considerable amount of thiol was found as a side product in methylations with methyl chloride, but possibly dimethyl sulfate may be used instead.

Experimental. The electrolytic equipment has been described earlier.[1] The disulfides were either commercial products or prepared by literature methods. The DMF was dried over A4 molecular sieves. Boiling and melting points are uncorrected.

General procedure for electrolytic reductive alkylation and acylation. The disulfides (5.0 ml or 5.0 g) were reduced overnight at room temperature (cell surrounded by a water bath) in DMF (approx. 175 ml) containing 0.5 M lithium chloride. The reference electrode was Ag/AgCl/0.5 M LiCl in DMF, and working potentials and amounts of added electrophile are indicated in Tables 1 and 2. For some of the very easily reducible compounds the mercury cathode was polarized negatively before addition of the disulfide to avoid the formation of a black precipitate by reaction with the metal. The electricity consumption was usually very close to 2 F/mol of disulfide. After reduction an equal volume of benzene was added to the catholyte which was then washed 3 times with 1.5 l of water to remove the DMF. For experiments in acetonitrile the solvent was evaporated *in vacuo* before the extraction with benzene. The organic layer was dried over anh. magnesium sulfate, the solvent removed *in vacuo*, and the residue either fractionated *in vacuo* or recrystallized from ethanol. In a few experiments with diphenyl- and dibenzyldisulfide about 600 ml of water were added to the catholyte which was then extracted continuously with petroleum ether (16–20 h) and worked up as above. The extraction procedure was checked with benzyl methyl sulfide giving 95 % recovery. The prepared sulfide derivatives were known from the literature and identified by comparison with authentic specimens or literature data. Optimization of yields has not been attempted.

S-*Methyl(2-thiomethyl)thiobenzoate.* For this compound our m.p. (53–54 °C) did not agree with the reported[3] one (121–122 °C). NMR-spectrum (60 MHz, CCl_4, TMS): δ 2.35 (s, 3 H); δ 2.40 (s, 3 H); δ 6.9–7.9 (multiplet, 4 H). Strong IR-absorptions (KBr, cm^{-1}): 1660, 1205, 905. (Found: C 54.47; H 5.13; S 32.07. Calc. for $C_9H_9OS_2$: C 54.54; H 5.09; S 32.30). For comparison the NMR (CCl_4) *S*-methyl signals are given for S-*methylthiobenzoate:* δ 2.40 (s, 3 H) and S-*methyl(2-methylthio)dithiobenzoate*: δ 2.32 (s, 3 H) and δ 2.69 (s, 3 H).

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Radiation Effects on the Glycosidic Bond in Some Crystalline Disaccharides

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Irradiated crystalline disaccharides have been analyzed by gas chromatography of the trimethylsilyl derivatives for the presence of constituent monosaccharides after dissolving in pyridine or water. Sucrose yields D-fructose and α-D-glucose in equal amounts, with initial G-values of 0.8. Maltose monohydrate yields equal amounts of α- and β-D-glucose with $G \approx 0.15$, and trehalose dihydrate yields α-D-glucose with $G = 0.8$. By means of these results and previously reported degradation yields, it is demonstrated that the major part of the radiation damaged molecules in sucrose, maltose monohydrate, and trehalose dihydrate has intact glycosidic bonds. No detectable amounts of D-glucose or D-galactose were found in irradiated lactose monohydrate.

The susceptibility of the glycosidic bond in crystalline disaccharides to ionizing radiations has been mentioned in the early literature,[1–4] but no conclusive data have yet been reported.

The total degradation yields for four crystalline disaccharides have been reported in a previous communication [5] together with degradation yields, measured as degradation of their constituent monosaccharides, for three of these disaccharides (sucrose, maltose monohydrate, and trehalose dihydrate). The results showed that the damage to these disaccharides is mainly associated with only one of the monosaccharide moieties of any degraded disaccharide molecule. These results give rise to two possible interpretations. The glycosidic bond may be either intact, joining the damaged monosaccharide moiety (or part of it) with the undamaged one, or broken, in which case a constituent monosaccharide is released. We have now measured the radiation induced release of constituent monosaccharides for sucrose, maltose monohydrate, trehalose dihydrate, and lactose monohydrate, and the present results give information about the radiation sensitivity of the glycosidic bonds.

EXPERIMENTAL

The ^{60}Co γ-irradiation and the disaccharides, sucrose, β-maltose monohydrate, α,α-trehalose dihydrate, and α-lactose monohydrate, have previously been described.[5]

For the determination of released constituent monosaccharides in the irradiated crystalline disaccharides, a known amount of anhydrous pyridine was added to a weighed amount of the irradiated or unirradiated sample to give a concentration of approximately 10 mg carbohydrate/ml pyridine. The samples were either directly dissolved in pyridine or first dissolved in water and subsequently freeze-dried.

The trimethylsilyl derivatives were prepared by addition of hexamethyldisilazane and trimethylchlorosilane to the pyridine solution, as described by Sweeley *et al.*[6] As reference samples unirradiated disaccharides, and occasionally irradiated disaccharides, were used to which were added known small amounts of the appropriate monosaccharides dissolved in pyridine or water.

The pyridine solutions of the derivatives were analyzed by gas chromatography on a Perkin Elmer F11 with FID detection.[7] Columns were: 1.4 m × 2.2 mm of 2 % SE 30 on Chromsorb W 60 – 80 mesh at 160 °C and 1.2 m × 2.2 mm of a 2:1 mixture of 8 % QF 1 and 4 % SF 96 on Gas Chrom P 100 – 120 mesh at 140 °C.

RESULTS

For the investigated disaccharides, with the exception of lactose monohydrate, irradiation gave rise to small amounts of the constituent

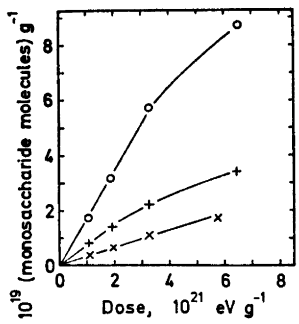

Fig. 1. γ-Radiation induced release of monosaccharides from crystalline disaccharides *vs.* dose: O, D-fructose + α-D-glucose from sucrose; +, α-D-glucose from trehalose dihydrate; ×, α- + β-D-glucose from maltose monohydrate.

monosaccharides which did not appear in unirradiated samples. Sucrose yielded D-fructose and α-D-glucose in equal amounts. Maltose monohydrate yielded α- and β-D-glucose in equal amounts, whereas trehalose dihydrate only gave rise to α-D-glucose. The yield-dose curves for the formation of these free constituent monosaccharides are given in Fig. 1 and the initial G-values are given in Table 1. Neither α- and β-D-glucose nor α- and β-D-galactose were present in detectable amounts in irradiated lactose monohydrate (Table 1).

There were no detectable differences in the gas chromatograms or yields of monosaccharides between samples analyzed directly after irradiation, and samples analyzed after dissolving in water and subsequent freeeze-drying, with the exception that mutarotation occurred for D-glucose when the aqueous solutions were not frozen immediately after dissolving the sample.

Table 1. Initial G-values for the release of constituent monosaccharides from crystalline disaccharides.

	G-value			
	D-Fructose	α-D-Glucose	β-D-Glucose	β-D-Galactose
Sucrose	0.8	0.8		
Maltose.H_2O		≈0.15	≈0.15	
Trehalose.$2H_2O$		0.8		
Lactose.H_2O		<0.1		<0.1

DISCUSSION

The fact that D-glucose released from sucrose, maltose monohydrate, and trehalose dihydrate are the anomers which would be expected from the spatial configuration of the disaccharide, shows that the breakage of the glycosidic bond is concomitant with retention of the geometry of the carbon atom involved in the bond. An inversion, *e.g.* from α- to β-D-glucose in sucrose, of a detectable magnitude ($G \approx 0.1$) would have been noticed. The monosaccharides are probably already present as such in the solid state, as their formation is independent of the method used to dissolve the irradiated sample. The pyranoside and furanoside forms of D-fructose have been resolved by GLC of their trimethylsilyl derivatives.[8] They are, however, not separated by the experimental conditions used in the present investigation (*cf.* Ref. 6) which were employed to separate and quantitate the small amounts of released constituent monosaccharides.

Lactose monohydrate behaves differently from the three other investigated disaccharides. The initial total degradation yield for lactose monohydrate with $G \approx 50$ is also exceptionally high compared with the G-values of 6 – 8 for the other disaccharides.[5] The absence of released glucose and galactose in irradiated lactose monohydrate is in agreement with data given by von Sonntag and Dizdaroglu.[9]

The G-values for the radiation induced degradation of sucrose, trehalose dihydrate, and maltose monohydrate have been determined to be 6, 7, and 8, respectively and the G-values for the degradation of their constituent monosaccharides have been determined to be the same or slightly higher,[5] *i.e.* for sucrose: G(−glucose) + G(−fructose) = 6, for trehalose dihydrate: G(−glucose) = 7, and for maltose monohydrate: G(−glucose) = 10.

From these data and the present results on the release of constituent monosaccharides (Table 1), it can be calculated that radiation damaged molecules of the type S−O−S*, in which one monosscharide moiety (S) is damaged but has an intact glycosidic bond, account for more than 70 % of the degradation in sucrose and maltose monohydrate and for about 90 % in trehalose dihydrate. It can also be calculated that processes leading to breakage

of the glycosidic bond have $G \leq 1.6$ in sucrose, $G \leq 2.3$ in maltose monohydrate, and $G \leq 0.8$ in trehalose dihydrate. This result makes it difficult to assign the glycosidic bond as origin and/or trapping site of the free radicals as these are formed with G-values of $3-5$.[10,11]

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Transformation of Steroids by Cell-free Preparations of *Penicillium lilacinum* NRRL 895. V. Properties of 20-Oxopregnane Side Chain Cleavage and 20(α+β)-Oxidoreductase Activities

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Assay conditions for the 20-oxopregnane side chain cleavage and the 20(α + β)-oxidoreduction in cell-free preparations from *P. lilacinum* have been determined. Progesterone and 17α-hydroxyprogesterone but not 17α-acetoxyprogesterone were substrates for the two types of enzymes. Side chain cleavage showed an absolute requirement for NADPH and may involve participation of flavins and iron-sulphur protein. The reaction was intensitive to cyanide, azide, fluoride, and carbon monoxide, indicating that no cytochromes take part in the oxygenation of the substrate. Reduction at C-20 required NADH or NADPH with a preference for NADH. NADPH favoured formation of the 20α-alcohol while NADH favoured formation of the 20β-epimer. Clomiphene citrate selectively inhibited the 20α-reduction.

Oxidation of ketones to esters (Baeyer-Villiger oxidations) are well known in microbial metabolism. Long chain aliphatic ketones, simple cyclic ketones, monoterpenes and steroids serve as substrates for this reaction.[1] While the electron transport system in the monoterpene biooxidation has been thoroughly studied,[2-6] little is known about the corresponding systems utilized in steroid oxidations. Prairie and Talalay studied the lactonization of ring D in 17-oxosteroids by a partially purified enzyme from *P. lilacinum*.[7] They found that atmospheric oxygen was incorporated in the testololactone formed from 4-androstene-3,17-dione. The reaction showed an absolute requirement for NADPH and was insensitive towards cyanide, chelating agents and several metal ions. The C_{17-20}-lyase and steroid esterase activities in purified enzyme preparations from *Cylindrocarpon radicicola* have been studied by Rahim and Sih.[8] Esterase-free lyase preparations transformed progesterone into testosterone acetate in good yield and Nakano and co-workers demonstrated that atmospheric oxygen was incorporated in the 17β-position.[9] The reaction required NADPH and the effects of metal ions and inhibitors were similar to those observed for the ring D lactonization.

The 20-oxopregnane side chain cleavage in cell-free extracts from *P. lilacinum* has recently been shown to proceed *via* the same pathway as in *C. radicicola* and other microorganisms.[8,10-15] This reaction is accompanied by reduction of the 20-oxo group, and it has been suggested that the 20-reductase(s) compete with the side chain cleaving enzyme for the C_{21} steroid substrate.[16,17] The present paper describes assay conditions for the 20-oxopregnane side chain cleavage as well as 20(α + β)-reduction. Effects of metal ions, inhibitors, and substrate structure have also been studied.

MATERIALS AND METHODS

Abbreviations and trivial names. GLC: gas liquid chromatography; GC-MS: gas chromatography-mass spectrometry; silyl: trimethylsilyl; TLC: thin layer chromatography; UV: ultra-

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violet. Clomiphene citrate: 1(*p*-2-diethylaminoethoxyphenyl)-1,2-diphenyl-2-chloroethylene dihydrogen citrate; progesterone: 4-pregnene-3,20-dione; 17α-acetoxyprogesterone: 17α-acetoxy-4-pregnene-3,20-dione; 17α-hydroxyprogesterone: 17α-hydroxy-4-pregnene-3,20-dione; testololactone: 17α-oxa-4-androstene-3,17-dione; testosterone: 17β-hydroxy-4-androsten-3-one.

Radioactive steroids. [7-^{3}H]Progesterone (specific activity 16 Ci/mmol) and [21-^{14}C] progesterone (specific activity 0.050 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass, They were purified by TLC before use.

[7-^{3}H,21-^{14}C]17α-Hydroxyprogesterone was prepared by enzymatic 17α-hydroxylation of labelled progesterone.[18] The following procedure was used: Five male Sprague-Dawley rats (200 – 230 g) were killed by a blow to the head. The testes were removed, decapsulated and washed with ice cold 0.06 M Tris-HCl pH 7.2, containing 5 g of NaCl, 0.3 g of KCl, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4.7H_2O$, and 2 g of glucose per litre. The testes were homogenized with one volume of the medium in a Turmix household mixer for 60 s at +4 °C. Twenty ml of the homogenate were transferred into a beaker and 50 μCi of [7-^{3}H]progesterone + 20 μCi of [21-^{14}C]-progesterone in 30 μl of ethanol and 20 mg of NADPH were added. The mixture was stirred in air at 37° for 45 min. The reaction was terminated by the addition of 1 ml acetic acid. After addition of 50 μg each of unlabelled progesterone, 4-androsten-3,17-dione, 17α-hydroxyprogesterone, 20α-hydroxy-4-pregnen-3-one, and testosterone, the mixture was extracted three times with one volume of ethyl acetate. The ethyl acetate extract was washed with 1 M NaOH, 8 % $NaHCO_3$ and water and was evaporated to dryness.

The extract was defatted by partition between 70 % methanol and hexane and subjected to TLC on Silica gel GF_{254} (system I). The zone containing 17α-hydroxyprogesterone + 20α-hydroxy-4-pregnen-3-one was isolated, acetylated with acetic anhydride in pyridine and subjected to a second TLC in the same system. The ^{3}H radioactivity in the 17α-hydroxyprogesterone zone corresponded to a yield of 25 %. The 17α-hydroxyprogesterone was rechromatographed on Al_2O_3 GF_{254} 0.5 % ethanol in benzene as solvent. It moved as a single homogenous band. Its radiochemical homogeneity was established by crystallization to constant specific activity.

In the substrate solutions the radioactive compounds were diluted with unlabelled steroids dissolved in ethanol. For progesterone the final radioactivity corresponded to 18 000 – 100 000 cpm ^{3}H per 0.319 μmol steroid in 10 μl of ethanol. The corresponding figure for 17α-hydroxyprogesterone was 2 500 cpm ^{3}H 0.319 μmol steroid in 10 μl of ethanol.

Other reagents. Progesterone, 20α-hydroxy-4-pregnen-3-one, and 20β-hydroxy-4-pregnen-3-one were purchased from Ikapharm Ltd, Ramat-Gan, Israel; 5α-cholestane, 17α-hydroxyprogesterone, testosterone, and 4-androstene-3,17-dione from Sigma Chemical Co, St Louis, Mo, and 17α-acetoxyprogesterone, 17α,20α-dihydroxy-4-pregnen-3-one, and 17α,20β-dihydroxy-4-pregnen-3-one from Steraloids Inc., Pawling, N. Y. All steroids were checked for purity by TLC and/or GLC.

Clomiphene citrate was kindly donated by Draco, AB, Lund, Sweden. It contained 60 % of the *cis* and 40 % of the *trans* epimer.

Table 1. Variation between individual samples in duplicate incubations expressed as S. D. $=\sqrt{\sum d^2/2N}$. N = number of duplicate incubations.

Steroid	Mol-% in sample mean range	N	S.D., mol-%
Progesterone	68.7 (54.4 – 75.8)	26	± 1.5
»	88.5 (77.9 – 98.6)	31	± 0.9
20(α+β)-Hydroxy-4-pregnen-3-one	3.1 (0.4 – 5.0)	20	± 0.5
»	7.3 (5.1 – 9.6)	15	± 0.7
»	15.8 (10.1 – 27.0)	30	± 1.8
Testosterone	1.9 (0.1 – 5.0)	24	± 0.6
»	7.4 (5.3 – 9.4)	21	± 0.6
»	14.9 (10.6 – 30.0)	11	± 1.3
4-Androstene-3,17-dione	2.4 (0.9 – 5.0)	36	± 0.4
»	7.2 (5.2 – 11.9)	13	± 0.4
Testosterone acetate	1.4 (0.3 – 7.1)	16	± 0.4
Testololactone	1.0 (0.2 – 1.9)	32	± 0.2
Total C_{19} steroids	3.0 (1.5 – 5.0)	28	± 0.5
»	7.4 (5.1 – 9.9)	27	± 0.6
»	15.1 (10.1 – 32.2)	33	± 0.9

Other chemicals were of reagent grade and were treated as described previously.[19]

Chromatographic systems. TLC on Silica gel GF_{254} (system I) and on Al_2O_3 GF_{254} (0.5 % ethanol in benzene as solvent); GLC on OV-17 and GC-MS were carried out as previously described.[19] Steroid silyl ether mixtures were quantitatively analyzed by GLC using 2.5 % XE-60 as stationary phase.

Growth of organism and preparation of cell-free extracts. P. lilacinum NRRL 895 was grown on Czapek-Dox medium, induced with progesterone, washed and frozen as described previously.[19] Cell-free extracts were prepared by high speed grinding with glass beads followed by centrifugation at 100 000 *g*.[20,21] The crude 100 000 *g* supernatant contained endogenous cofactors sufficient for a limited degree of C_{17-20}-lyase activity and minor amounts of testosterone. Extracts free from cofactors and steroids were prepared by gel filtration through a 300 mm × 15 mm i.d. Sephadex G-25 column connected to a LKB Uvicord II UV recorder and a fraction collector. Extracts free from steroids were prepared by passing the sample through an Amberlite XAD-2 column.

Incubations of steroids with cell-free extracts. The procedures for incubation and extraction of the reaction mixture have been described previously.[10] The steroid substrates were added in 10 µl of ethanol per ml of enzyme preparation. Inhibitors and metal salts were added as solutions in 0.06 M Tris-HCl adjusted to pH 7.2. If not otherwise stated, the transformations were carried out using crude 100 000 *g* supernatants with [7-^{3}H,21-^{14}C]progesterone as substrate for 60 min at 26 – 28 °C.

Steroid analysis. Quantitative analysis was made by TLC and liquid scintillation counting as previously described.[10] The variation between individual samples in duplicate incubations is given in Table 1. When [7-^{3}H,21-^{14}C] progesterone was the substrate, blank values due to tailing or non-enzymatic reactions were rather small. Thus, after incubation with boiled extracts + NADPH, 98.0 % of the total ^{3}H radioactivity on the TLC plate was found in the progesterone fraction, 0.9 % in the 4-androstene-3,17-dione fraction, 0.3 % in the 20($\alpha+\beta$)-hydroxy-4-pregnen-3-one fraction, 0.2 % in the testosterone fraction, and 0.6 % in the "testololactone" fraction. However, with [7-^{3}H,21-^{14}C]17α-hydroxyprogesterone as substrate there were considerable blank values, especially in the zone more polar than 17α-hydroxyprogesterone. When unacetylated blank samples were chromatographed, 0.9 % of the ^{3}H radioactivity was found in the 4-androstene-3,17-dione fraction, 88.4 % in the 17α-hydroxyprogesterone fraction, 6.6 % in the testosterone fraction, 3.4 % in the 17α,20($\alpha+\beta$)-dihydroxy-4-pregnen-3-one fraction and 0.6 % in the most polar fraction. Due to contamination of the testosterone fraction with substrate, samples from incubations with [7-^{3}H,21-^{14}C]17α-hydroxyprogesterone were analyzed before and after acetylation. Blank corrections were made in all assays.

The reactions were terminated at a stage when still large amounts of C_{21} steroids remained in the mixture. This will explain the low or insignificant amounts of testololactone detected.[15]

Protein assays were made by the biuret method.[22]

Identifications. The steroid metabolites were identified by their chromatographic and mass spectrometric properties as free steroids, acetates, and silyl ethers and by their ^{3}H/^{14}C ratios.[10,19] In the GC-MS, 17α,20α-dihydroxy-4-pregnen-3-one and its 20β-epimer were analyzed as 20-acetates and as *O*-methyloxime silyl ether derivatives.

RESULTS

Assay conditions. The C_{17-20}-lyase activity had its optimum between pH 7.2 and 7.6. 20-Reduction was maximal at pH 6.4 (Fig. 1). The time-course of the transformation of progesterone is shown in Fig. 2. The concentration of 20($\alpha+\beta$)-hydroxy-4-pregnen-3-one was maximal at about 60 min and then decreased. The formation of C_{19} steroids still continued after 180 min.

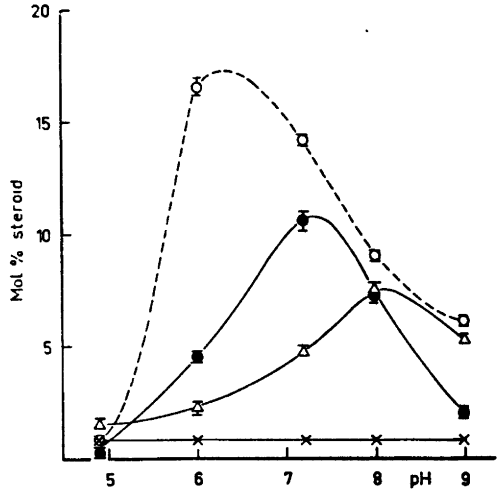

Fig. 1. pH/activity relationship for the side chain cleavage and the 20($\alpha+\beta$)-reduction of progesterone by cell-free preparations from *P. lilacinum*. Crude 100 000 *g* supernatants. Initial concentration of progesterone 2.90×10^{-4} M and of exogenous NADPH 1.21×10^{-3} M. 20($\alpha+\beta$)-Hydroxy-4-pregnen-3-one ○; testosterone ●; 4-androstene-3,17-dione△; "Compound T" (tentatively identified as testololactone) ×.

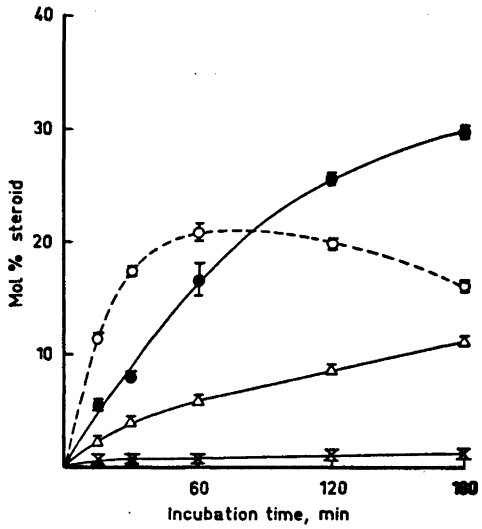

Fig. 2. Time-course of the side chain cleavage and the 20(α+β)-reduction of progesterone by cell-free preparations from *P. lilacinum*. Crude 100 000 *g* supernatant. Initial concentration of progesterone 2.90×10^{-4} M and of exogenous NADPH 1.34×10^{-3} M. 20(α+β)-Hydroxy-4-pregnen-3-one ○; testosterone ●; 4-androstene-3,17-dione △; "Compound T" (tentatively identified as testololactone) ×.

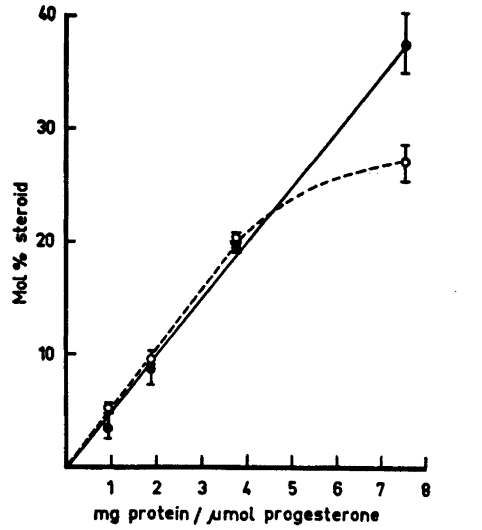

Fig. 3. Effect of the enzyme/substrate ratio on the side chain cleavage and the 20(α+β)-reduction of progesterone by cell-free preparations from *P. lilacinum*. Crude 100 000 *g* supernatant. Initial concentration of exogenous NADPH 1.10×10^{-3} M and of progesterone 2.66×10^{-4} M in the first three points and 1.33×10^{-4} M in the last point (7.52 mg protein/μmol progesterone). Total side chain cleavage ●; total 20-reduction ○.

The side chain cleavage as well as the 20-reduction was linear with the enzyme/substrate ratio up to about 4 mg/μmol (Fig. 3).

Substrate specificity. Progesterone and 17α-hydroxyprogesterone were substrates for the C_{17-20}-lyase and the 20(α+β)-oxidoreductase activities (Table 2). 17α-Acetoxyprogesterone yielded no detectable metabolites (by GLC and TLC).

The metabolites of 17α-hydroxyprogesterone were identified after isolation by TLC in system I (triple run). One half of the 17α,20-dihydroxy-

Table 2. Transformation of progesterone and 17α-hydroxyprogesterone by cell-free preparations from *P. lilacinum*.

Experiment	Steroid composition of reaction mixture (mol %)							
	Progesterone	20(α+β)-Hydroxy-4-pregnen-3-one	17α-Hydroxyprogesterone	17α20(α+β)-Dihydroxy-4-pregnen-3-one	Testosterone	4-Androstene-3,17-dione	Testololactone	Total C_{19}steroids
1/P[b]	73.1	11.7	—	—	9.6	5.1	0.5	15.2
1/17[b]	—	—	77.5	7.8	1.8	12.9	n.s.	14.7
2/P[a]	81.8	3.9	—	—	11.6	2.2	0.5	14.3
2/17[a]	—	—	70.6	19.1	2.0	8.3	n.s.	10.3
3/P[a]	82.5	7.8	—	—	7.1	2.1	0.5	9.7
3/17[a]	—	—	82.4	10.1	2.1	5.8	n.s.	7.9

[a] Crude 100 000 *g* supernatant. [b] Crude 100 000 *g* supernatant after passing Amberlite XAD-2. P indicates progesterone as substrate, 17 17α-hydroxyprogesterone. Initial concentrations of progesterone and 17α-hydroxyprogesterone 2.90×10^{-4} M and of exogenous NADPH 1.33×10^{-3} M.

Table 3. R_F- and t_R values for the 20-epimeric 17α,20-dihydroxy-4-pregnen-3-ones as free alcohols, 20-acetates and O-methyloxime silyl ether derivatives.

	R_F-value, system I		t_R-value (5α-cholestane = 1.00)
	Double run	Triple run	1.5 % SE-30, column temp. 240 °C, carrier gas flow 32 ml/min
17α,20α-Dihydroxy-4-pregnen-3-one	0.12	0.20	
17α-Hydroxy,20α-acetoxy-4-pregnen-3-one	0.37		1.47
O-Methyloxime silyl ether derivative of 17α,20α-dihydroxy-4-pregnen-3-one			1.49
17α,20β-Dihydroxy-4-pregnen-3-one	0.16	0.28	
17α-Hydroxy,-20β-acetoxy-4-pregnen-3-one	0.29		1.39
O-Methyloxime silyl ether derivative of 17α,20β-dihydroxy-4-pregnen-3-one			1.41

4-pregnen-3-one fraction was acetylated and rechromatographed in the same system (double run). R_F-Values and t_R-values are given in Table 3. The "atypical" behaviour of the 20α-acetate with greater chromatographic mobility than the 20β-epimer is noteworthy. TLC and GLC behaviour and mass spectra of the metabolites were in close agreement with those of authentic reference compounds.

Cofactor requirements. The crude 100 000 *g* supernatant contained endogenous cofactors sufficient for a certain degree of C_{17-20}-lyase activity. In such extracts this activity was stimulated by NADPH as well as by NADH (Table 4). Extracts purified by gel filtration were inactive without cofactors. In such extracts the C_{17-20}-lyase activity showed an absolute requirement for NADPH. Addition of FMN

Table 4. Effect of exogenous cofactors on the transformation of progesterone by cell-free preparations of *P. lilacinum*.

Experiment	Initial concentration of exogenous cofactors, mM			Initial concentration of progesterone, mM	Mol % total side chain cleavage	Mol % 20 (α+β)-reduction
	NADPH	NADH	FMN			
4[a]	–	–	–	0.266	6.7	n.s.
4	0.55	–	–	»	15.9	12.7
4	1.10	–	–	»	17.9	19.8
4	2.20	–	–	»	16.9	21.9
5[b]	–	–	–	0.228	2.3	n.s.
5	–	0.50	–	»	3.3	10.7
5	–	1.00	–	»	4.7	18.2
5	0.52	–	–	»	4.5	7.0
5	1.04	–	–	»	4.2	10.8
5	1.04	–	0.90	»	6.4	7.6
6[c]	–	–	–	0.266	n.s.	n.s.
6	–	1.06	–	»	n.s.	21.3
6	1.10	–	–	»	4.8	11.2
6	1.10	–	1.04	»	6.2	8.9
7[c]	1.10	–	–	0.266	7.1	8.8
7	1.10	–	0.11	»	8.6	7.4
7	1.10	–	1.10	»	8.8	7.2

[a] Crude 100 000 *g* supernatant. [b] Crude 100 000 *g* supernatant after passing Amberlite XAD-2. [c] Gel filtrated 100 000 *g* supernatant.

Table 5. Influence of cofactors and of clomiphene citrate on the 20α and 20β reduction of progesterone by cell-free preparations from *P. lilacinum*.

Experiment	Initial concentration of exogenous cofactors, mM		Clomiphene citrate added, mM	Steroid composition of reaction mixture (mol-%)			
	NADPH	NADH		20α-Hydroxy-4-pregnen-3-one	20β-Hydroxy-4-pregnen-3-one	Total 20-reduction	Total side chain cleavage
5[b]	0.52	—	None	4.5	2.5	7.0	4.5
5	1.04	—	»	7.3	3.5	10.8	4.2
5	—	0.50	»	4.3	6.4	10.7	3.3
5	—	1.00	»	6.9	11.3	18.2	4.7
6[c]	1.10	—	»	6.2	5.0	11.2	4.8
6	—	1.06	»	4.9	16.4	21.3	n.s.
8[a]	1.21	—	»	7.2	6.1	13.3	6.7
8[d]	1.21	—	»	5.1	2.7	7.8	n.s.
8[d]	1.21	—	3	n.s.	6.6	6.6	n.s.
8[d]	—	1.25	None	8.2	20.0	28.2	n.s.
8[d]	—	1.25	3	n.s.	18.5	18.5	n.s.

[a] Crude 100 000 *g* supernatant. [b] Crude 100 000 *g* supernatant after passing Amberlite XAD-2. [c] Gel filtrated 100 000 *g* supernatant. [d] Protein fraction obtained from 100 000 g supernatant by precipitation with 80 % saturated ammonium sulphate, redissolving in Tris buffer and dialysis. Initial concentration of progesterone in experiment 5 2.28×10^{-4} M, in experiments 6 and 8 2.66×10^{-4} M.

caused a slight increase in the stimulatory effect of NADPH (Table 4).

The 20-reduction in crude 100 000 *g* supernatants and in purified extracts required NADPH or NADH (Tables 4 and 5). NADH was more effective in stimulating the 20-reduction than was NADPH. NADH favoured formation of the 20β-epimer in all types of extracts. Clomiphene citrate selectivity inhibited the 20α-reduction.

Effects of metal ions. The C_{17-20}-lyase activity was strongly suppressed by Hg^{2+}, Cu^{2+} and

Table 6. Effect of metal ions on the transformation of progesterone by cell-free preparations from *P lilacinum*.

Experiment	Metal added	Mol-% total side chain cleavage	Mol-% 20(α+β)-reduction
9[a]	None	17.0	5.3
9	10^{-3} M Hg^{2+}	n.s.	n.s.
10[a]	None	14.5	13.9
10	10^{-3} M Fe^{2+}	14.5	10.8
10	10^{-3} M Fe^{3+}	12.4	11.0
10	10^{-3} M Ni^{2+}	10.5	13.8
10	10^{-3} M Co^{2+}	11.2	8.9
10	10^{-3} M Mn^{2+}	14.2	10.8
10	10^{-3} M Cu^{2+}	1.5	3.4
10	10^{-3} M Zn^{2+}	3.3	5.5
11[b]	None	5.8	12.3
11	10^{-4} M Fe^{2+}	6.2	11.4
11	10^{-3} M Fe^{2+}	8.2	10.1
11	10^{-3} M Fe^{3+}	6.1	11.0
11	10^{-3} M Ni^{2+}	3.4	8.0
11	10^{-3} M Co^{2+}	5.7	7.5
11	10^{-3} M Mn^{2+}	6.9	8.0

[a] Crude 100 000 *g* supernatant. [b] Gel filtrated 100 000 *g* supernatant. Initial concentrations of progesterone 2.66×10^{-4} M and of exogenous NADPH 1.21×10^{-3} M.

Table 7. Effect of inhibitors on the transformation of progesterone by cell-free preparations of *P. lilacinum*.

Experiment	Inhibitor (10^{-3} M)	Mol % total side chain cleavage	% inhibition of side chain cleavage	Mol % 20 ($\alpha+\beta$)-reduction	% inhibition of 20($\alpha+\beta$)-reduction
4	None	17.9	–	19.8	–
4	Ferron	3.5	80	8.3	58
4	1,10-phenantroline	9.7	46	17.0	n.s.
4	Phenazine metosulphate	n.s.	>99	2.2	89
4	10^{-3} M KCN	13.3	26	11.9	40
12	None	12.9	–	15.2	–
12	Ferron	8.4	35	9.8	35
12	*p*-Hydroxymercuribenzoate	2.0	85	6.2	59
12	Methylene blue	6.2	51	4.2	72
13	4 % O_2+96 % N_2	13.3	–	18.3	–
13	40 % CO+4 % O_2+56 % N_2	14.2	n.s.	14.6	21

10^{-3}M concentrations of KCN, NaN_3, NaF, EDTA, and 8-hydroxyquinoline had no significant effects on side chain cleavage or 20-reduction. Crude 100 000 *g* supernatants were used. Initial concentration of progesterone was 2.66×10^{-4}M and of exogenous NADPH 1.10×10^{-3} M. The incubations in experiment 13 were performed in darkness.

Zn^{2+} and to a lesser degree also by Ni^{2+} (Table 6). Fe^{2+} slightly stimulated the activity in extracts purified by gel filtration.

The 20($\alpha+\beta$)-reduction was strongly inhibited by Hg^{2+}, Cu^{2+} and Zn^{2+}. A slight suppression of the activity was achieved by Ni^{2+}, Co^{2+} and Mn^{2+} ions.

Effects of inhibitors. The enzyme preparation was preincubated with inhibitors 15 min before the addition of steroid and NADPH. The effects of inhibitors are shown in Table 7. The C_{17-20}-lyase activity was insensitive towards CO and 10^{-3} M concentrations of CN^-, N_3^-, and F^-. Methylene blue, phenazine metosulphate, and *p*-hydroxymercuri benzoate were strong inhibitors. Ferron and 1,10-phenantroline had a significant inhibitory effect whereas EDTA and 8-hydroxyquinoline were inactive.

The reduction at C-20 was inhibited by 10^{-3} M CN^- and by ferron, phenazine metosulphate, methylene blue, and *p*-hydroxymercuri benzoate, whereas the other inhibitors tested were without effects.

DISCUSSION

Progesterone and 17α-hydroxyprogesterone but not 17α-acetoxyprogesterone were substrates for the side chain cleavage in *P. lilacinum*. Side chain splitting enzymes from microorganisms in general have a very broad substrate specificity. It has previously been reported that whole cells of *P. lilacinum* split the side chain from different 20-oxosteroids in the 5α-pregnane, 5β-pregnane, 4-pregnene, 5-pregnene and 1,4-pregnadiene series.[11,23]

Crude 100 000 *g* supernatants contained endogenous cofactors sufficient for a limited degree of side chain cleavage, and addition of exogenous NADPH or NADH further increased the activity. In contrast to crude extracts, extracts purified by gel filtration showed an absolute requirement for NADPH. The stimulatory effect of NADH in crude extracts (see also paper I in this series [19]) might be explained by hydrogen transfer to endogenous $NADP^+$, thereby increasing the NADPH levels. The occurrence of transhydrogenases in microorganisms is well known.[24] It should be mentioned that stimulation by NADH due to transhydrogenation has been reported for the side chain cleavage of cholesterol by adrenal mitochondria.[25]

From a wealth of information on microbial and mammalian oxygenases it might be assumed that flavin participates in the side chain cleavage in *P. lilacinum*, at least in the oxidation of NADPH. In accordance with this assumption FMN caused a slight increase in the stimulatory effect of NADPH. It should be mentioned that participation of FMN in the oxidation of NADH as well as in the oxygenation of the substrate has also been established in the NADH-de-

pendent lactonization of camphor by highly purified enzymes from *Pseudomonas putida*.[6]

An iron-sulphur protein might also participate in the side chain cleavage studied. Thus heavy metals known to combine with the sulphur moiety as well as substances reacting with Fe^{2+} inhibited the reaction. Ni^{2+} was also inhibitory, which might be due to a similarity in the ionic radii of Ni^{2+} and Fe^{2+}.[26] A similar effect can be expected from Co^{2+} and in fact inhibition by Co^{2+} was observed in one experiment. Addition of Fe^{2+} slightly stimulated the side chain cleavage in purified extracts but was ineffective in crude 100 000 *g* supernatants. It is noteworthy that the effects of Fe^{2+} and Fe-reacting agents differ from those presented for the ring D lactonization in *P. lilacinum* enzyme preparations and the side chain cleavage in *C. radicicola*.[7,8] In those studies Fe^{2+} and 1,10-phenantroline were inactive. An explanation for the discrepancy might be found in the higher degree of purity of the enzymes used in those studies. Early studies by Gunsalus and co-workers indicated the participation of a non-heme iron protein in the camphor lactonization.[3] Thus addition of Fe^{2+} generally stimulated and bipyridine inhibited the reaction. The effect of Fe^{2+} was, however, less reproducible. In a later study using a reconstituted, extensively purified system devoid of iron, added Fe^{2+} and bipyridine were without effect.[6] The lack of effect of Fe^{2+} in the purified system could be due to the "ferredoxin-flavodoxin replacement phenomenon", *i.e.* replacement of the non-heme iron protein in the electron transport chain by an iron-free flavoprotein such as flavodoxin.[6,27]

Cytochrome P-450 has been shown to be the terminal oxidase protein in the mammalian 20-oxopregnane side chain cleavage as well as in a large number of mammalian and microbial hydroxylations.[28–30] However, the insensitivity to heme-reacting inhibitors excludes participation of cytochromes in the side chain cleavage of *P. lilacinum*. In the camphor lactonizing system no heme-proteins have been found and the reaction catalyzed by this system was insensitive to CO, CN^- and N_3^-.[3] Insensitivity to CN^- and N_3^- has also been demonstrated for 20-oxopregnane side chain cleavage by whole cells of *Nocardia restrictus*[31] and by enzyme preparations from *C. radicicola*.[8] D-Ring lactonization in enzyme preparations from *P. lilacinum*[7] and lactonization of cyclohexanone in cell-free extracts from *Nocardia opaca*[32] are also known to be insensitive towards CN^- and N_3^-. Obviously the "biochemical Baeyer-Villiger oxidations" belong to a class of oxygenases which do not utilize cytochromes as terminal oxidase.

The side chain cleavage occurred with a concomitant and efficient reduction in the C-20 position. Crude 100 000 *g* supernatants contained endogenous cofactors sufficient for a limited degree of side chain cleavage but not for a simultaneous 20-reduction (Table 4, see also paper I in this series[19]). Addition of exogenous NADH of NADPH increased the side chain cleavage and simultaneously 20-reduced metabolites appeared. Initial exogenous cofactor levels higher than those necessary for a maximal side chain cleavage did not affect that reaction but further stimulated the 20-reduction. In the early stages of incubations excess of NADPH was present and the amounts of 20-alcohols and C_{19} steroids increased simultaneously (Fig. 2). When a larger part of the NADPH was oxidized, the concentration of 20-alcohols levelled off and later even decreased while the C_{19} steroid concentration still increased. El-Tayeb and co-workers suggested a competition between C_{17-20}-lyase and 20-oxidoreductase enzymes for the 20-oxo-C_{21} steroids.[16,17] The results given above show that the level of reduced pyridine nucleotide is an important limiting factor for the 20-reduction. With an excess of NADPH or NADH also "good" lyase substrates such as progesterone yield considerable amounts of 20-alcohols together with the side chain cleavage products.

Purified microbial 20-oxidoreductases are known to possess a pronounced pyridine nucleotide specificity. NADPH is the specific cofactor for the 20α-oxidoreductase in *Actinomyces roseochromogenus* and the 20β-oxidoreductase in *Curvularia lunata*, while the crystallized 20β-oxidoreductase from *Streptomyces hydrogenans* is NADH-specific.[33–35] The 20(α + β)-reduction in *P. lilacinum* was more effeciently stimulated by NADH than NADPH. NADH favoured formation of the 20β-epimer. Interestingly clomiphene citrate selectively inhibited the 20α-reduction.

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Fungal Extractives. VIII.* Two Sesquiterpene Furans from *Lactarius*

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The structures and relative configurations of two sesquiterpene furans (*8* and *9*) isolated from *Lactarius vellereus*, *L. pergamenus*, and *L. helvus* have been determined with the help of standard spectroscopic methods and computer analysis of lanthanide-induced chemical shifts. Evidence that *8* and *9* are artifacts formed during the isolation procedure is presented.

Hydroazulenic sesquiterpenes with a *gem*-substituted cyclopentane ring were reported (without stereochemical details) for the first time by Nozoe *et al.*[2] (compounds *1* and *2*). Five more compounds with this carbon skeleton have since been reported: Velleral[3] (*3*) and two lactones[4,5] (*4* and *5*) from *Lactarius vellereus* and *L. pergamenus*; lactarorufins A[6] (*6*) and B[7] (*7*) from *L. rufus*. The hydroazulene *2* has also been found in *L. necator* and its relative configuration determined (Fig. 1).[8]

* Part VII, see Ref. 1.

We now report the structures and relative configurations of two sesquiterpene furans (*8* and *9*) from *L. vellereus*, *L. pergamenus*, and *L. helvus* (Russulaceae).

Compound *8* was shown by ^{13}C NMR (15 C and 19 H) and mass spectrometry (M^+ at *m/e* 232) to have the molecular formula $C_{15}H_{20}O_2$. Its IR spectrum revealed the presence of a hydroxyl group, a *gem*-dimethyl group (ν_{max} 1390 and 1385 cm^{-1}) and a furan ring (ν_{max} 1540 and 880 cm^{-1}). The ^1H NMR spectrum showed that the furan ring is disubstituted with substituents in the 3 and 4 positions (signals at δ 7.37 and 7.10 ppm)[9,10] and that the hydroxyl group is attached to a tertiary carbon atom next to the furan ring (two doublets centered at δ 4.34 ppm with $J_1 = 11.0$ and $J_2 = 1.4$ Hz). In addition to the signals from the *gem*-dimethyl group there was a broadened three-proton signal (δ 1.71 ppm), which was assigned to a methyl

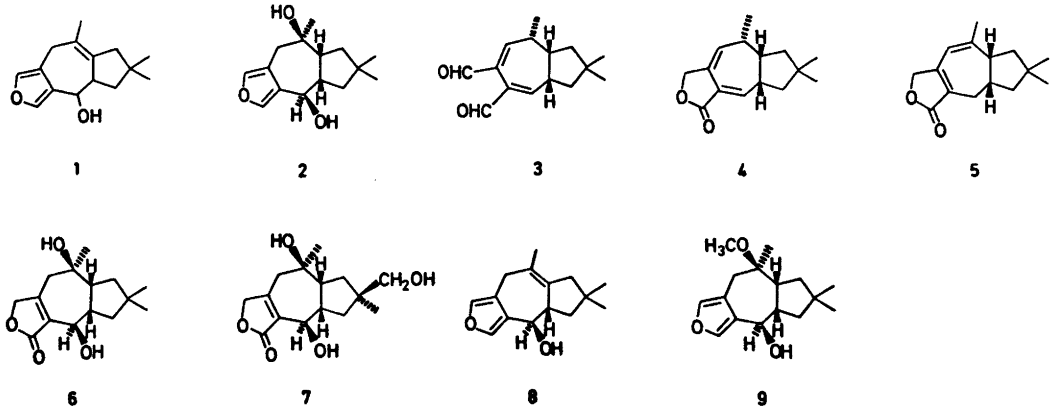


Fig. 1.

group situated on a double bond. From the ^{13}C NMR data (Table 1) it was established that *8* is a tricyclic compound with a tetrasubstituted double bond, with three primary, three secondary, two tertiary and one quaternary carbon atoms and, in addition, with furan ring carbon atoms. Extensive ^{1}H NMR decoupling experiments established the structure and relative configuration of *8* (Fig. 2). The ^{1}H NMR shifts

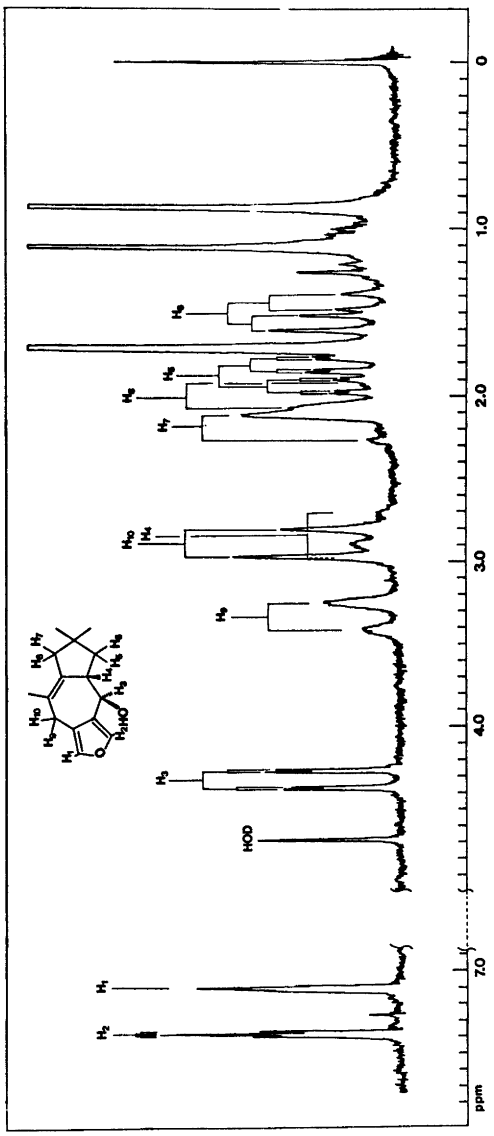
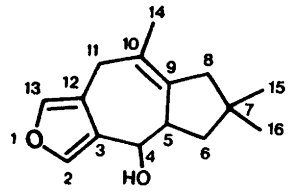


Fig. 2. ^{1}H-NMR spectrum of compound *8* (100 MHz; $CDCl_3/D_2O$).

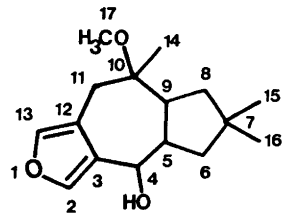
Table 1. ^{13}C NMR data for compounds *8* and *9* (25.2 MHz; $CDCl_3$).

Chemical shift (ppm from TMS)	Signal multiplicity[a]	Assignment (carbon No.)



8

140.4	d	2, 13
137.7	d	
136.7	s	9 or 10
128.8	s	3, 12
127.5	s	
120.7	s	9 or 10
70.4	d	4
48.4	d	5
46.3	t	6, 8
45.6	t	
37.0	s	7
29.5	t	11
29.5	q	15, 16
26.6	q	
21.9	q	14



9

141.8	d	2, 13
139.7	d	
127.3	s	3, 12
127.3	s	
80.2	s	10
66.7	d	4
48.8	q	17
46.8	d	5, 9
46.8	d	
45.3	t	6, 8
45.0	t	
36.6	s	7
29.9	q	15 or 16
28.0	t	11
27.7	q	15 or 16
24.4	q	14

[a] s=singlet, d=doublet, t=triplet, q=quartet; obtained by "off-resonance" decoupling.

reported here for *8* are in close agreement with those reported for *1*[2], except for the furan protons which appear at δ 7.37 and 7.10 ppm (in $CDCl_3$) instead of at 7.72 and 7.04 ppm (solvent not reported). The specific rotation for *8* is +123° (in methanol) instead of +69.5° (solvent not reported) for *1*.[2] In spite of these differences it seems probable that the compounds *1* and *8* are identical.

Compound *9* was shown by the same techniques as used for *8* to be a tricyclic molecule with a 3,4-disubstituted furan ring and a secondary alcohol group next to this ring. The molecular formula ($C_{16}H_{24}O_3$) implied a formal addition of one molecule of methanol to *8*. Compound *9* is devoid of sp^2 carbon atoms other than those constituting the furan ring (^{13}C NMR data in Table 1) and possesses four primary, three secondary, three tertiary, and two quaternary carbon atoms. From these data, including ^1H NMR chemical shifts and integrals, a probable structure could be constructed. There are eight configurational isomers I–VIII (Fig. 3) of this structure to be considered.

Extensive decoupling experiments did not solve the stereostructure of *9*, but revealed two important facts: The protons H_3 and H_4 (see formula I in Table 2) showed *vicinal* coupling of $J = 4.0$ Hz reflecting an approximate dihedral angle of either 50° or 135° as judged from the Karplus curves;[11] the protons H_3 and H_2 were coupled with $J < 0.5$ Hz. The dihedral angle in this *cis*-allylic coupling system should then be close to 0°.[12,13] (The decoupling experiments were run in $CDCl_3$ with D_2O added. The coupling constants were unchanged in CCl_4 solution). The IR spectrum of *9* (0.0027 M solution in CCl_4) showed only one band (3430 cm^{-1}) in the hydroxyl stretching region. This indicates an intramolecular $OH\cdots OCH_3$ bond and thus that the hydroxy and methoxy groups are on the same side (*cis*) of the seven-membered ring. The intramolecular hydrogen bond in combination with the two possible dihedral angles between H_3 and H_4 requires the bridgehead hydrogens to be *cis*. This still, however, leaves two possible isomers, compounds I and IV.

Another approach to the stereochemical problem was tried. A ^1H NMR spectrum of *9* in CCl_4 containing Yb(fod)$_3$, (fod = 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionate), showed induced chemical shifts but these did not establish the relative configuration of *9*. A newly developed computer program [14] (*cf.* also Ref. *15*) was used to calculate the expected lanthanide-induced chemical shifts for isomers I–VIII. Since conformational changes may occur in *9* in the presence of the Yb(fod)$_3$ complex, no assumptions regarding hydrogen bonding or dihedral angles were made. From Dreiding models the Cartesian coordinates in an arbitrary coordinate system of the hydrogens $H_1 - H_{23}$ were determined. Twenty-eight cases were included (four conformers of each of the *cis*-ring junction isomers I–IV, and three of each *trans*-ring junction isomer V–VIII). These sets of figures together with the experimental lanthanide-induced chemical shift values

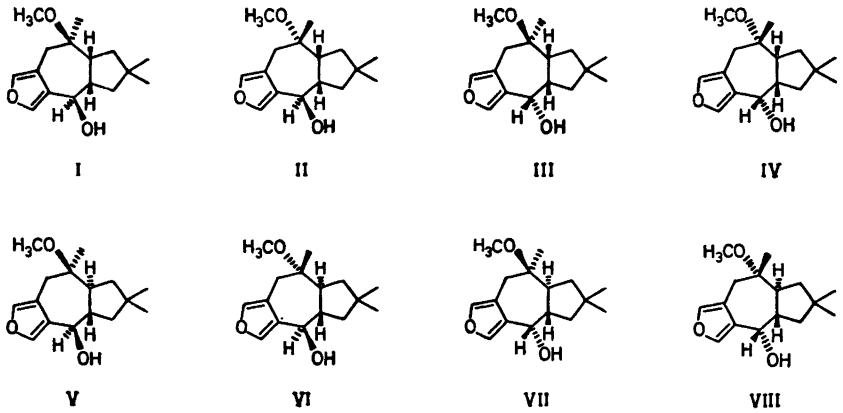


Fig. 3.

were used as input parameters in the program. Association of the shift reagent with the furan oxygen was neglected [16] and an association with the methoxy oxygen could be ruled out by the relatively small observed shift difference for the methyl group ($H_{12}-H_{14}$). The shifts of the methyls and the methoxy group were calculated as mean values of their proton shifts and a single position was chosen for the freely rotating methoxy group. Unequivocal assignments of the protons H_4 and H_9, H_{10} and H_{11}, and of the methyl group protons $H_{15}-H_{17}$ and $H_{18}-H_{20}$ were not possible and permutations of these three assignment pairs were therefore made. Only approximate values for the experimentally-induced chemical shifts of the four methylene protons (H_5-H_8) of the cyclopentane ring could be estimated and these were used with reduced weight in the computer program. A threefold potential barrier for oxygen–carbon rotation was assumed (in analogy with the calculations in Ref. 15) and the relative populations of the three rotamers of the hydroxy group were adjusted to best fit.

Four isomers (III, IV, VII, VIII) could be excluded in the initial calculations. Cases which gave agreement factors (R) [17] higher than 25 %, and cases with R-values between 20 % and 25 % and ytterbium–oxygen distances (d) far outside ($d > 5.77 \ldots . 1.87 > d$ Å) reasonable limits (*ca.* 3.2–2.2 Å)[18] were rejected. There remained eight cases, two conformers of each isomer I, II, V, and VI, with R-values in the interval 13.6–19.5 % and with acceptable Yb–O distances. It was realised that there need not necessarily be only a single conformer present even though this was the case in the absence of the Yb complex (^{1}H NMR, ^{13}C NMR, IR). Mixed conformer populations of the four isomers were therefore used in the second calculation. One isomer (I) gave a very low agreement factor (R = 6.0 %; d = 2.22 Å) for a mixture of the boat and chair conformations in a ratio of *ca.* 55:45 (Fig. 4). Agreement factors for the three other isomers (II, V, VI) did not improve in this second calculation and could be rejected with high statistical significance (> 99.5 %).[17,19] Experimental and calculated shifts for I are presented in Table 2. Isomer I is one of the two derived by the independent reasoning above. In view of the complexity of the twentythree proton system some uncertainty in the calculation results cannot be excluded. However, in combination with the spectroscopic evidence the calculations firmly establish the relative configuration of the furan alcohol *9*.

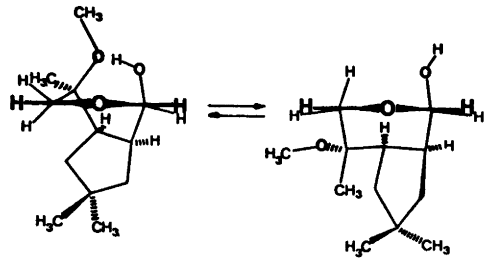
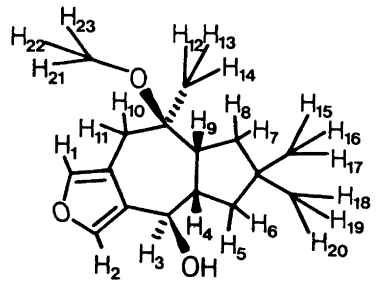


Fig. 4.

Table 2. Yb(fod)$_3$-induced and calculated chemical shifts of I.



1

Proton No.	Induced chemical shifts (Hz) Observed	Calculated
1	60	71
2	86	85
3	430	424
4	230	232
5	75[a]	57
6	75[a]	126
7	75[a]	31
8	75[a]	73
9	40	40
10	90	82
11	103	111
12–14	57	52
15–17	27	13
18–20	36	52
21–23	79	78

[a] Only roughly estimated shifts (± 50 Hz).

Interestingly the stereostructure found for *9*, with the *cis*-fused hydroazulene ring system and

the methyl group [$-CH_{3(12-14)}$] *trans* to the bridgehead hydrogens is the same as in other basidiomycete sesquiterpenes (see, *e.g.*, Fig. 1) and has the same relative configuration of the hydroxyl bearing carbon as in compounds *2*, *6*, *7*, and *8*.

It should be pointed out that compounds *8* and *9* may not be native to the *Lactarius* species investigated. The procedure used for the isolation of *8* and *9* was different from that used for the dialdehydes velleral (*3*), isovelleral,[10] and the two lactones *4* and *5*. *Methanol* was used instead of hexane [3] for extraction. Carbon tetrachloride extraction of the methanolic phase gave *8* and *9*, but neither the two dialdehydes (*cf.* Ref. 20) nor the two lactones were detected (TLC). On the other hand *8* and *9* were not obtained when hexane was used for extraction. It thus seems probable that *8* and *9* were formed during the work-up process. In order to test this possibility the mushrooms were ground with *ethanol* instead of methanol. From this extract there were isolated *8* and an *ethyl* ether (*10*), homologous to *9*. The methyl ether *9* was not detected. This finding is a strong indication that compound *9* at least, and probably also *8* are artifacts. Their formation by an enzymatically-assisted reaction sequence would seem more plausible than a pure chemical one since no stereoisomers of *8* and *9* were detected. The nature of the precursors of these compounds is of course important but, at this stage it is premature to consider this topic.

EXPERIMENTAL

The ^1H NMR spectra were recorded on a Varian XL-100 instrument with ^{13}C NMR capability and Fourier transform equipment. Mass spectra were recorded on an LKB 1100 instrument.

Isolation procedure. Fresh fungi (*Lactarius vellereus, L. pergamenus, L. helvus*) were ground with methanol and the mixture was pressed with Celite in a fruit press (Hafico). The aqueous filtrate was evaporated at room temperature to half its volume, diluted with water to the original volume and then extracted with three portions of carbon tetrachloride. The residue obtained on evaporation of the solvent was chromatographed on a silica gel column. Elution with benzene – ether (9:1) and then light petroleum – ether (2:1) gave the furan compounds *8* and *9*.

Furan alcohol 8. Recrystallisation from hexane at −20 °C gave *8*, m.p. 34 – 44 °C; $[\alpha]_D^{22}$ +123° (*c* 0.6, methanol), $[\alpha]_D^{22}$ +123° (*c*. 0.6, chloroform); IR: ν_{max} ($CHCl_3$) 3600, 1535 (furan), 1385 and 1368 (*gem*-CH_3), 1049 and 875 (furan) cm^{-1}; UV, nm (ε): λ_{max} (ethanol) 208.5 (ε 8200); ^1H NMR: δ_{TMS} ($CDCl_3/D_2O$) 7.37 (1H, d of d $J_1 = 1.7$ and $J_2 = 1.4$ Hz; $-CHOD-fur-H$), 7.10 (1 H, m; $-CH_2-fur-H$), 4.34 (1 H, d of d $J = 11.0$ and 1.4 Hz; $fur-CHOD-CH-$), 3.35 (1 H, d broad $J = 17.0$ Hz; $fur-HCH-C=C-$), 2.91 (1 H, d $J = 17.0$ Hz; $fur-HCH-C=C-$), 2.86 (1 H, m; $-CHOD-CH-$), 2.19 2.01)1 H each, d of d broad $J = 16.0$ Hz; $=C-HCH-C-$), 1.88 (1 H, m $J = 13.0$, 8.0 and 1.5 Hz; $-CH-HCH-C-$), 1.51 (1 H, d of d $J = 13.0$ and 9.0 Hz; $-CH-HCH-C-$), 1.71 (3 H, s broad; $CH_3-C=C-$), 1.11 0.87 (3 H each, s; *gem*-CH_3-CH_3) ppm. ^{13}C NMR data see Table 1. MS (70 eV): *m/e* 232 (M^+ 1 %; $C_{15}H_{20}O_2$), 214 (100), 199 (80), 197 (28), 158 (57), 129 (29), 128 (30).

Furan alcohol 9. Recrystallisation from ether at −20 °C gave *9*, m.p. 65 – 66 °C; $[\alpha]_D^{22}$ +6.0° (*c* 0.6, methanol); IR: ν_{max} ($CHCl_3$) 3360, 1538 (furan), 1390 and 1375 (*gem*-CH_3), 1110, 1060, 880 (furan) cm^{-1}; UV, nm (ε): λ_{max} (ethanol) 216 (4400); ^1H NMR: δ_{TMS} ($CDCl_3/D_2O$) 7.38 (1 H, d $J = 1.6$ Hz; $-CHOD-fur-H$), 7.17 (1 H, m $-CH_2-fur-H$), 4.61 (1 H, d $J = 4.0$ Hz; $fur-CHOD-CH-$), 3.21 (3 H, s; $-OCH_3$), 2.97 (1 H, m t $J = 18.0$, 1.2 and 1.0 Hz; $fur-HCH-C-$), 2.78 (1 H, d of d $J = 18.0$ and 1.4 Hz; $fur-HCH-C-$), 2.80 2.68 (1 H each, m; bridgehead protons), 1.70 (1 H, d of d $J = 10.5$ and 8.0; $-CH-HCH-C-$), 1.10 – 1.55 (3 H, m), 1.19 (3 H, s; $CH_3-C(OCH_3)-$), 1.06 (6 H, s; *gem*-CH_3-CH_3) ppm. ^{13}C NMR data, see Table 1. MS (70 eV): *m/e* 246 ($C_{16}H_{24}O_3$, $M^+ - H_2O$ 63 %), 231 (36), 215 (63), 214 (100), 199 (100), 158 (72).

Furan alcohol 10. Fresh fungi (*L. vellereus*) were ground with ethanol and the mixture was treated as described under *Isolation procedure*. Compound *10* was isolated by chromatography on a silica gel column with light petroleum – ether (3:1) as eluent. *10* was obtained as a viscous oil, $[\alpha]_D^{22}$ +5.6° (*c* 0.7, ethanol) with physical properties similar to those described for *9*, except for changes due to the ethoxy group.

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Oxidation of Lignin Model Compounds with Chlorine Dioxide and Chlorite. Reactions with Stilbenes

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The reaction products from the oxidation of several different types of stilbenes with chlorine dioxide have been isolated and characterised. *trans*-Stilbene was oxidised to α,α'-epoxy-bibenzyl which then underwent rearrangement or was attacked by nucleophiles present in the reaction mixture. 4-Hydroxy-3,3',4'-trimethoxy-*trans*-stilbene was attacked mainly on its phenolic hydroxyl group, resulting in formation of polymers. Some veratraldehyde and veratric acid, as well as vanillin and vanillic acid was also formed by attack at the double bond. *cis*-2,3-Diphenyl-prop-2-en-1-ol gave mainly allylic oxidation products, whereas *cis*-2,3-di-(3,4-dimethoxyphenyl)-prop-2-en-1-ol mainly underwent oxidative cleavage into veratraldehyde and 3,4-dimethoxyphenacyl alcohol. The reaction paths are discussed.

Most of the model studies [1-4] of the reaction between lignin and chlorine dioxide have been performed with lignin model compounds which contain free phenolic groups, *e.g.* vanillin and guaiacol. Such studies are informative for the reactions of chlorine dioxide when used in the first bleaching stage of sulfate pulp. However, for the final stages of bleaching, where the main part of the chlorine dioxide reacts, these model compounds are of less relevance, since most of the phenolic groups in lignin evidently are oxidised during the earlier stages.

Final bleaching with chlorine dioxide gives pulps with high brightness. Chlorine dioxide thus reacts with the colour-giving elements of lignin. The chlorine dioxide oxidation of model compounds of such elements are therefore of greater interest than the reaction of phenols for the understanding of how the lignin residues react during the final bleaching. They may also be of interest for understanding the reactions of a first bleaching stage with chlorine dioxide.

The structure of these colour-giving elements are, however, unknown. They may include conjugated systems composed of carbonyl groups, double bonds, or aromatic as well as quinoid structures.

On this basis we have studied how chlorine dioxide reacts with models containing one type of these elements, *viz.* double bonds conjugated with benzene nuclei. Four different stilbene derivatives were chosen as models.

The action of chlorine dioxide on organic compounds is generally a complex process as the chlorine dioxide is primarily reduced to chlorine and chlorite [5,6] which may both react with the starting material, the intermediates, or the reaction products. Complicated reaction products are therefore expected from the chlorine dioxide oxidation of stilbenes.

RESULTS AND DISCUSSION

The stilbene derivatives chosen were *trans*-stilbene, *cis*-2,3-diphenyl-prop-2-en-1-ol, *cis*-2,3-di-(3,4-dimethoxyphenyl)-prop-2-en-1-ol, and 4-hydroxy-3,3',4'-trimethoxy-*trans*-stilbene.

Treatments with chlorine dioxide were carried out in aqueous *t*-butanol or dioxane solutions and in non-aqueous solutions (chloroform or carbon tetrachloride).

In a typical experiment chlorine dioxide solution was added in darkness at room temperature to the stilbene solution. The reaction products obtained were first separated into two fractions containing acidic and neutral compounds, respectively. The neutral part was fractionated

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further by column chromatography. If a subfraction contained two or more compounds, their respective yields were determined from the peak areas of the gas chromatograms ("GLC:s") or of the NMR spectra. The acid fractions were analysed by spectral methods and by GLC/MS after methylation.

trans-*Stilbene*. The chlorine dioxide treatment of *trans*-stilbene in carbon tetrachloride solution yielded the following main products: α-chlorobenzyl phenyl ketone (yield 43 %), *trans*-α,α'-epoxy-bibenzyl (yield 36 %), and α,α'-dichlorobibenzyl (yield 7 %). The presence of small amounts of benzil in the reaction mixture was also observed. The yields were estimated from the peak areas of the NMR spectrum and were substantiated by the isolation of the chloro ketone and the dichloro compound in amounts corresponding to those mentioned above. The isolated dichloro compound consisted mainly of the *racem.* form like the product obtained after chlorination of *trans*-stilbene.[7] The epoxide had mostly decomposed during the working-up procedure.

The first step in the oxidation of *trans*-stilbene in carbon tetrachloride is most likely an addition of chlorine dioxide to the aliphatic double bond, but subsequent steps are less obvious. The chloro ketone was evidently not formed *via* the epoxide since no chloro ketone was found after treatment of the epoxide with chlorine dioxide, chlorine, or a mixture of them.

When *trans*-stilbene was treated with chlorine dioxide in aqueous dioxane solution the formation of α-chloro-α'-hydroxybibenzyl (yield 44 %), diphenylacetic acid (15 %), α,α'-dihydroxybibenzyl (12 %, the *erythro* and *threo* isomers in about equal amounts), benzoic acid (5 %), benzoin (3 %), benzil (3 %), α,α'-dichloro-bibenzyl (3 %), and α-chloro-benzyl phenyl ketone (2 %) was detected.

The presence of *trans*-α,α'-epoxy-bibenzyl was not observed. However, this does not exclude the possibility that it was formed as an intermediate since it was found to be unstable in the reaction mixture. The α-chloro-α'-hydroxybibenzyl and α,α'-dihydroxybibenzyl may have been formed partly *via* the epoxide. Parts of the epoxide may also have rearranged into the diphenyl acetaldehyde which was then oxidised (probably by chlorite [10]) to the diphenylacetic acid.

cis-*2,3-Diphenyl-prop-2-en-1-ol*. When this stilbenol was treated with chlorine dioxide in carbon tetrachloride solution the main products were two allylic oxidation products *viz.* *cis*-2,3-diphenyl acrolein (yield 25 %) and *cis*-2,3-diphenyl-acrylic acid (yield 19 %). The other products found were benzaldehyde (11 %), 2,3-diphenyl-2-chloro-propan-1-ol-3-one (5 %), α-chloro-benzyl phenyl ketone (4 %), and *trans*-stilbene (0.5 %). *cis*-2,3-Diphenyl-acrylic acid might well have been formed by chlorite oxidation [10] of *cis*-2,3-diphenyl-acrolein. An addition of formaldehyde, which reacts rapidly with chlorite, but only slowly with chlorine dioxide [6] would then suppress the formation of the acid in favour of the aldehyde. We therefore investigated the possibility that chlorine dioxide in the presence of formaldehyde could be used for oxidation of allylic carbinol groups to aldehyde groups. In agreement with the assumption above, the yield of *cis*-2,3-diphenyl acrolein increased to 38 % in the presence of formaldehyde if the oxidation was carried out as described above in carbon tetrachloride solution. If the oxidation was performed in aqueous solution its yield was 27 % in the presence of formaldehyde and only 8 % in the absence of formaldehyde. Chlorine dioxide plus formaldehyde may then in special cases be a cheap reagent for oxidation of allyl alcohols to acroleins.

Chlorine dioxide oxidised *cis*-2,3-di-(3,4-dimethoxyphenyl)-prop-2-en-1-ol in aqueous *t*-butanol solution to 3,4-dimethoxy-phenacyl alcohol (yield 42 %), veratraldehyde (24 %), veratric acid (25 %), and 2,3-dichloro-2,3-di-(3,4-dimethoxyphenyl)-propan-1-ol (20 %). The same products were predominant when the oxidation was carried out in chloroform solution.

Thus, this oxidation involved mainly a cleavage of the aliphatic double bond. No evidence for an allylic oxidation was observed, which clearly distinguished the oxidation of this stilbene derivative from the oxidation of that containing no methoxyl group, *viz.* *cis*-2,3-diphenyl-prop-2-en-1-ol. The methoxylated model compound reacted faster with chlorine dioxide than the one without methoxyl groups. The methoxyl groups evidently promote chlorine dioxide addition to the double bond, rather than oxidation in the allylic position.

4-Hydroxy-3,3',4'-trimethoxy-trans-*stilbene.* The chlorine dioxide oxidation of this stilbene in chloroform solution gave only a low yield of identifiable products. These products were vanillic and veratric acid (total acid yield 10 %), veratraldehyde, monochlorinated veratraldehyde, and vanillin (each aldehyde in a yield less than 1 %). The main part of the reaction product consisted of a dark, probably polymeric material. The possibility that chlorine dioxide induced vinylic polymerization involving free radicals was excluded since only one mol of stilbene was consumed per mol chlorine dioxide (determined by UV-absorption spectrometry). This polymerisation was probably caused by oxidation of the phenolic hydroxyl group (*cf.* the polymerisation caused by chlorine dioxide oxidation of other phenols).[1-5]

No general pattern was found in the reactions of the four stilbenes with chlorine dioxide. Epoxidation, allylic oxidation, splitting of the aliphatic double bond, α-chloro ketone formation *etc.* were observed. The phenolic stilbene seemed to be attacked mainly at the free phenolic group.

The products from the oxidation of the methoxylated stilbenes with chlorine dioxide, however, suggest that the conjugated double bonds which possibly are present in the residual lignin, may be cleaved by the dioxide, forming carbonyl groups.

EXPERIMENTAL

General. Unless otherwise stated, the following methods were used. The chlorine dioxide treatment of the stilbenes were carried out at room temperature in darkness. NMR-spectra (in deuterio chloroform) were obtained on a Perkin-Elmer R12 (60 MHz); IR-spectra (in chloroform) on a Perkin-Elmer 237; and UV-spectra, on a Beckman DK-2; GLC on a Perkin-Elmer 800, 900 or F 30; and GLC/MS on a Perkin-Elmer 270. TLC:s were run on silica gel plates with chloroform, chloroform/methanol or toluene as solvents.

trans-Stilbene and chlorine dioxide

Oxidation by chlorine dioxide in carbon tetrachloride. Chlorine dioxide (9 mmol) in carbon tetrachloride (158 ml) was added to *trans*-stilbene (5 mmol, 900 mg) in carbon tetrachloride (10 ml). After 20 h, the reaction mixture gave the following NMR signals (in carbon tetrachloride solution) (their assignments are given in brackets): δ 3.83 s (*trans*-α,α'-epoxy-bibenzyl) relative peak area 10; δ 5.21 s (α,α'-dichloro-bibenzyl) area 2; δ 6.28 s (α-chloro-benzyl phenyl ketone) area 6; δ 7.0 – 7.6 m (aromatic protons of the products and of unreacted stilbene) area 125; δ 7.8 – 8.0 m (α-chloro-benzyl phenyl ketone) area 14. Calculations from the peak areas showed that the mixture consisted of about 46 mol % α-chloro-benzyl phenyl ketone, 36 % *trans*-α,α'-epoxy-bibenzyl and 7 % α,α'-dichloro-bibenzyl.

The mixture was chromatographed on a silica gel column with toluene as eluent. (The reaction mixture was found to contain only negligible amounts of acids). Five fractions were collected.

The following NMR signals of fraction 1 (evaporation residue 109 mg) were observed: δ 5.20 s (assigned to both the *racem.* and the *meso* form of α,α'-dichloro-bibenzyl) relative peak height 3; δ 6.88 s (unassigned) height 0.7; δ 7.14 s (assigned to the *racem.* form of α,α'-dichloro-bibenzyl) height 12.4; δ 7.20 – 7.45 m (assigned to *trans*-stilbene); δ 7.35 s (assigned to the *meso* form of α,α'-dichloro-bibenzyl), height 4; and δ 7.7 s (assigned to *trans*-stilbene) height 3. TLC of the fraction showed two spots which were indistinguishable from those of *trans*-stilbene and α,α'-dichloro-bibenzyl (the D,L and the *meso* forms of the latter were not separated).

The TLC and the NMR signals of fraction 2 (33 mg) were indistinguishable from those of an authentic sample of *trans*-stilbene epoxide and in accordance with those given by Ceccarelli *et al.*[11]

Fraction 3 (42 mg) was a mixture of the components in fractions 2 and 4.

Fraction 4 (422 mg) showed NMR and IR spectra indistinguishable from those of an authentic sample of α-chloro-benzyl phenyl ketone, and had a m.p. 65 – 67.5 °C (lit.[12] 68.5 °C).

Fraction 5 (122 mg) consisted of about two thirds diphenylacetaldehyde as shown by comparing its IR and NMR spectra with those of an authentic sample. The presence of NMR signals at δ 7.7 – 8.0, together with a GLC analysis of the crude product of another run, indicated the presence of small amounts of benzil.

Oxidation by chlorine dioxide in aqueous dioxane solution. A dioxane (350 ml)/water (265 ml) solution of *trans*-stilbene (10 mmol, 1.80 g) and chlorine dioxide (19 mmol) was kept for 2.5 days and was then partly evaporated to remove chlorine dioxide and most of the dioxane. The resulting solution was divided by extraction with a sodium hydrogen carbonate solution into an acid (0.31 g) and a neutral fraction (1.71 g).

The NMR spectrum of the acid fraction was run both before and after methylation with

diazomethane in absolute methanol. The methylated fraction was also analysed by GLC (3 % OV-17). By comparing the retention times and NMR signals obtained with those from benzoic and diphenyl- acetic acid the fraction was found to consist almost exclusively of these acids. Their yields were estimated from the NMR signals.

1.40 g of the neutral fraction was fractionated on a silica gel column with chloroform as eluent. The following fractions were taken:

Fraction 1 (74 mg) consisted of α,α′-dichlorobibenzyl (mainly the D,L form) as shown by NMR (*cf.* the experiment in carbon tetrachloride solution).

Fraction 2 (155 mg) was a mixture of α-chloro-benzyl phenyl ketone, benzil and an unidentified compound. Benzil and the chloro ketone were identified from GLC (OV-17, 145 °C, $R_T = 23$ and 31 min, respectively) and by comparing the NMR and IR spectra cf these fractions with those of authentic compounds. The yields of the two compounds were estimated by GLC and their NMR integral signals.

Fraction 3 (772 mg) gave NMR signals at δ 7.20, 7.18, 7.09, 7.05 (four s, 10 H); δ 4.95 (s, 1 H); δ 4.88 (s, 1 H) and δ 3.21 (broad s, 1 H). Its IR spectrum showed strong absorption at 3550 cm^{-1} and 3400 cm^{-1} due to the presence of hydroxyl groups. The spectral and chromatographic properties of this fraction were equal to those of the mixture of the *erythro* and *threo* forms of α-chloro-α′-hydroxy-bibenzyl obtained by chlorination of *trans*-stilbene in a dioxane/water system.

Fraction 4 (60 mg). Its TLC and spectra (NMR and IR) were indistinguishable from those of an authentic sample of benzoin. Its m.p. was 131.4 – 132.5 °C (lit.[13] 137 °C).

Fraction 5 (103 mg). Two of its TLC spots were coloured red when sprayed with lead tetraacetate solution followed by Schiff's reagent (suggesting the presence of α-diols). Its IR spectrum showed absorption (3590 cm^{-1}) indicating hydroxyl groups. Its NMR spectrum showed the following main peaks: δ 7.21 (s, 10 H); δ 4.76 (s, 2 H); and 2.7 (broad s, 2 H), which correspond to the reported[14] data for *meso*-α,α′-dihydroxy-bibenzyl. An NMR signal at δ 4.61 was assigned to D,L-α,α′dihydroxy-bibenzyl (see fraction *6*).

Fraction 6 (104 mg). Its IR spectrum showed absorption at 3590 cm^{-1} and its TLC spots showed positive reaction with lead tetraacetate followed by Schiff's reagent. Its NMR spectrum showed signals at δ 7.20 – 7.05 (4 s, 10 H); 4.62 (s, 2 H) and 3.0 (broad s, 2 H) and was assigned to D,L-α,α′-dihydroxy-bibenzyl.

cis-2,3-Diphenyl-2-propen-1-ol and chlorine dioxide

A carbon tetrachloride solution of *cis*-2,3-diphenyl-2-propen-1-ol (6.2 mmol) and chlorine dioxide (6.6 mmol) was reacted for 18 h and was then washed with ice water and evaporated to dryness. The residue (1.6 g) was separated into neutral and acid components as usual by partition between sodium hydrogen carbonate solution and ether.

The acidic part consisted of *cis*-2,3-diphenyl-acrylic acid (yield 19 %, m.p. 174 – 175 °C, lit.[15] 172 – 173 °C, NMR indistinguishable from that of an authentic sample) contaminated with small amounts of benzoic acid which was observed by GLC/MS analyses of the fraction after diazomethane methylation.

An aliquot (90 %, *i.e.* 1.2 g) of the neutral fraction was eluted with chloroform from a silica gel column; five fractions (together 1.03 g) were collected.

Fraction 1 (400 mg) was rechromatographed on a silica gel column with hexane/toluene 1:1 as eluent. The separation was still incomplete, but *trans*-stilbene (0.5 %), α-chloro-benzyl phenyl ketone (4 %) and benzaldehyde (11 %) and small amounts of *cis*-2,3-diphenyl-acrolein were identified by NMR signals and TLC. The yields were estimated from the NMR integrals.

Fraction 2 (309 g) consisted of *cis*-2,3-diphenyl-acrolein (yield 25 %) m.p. 93.5 – 94.5 °C, lit.[16] 95 °C. NMR: δ 9.67 (s, 1 H); δ 7.4 – 7.1. (m, 11 H). TLC and GLC (OV-17, 3 %, 170 °C) showed that the fraction was practically pure. MS: *m/e* 208 (molecular ion, base peak), 207 (50 % of the base peak height), 192 (20), 179 (70), 178 (75), 165 (30), 152 (20), 105 (30) and 103 (45).

Fraction 3 (89 mg) contained according to NMR and TLC mainly *cis*-2,3-diphenyl-acrolein (see fraction *2*) and 2,3-diphenyl-2-chloro-propan-1-ol-3-one (see fraction *4*) but also an unidentified compound.

Fraction 4 (88 mg). Its IR absorption (3580 and 3450 cm^{-1} for hydroxyl group and 1675 cm^{-1} for keto group conjugated with double bond) and its NMR spectrum [δ 7.8 – 7.0 (m, 12 H); δ 4.1 (m, 2 H) and δ 2.95 (broad s, 1 H)] indicated that it consisted of 2,3-diphenyl-2-chloro-propan-1-ol-3-one (5 %).

Fraction 5 (144 mg) was a complicated mixture which contained, besides unidentified material, the chloro ketone from fraction 4 and small amounts of the starting material.

In other runs, *cis*-2,3-diphenyl-prop-2-en-1-ol (5.2 mmol) was reacted with chlorine dioxide (5.2 mmol) in the presence and in the absence of formaldehyde (6 mmol). The reaction was carried out in carbon tetrachloride (as above) and in a mixture of *t*-butanol/water (7:10). The reaction product was analysed by GLC. For results, see text.

cis-2,3-Di-(3,4-dimethoxyphenyl)-prop-2-en-1-ol.[17]

Oxidation by chlorine dioxide in aqueous t-butanol solution. Chlorine dioxide (1 mmol) in

water (18.5 ml) was added to *cis*-2,3-di-(3,4-dimethoxyphenyl)-prop-2-en-1-ol (1 mmol) in *t*-butanol (18.5 ml). After one hour the reaction mixture was partly evaporated to remove most of the *t*-butanol, diluted with water and extracted with methylene chloride.

The acidic part (44 mg, yield 25 % calc. as veratric acid) of the extract was methylated with diazomethane and analysed with GLC/MS (OV-1). It consisted of methyl veratrate contaminated with a small amount of veratraldehyde.

The neutral part (280 mg) was fractionated with chloroform on a silica gel column. The yields were estimated from the NMR integral signal of the raw product and were in agreement with the fraction weights. The following fractions were collected:

Fraction 1 (40 mg). Its NMR, GLC, MS, and TLC properties were indistinguishable from those of an authentic sample of veratraldehyde.

Fraction 2 (23 mg) was a mixture of veratraldehyde, an unknown compound and 3,4-dimethoxy-phenacyl alcohol (see fraction *3*).

Fraction 3 (82 mg) consisted according to TLC mainly of one substance which from its NMR and IR spectra appeared to be 3,4-dimethoxy-phenacyl alcohol [NMR: δ_a 7.42 (d, 1 H); δ_b 7.40 (q, 1 H); δ_c 6.85 (d, 1 H); δ_d 4.80 (broad s, 2 H); δ_e 3.90 (s, 6 H); δ_f 3.55 (broad s, 1 H, disappeared upon addition of D_2O). $J_{ab}=1.5$ Hz, $J_{bc}=8.5$ Hz. IR: a broad absorption at 3450 cm^{-1}, a sharp absorption at 1670 cm^{-1} and doublets at 1590 cm^{-1} and 1510 cm^{-1}]. The NMR and TLC of the acetylated fraction were indistinguishable from those of an authentic sample of 3,4-dimethoxyphenacyl acetate.[18]

Fraction 4 (27 mg) consisted of a mixture of 3,4-dimethoxy-phenacyl alcohol and the dichloro-compound from fraction *5* below.

Fraction 5 (30 mg) had the same NMR spectrum [δ 6.75 (m, 3 H); δ 4.95 (s, 1 H); δ 4.35 (s, 2 H) and δ 3.80 (s, 6 H)] as 2,3-dichloro-2,3-di-(3,4-dimethoxyphenyl)-propanol, which was obtained by chlorination of *cis*-2,3-di-(3,4-dimethoxyphenyl)-prop-2-en-1-ol in carbon tetrachloride solution (see below).

Fraction 6 (37 mg) was a mixture of constituents which were not identified, except for a small amount of the dichloro compound of fraction 5.

Oxidation by chlorine dioxide in chloroform solution. An experiment similar to that above was carried out in chloroform. The reaction products were veratric acid (22 %) and a neutral part which did not differ significantly (as shown by TLC and NMR) from the reaction products obtained when the experiment was done in *t*-butanol/water.

Chlorination. cis-2,3-Di-(3,4-dimethoxyphenyl)-prop-2-en-1-ol was also reacted with chlorine. When the reaction was performed in carbon tetrachloride, the residue gave after chromatography on silica gel with chloroform, a fraction (56 %) which was identical with the dichloro compound from fraction 5 above, according to NMR signals and TLC.

When performed in *t*-butanol/water, the chlorination gave the chlorohydrine as the main product.

4-Hydroxy-3,3',4'-trimethoxy-stilbene[19] and chlorine dioxide

To the stilbene (2 mmol) in chloroform (20 ml) was added chlorine dioxide (2.6 mmol) in chloroform (40 ml). The reaction mixture immediately turned reddish, and subsequently yellow. After 20 min the mixture was evaporated to dryness and a viscous oil (727 mg) was obtained which was analysed by GLC (5 % OV-1, 100→200 °C, +4 °C/min) and MS. Veratraldehyde, vanillin, monochlorinated veratraldehyde, and veratric acid were identified. All yields were less than 1 %. The acidic part (34 mg, 9 % if calculated as veratric acid) was methylated with diazomethane and analysed with GLC (5 % OV-1, 180 °C) and MS. Methyl veratrate was by far the predominant compound. IR analysis gave the same result. The ester was contaminated with small amounts of veratraldehyde, monochlorinated veratraldehyde, and monochlorinated methyl veratrate (GLC/MS). The neutral fraction consisted of dark polymeric material.

In other experiments, chloroform solutions of the stilbene (10 mM) and chlorine dioxide (5 mM) were mixed in different proportions (10/1, 10/2, 9/4 and 1/1 ml/ml) and were diluted to 25 ml. After 10 min and after 24 h, samples of the solutions were diluted 100 fold and UV-spectra were taken. For results, see text.

α,α'-Epoxy-bibenzyl[20] and chlorine dioxide, chlorine, or a mixture of chlorine dioxide and chlorine

A carbon tetrachloride solution (50 ml) of α,α'-epoxy-bibenzyl (1 mmol) and chlorine dioxide (1 mmol) was reacted for 20 h at room temperature in darkness. Two similar experiments were performed with chlorine (1 mmol) and a mixture of chlorine dioxide (1 mmol) and chlorine (0.5 mmol), respectively.

The reaction mixtures were partly evaporated and analysed by NMR.

Only negligible alternations seemed to have occurred, and no α-chloro-benzyl phenyl ketone was detected.

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Conversion of Penta-*O*-acetyl-1,2-*O*-isopropylidene-*aldehydo*-D-glucose into Tri-*O*-acetyl-2,3-dideoxy-*aldehydo*-D-*erythro*-hex-2-enose

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Acetolysis of 3,5,6-tri-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranose gave 1-*R*-1,3,4,5,6-penta-*O*-acetyl-1,2-*O*-isopropylidene-*aldehydo*-D-glucose (*2*) and a small amount of the 1-*S*-isomer (*3*). Treatment of (*2*) with hydrogen bromide in acetic acid yielded mainly *trans*-4,5,6-tri-*O*-acetyl-2,3-dideoxy-*aldehydo*-D-*erythro*-hex-2-enose (*4*). Besides, a small amount of 2,3,5,6-tetra-*O*-acetyl-D-glucofuranose was formed. The mechanism of the formation of (*4*) is discussed.

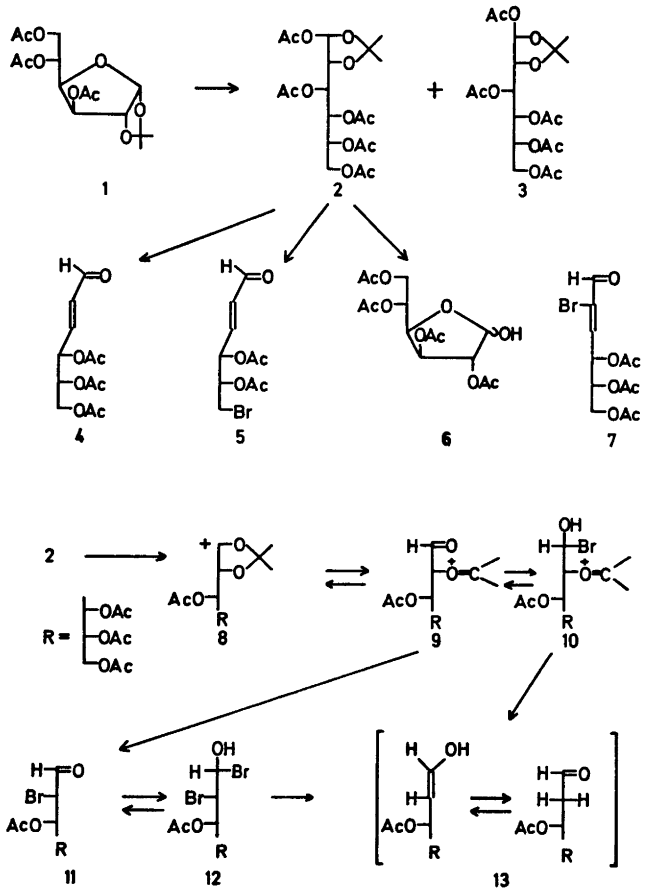

Table 1. Proton NMR spectra in deuteriochloroform. Chemical shifts are in ppm and observed 1st order coupling constants in Hz.

Compound	H1	H2	H3	H4	H5	H6	H6′	J_{12}	J_{23}	J_{24}	J_{34}	J_{45}	J_{56}	$J_{56'}$	$J_{66'}$
2	6.25	4.35	5.38	5.46	5.14	4.15	4.24	2.2	4.9		2.9	8.5	3.0	4.0	12.5
3	6.36	4.18	5.62	5.28	5.04	4.2–4.3		3.8	7.2		1.7	9.0	3.1	3.1	
4	9.60	6.30	6.75	5.80	5.30	4.32	4.22	7.2	15.4	1.5	4.9	4.9	4.4	6.0	12.2
5	9.58	6.30	6.72	5.81	5.28	3.4–3.6		7.2	15.4	1.4	5.0	5.0	4.6	6.0	
7	9.26		7.04	6.05	5.45	4.20	4.30				7.8	4.8	4.6	5.8	12.0

Both Brigl and Zerrweck [1] and Schlubach *et al.*[2] have shown that acetolysis of tri-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranose (*1*) gives a crystalline product in good yield, and several structures were proposed for this product. In a recent paper Magnani and Mikuriya [3] have shown by NMR spectroscopy that the product is an *aldehydo*-D-glucose derivative, (*2*) or (*3*).

We have also studied the acetolysis of (*1*) and obtained the same product as that described by the above mentioned authors. In addition, we isolated a minor amount of an isomeric material. Proton NMR spectra of the two products (Table 1) show that they both contain an isopropylidene group and five *O*-acetyl groups in agreement with structures (*2*) and (*3*), as found by Magnani and Mikuriya [3] for the major product. The low field position of H4 in both products rule out the furanose structure proposed by Schlubach *et al.*[2] The main product (*2*) has $J_{12}=2.2$ Hz whereas J_{12} of the minor product (*3*) is 3.8 Hz. This indicates a *trans* orientation of H1 and H2 in (*2*) and a *cis* orientation in (*3*). On this basis the configuration of C1 in (*2*) is assumed to be *R* and that of (*3*) is *S*. ^{13}C NMR data (Table 2) further confirm the structures.

In connection with other work (*2*) was treated with hydrogen bromide in acetic acid for 24 h. This gave an unstable mixture which was hydrolysed with water and silver carbonate. From the product thus obtained was isolated 45 % of the unsaturated aldehyde (*4*). Besides, small amounts of the 6-bromo-aldehyde (*5*) and of tetra-*O*-acetyl-D-glucofuranose (*6*) were obtained. The structure of (*4*) was proved through ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) and

Table 2. ^{13}C NMR spectra in deuteriochloroform. Chemical shifts are in ppm relative to internal tetramethylsilane.

Compound	C1	C2	C3	C4	C5	C6	O–C–O	C–$(CH_3)_2$		H_3C–C=O
1	104.6	88.7	74.2	76.3	67.1	62.9	112.1	26.5	25.9	20.3
2	95.8	80.7	68.5[a]	68.2[a]	68.0[a]	61.4	113.0	26.5	26.3	20.6 20.1
3	93.3	77.5	68.5[a]	68.1[a]	67.6[a]	61.2	111.9	27.8	25.7	20.3
4	191.6	132.9	147.6	70.5[a]	70.1[a]	61.0				
5	191.6	133.6	147.8	72.0[a]	71.2[a]	28.9				
7	184.6	130.4	144.7	70.8[a]	70.3[a]	61.4				

[a] Assignment may be reversed.

by comparison with an authentic sample prepared according to Fraser-Reid and Radatus.[4]

The first step in the reaction of (*2*) with hydrogen bromide in acetic acid is probably a protonation followed by loss of acetic acid to give (*8*) which could rearrange to (*9*). The latter type of ion has been described by Barton *et al.*[5] Since (*9*) contains a good leaving group it could react with bromide ions to give (*11*) which, in the presence of HBr, would be in equilibrium with (*12*). NMR spectra directly on the reaction mixture show no aldehydic protons, indicating that (*12*) is favoured; this also applies to the equilibrium between (*9*) and (*10*) (see below). Reaction of the latter with bromide ions could result in elimination of bromine and formation of (*13*). Alternatively, (*9*) would be in equilibrium with (*10*) which, by a similar reaction with bromide ions, could give (*13*), bromine, and acetone. It is also possible that (*10*) could yield (*11*) by migration of bromine from C1 to C2 with simultaneous displacement of acetone. Bromoacetone was found in the reaction product, and it is probably formed in a secondary reaction between the bromine eliminated from (*10*) or (*12*) and acetone.

The 2-deoxy-aldehyde (*13*) is probably the final product from the reaction of (*2*) with hydrogen bromide in acetic acid. It loses acetic acid to some extent when the reaction mixture is worked up, and the elimination is completed by the subsequent treatment with water and silver carbonate.

When (*2*) was allowed to react with hydrogen bromide in acetic acid for only 1 h the 2-bromo-aldehyde (*7*) could be isolated together with a rather large amount of (*6*). The latter is probably formed by hydrolysis of the ions (*9*) or (*10*) during work up, followed by acyl-migration and ring-closure. After 24 h reaction (*7*) could not be found. Since it must arise from (*11*) by elimination of acetic acid during work up the latter would appear to be present in the initial stage of the reaction, as proposed above, but disappears later as it is converted into (*13*) *via* (*12*).

When pure (*7*) was treated with HBr in acetic acid for 24 h in the presence of acetone it gave (*4*) after work up. In the absence of acetone the main product was unreacted (*7*) and only small amounts of (*4*) were formed. The first step must be addition of HBr to (*7*) followed by elimination of bromine which then reacts with acetone.

The conclusion is that (*13*) is formed from (*9*) or (*10*) *via* the 2-bromo-compound (*11*), or to some extent, directly from (*10*) by elimination of bromine and acetone.

Pure (*7*) was prepared in good yield by reaction of (*4*) with bromine followed by elimination of HBr by treatment with silver carbonate and water.

EXPERIMENTAL

Melting points are uncorrected. Proton NMR spectra were obtained on Varian A-60 and HA-100 instruments, ^{13}C spectra on a Bruker WH-90 instrument. Thin layer chromatography (TLC) was performed on silica gel PF_{254} (Merck); for preparative work 1 mm layers on 20 × 40 cm plates were used. Spots were visualized with UV light or by charring with a hot wire.

*Acetolysis of tri-*O*-acetyl-1,2-*O*-isopropylidene-α-*D*-glucofuranose.* A mixture of (*1*) (40.0 g) and powdered, anhydrous $ZnCl_2$ (11.0 g) was dissolved in acetic anhydride (80 ml) by stirring at 0 °C. The solution was kept at +5° for 24 h. Ice and water was then added and the mixture was stirred until the oily precipitate had crystallized. The product was then filtered off, washed with water and dried. The crude material (40 g) was shown by NMR spectroscopy to be a mixture of (*2*) and (*3*) in a *ca.* 12:1 ratio. Two recrystallizations from ethanol gave 29.0 g (56 %) of 1-*R*-1,3,4,5,6-penta-*O*-acetyl-1,2-*O*-isopropylidene-*aldehydo*-D-glucose (*2*), m.p. 136–138 °C. Further recrystallizations from ethanol and from ether-pentane did not change the m.p., $[\alpha]_D^{22}$ +58.3° (*c* 3.0, $CHCl_3$). (Reported[1] m.p. 141 °C, $[\alpha]_D$ +60.5°).

In two experiments fractional crystallization of the material in the mother liquor gave a small amount of the 1-*S*-isomer (*3*), m.p. 139–140 °C, $[\alpha]_D^{20}$ −19.0° (*c* 1.4, $CHCl_3$). (Found: C 50.94; H 6.22. Calc. for $C_{19}H_{28}O_{12}$; C 50.88; H 6.29). Attempts to repeat the isolation of (*3*) from the mother liquors of other preparations of (*2*) were unsuccessful.

Preparation of (*2*) from (*1*) by treatment with acetic anhydride and sulfuric acid, as described by Schlubach *et al.*,[2] gave lower yields than those obtained when zinc chloride was used.

Reaction of (*2*) with hydrogen bromide in acetic acid

For 24 h. The isopropylidene-derivative (*2*) (820 mg) was dissolved in 8 ml of glacial acetic acid containing 30 % HBr and the solution was kept at room temp. for 24 h. It was then diluted with dichloromethane and washed with water and aqueous sodium hydrogencarbonate, dried ($MgSO_4$) and evaporated. The residue in acetone

(6 ml) was stirred over night with water (0.5 ml) and silver carbonate (1.0 g). Filtration through activated carbon and evaporation gave a crude product which was separated into 3 fractions by preparative TLC using ether-pentane (3:1) as eluent. The fastest moving fraction gave 35 mg (6.5 %) of the 6-bromo-compound (*5*) as a *cis-trans* mixture in a 2/5 ratio. The product was a syrup and the two isomers could not be separated, λ_{max} 217 nm (ethanol). Proton and ^{13}C NMR data are given for the *trans* isomer in Tables 1 and 2.

The next fraction gave 220 mg (45 %) of 4,5,6-tri-*O*-acetyl-2,3-dideoxy-*aldehydo*-D-*erythro-trans*-hex-2-enose (*4*) as a syrup, $[\alpha]_D^{20}$ +25.3° (*c* 4.3, $CHCl_3$), λ_{max} 217 nm (ethanol) (reported [4] $[\alpha]_D$ +12.0°, λ_{max} 217 nm). A 2,4-dinitrophenylhydrazone had m.p. 111–112 °C (reported [4] m.p. 108–109 °C). Proton and ^{13}C NMR data were identical with those of an authentic sample prepared according to Ref. 4.

The slowest moving fraction gave 53 mg (9 %) of tetra-*O*-acetyl-D-glucofuranose (*6*) which was acetylated to give the pentaacetate as a 1:2 mixture of the α- and β-anomers. Proton NMR spectra were identical with those of previously described compounds.[6]

Isolation of bromoacetone. A solution of (*2*) (5.0 g) in HBr–HOAc (8 ml) was kept for 24 h at room temp. Dichloromethane (75 ml) was then added and the solution was washed twice with water and once with aqueous $NaHCO_3$ and dried ($MgSO_4$). The dichloromethane was distilled off through a Vigreux column and the residue was distilled at 50 mmHg pressure. A fraction of 1.0 g was collected, b.p. 40 °C. This lachrymatory product consisted of a mixture of bromoacetone and acetic acid. A proton NMR spectrum gave signals at δ 2.4 and 4.0 and a ^{13}C spectrum at 27.1, 35.7, and 199.5 ppm. Both spectra were identical with those of an authentic sample.

B. For 1 h. A solution of (*2*) (1.0 g) in HBr-HOAc (10 ml) was kept for 1 h at room temp. and then worked up. The product was treated with water and silver carbonate as described above. The crude product (737 mg) was purified by preparative TLC (ether-pentane 3:1). The main fraction (332 mg) was a mixture of (*4*) and the 2-bromo-compound (*7*) as seen from an NMR spectrum. The ratio between (*4*) and (*7*) varied from 1:2 to 1:5 in different experiments. It was not possible to separate the two compounds and (*7*) was therefore only identified by comparing proton NMR spectra of the mixture with those of pure (*7*) and (*4*).

Another fraction gave 170 mg (22 %) of (*6*), characterized through its NMR spectrum.

4,5,6-Tri-O-*acetyl-2-bromo-2,3-dideoxy*-aldehydo-D-erythro-*hex-2-enose* (*7*). To a solution of (*4*) (350 mg) in tetrachloromethane (5 ml) was added a solution of bromine (0.08 ml) in tetrachloromethane (1.4 ml) and the solution was kept for 10 min at room temp. Silver carbonate (2.0 g) and acetonitrile (15 ml) were then added and the mixture was stirred for 1 h. The silver salts were filtered off, the solvent was removed and the residue was dissolved in dichloromethane and washed with aqueous $NaHCO_3$, dried and evaporated. Preparative TLC (ether-pentane 3:1) gave 195 mg (43 %) of (*7*) as a syrup, $[\alpha]_D^{20}$ +24.5° (*c* 0.84, $CHCl_3$), λ_{max} 235 nm (ethanol). The compound was unstable and a satisfactory analysis could therefore not be obtained. NMR spectral data (Tables 1 and 2) were in agreement with the structure. A mass spectrum showed no molecular peak, but two peaks at *m/e* 290 and 292, resulting from loss of acetic acid and showing that bromine was present.

Conversion of (*7*) *to* (*4*). A mixture of (*7*) (260 mg), HBr in acetic acid (3 ml), and acetone (0.2 ml) was kept for 24 h at room temp. It was then worked up and treated with silver carbonate and water as described above. The product was separated into two fractions by preparative TLC. The fast moving fraction gave 20 mg (8 %) of the 6-bromo-aldehyde (*5*). The slow moving fraction gave 107 mg (49 %) of (*4*). Both products were characterized through their NMR spectra.

Microanalyses were performed by Novo Microanalytical Laboratory.

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Diels-Alder Reactions of 2,4-Cyclohexadienones.* III.** Formation of Stereoisomers in the Dimerization of an *o*-Quinol Acetate

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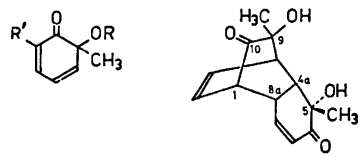
o-Quinol acetate *4*, which at 120 °C gives the *endo* Diels-Alder dimer *8*, at temperatures of 160 – 180 °C gives the C-5 stereoisomeric dimer *9* in addition to *8*. On heating at 160 – 180 °C *8* is also converted to *9*. When treated with ethanolic KOH, the diacetates *8* and *9* are hydrolyzed to give the stereoisomers *10* and *12*, respectively, the phenol *11* being formed as an additional product in both cases. The non-acetylated dimer *12* isomerizes at 160 – 180 °C to give dimer *10*. These results indicate that of the two acetylated dimers isomer *9* is thermodynamically more stable than isomer *8*, whereas the reverse seems to be the case for the corresponding non-acetylated dimers *12* and *10*, respectively. This behaviour can be understood by considering the bulkiness of the substituents at C-5 of the two types of dimers.

Acid hydrolysis of dimer diacetate *8* initially gives dimer *10*, which subsequently undergoes proton-catalyzed opening of the bicyclooctenone ring system to give phenol *13*. Similar treatment of the dimer diacetate *9* with acid effects, in addition to ester hydrolysis, aromatization of the unbridged ring followed by rearrangement of the bicyclooctenone system with the formation of phenol *16*.

In a number of papers the spontaneous Diels-Alder dimerization of 2,4-cyclohexadienones has been reported.[1–7] The reactions were found to proceed with a high degree of selectivity, only one of several conceivable dimerization products being obtained in each case. By using chemical, photochemical, and spectrometric methods,[4–8] as well as X-ray diffraction analysis,[9,10] all dimers so far investigated were shown to be *endo* forms with the same stereochemical and structural orientation.

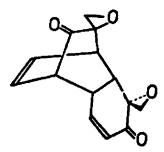
The stereochemical orientation at C-5 and C-9 was interpreted as being due to steric approach control [11] in the dimerization, the most bulky substituent being directed away from the reaction center. This is illustrated by formula *5* for the dimer of the parent *o*-quinol (*1*) (6-hydroxy-6-methyl-2,4-cyclohexadienone) and by formula *7* for the dimer of the corresponding spirooxirane (*6*). Analogous configurations have been proposed for dimers of 2,4-cyclohexadienones carrying halogen atoms instead of hydroxyl groups in the 6-position.[12,13]



1 R=R'=H
2 R=Ac ; R'=H
3 R=H ; R'=CH_3
4 R=Ac ; R'=CH_3

5

6



7

Contrary to *o*-quinols (type *1*) and spiro-epoxydienones (type *6*), *o*-quinol acetates such as *2* and *4* are comparatively stable at room temperature, but dimerize when heated at

* Part XIII in the series "Periodate Oxidation of Phenols".

** Part II, see Ref. 7.

120 °C.[14,3] Hydrolysis of the dimeric *o*-quinol acetates gave the free dimers which were identical with those obtained by spontaneous dimerization of the corresponding *o*-quinols (*1* and *3*), and, *vice versa*, acetylation of the latter dimers gave the dimerization products of the corresponding *o*-quinol acetates *2* and *4*. Since the structures of the non-acetylated dimers (*5* and *10*) were known,[6,10] these interconversions also established the structures of the dimeric diacetates, the dimer of *o*-quinol acetate *4*, for instance, possessing structure *8*.

FORMATION OF STEREOISOMERS IN THE DIMERIZATION OF *o*-QUINOL ACETATE *4*

In earlier work [3] it has been observed that in the dimerization of *o*-quinol acetate *4* at 120 °C which mainly yields the *endo* dimer *8*, m.p. 159 – 160 °C, small amounts of an isomeric by-product of m.p. 182 – 184 °C are also formed. It has now been found that the yield of the higher-melting compound increases if the heating temperature is raised above 120 °C (see below). According to a recent X-ray investigation [15] the product of m.p. 182 – 184 °C is the C-5 stereoisomer *9* of dimer *8*. Dimer *8* is a Diels-Alder adduct of two sterically identical molecules of monomer *4* ($S+S$ and $R+R$ enantiomers, respectively). The structure of *9* indicates that it is formed by Diels-Adler addition of $S+R$ enantiomers and of $R+S$ enantiomers of the monomer.

Fig. 1 shows the influence of temperature and heating time on the weight proportions of monomer *4*, dimer *8* and dimer *9* in the reaction mixtures. The proportions of the latter isomer increases with increasing temperature, optimum yields being obtained within the range of 160 – 180 °C. In a preparative experiment *9* was obtained in a yield of 60 % after heating of monomer *4* for 2.5 h at 165 °C.

At higher temperatures dimer *9* undergoes retro Diels-Alder reaction, cleavage being nearly complete; heating of *9* for 30 min at 205 °C thus gave 84 % of monomer *4*. Under the same conditions, the earlier reported [3] thermal cleavage of dimer *8* similarly produced the monomer in a yield of 79 %.

As indicated in Fig. 1, the reaction mixtures obtained on heating of monomer *4* further contained an "unidentified product", which predominated after 6 hours' heating at 185 °C (Fig. 1 c). The dark viscous oil obtained under the last-mentioned conditions probably was a mixture and was not further investigated. It may be noted that brief heating of *o*-quinol acetates at 450 °C has been reported [16] to produce monoacetates of substituted hydroquinones and catechols.

Dimer *9* was also formed when dimer *8* was heated just above its melting point (2.5 h at 165 °C); this isomerization can be interpreted to be due to retro Diels-Alder reaction of *8*, followed by recombination of the enantiomers of the monomer.

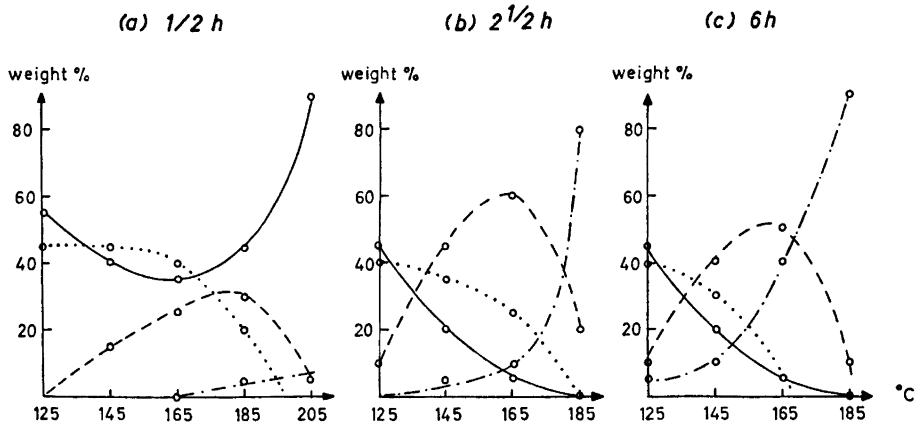


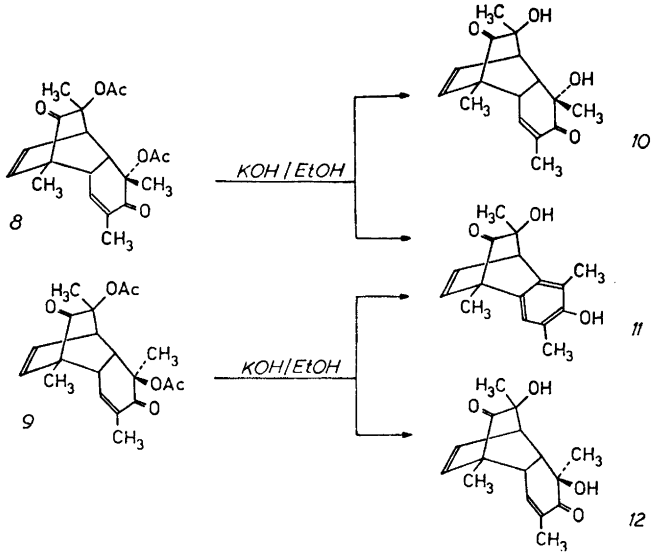
Fig. 1. Yields of products formed on heating of *o*-quinol acetate *4 vs.* temperature (*cf.* also Experimental). Compounds *4* (——), *8* (...), *9* (– – –) and unidentified product (–.–.–.).

These findings indicate that the sterically favoured configuration at C-5 is that present in compound *9*, this isomer being thermodynamically more stable than *8*. Evidently, the methyl group at C-5 is less bulky than the acetoxy group, although in the latter substituent free rotation around the C-5 – O linkage is possible.

The formation of isomer *8* from the monomeric *o*-quinol acetate (*4*) obviously is kinetically controlled; this may be connected with the *endo* oriented acetoxy group of the dienophile providing additional π-electrons to the reacting double bond system.

Contrary to the configuration of C-5 that of C-9 is retained in the heat isomerization of *8*. In the formation of both isomers the C-9 acetoxy group is oriented towards the reaction center and the C-9 methyl group away from it. This may possibly be understood on the basis of a proposal made by Williamson *et al.*,[17] according to which *syn-anti* isomerism of Diels-Alder reactions is governed not only by steric factors but also by electronic factors: if the dienophile is a dipole, electrostatic forces will direct the most polarizable bridge substituent into a position *anti* to the ethylene bridge. The configurations at C-9 of dimers *8* and *9* are in accord with this view, the methyl groups, which are the less polarizable substituents, heading towards the C-1, C-4 ethylene bridge.

The behaviour of dimer *9* on alkaline hydrolysis is noteworthy (Scheme 1). As reported earlier, treatment of isomer *8* with ethanolic potassium hydroxide gives the phenolic compound *11*, in addition to the expected hydrolysis product, *i.e.* the non-acetylated dimer *10*.[3] The two compounds were formed in about equal yields. The ease of the base-catalyzed elimination of acetic acid in compound *8* has been interpreted to be due to a favourable *trans* relationship of H-4a and AcO-5 with *anti* elimination taking place.[7] In the C-5 stereoisomer (dimer *9*) H-4a and AcO-5 are in *cis* position and elimination must consequently proceed by a *syn* mechanism. According to a recent monograph on elimination reactions,[18] *syn* elimination in 6-membered rings takes place much less readily than *anti* elimination. Alkaline hydrolysis of dimer *9* was therefore expected to give the non-acetylated dimer *12* in high yield. Surprisingly, however, phenol *11* was formed as the major reaction product (68 %) on treatment with ethanolic potassium hydroxide and only minor amounts (9 %) of dimer *12* were obtained. The ease of elimination in both *8* and *9* may be due to initial abstraction of a proton from C-8a to give enolates which readily aromatize by loosing HOAc.



Scheme 1.

THERMAL BEHAVIOUR OF DIMERS *10* AND *12*

For comparison with the thermal behaviour of the acetylated dimers *8* and *9*, that of the corresponding free dimers *10* and *12* was also investigated. Dimer *12* (m.p. 147 – 149 °C), when heated at temperatures between 160 and 180 °C, readily isomerized to compound *10*. For instance, after 1 h at 160 °C 83 % of isomer *10* were obtained and no starting material was detected in the reaction product. This result indicates that in the case of the non-acetylated dimers isomer *10* is thermodynamically more stable than isomer *12*, while the reverse is true for the corresponding diacetates (*8* and *9*, respectively), as shown by the experiments discussed above.

Apparently, the greater stability of dimer *10* as compared to dimer *12* is due to the fact that in the former isomer the smallest C-5 substituent, *i.e.* the OH group, is *endo* oriented. This, of course, indicates that steric approach control is essential in the dimerization of the monomeric *o*-quinol (*3*) which arises when dimer *12* is heated as described above.

It may also be mentioned that no formation of an *exo* form of a 2,4-cyclohexadienone dimer has hitherto been reported.

Dimer *10* proved to be unstable when heated at 210 °C, *i.e.* 15 °C above its melting point, an isomeric compound of m.p. 163 – 165 °C being formed as a major product. Dimer *12* on similar heat treatment gave the same compound, the reaction obviously passing via isomer *10*. Wessely *et al.*[19,20] reported that at 280 °C dimer *10* gives small amounts of 6-hydroxy-5,6-dimethyl-2,4-cyclohexadienone as the product of an acyloin rearrangement of the primarily formed isomer *3*. It was recently found in this laboratory that a major product of the thermolysis at 280 °C is the above-mentioned compound of m.p. 163 – 165 °C.[21] Its structure is still unknown.

ACID-CATALYZED FORMATION OF PHENOLS *13* AND *16* FROM DIMER ACETATES *8* AND *9*, RESPECTIVELY

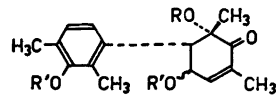
As mentioned earlier, dimer *12* is obtained in a very poor yield (9 %) by alkaline hydrolysis of its diacetate *9*, the phenolic compound *11* being formed predominantly. In an attempt to find a better method for the preparation of *12*, diacetate *9* was subjected to acid hydrolysis (7 h reflux with aqueous-ethanolic sulfuric acid) as previously used[14] for the preparation of dimer *5* from its diacetate. Under these conditions, *9* gave the desired *o*-quinol dimer *12* in a yield of 45 %. In addition, a phenolic compound (26 %, m.p. 205 – 206 °C) was obtained; it was not identical with phenol *11*.

Similar acid treatment of diacetate *8* produced, in addition to *o*-quinol dimer *10* (28 %), a phenolic product (46 %, m.p. 139 – 140 °C) which, unexpectedly, differed from both the aforementioned phenols.

The structures of the two new phenols are discussed in the following sections.

Phenol, m.p. 139 – 140 °C, from dimer diacetate 8. The compound, $C_{16}H_{20}O_4$, is isomeric with the dimeric *o*-quinols (*10*, *12*). Its UV and IR spectra indicated the presence of an aromatic ring, an α,β-conjugated keto group and hydroxyl groups. Treatment with Ac_2O/pyridine gave a diacetate showing the IR characteristics of both an aryl ester and an alkyl ester group, as well as remaining hydroxyl absorption. Acid-catalyzed acetylation[22] produced a triacetate, indicating that the last-mentioned hydroxyl group was a tertiary one. From the shift of the IR absorption peak due to the conjugated CO group from 1680 cm^{-1} (untreated phenol) and 1675 cm^{-1} (diacetate) to 1704 cm^{-1} (triacetate) it could be concluded that the tertiary hydroxyl group is located adjacent to the CO group (*cf.* Ref. 4, p. 2056).

These findings, as well as NMR data (see Exptl.), suggested structures *13*, *13a*, and *13b*



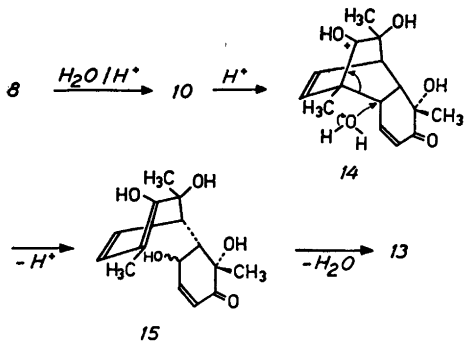
13 R=R'=H
13a R=H; R'=Ac
13b R=R'=Ac

for the phenol, its diacetate, and its triacetate, respectively.

It was found that the yield of dimer *10*, obtained in addition to the phenol *13*, decreased with increasing heating time, the yield of *13* simultaneously increasing. Furthermore, 7 h treatment of *10* under the hydrolysis conditions produced phenol *13* in a yield of 66 %, 15 % of unreacted *10* being recovered. These findings

indicated that dimer *10* is an intermediate in the formation of phenol *13*, the initial ester hydrolysis (*8*→*10*) being faster than the subsequent consumption of *10*.

The mechanism shown in Scheme 2 is proposed for the conversion of dimer *8 via 10* to phenol *13*. It is believed that protonation

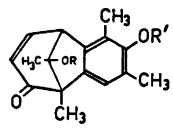


Scheme 2.

of the keto group at C-10 initiates ring opening at C-8a, a hydroxyl group being introduced at the latter carbon atom, as indicated in formula *14*. Acid-catalyzed elimination of water from the cyclohexadienol ring of the resulting intermediate *15* leads to the phenolic end-product *13*.

For the suggested conversion *14*→*15*, a few analogies have been found in the literature, *viz.* the acid-catalyzed formation of isocamphorquinone from camphorquinone [23] and the conversion of *endo* bornylamine into α-terpineol.[24]

Phenol, m.p. 205–206 °C, from dimer diacetate 9. This compound had the composition $C_{16}H_{18}O_3$ and was thus isomeric with phenol *11*. The strong red-shift of the UV maxima of its ethanolic solution which took place on addition of alkali indicated the presence of a phenolic group. The IR spectrum of the compound revealed a conjugated carbonyl group in addition to hydroxyl groups and an aromatic ring. Treatment with Ac_2O/pyridine gave a monoacetate, exhibiting IR absorptions at 1752 cm^{-1} (aryl ester) and at 3480 cm^{-1} (OH). Acetylation with $Ac_2O/HClO_4$ [22] esterified also the last-mentioned hydroxyl group, which must be tertiary. On the basis of these results, as well as of NMR data, structures *16*, *16a*, and *16b* are proposed for the phenol of m.p. 205–206 °C, its monoacetate, and its diacetate, respectively.



16 R=R'=H
16a R=H; R'=Ac
16b R=R'=Ac

Phenol *16* was also obtained by acid hydrolysis of the diacetate *17* of phenol *11*. However, the non-acetylated *o*-quinol dimer *12*, as well as phenol *11*, proved to be stable under the conditions of the acid hydrolysis. These findings suggest the following pathway for the formation of phenol *16* (Scheme 3).

Elimination of acetic acid from *9* accompanied by aromatization gives monoacetate *18*, the latter also being formed by partial hydrolysis of diacetate *17*. Protonation of the keto group of *18* induces two consecutive 1,2-shifts resulting in the formation of monoacetate *21* which is finally hydrolyzed to give phenol *16*.

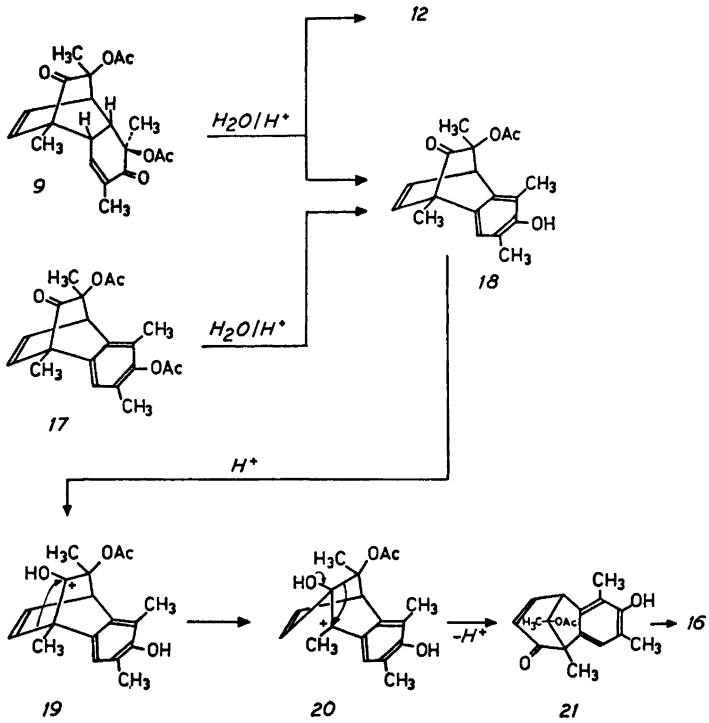
The assumption that the rearrangement takes place before the tertiary acetoxyl group is hydrolyzed, is based on the fact that neither the non-acetylated dimer *12*, which is the major product of the acid hydrolysis of *9*, nor the non-acetylated phenol *11* is affected under the conditions used. The role of the tertiary acetoxyl group is not clear; it might be considered that its $-I$ effect causes the electron density at C-10 in *19* to become sufficiently low for the Wagner-Meerwein type rearrangement *19*→*20* to occur. The intermediate *20* is a well-stabilized tertiary *p*-hydroxybenzylium ion.

It seems remarkable that diacetate *8*, contrary to its isomer *9*, under the acidic conditions used does not undergo elimination of acetic acid with aromatization of the unbridged ring, in competition with ester hydrolysis (*cf.* p. 859). Similar to the base-catalyzed eliminations, acid-catalyzed *syn* elimination of acetic acid seems to proceed more readily than the corresponding *anti* elimination.

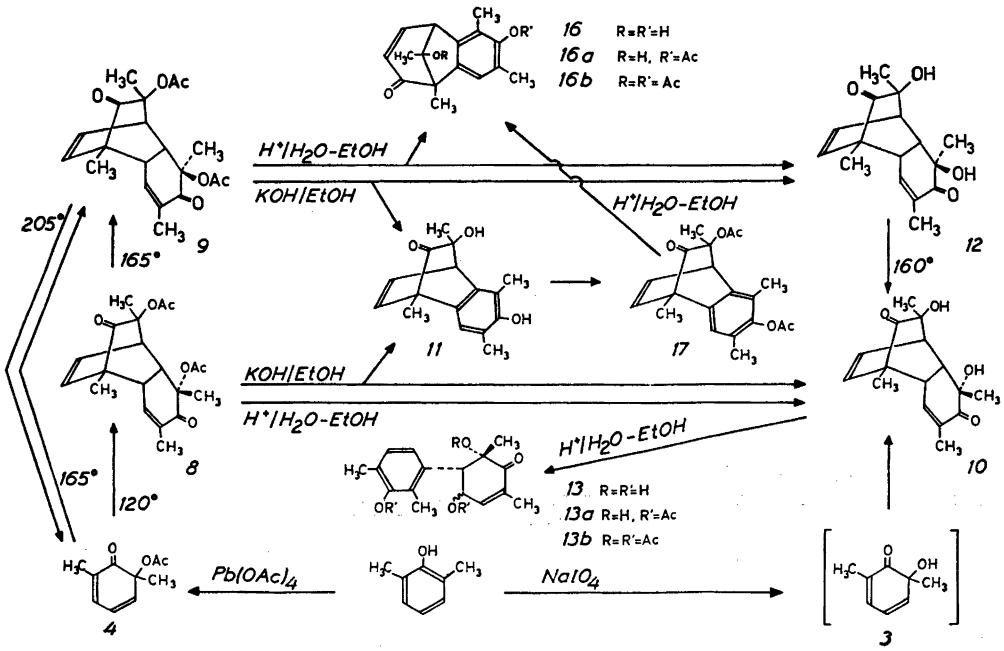
The reactions discussed in this paper are summarized in Scheme 4.

EXPERIMENTAL

Ultraviolet spectra were recorded on a Cary Model 14 spectrophotometer; IR and NMR spectra were obtained using Beckman 9A and Varian A-60 instruments, respectively. Chemical shifts are given in δ (ppm) units with TMS being used as internal standard.



Scheme 3.



Scheme 4.

6-Acetyloxy-2,6-dimethyl-2,4-cyclohexadienone (4) was prepared using the method reported by Wessely *et al.*[19] with slight modifications. Lead tetraacetate (57.5 g, 0.13 mol), which had been freed from acetic acid by washing with ether, was added to a solution of 2,6-dimethylphenol (13.6 g, 0.11 mol) in chloroform which was cooled with ice-water. After 16 h at room temperature the mixture was filtered and the filtrate brought to dryness. The residue was treated with ether (40 ml) and, after cooling the solution in the refrigerator for 4 h, undissolved material was filtered off. The solvent was evaporated, leaving an oil which crystallized when kept in the refrigerator for 24 h. Recrystallization from ether-light petroleum (b.p. 60–80 °C) gave yellow crystals of *4*, m.p. 35–36 °C (lit.[19,25] 36 °C) in a yield of 76 %. NMR ($CDCl_3$): δ 1.38 (s, 3 H, CH_3), 1.93 (d, 3 H, olefinic CH_3), 2.08 (s, 3 H, CH_3CO), 6.11 and 6.19 (broad singlets, 1 H each, H-4 and H-5), 6.69 (m, 1 H, H-3).

Dimer diacetate 8. *o*-Quinol acetate *4* (10 g) was heated under N_2 for 1 h at 120 °C. The resulting oil was dissolved in ether (50 ml) and, after 24 h at room temperature, the crystalline product deposited was recrystallized from ethanol to give 48 % of *8*, m.p. 159–160 °C (lit.[3] 159–160 °C). NMR ($CDCl_3$): δ 1.42, 1.50 and 1.73 (singlets, 3 H each, 3 CH_3), 1.83 (t, 3 H, olefinic CH_3), 2.10 and 2.17 (singlets, 3 H each, 2 CH_3CO), 3.00 (broad d, 1 H, H-8a. Coupling with H-4a gives rise to the doublet, which is further split by coupling with H-8 and by homoallylic coupling with CH_3-7), 3.48 (dd, 1 H, H-4a), 3.77 (td, 1 H, H-4), 5.64 (dd, 1 H, H-2), 6.23 (broad signal, 1 H, H-8), 6.29 (dd, 1 H, H-3). $J_{2,3}=7.8$ Hz, $J_{2,4}=1.5$ Hz, $J_{3,4}=7.0$ Hz, $J_{4,4a}=1.5$ Hz, $J_{4a,8a}=8.1$ Hz.

Dimer diacetate 9. *o*-Quinol acetate *4* was heated under N_2 for 2.5 h at 165 °C and the resulting oily product purified as described for isomer *8*. M.p. 182–184 °C; yield, 60 %. Elemental analysis, see Ref. 3, p. 1595. UV (ethanol): λ_{max}, nm (log ε) 209 (3.82) (β,γ-enone), 238 (3.80) (α,β-enone), 310 (2.18) (α,β- and β,γ-enones). IR (KBr), see Ref. 3. NMR ($CDCl_3$): δ 1.36 (s, 3 H, CH_3), 1.50 (s, 6 H, 2 CH_3), 1.80 (broad s, 3 H, olefinic CH_3), 1.95 and 2.12 (singlets, 3 H each, 2 CH_3CO), 2.90 (broad s, 2 H, H-4a and H-8a), 3.84 (td, 1 H, H-4), 5.60 (dd, 1 H, H-2), 6.14 (dd, 1 H, H-3), 6.17 (broad s, 1 H, H-8). $J_{2,3}=8.2$ Hz, $J_{2,4}=1.6$ Hz, $J_{3,4}=6.9$ Hz.

Estimation of yields of products formed by heat treatment of o-quinol acetate 4 (see Fig. 1). Monomer *4* was heated under N_2 for 0.5, 2.5, and 6 h, in each instance at temperatures of 125, 165, and 185 °C; an experiment with a heating time of 0.5 h at 205 °C was also carried out. After rapid cooling the reaction mixtures were dissolved in $CDCl_3$ and NMR spectra were recorded. The proportions of *4*, *8*, and *9* were estimated from the integrals of the signals of the various CH_3 and CH_3CO groups of the compounds present. The "unidentified product", increasing in amount with increasing temperature and heating time, is characterized by broad signals between δ 2.10 and 2.35, as shown for a product obtained on 6 h heating of *4* at 185 °C. It was assumed to have the same empirical formula as the three known products.

Isomerization of diacetate 8 *to diacetate* 9. Compound *8* (5.0 g) was heated under N_2 at 165 °C for 2.5 h. Ether (50 ml) was added, and *9* (3.0 g, 60 %) was collected after 24 h. The product was identical with that obtained from *4* (see above) by m.p., mixed m.p. and spectral properties.

Thermal degradation of dimer diacetates 8 *and* 9. Heating of the compounds under N_2 at 205 °C for 30 min, followed by rapid cooling, gave *o*-quinol acetate *4* in yields of 79 and 84 %, respectively.

Alkaline hydrolysis of dimer diacetate 8 with ethanolic KOH was carried out as described in Ref. 3. The reaction products *10* and *11* gave the following NMR spectra. *o-Quinol dimer* 10. NMR ($CDCl_3$): δ 1.25, 1.31 and 1.33 (singlets, 3 H each, 3 CH_3), 1.85 (t, 3 H, olefinic CH_3), 2.86 (s, 1 H, OH, exchangeable with D_2O), 2.90 (m, 1 H, H-8a. The signal is split by vicinal coupling to H-4a and H-8 and homoallylic coupling to CH_3-7), 3.30 (dd, 1 H, H-4a), 3.41 (td, 1 H, H-4), 4.06 (s, 1 H, OH, exchangeable with D_2O), 5.51 (dd, 1 H, H-2), 6.27 (dd, 1 H, H-3), 6.31 (broad signal, H-8). $J_{2,3}=7.0$ Hz, $J_{2,4}=1.5$ Hz, $J_{3,4}=6.0$ Hz, $J_{4,4a}=2.0$ Hz, $J_{4a,8a}=7.0$ Hz.

Phenol 11. NMR ($CDCl_3$): δ 1.47 and 1.70 (singlets, 3 H each, 2 CH_3), 2.02 (s, 1 H, OH), 2.21 and 2.32 (singlets, 3 H each, 2 aromatic CH_3), 4.30 (dd, 1 H, H-4), 4.98 (s, 1 H, OH), 6.22 (dd, 1 H, H-2), 6.66 (t, 1 H, H-3), 6.89 (s, 1 H, H-8).

Alkaline hydrolysis of dimer diacetate 9. A solution of *9* (5.0 g) in 10 % ethanolic KOH (200 ml) was kept under N_2 for 16 h at room temperature. Ethanol was removed under vacuum, water being added during the evaporation, and the aqueous solution (about 100 ml) was extracted with three 50 ml portions of chloroform. The dried extract on evaporation gave an oil which was purified on a silica gel column using acetone-hexane (2:1) as eluent. *o-Quinol dimer* 12, $R_F=0.50$, m.p. 147–149 °C (from ethanol), was obtained in a yield of 9 %. (Found: C 69.50; H 7.32. Calc. for $C_{16}H_{20}O_4$: C 69.54; H 7.30.) UV (ethanol): λ_{max}, nm (log ε) 211 (3.91), 238 (3.87), 310 (2.13). IR (KBr): ν_{max}, cm^{-1} 1672 (conj. CO), 1722 (CO), 3480 and 3500 (OH). NMR ($CDCl_3$): δ 1.31 (s, 6 H, 2 CH_3), 1.38 (s, 3 H, CH_3), 1.86 (t, 3 H, olefinic CH_3), 2.75 (broad signal, 2 H, H-4a and H-8a), 3.27 (s, 2 H, 2 OH, exchangeable with D_2O), 3.35 (broad d, 1 H, H-4), 5.50 (dd, 1 H, H-2), 6.30 (broad signal, 2 H, H-3 and H-8).

The alkaline aqueous phase was neutralized with acetic acid and extracted with chloroform.

Evaporation of the chloroform phase and treatment of the residue with ether gave 68 % of a product of m.p. 172 – 173 °C, identical by m.p., mixed m.p. and spectral properties with phenol *11*.[3]

Thermal isomerization of o-*quinol dimer* 12 *to* o-*quinol dimer* 10. A sample of compound *12* (3.0 g) was heated under N_2 for 1 h at 160°C. After cooling, ether (40 ml) was added. The crystalline product obtained after recrystallization from ethanol had m.p. 194 – 196 °C. Yield, 83 %. The product was identical by m.p., mixed m.p. and spectral properties with dimer *10*.[3]

Acid hydrolysis of dimer diacetate 8. A solution of *8* (2.0 g) in a mixture of ethanol (75 ml) and 10 % aqueous sulfuric acid (75 ml) was heated under reflux for 7 h in an atmosphere of nitrogen. The reaction mixture was extracted with three 75 ml portions of chloroform, the combined organic phases were concentrated to half their volume and then extracted with 3 × 50 ml of 2.5 M aqueous NaOH. The chloroform phase was dried and evaporated. Addition of ethanol (5 ml) to the semi-solid residue gave 28 % of crude *o*-quinol dimer *10*, m.p. 191 – 193 °C. Recrystallization from ethanol raised the m.p. to 195 – 196 °C (lit.[3] 194 – 196 °C).

The combined aqueous alkaline phases were neutralized with aqueous HCl and extracted twice with 100 ml portions of chloroform. The residue obtained after removal of the chloroform, when treated with ether gave a crystalline product which was recrystallized from benzene yielding 46 % of *4,6-dihydroxy-2,6-dimethyl-5-(3-hydroxy-2,4-dimethylphenyl)-2-cyclohexenone* (13), m.p. 139 – 140 °C. (Found: C 69.57; H 7.31. Molecular ion, M = 276. Calc. for $C_{16}H_{20}O_4$: C 69.54; H 7.30. Molecular ion, M = 276.). UV (ethanol): λ_{max}, nm (log ε) sh 239 (3.81), 276 (3.22). IR (KBr): ν_{max}, cm^{-1} 1492 and 1580 (arom. ring), 1680 (conj. CO), 3420 and 3520 (OH). NMR ($CDCl_3$): δ 1.48 (s, 3 H, CH_3-6), 1.95 (t, 3 H, CH_3-2), 2.15 and 2.22 (singlets, 3 H each, 2 aromatic CH_3), 2.10, 3.52 and 4.98 (broad singlets, 1 H each, 3 OH, exchangeable with D_2O), 4.11 (d, 1 H, H-5), 4.76 (broad signal, 1 H, H-4), 6.66 (broad signal, 1 H, H-3), 6.57 and 6.71 (doublets, 1 H each, 2 aromatic H, J = 8.0 Hz). $J_{CH_3\text{-}2,H\text{-}3}$ and $J_{CH_3\text{-}2,H\text{-}4}$ = 1.5 Hz; $J_{4,5}$ = 6.0 Hz.

Acid treatment of o-*quinol dimer* 10. The same procedure as described above for the acid hydrolysis of dimer acetate *8* gave unreacted *10* (15 %) and phenol *13* (66 %).

Diacetate 13a. From phenol *13* with Ac_2O/pyridine. Yield, 81 %; m.p. 136 – 137 °C (ethanol). (Found: C 66.62; H 6.73. Calc. for $C_{20}H_{24}O_6$: C 66.65; H 6.71), IR (KBr): ν_{max}, cm^{-1} 1675 (conj. CO), 1739 and 1752 (alkyl and aryl ester), 3480 (OH). The NMR signals of the CH_3CO groups are located at δ 1.78 and 2.29 ($CDCl_3$).

Triacetate 13b. From phenol *13* with Ac_2O/$HClO_4$.[22] Yield, 84 %; m.p. 157 – 158 °C (ethanol). (Found: C 65.66; H 6.49. Calc. for $C_{22}H_{26}O_7$: C 65.66; H 6.51). IR (KBr): ν_{max}, cm^{-1} 1704 (conj. CO), 1738 (2 alkyl ester CO) and 1755 (aryl ester CO). The NMR signals of the CH_3CO groups are located at δ 1.92, 1.94 and 2.31 ($CDCl_3$).

Acid hydrolysis of dimer diacetate 9. A 2 g sample of diacetate *9* was treated as described above for isomer *8*. The neutral fraction gave *o*-quinol dimer *12*, m.p. 147 – 149 °C, identical with the product of the same melting point obtained on alkaline hydrolysis of *9* by mixed m.p. and spectral properties.

The aqueous alkaline phase after neutralization was extracted with chloroform, and the dried extract concentrated to a volume of 30 ml. From the solution colourless crystals of *2,3-dihydro-2,5-dihydroxy-1,2,4,6-tetramethyl-1,3-propeno-1H-inden-10-one* (16) deposited. After recrystallization from acetone-chloroform, m.p. 205 – 206 °C; yield, 26 %. (Found: C 74.42; H 7.03. Molecular ion, M = 258. Calc. for $C_{16}H_{18}O_3$: C 74.40; H 7.02. Molecular ion, M = 258). UV (ethanol): λ_{max}, nm (log ε) 241 (4.03), 301 (3.71). UV (0.1 M NaOH in 80 % aqueous ethanol): λ_{max}, nm (log ε) 268 (4.03), 332 (3.83), 373 (4.24). IR (KBr): ν_{max}, cm^{-1} 1470, 1582, 1593 (aromatic ring), 1655 (conj. CO), 3320 (broad, OH). NMR (DMSO-d_6): δ 1.12 and 1.43 (singlets, 3 H each, 2 CH_3), 2.17 (s, 6 H, 2 aromatic CH_3), 3.61 (d, 1 H, H-3), 4.84 (broad signal, 1 H, OH, exchangeable with D_2O), 5.56 (d, 1 H, H-9), 6.56 (dd, 1 H, H-8), 7.37 (s, 1 H, H-7), 8.67 (broad signal, 1 H, OH). $J_{3,8}$ = 3.5 Hz, $J_{8,9}$ = 6.0 Hz.

Monoacetate 16a. From phenol *16* with Ac_2O/pyridine. Yield, 73 %; m.p. 175 – 176 °C (benzene). (Found: C 71.83; H 6.67. Calc. for $C_{18}H_{20}O_4$: C 71.88; H 6.71). UV (ethanol): λ_{max}, nm (log ε) 232 (4.09), 237 (3.72), 303 (3.16). IR (KBr): ν_{max}, cm^{-1} 1670 (conj. CO), 1752 (arylester), 3480 (OH). The NMR signal of the CH_3CO group is located at δ 2.32 ($CDCl_3$).

Diacetate 16b. From *16* with Ac_2O/$HClO_4$.[22] Yield, 80 %; m.p. 143 – 144 °C (ethanol). (Found: C 70.01; H 6.50. Calc. for $C_{20}H_{22}O_5$: C 69.98; H 6.39). IR (KBr): ν_{max}, cm^{-1} 1685 (conj. CO), 1735 and 1750 (alkyl and aryl ester). NMR signals of the CH_3CO groups: δ 1.66 and 2.36 (DMSO-d_6).

Acid hydrolysis of diacetate 17. A solution of diacetate *17*[3] (0.5 g) in a mixture of ethanol (20 ml) and 10 % aqueous sulfuric acid (20 ml) was refluxed under N_2 for 7 h and then worked up as described above for the similar hydrolysis of *8*. The neutral fraction consisted of unreacted diacetate *17* (65 %). The phenolic fraction gave compound *16*, m.p. 205 – 206 °C. in a yield of 22 %.

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Dioxolanylium Ions Derived from Carbohydrates. I. Reactions with Water and with Bromide Ions

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Treatment of 3,4-*O*-benzylidene derivatives of methyl β-D-arabinopyranoside with triphenylmethyl fluoroborate gave benzoxonium ions which were stable in acetonitrile solution. Reaction of the benzoxonium ions with water gave hydroxy-benzoates with *cis*-opening of the dioxolanylium ring. Treatment with bromide ions lead to *trans*-opening and formation of bromodeoxypentopyranosides. Similar results were obtained with methyl 4-*O*-benzoyl-2,3-*O*-benzylidene-α-D-lyxopyranoside and with methyl 5-*O*-benzoyl-2,3-*O*-benzylidene-β-D-ribofuranoside.

The preparation of dioxolanylium ions from aldehydic acetals by hydride abstraction with triphenylmethyl fluoroborate or similar reagents is well known.[1–3] Dioxolanylium ions derived from carbohydrates have been obtained in a few cases using this method.[3–5]

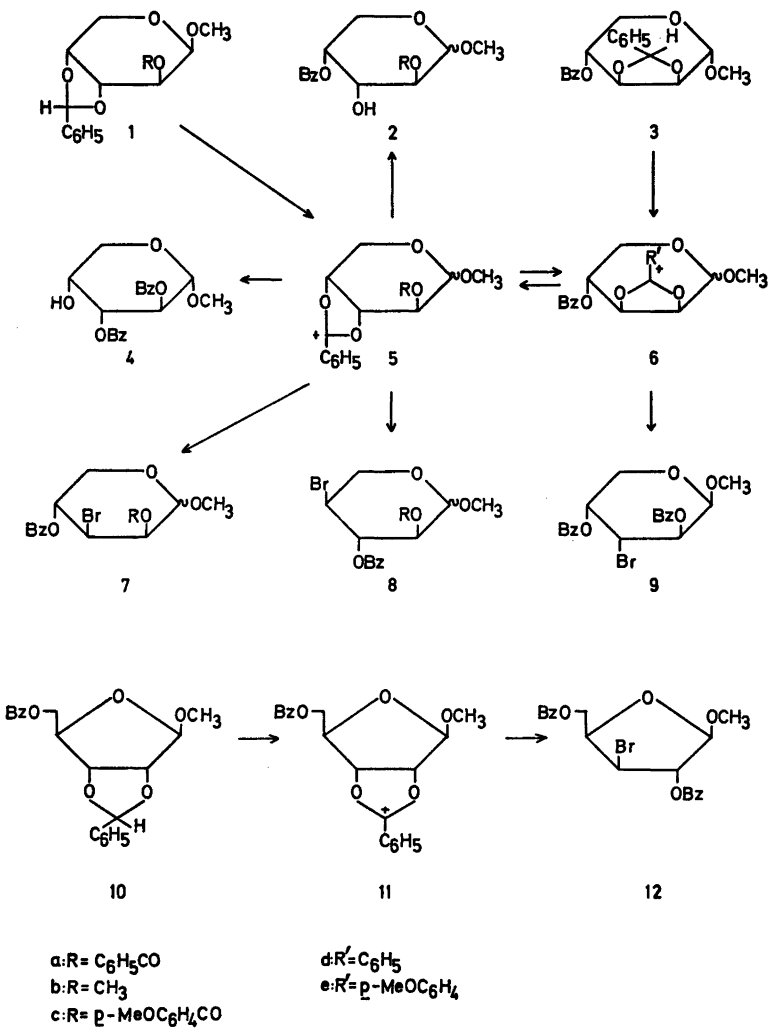
We have now investigated the reaction of a variety of carbohydrate benzylidene acetals with triphenylmethyl fluoroborate with the purpose of preparing 2-phenyl-dioxolanylium (benzoxonium) ions and studying their reactions with nucleophilic reagents. In the present paper the reactions of some pentose derivatives are described. A following paper will describe the behaviour of some hexose derivatives. When this work was almost completed a communication by Hanessian and Staub appeared in which closely related work is described.[6]

When methyl 2-*O*-benzoyl-3,4-*O*-benzylidene-β-D-arabinopyranoside (*1a*) was treated with triphenylmethyl fluoroborate in acetonitrile solution it was completely converted to a benzoxonium ion within a few hours and NMR spectra (Table 1) showed that it was the 3,4-benzoxonium ion (*β5a*) which was the main product. When moisture was excluded the ion was stable in acetonitrile solution for several days except that anomerization took place to some extent. The ion *β5a* is in equilibrium with the 2,3-benzoxonium ion (*β6d*),[3,4,7] derived from methyl lyxoside. However, the spectra indicated that the equilibrium was almost completely shifted towards *β5a*. This was confirmed by the reaction of methyl 3,4-*O*-benzylidene-2-*O*-methyl-β-D-arabinopyranoside (*1b*) with triphenylmethyl fluoroborate. This can of course only give the 3,4-benzoxonium ion (*β5b*) and the NMR spectrum (Table 1) of the ion formed was very similar to that of *β5a*.

A similar treatment of the lyxose derivative (*3*) would be expected to give initially the 2,3-benzoxonium ion (*α6d*), however, the product observed through NMR spectra was the 3,4-benzoxonium ion (*α5a*). Thus, the equilibrium between *5* and *6* is apparently largely shifted towards *5* in both anomers.

In order to get an NMR spectrum of a 2,3-benzoxonium ion methyl 3,4-*O*-benzylidene-2-*O*-*p*-methoxybenzoyl-β-D-arabinopyranoside (*1c*) was treated with triphenylmethyl fluoroborate. This gave a *p*-methoxybenzoxonium ion (*β6e*) in equilibrium with the 3,4-benzoxonium ion (*β5c*). Since a *p*-methoxy group stabilizes a benzoxonium ion [4] this equilibrium was shifted strongly towards *6* and only *ca.* 10 % of *5* was present. NMR data of both ions could be obtained from a spectrum of the mixture (Table 1) and the spectrum of *β6e* is quite different from that of *β5c* and from the spectra of the other 3,4-benzoxonium ions.

Treatment of the ribofuranose derivative *10* with triphenylmethyl fluoroborate gave the



benzoxonium ion *11* as already described by Hanessian and Staub;[6] its NMR data are presented in Table 1.

It is well known that dioxolanylium ions react rapidly with water to give *cis*-hydroxy esters [2] and this reaction has been described for the benzoxonium ion (*11*).[6] Addition of water to a solution of *β5a* in acetonitrile gave methyl 2,4-di-*O*-benzoyl-*β*-D-arabinopyranoside (*β2a*) as the only detectable product. This is in agreement with the results of King and Allbutt [8] who found that hydrolysis of a benzoxonium ion gives predominantly the product which has the *O*-benzoyl group axially oriented. In *β2a*, which preferentially adopts the $^1C_4$ conformation, the *O*-benzoyl group at C4 is axial. When *α5a* was hydrolysed, the main product was the 2,4-di-*O*-benzoate (*α2a*); but in this case a small amount of the 2,3-di-*O*-benzoate (*4*) was also isolated, possibly arising from acyl-migration. No products resulting from hydrolysis of *6* were found, indicating that the equilibrium between *5* and *6* is shifted strongly towards *5*, as also found from the NMR spectra. Hydrolysis of the 2-*O*-methylated ion (*β5b*) gave the 4-*O*-benzoate (*β2b*) as the only detectable product.

Reaction of benzoxonium ions with halide ions leads to *trans*-opening with formation of

Table 1. Proton NMR spectra of benzoxonium ions in deuterioacetonitrile. Chemical shifts are in ppm relative to TMS; coupling constants are in Hz.

Compound	H1	H2	H3	H4	H5	H5′	J_{12}	J_{23}	J_{34}	J_{45}	$J_{45'}$	$J_{55'}$	OCH_3	Conformation
β5a	5.17	5.63	6.27	6.15	4.53	4.35	4.2	6.0	8.4	~0	2.4		3.41	distorted $^1C_4$ or $^1H_0$
α5a	4.95	5.46	6.18–6.20		4.70	4.44	4.3	~2		~0	1.8		3.27 3.43	$^{2,5}B$
β5b	5.04	3.90	5.85–6.05		4.46	4.21	3.8	6.8		0	2.0	14.7	3.52 3.41	distorted $^1C_4$ or $^1H_0$
β6e	5.24	6.06	5.94	5.81	4.26	3.95	3.8	7.5	3.0	4.0	3.5	13.2	3.98 3.44	distorted $^1C_4$
β5c	5.18	5.60	6.27	6.16	4.55	4.35	4.0	6.0	8.5	~0	~2	~15.5	3.84	distorted $^1C_4$ or $^1H_0$
11	5.56	6.54 or 6.16		5.14	4.58	4.52	~0.5	6.8	0.6	6.3	6.7		3.38	

A ^{13}C NMR spectrum of *β5a* in deuterioacetonitrile gave the following chemical shifts (relative to TMS): C1, 94.7; C2, 67.6; C3 and C4, 87.1 and 84.3; C5, 55.6; $C_6H_5C^+$, 181.5; $-OCH_3$, 55.2.

bromo-benzoates.[2,3] Treatment of the ions described above with tetraethylammonium bromide in acetonitrile proceeded in accordance herewith. The benzoxonium ions disappear within a few minutes when bromide is added, but the formation of the bromo-deoxy compounds requires *ca.* 2 h. This may indicate that intermediate orthoester bromides are formed rapidly and that they rearrange more slowly to the bromo-deoxy compounds.

Reaction of *β5a* with bromide ions gave two products in equal amounts. One was the 4-bromo-L-xylose derivative (*α8a*), resulting from attack of a bromide ion on C4 of *β5a*. The other product was the 3-bromo-arabinoside (*9*) which is probably formed by attack of bromide on C3 of *β6d*. The latter ion is only present to a small extent, as discussed above, but if its reaction with bromide is more rapid than that of *5* formation of rather large amounts of *9* would be possible.

Reaction of the 2-*O*-methylated ion (*β5b*) with bromide gave *α8b* as the main product and a small amount of the 3-bromo-lyxose derivative (*β7b*). Both can arise from *β5b* by substitution with bromide ions at C4 and C3, respectively. The same two bromo-compounds were obtained when *1b* was treated with *N*-bromosuccinimide according to Hanessian and Plessas.[7] Reaction of *3* with triphenylmethyl fluoroborate followed by treatment with bromide ions gave *α7a* as the main product together with a small amount of *β8a*. Thus, in this case the ion *α6d* does not seem to be reactive enough, probably because the α-methoxy group at C1 exerts steric hindrance towards attack at C3 of *α6*. Similarly, the low reactivity of C3 of the *β*-anomer (*β5a*) may be explained through steric hindrance by the methoxy group at C1.

Treatment of the benzoxonium ion (*11*) with bromide ions gave a good yield of the 3-bromo-3-deoxy-D-xylofuranose derivative (*12*). Since anomerisation took place during the reaction with bromide a mixture of the two anomers of *12* was obtained. These were treated with hydrogen bromide and the furanosyl bromide thus obtained was reacted with methanol. This gave the pure *β*-anomer of *12*.

The reactions of the benzoxonium ions described above with other nucleophiles will be discussed in a forthcoming paper.

EXPERIMENTAL

Thin layer chromatography (TLC) was performed on silica gel PF_{254} (Merck); for preparative work 1 mm layers were used on 20 × 40 cm plates. NMR spectra were obtained on Varian A-60 or HA-100 instruments and on a Bruker WH-90 instrument.

BENZYLIDENE COMPOUNDS

Methyl 2-O-benzoyl-3,4-O-benzylidene-β-D-arabinopyranoside (1a). Finely powdered methyl *β*-D-arabinopyranoside (4.92 g), benzaldehyde (3.30 g), *p*-toluenesulfonic acid (100 mg), and chloroform (150 ml) were refluxed for 10 h with a Soxhlet extractor containing 30 g of 4 Å molecular sieves. The solution was then washed with aqueous sodium hydrogencarbonate and water and dried ($MgSO_4$). The solvent was evaporated and the residue was benzoylated with benzoyl chloride (4.2 ml) in pyridine (50 ml) to give a product which was crystallized from ether at −20 °C. Decantation of the ether and recrystallization from ethyl acetate–pentane gave 5–7 g (47–65 %) of *1a* as a mixture of diastereomers, m.p. 95–105 °C.[7,8]

Methyl 4-O-benzoyl-2,3-O-benzylidene-α-D-lyxopyranoside (3) was prepared in the same way from methyl α-D-lyxopyranoside (3.3 g) which gave 2.5 g (35 %) of a mixture of the diastereomeric benzylidene acetals. Fractional crystallization from ethyl acetate-cyclohexane yielded small amounts of pure *exo* and *endo* isomers of (3). The H-*endo* isomer had m.p. 101–103 °C, $[\alpha]_D^{22}$ −22.5° (*c* 1.0, $CHCl_3$). (Found: C 67.38; H 5.84. Calc. for $C_{20}H_{20}O_6$: C 67.40; H 5.66). The H-*exo* isomer had m.p. 93–94 °C, $[\alpha]_D^{22}$ −44.8° (*c* 1.5, $CHCl_3$). (Found: C 67.51; H 5.72).

Methyl-3,4-O-benzylidene-2-O-p-methoxybenzoyl-β-D-arabinopyranoside (1c). Crude methyl 3,4-*O*-benzylidene-*β*-D-arabinopyranoside (1.15 g) was treated with *p*-methoxybenzoyl chloride (1.27 g) in pyridine to give 1.7 g of a mixture of *endo*- and *exo*-H product. Preparative TLC (ether–pentane 1:1) gave two fractions. The fast moving component was the *endo*-H product (*1c*) (645 mg) which was crystallized from ethyl acetate–pentane to give 290 mg of pure material, m.p. 97–99 °C, $[\alpha]_D^{21}$ −161.7° (*c* 1.1, $CHCl_3$). (Found: C 65.28; H 5.70. Calc. for $C_{21}H_{22}O_7$: C 65.27; H 5.74). The slow moving fraction, obtained as a syrup, was the *exo*-H product contaminated with some of the *endo*-compound.

Methyl 5-O-benzoyl-2,3-O-benzylidene-β-D-ribofuranoside (10). A similar treatment of methyl *β*-D-ribofuranoside[10] (1.64 g) gave 3.3 g of crude *10* which was crystallized from ether (10 ml)–pentane (35 ml) to give 2.5 g (70 %) of the H-*exo* product, m.p. 70–72 °C. Further recrystallization from ether–pentane gave the

pure product, m.p. 73 – 75 °C, $[\alpha]_D^{27}$ –27.4° (*c* 2.4, $CHCl_3$). (Found: C 67.55; H 5.71. Calc. for $C_{20}H_{20}O_6$: C 67.40; H 5.66).

Conversion of benzylidene derivatives to hydroxy-benzoates

General procedure. The appropriate benzylidene derivative (1 part) was treated with a 10 – 25 % molar excess of triphenylmethyl fluoroborate in dry acetonitrile (10 parts) at room temp. for the time specified below. The solution was then poured into aqueous sodium hydrogencarbonate, the mixture was extracted with chloroform, and the chloroform solution was dried and evaporated. Preparative TLC with ether – pentane (2:1) as eluent gave triphenylmethane moving with the solvent front, closely followed by triphenylcarbinol and the product(s).

Methyl 2-O-benzoyl-3,4-O-benzylidene-β-D-arabinopyranoside (*1a*) (809 mg) was treated with triphenylmethyl fluoroborate for 5 h after which time it was completely converted to the ion *β5a* as seen from an NMR spectrum. Hydrolysis and chromatography as described above gave 569 mg (67 %) of methyl 2,4-di-*O*-benzoyl-β-D-arabinopyranoside (*β2a*), m.p. 143 – 146 °C. Recrystallization from ethyl acetate – pentane gave the pure product, m.p. 147 – 149 °C, $[\alpha]_D^{25}$ –240.4° (*c* 2.3, $CHCl_3$). (Found: C 64.36; H 5.23. Calc. for $C_{20}H_{20}O_7$: C 64.51; H 5.41). Acetylation with acetic anhydride in pyridine gave the known methyl 3-*O*-acetyl-2,4-di-*O*-benzoyl-β-D-arabinopyranoside, m.p. 86 – 88 °C (reported [11] m.p. 86 – 87 °C). A mixed melting point with an authentic sample gave no depression.

Direct crystallization of *β2a* from ethyl acetate-pentane without previous chromatographic separation gave a 40 % yield of a product with m.p. 140 – 146 °C.

Methyl 4-O-benzoyl-2,3-O-benzylidene-α-D-lyxopyranoside (3) (474 mg) was treated with triphenylmethyl fluoroborate for 7 h. Hydrolysis and chromatography gave two products. The fast moving compound was methyl 2,4-di-*O*-benzoyl-α-D-arabinopyranoside (*α2a*) (206 mg, 42 %). The slow moving fraction gave 41 mg (8 %) of methyl 2,3-di-*O*-benzoyl-α-D-arabinopyranoside (*4*). The products were identified through their NMR spectra (Table 2). On benzoylation they gave identical products as seen from NMR spectra. The two benzoylated products were mixed and recrystallized from methanol to give 191 mg of methyl tri-*O*-benzoyl-α-D-arabinopyranoside, m.p. 144 – 145 °C (reported [12] m.p. 146 °C). A mixed m.p. with an authentic sample gave no depression.

Methyl 3,4-O-benzylidene-2-O-methyl-β-D-arabinopyranoside (1b) [13] (560 mg) was treated with triphenylmethyl fluoroborate for 2 h. Hydrolysis and chromatography gave 274 mg (46 %) of methyl 4-*O*-benzoyl-2-*O*-methyl-β-D-arabinopyranoside (*β2b*) which slowly crystallized from pentane. Recrystallization from ethyl acetate – pentane gave the pure product, m.p. 107 – 108 °C, $[\alpha]_D^{21}$ –204.0° (*c* 0.9, $CHCl_3$). Benzoylation gave the known methyl 3,4-di-*O*-benzoyl-2-*O*-methyl-β-D-arabinopyranoside, m.p. 72 – 74 °C (reported [14] m.p. 75 – 77 °C). A mixed m.p. with an authentic sample gave no depression.

Conversion of benzylidene derivatives to bromo-deoxy compounds

General procedure. The benzoxonium ion was prepared as described above in acetonitrile solution. To this solution was added dry tetraethylammonium bromide (3 molar equiv.) and the mixture was stirred until it was homogeneous and then kept for 2 h at room temp. The solution was then stirred for 5 min with aqueous sodium hydrogencarbonate and extracted with chloroform. The chloroform solution was washed with water, dried and evaporated. Preparative TLC (ether – pentane 1:2) gave triphenylmethane, moving with the solvent front, and triphenylcarbinol, usually followed closely by the product(s).

Methyl 2-O-benzoyl-3,4-O-benzylidene-β-D-arabinopyranoside (1b) (935 mg) by this treatment gave two products. The fast moving fraction (380 mg, 33 %) was methyl 2,3-di-*O*-benzoyl-4-bromo-4-deoxy-α-L-xylopyranoside (*α8a*). Crystallization from cyclohexane gave the pure product, m.p. 109 – 110 °C, $[\alpha]_D^{25}$ –129.7° (*c* 1.1, $CHCl_3$). (Found: C 55.32; H 4.31; Br 18.50. Calc. for $C_{20}H_{19}BrO_6$: C 55.18; H 4.40; Br 18.36).

The slower moving product (351 mg, 31 %) was methyl 2,4-di-*O*-benzoyl-3-bromo-3-deoxy-β-D-arabinopyranoside (*9*) which crystallized slowly. Three recrystallizations from cyclohexane gave a product with m.p. 82 – 86 °C, $[\alpha]_D^{25}$ –267.8° (*c* 1.0, $CHCl_3$). (Found: C 55.34; H 4.37; Br 18.54). The structures of the two bromides were established from their NMR spectra (Table 2). Separation of the mixture of bromides described by Hanessian and Plessas [7] gave products identical with those described above.

In addition to *α8a* and *9* 135 mg (14 %) of *4* was also isolated.

Methyl 3,4-O-benzylidene-2-O-methyl-β-D-arabinopyranoside (1b) (925 mg) gave by the same treatment two products. The fast moving fraction (63 mg, 5 %) was methyl 4-*O*-benzoyl-3-bromo-3-deoxy-2-*O*-methyl-β-D-lyxopyranoside (*β7b*) as a syrup, $[\alpha]_D^{21}$ –33.7° (*c* 1.3, $CHCl_3$). (Found: C 48.78; H 4.97; Br 23.37. Calc. for $C_{14}H_{17}BrO_5$: C 48.71; H 4.97; Br 23.15). The slower moving fraction (494 mg, 41 %) was methyl 3-*O*-benzoyl-4-bromo-4-deoxy-2-*O*-

Table 2. Proton NMR spectra of benzylidene derivatives and products prepared from them. Chemical shifts are in ppm relative to tetramethylsilane; coupling constants in Hz.

Compound	Solvent	H1	H2	H3	H4	H5	H5′	OCH_3	J_{12}	J_{23}	J_{34}	J_{45}	$J_{45'}$	$J_{55'}$		Predominant conformation
1c endo-H	$CDCl_3$	5.04	5.30	4.80	4.30	4.10	3.97	3.82 3.40	3.4	8.2	5.3	~0.5	2.2	13.2	benzylidene H 6.26	
1c exo-H	»	5.02	5.20	4.67	4.39	4.18	4.06	3.82 3.40	3.4	7.6	6.2	1.0	2.7	13.4	benzylidene H 5.93	
3 endo-H	»	4.86	4.23	4.59	5.39	3.92	3.78	3.46	2.6	5.2	6.4	5.0	7.6	11.5	benzylidene H 6.26	
3 exo-H	»	4.84	4.24	4.51	5.23	3.88	3.82	3.45	3.2	5.9	5.2	4.8	6.0	11.8	benzylidene H 5.94	
10 exo-H	»	5.18	4.76	4.86	4.73	4.42	4.41	3.36	<0.5	6.3	<0.5	~6.4	~7.7		benzylidene H 5.80	
α2a	»	4.64	5.35	4.18	5.42	4.15	3.78	3.50 3.41	4.9	7.0	3.2	6.2	3.3	12.5		
β2b	»	4.95	3.65	4.18	5.38	3.80		3.52	3.5	10.0	3.5	<2	<2			
4	»	4.58	5.62	5.39	4.32	4.10	3.75	3.50	5.8	8.3	3.2	4.5	2.3	12.4		
α7a	»	4.83	5.49	4.73	5.64	4.12	3.86	3.43 3.44	2.2	3.3	10.7	5.5	10.5	11.0		$^4C_1$
β7b	»	4.72	3.57	4.53	5.45	4.06	3.65	3.55	2.6	3.2	9.8	4.9	9.0	10.8		$^4C_1$
α8a	Benzene-d_6	5.11	5.25	6.31	3.53–3.90			2.95 3.44	3.5	9.8	9.9	7.5	9.3	11.6		$^1C_4$
α8b	Acetone-d_6	5.14	3.63	5.78	4.35	3.97	3.96	3.53	3.4	9.8	9.8	7.6	9.1	11.2		$^1C_4$
β8a	$CDCl_3$	4.61	5.32	5.72	3.7–4.4			3.50	7.6	9.3	~9					$^1C_4$
9	»	5.13	5.59	4.81	5.55	4.05	3.95	3.42	3.4	10.7	3.4	1.5	1.8	12.7		$^1C_4$
α12	»	5.40	5.56	4.3–4.9				3.40	4.5	5.5						
β12	Benzene-d_6	4.95	5.70	4.10	4.4–4.7			3.23	<0.5	1.6	5.2					
[a]	»	6.95	5.60	4.5–5.1					4.6	6.5						

[a] 2,5-Di-*O*-benzoyl-3-bromo-3-deoxy-α-D-xylofuranosyl bromide.

methyl-α-L-xylopyranoside (*α8b*), m.p. 72–74 °C. Two recrystallizations from ether-pentane gave a product with m.p. 73–74 °C, $[\alpha]_D^{21}$ −76.2° (*c* 1.3, $CHCl_3$). (Found: C 48.87; H 5.07; Br 23.02). The structures of the two products were derived from their NMR spectra (Table 2).

Methyl-2,3-O-benzylidene-4-O-benzoyl-α-D-lyxopyranoside (3) (587 mg) gave two products. The fast moving material (351 mg, 49 %) was methyl 2,4-di-*O*-benzoyl-3-bromo-3-deoxy-α-D-lyxopyranoside (*α7a*) which was crystallized from ether–pentane, m.p. 103–105 °C, $[\alpha]_D^{25}$ −125.4° (*c* 1.3, $CHCl_3$). (Found: C 55.26; H 4.25; Br 18.25. Calc. for $C_{20}H_{19}BrO_6$: C 55.18; H 4.40; Br 18.36). The slower moving fraction (44 mg, 6 %) was identified, after rechromatography, through its NMR spectrum as impure methyl 2,3-di-*O*-benzoyl-4-bromo-4-deoxy-β-L-xylopyranoside (*β8a*).

Methyl 2,3-O-benzylidene-5-O-benzoyl-β-D-ribofuranoside (10) (576 mg) gave after treatment with triphenylmethyl fluoroborate followed by reaction with tetraethylammonium bromide and chromatography 521 mg (74 %) of methyl 2,5-di-*O*-benzoyl-3-bromo-3-deoxy-D-xylofuranoside as a mixture of anomers in an α:β ratio of 3:2, as seen from an NMR spectrum. Treatment with 30 % hydrogen bromide in glacial acetic acid and work up in the usual way gave 520 mg of 2,5-di-*O*-benzoyl-3-bromo-3-deoxy-α-D-xylofuranosyl bromide, characterized through its NMR spectrum. The bromide was stirred over night with silver carbonate (1.0 g) in methanol (10 ml) and the product thus obtained was benzoylated with benzoyl chloride (0.3 ml) in pyridine (10 ml). Preparative TLC (ether–pentane 1:2) gave 276 mg (60 %) of methyl 2,5-di-*O*-benzoyl-3-bromo-3-deoxy-β-D-xylofuranoside (*12*) as a syrup, $[\alpha]_D^{21}$ +31.8° (*c* 3.0, $CHCl_3$). (Found: C 55.02; H 4.47; Br 18.54. Calc. for $C_{20}H_{19}BrO_6$: C 55.18; H 4.40; Br 18.36). Besides, 68 mg (12 %) of a mixture of the anomeric 1,2,5-tri-*O*-benzoyl-3-bromo-3-deoxy-D-xylofuranoses was isolated. The products were characterized through their NMR spectra.

Microanalyses were performed by Dr. A. Bernhardt, Mikroanalytisches Laboratorium or by Novo Microanalytical Laboratory.

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Effects of Salts on the Partition of Proteins in Aqueous Polymeric Biphasic Systems

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1. The influence of salts on the partition of ovalbumin and lysozyme in an aqueous biphasic system containing dextran and poly(ethylene glycol) has been studied. The logarithm of the partition coefficient of protein is almost linearly related to the net charge of the protein. The slope of the line varies with the salt included in the system.
2. The separatory capacity of this biphasic system has been characterized by comparing the partition of the two proteins. The separatory capacity varies markedly with the salt used and can be divided into two independent parts. One part depends on the difference in the net charge of the two proteins and is sensitive to cosolute electrolytes. The other part is determined by the solvation properties of the phases for proteins and is independent of the electrolytes in the system. The ions of the salt included in the system function independently in determining the charge-sensitive part of the separatory capacity.
3. The data on partition of a protein and of the salt that steers the protein partition conform to an earlier proposed model. According to this, the unequal affinity of the ions of the salt causes an interfacial potential which in turn affects the partition of charged macromolecules.
4. The interfacial potential has been experimentally determined by independent methods. Its value is in the range of 0 – 7 mV.

Biological materials such as proteins, nucleic acids, cell organelles, and even cells can be included, without changes in their biological activity, in the aqueous polymeric biphasic systems introduced by Albertsson.[1] Substances, depending on their molecular structure or surface characteristics, usually partition unequally between the two liquid phases of the system. The latter can therefore be used for separation of biomaterials. The partition behaviour of both biopolymers and particles is sensitive to the presence of salts in the system. It is further affected in different ways by salts which are chemically very similar, *e.g.* LiCl and NaCl.[1] Various alkali salts have been used to steer the partition of biomaterials in the biphasic system. It has been observed that salts also affect the resolving power of the systems.[2] In a previous communication[3] it was shown that alkali salts partition unequally between the two phases.

A theoretical model to describe the influence of salt on the partition of proteins has been formulated by Albertsson.[4] According to this model, the presence of several kinds of ions which differ in their relative affinity for the two liquid phases gives rise to an electrical potential across the interface between the phases. This proposed interfacial potential, ψ, that arises when the salt $A_{\nu_+}B_{\nu_-}$ is included in the system is given by

$$\psi = \frac{RT}{(\nu_+ + \nu_-)F} \ln \frac{K_-}{K_+} \qquad (1)$$

where R is the gas constant, T the absolute temperature and F the Faraday constant. K_+ and K_- respresent hypothetical partition coefficients which the positive and the negative ion, respectively, should have if every single ion could partition independently of other electrically charged species in the sytem. K_+ and K_- are related to the resultant partition coefficient of the salt, K_s, by

$$\ln K_s = \frac{1}{\nu_+ + \nu_-} \ln (K_+^{\nu_+} K_-^{\nu_-}) \qquad (2)$$

If a protein with the net charge Z is partitioned together with an excess of salt, the partition coefficient of the former, K_p, is given by

$$\ln K_p = \ln K_p^0 + \frac{Z}{\nu_+ + \nu_-} \ln \frac{K_-}{K_+} \quad (3)$$

where K_p^0 is the partition coefficient of the protein, when partitioned with an excess of a salt for which $K_- = K_+$, which means that $\psi = 0$.

The aim of the present work was to study the mechanism of the salt effect and to test the theoretical model. For this purpose two well known proteins, ovalbumin and lysozyme, for which the charge can be predicted from pH, have been partitioned in the biphasic systems.

The separatory effect of different salts included in the biphasic system has also been determined by comparing the partition of the two proteins under identical conditions, and the interfacial potential has been measured.

The results are in agreement with the model and the partition of proteins as regards the salt effect can therefore be explained and predicted in a rational way.

MATERIAL AND METHODS

Chemicals. Dextran T 500, mol. wt. 500 000, was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Poly(ethylene glycol), mol. wt. 4000, was obtained from Union Carbide Chemicals Co., New York, as Carbowax 4000. Salts, acids, and bases were of analytical grade. Water was double distilled in quartz. Proteins were obtained from Sigma Chemical Co., St. Louis, Missouri: hen ovalbumin, grade V, and lysozyme from egg white, grade I.

Biphasic systems were obtained by mixing aqueous solutions of dextran, 20 % (w/w) and poly(ethylene glycol), 40 % (w/w) with protein and/or salt solutions in test tubes. The protein solutions, 5 g/l lysozyme or 10 g/l ovalbumin, were predialyzed against 5 mM potassium phosphate buffer, pH 6.8, at 3 °C overnight. The pH of the solutions of neutral salts was adjusted to 6.8 by adding minute amounts of HCl or NaOH solutions. Ammonium salts were adjusted to the same pH by adding dilute ammonia.

The biphasic systems were mixed and placed in a thermostat bath, 25 ± 0.2 °C. After attaining the bath temperature, the system was mixed by inverting 40 times and then left for one hour in the thermostat bath to separate. The pH measured directly in the upper phase by means of a glass electrode was close to 6.70.

Partition coefficient of a protein. The protein concentration in each phase was measured in terms of absorbance. From each phase an aliquot was withdrawn with a 500 µl constriction pipette. The phase was transferred to 2.000 ml water and the inside of the pipette was washed ten times with the diluted phase to remove all of the viscous solution. The same pipette was used for both phases to ensure equal volumes. The absorbance of the diluted phases was measured at 280 nm with a Zeiss spectrophotometer, PMQ II, using a blank prepared from a system containing no protein, but the same concentration of salt. The partition coefficient, K_p, was calculated as the ratio between the absorbance determined for the upper and lower phase, respectively.

The partition coefficient as function of the net charge of the protein was determined by preparing 20 g phase system containing protein and neutral salt but no buffer. The system was titrated with either NaOH, Tris, HCl or H_3PO_4 solution, 0.1 – 1.0 M, at constant temperature, 25 ± 0.2 °C. After each addition the system was shaken and two aliquots, 2 and 1 ml were withdrawn. The 2 ml portion was allowed to settle and the partition coefficient, K_p, was determined as above. The 1 ml portion was diluted with 1.5 ml water and pH of the diluted solution was measured using a pH-meter (Radiometer, pHM-26) equipped with a glass electrode (Radiometer GK 2026C).

The partition coefficients of salt, K_s, was determined for chlorides, bromides, iodides, and thiocyanates by potentiometric titration using a Radiometer titrigraph fitted with one silver and one Hg-Hg_2SO_4-electrode (Radiometer, type P4011 and K 601). 1.000 ml upper or lower phase was mixed with 5 ml water and titrated with 15 mM $AgNO_3$. Partition of fluoride, sulfate, nitrate, and perchlorate was determined by direct conductometric measurements. 2.000 ml of one phase was mixed with 2.000 ml of the opposite phase from a phase system containing no salt and with 10.00 ml water. The conductivity of the solution was measured at 25 °C with a Metrohm conductometer E 382. Corrections were made by subtracting the conductivity of a similarly diluted mixture of phases from a system without salt. The conductivity is assumed to be proportional to the salt concentration in these diluted solutions. To obtain equal volumes of the two phases the same method as described earlier for dilution of the phases was used.

Measurements of interfacial potential. The arrangement for measuring the difference in interfacial potential is shown in Fig. 1. The potential was measured with a Radiometer TTT2 titrator used as mV-meter and connected with a Servogor potentiometric recorder, type RE 520. The whole apparatus was kept in a thermostated room at 25 °C. The measured potential was corrected for the potential that appeared between two identical systems, both containing potassium chloride.

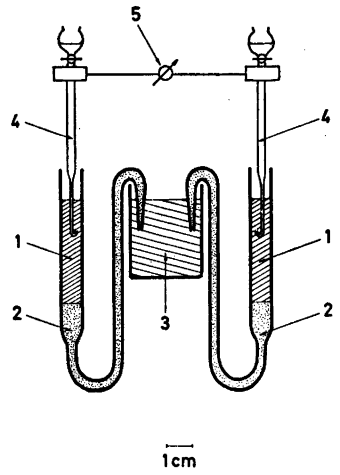

Fig. 1. Arrangement for measuring the interfacial potential difference between two biphasic systems containing different salts. Each U-tube-shaped vessel contains a single biphasic system where (1) is the upper phase and (2) the lower phase. The two lower phases are brought into electrical contact *via* a salt bridge consisting of 0.5 M NH_4NO_3 in 20 % (w/w) dextran solution (3). Calomel electrodes, Radiometer type K 100, (4), with open liquid junction and containing 12 % (w/w) poly(ethylene glycol) saturated with KCl are inserted in the upper phases. The potential difference between the two calomel electrodes is measured with the aid of a millivoltmeter (5).

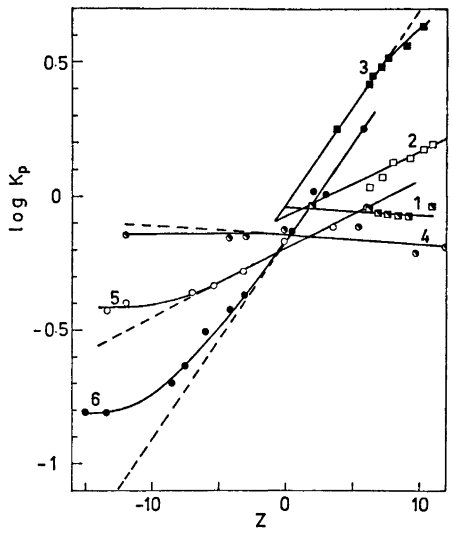

Fig. 2. Relationship between protein partition coefficient, K_p, and the net charge of the proteins, Z. The latter was obtained from titration curves and isoelectric points for the proteins. 1, lysozyme with 0.05 M K_2SO_4; 2, lysozyme with 0.1 M KF; 3, lysozyme with 0.1 M KBr; 4, ovalbumin with 0.05 M K_2SO_4; 5, ovalbumin with 0.1 M KF; and 6, ovalbumin with 0.1 M KBr, partitioned in systems containing 8 % (w/w) dextran and 8 % (w/w) poly(ethylene glycol) at 25 °C. The concentrations of protein were 1 g/l for lysozyme and 2 g/l for ovalbumin. Broken lines indicate linear extrapolations.

RESULTS AND DISCUSSION

Dependence of protein partition on the electrical charge of the protein. Ovalbumin or lysozyme were partitioned in a biphasic system containing either K_2SO_4, KF, or KBr. The variation of the protein partition coefficient with the protein net charge is shown in Fig. 2. The net charges were estimated from the measured pH values by using the titration curves of the proteins given by Tanford and Wagner [5] and Kenchington.[6] The logarithm of the partition coefficient of the protein is found to be linearly related to its net charge, Z. The slopes of the lines for the two proteins are almost identical when partition was carried out in the presence of a given salt. Some deviations from linearity are observed for ovalbumin when it is negatively charged. On the other hand, the slope of the line varies with the salt. This is in agreement with eqn. (3). For a single protein, the lines obtained when it is partitioned with different salts intersect where the net charge $Z = 0$. Albertsson *et al.*[7] have taken advantage of this fact and from the plot of log K_p *versus* pH estimated the isoelectrical points for a series of proteins.

The deviation from a straight line may indicate either a change in molecular weight of the protein due to association or dissociation, or changes in its effective charge due to interaction with ions other than H^+ and OH^-. The deviation could also be due to a difference in the extent of ionization of the protein in water as compared with that in polymer solutions. The effective Z may consequently differ from that calculated from titration curves in aqueous solutions. Deviations may also result from conformational changes which affect the K_p^0 in eqn. (3). Sasakawa and Walter [8] have shown that K_p at the isoelectric point. *i.e.* K_p^0, varies

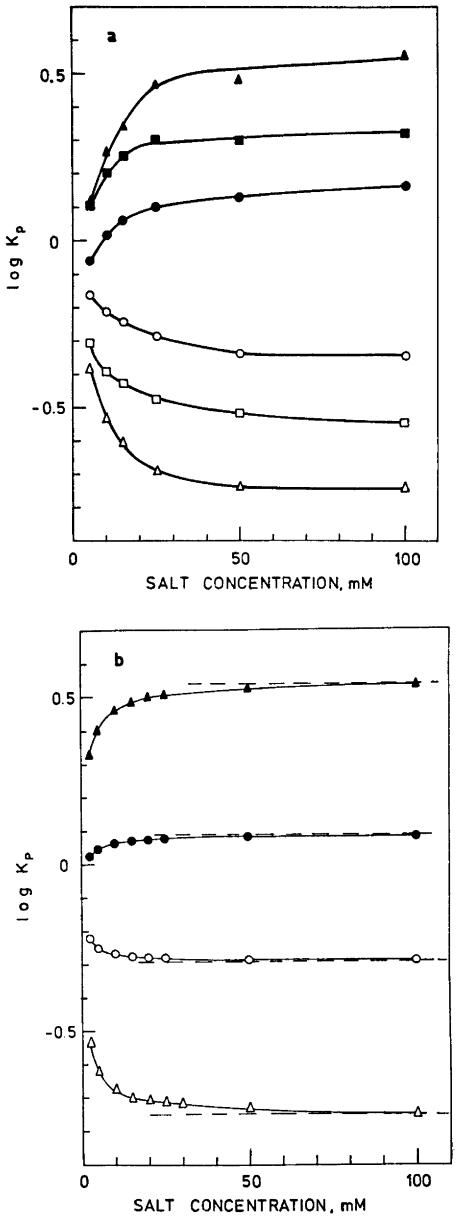

Fig. 3. Variation of protein partition coefficient, K_p, with salt concentration.
a. Lysozyme, 1 g/l (black spots) and ovalbumin, 2 g/l (white spots) were partitioned at 25 °C in a system containing 8 % (w/w) dextran, 8 % (w/w) poly(ethylene glycol), 1 mM sodium phosphate buffer, pH 6.8, and various concentrations of KF, ○; potassium acetate, □; and KBr, △.
b. Theoretical curves for lysozyme, black spots ($Z = 7.3$, $K_p^0 = 0.89$ and $C_p = 0.0705$ mM) and ovalbumin, white spots ($Z = -7.3$, $K_p^0 = 0.71$ and $C_p = 0.0445$ mM) when partitioned with KBr, △, or KF, ○. $K_+ = 0.828$ (K^+), $K_- = 1.202$ (Br^-) or 0.904 (F^-). The broken lines indicate the limit partition of the proteins at infinite salt concentration.

strongly with the protein and is also related to the molecular weight of the protein.

When K_2SO_4 is included in the system used here, the partition of a given protein is almost independent of Z. This salt therefore gives a system in which the interfacial potential is close to zero.

Dependence of protein partition on the concentration of salt. When ovalbumin and lysozyme are partitioned at very low salt concentrations their partition coefficients are close to one, but with increasing salt concentration K_p asymptotically approaches limiting values, Fig. 3a. At pH = 6.7 and at high salt concentration the positively charged lysozyme and the negatively charged ovalbumin have affinity for opposite phases.

The dependence of K_p on the salt concentration, according to the theoretical model, can be derived from eqn. (1), Fig. 3b, by combination with expressions for electroneutrality and mass balance which yields eqn. (4).

$$\frac{1 - 1/(1 + K_- y) + Z(2C_p - x)/2C_s}{Zx/2C_s + 1/(1 + K_- y)} = \frac{K_+}{y} \qquad (4)$$

where

$$y = [(2C_p - x)/xK_p^0]^{1/z}$$

C_p, and C_s are the respective molar concentrations of protein and salt in the system, and x is the molar concentration of protein in the lower phase. K_p^0, K_+ and K_- have been defined in connection with eqn. (1) – (3).

The validity of (4) is restricted to a system where the two phases have equal volume and the salt is a 1 – 1-electrolyte. Furthermore the protein must be negatively charged. Z is the *numerical* value of the protein net charge. Eqn. (4) was numerically scanned over a range of x values $0 < x < 2C_p$, with an increment of 0.0001 mM to obtain the value of x that best fitted the equation. This was done with aid of a Hewlett Packard electronic calculator 9100. K_p is related to x *via* eqn. (5).

$$K_p = (2C_p - x)/x \qquad (5)$$

The theoretical and experimental curves, Fig. 3, have the same shape. At very low salt concentrations the presence of 1 mM phosphate buffer will increasingly affect the partition of the protein as salt concentration decreases. This has not been corrected for in the calculations. The contribution of the protein to the interfacial potential will grow smaller and smaller as the concentration ratio between salt and protein increases. Therefore the partition of the protein will be increasingly determined by the partition of the salt ions when the concentration of the latter increases. The limiting partition coefficient for a protein in the presence of excess salt is given by eqn. (3). It is evident that to obtain the largest possible difference in the partition of the two proteins the salt concentration must be sufficiently high. In the present experiments the protein concentration in the systems was 2 g/l or less. When 25 mM 1 – 1-electrolyte is used, the difference in log K_p for the two proteins was about 90 % of the maximal one.

Above a minimum salt concentration the separation effect is almost independent of the salt concentration. This is in contrast to other separation methods which utilize charge differences between proteins, *e.g.* ion exchange chromatography or electrophoresis. In these separation procedures the ionic strength plays an important role in determining the separation. In partition, however, ionic strength can be chosen within a very wide range of values without affecting the degree of separation. Partition at high ionic strength may therefore be used for resolving proteins or subunits which otherwise interact strongly.

The separatory effect of various salts. The degree of separation of two substances by partition is measured by a separation factor, β. This is defined as the ratio between the partition coefficients of the two substances [9] and the order of the coefficients is chosen so that $\beta \geq 1$. It has here been found more suitable to use a logarithmic expression.

According to eqn. (3) the partition coefficient of a protein can be split into two parts; one, K_p^0, depending on the relative solvation of the protein in the two phases, the other part, $K_p^{el}[=(K_-/K_+)^{z/(\nu_+ + \nu_-)}]$, depending on the net charge of the protein. The difference in log K_p, Δ log K_p, of *two* proteins partitioned under equal conditions can be split up in the same way:

$$\Delta \log K_p = \Delta \log K_p^0 + \Delta \log K_p^{el} \quad (6)$$

where Δ log K_p^{el} depends on the difference in net charge between the two proteins. The value Δ log K_p is a measure of the separatory capacity of the biphasic systems and is related to the separation factor β, *via* log $\beta = |\Delta \log K_p|$. The part of the separatory capacity that depends

Table 1. The charge-dependent part of the separatory capacity, Δ log K_p^{el}, obtained when ovalbumin or lysozyme, respectively, was partitioned with different salts in a biphasic system containing 8 % (w/w) dextran and 8 % (w/w) poly(ethylene glycol) at 25 °C.

Salt	Salt concentration (mM)	Δ log K_p^{el}	Salt	Salt concentration (mM)	Δ log K_p^{el}
KF	25	−0.25	NaF	25	−0.23
KCl	25	−0.87	NaCl	25	−0.83
KBr	25	−1.06	NaBr	25	−1.04
KI	25	−1.36	NaI	25	−1.26
KSCN	25	−1.49	NaSCN	25	−1.36
K_2SO_4	20	0.04	Na_2SO_4	20	0.05
$KClO_4$	25	−1.73	$NaClO_4$	25	−1.58
NH_4F	25	0.04	LiF	20	0.29
NH_4Cl	25	−0.60	LiCl	25	−0.35
NH_4Br	25	−0.81	LiBr	25	−0.54
NH_4I	25	−1.14	LiI	25	−0.86
NH_4SCN	25	−1.21	LiSCN	25	−0.92
$(NH_4)_2SO_4$	20	0.22	Li_2SO_4	20	0.31
KNO_3	100	−0.81	$LiClO_4$	25	−1.15

on the net charges of the proteins should, according to the theory, be the difference between $\Delta \log K_p$ and $\Delta \log K_p^0$. $\Delta \log K_p$[$=\log K$ (ovalbumin) $-\log K$ (lysozyme)] for the protein pair ovalbumin and lysozyme (partitioned separately) at pH 6.7 has been determined using biphasic systems which contained different salts. $\Delta \log K_p^0 = 0.10$ for this pair of protein has been determined from Fig. 2, as the difference in $\log K_p$ at $Z=0$ when the proteins are partitioned in the presence of K_2SO_4. The resulting $\Delta \log K_p^{el}$ values are given in Table 1.

When the salt used is a 1 – 1-electrolyte, the $\Delta \log K_p^{el}$ values obtained, can be expressed as the sum of two constant terms, $\Delta \log K_C$ and $\Delta \log K_A$, which are characteristic for the cation and anion respectively,

$$\Delta \log K_p^{el} = \Delta \log K_C + \Delta \log K_A \quad (7)$$

Setting $\Delta \log K_C$ for the potassium ion arbitrarily equal to zero, $\Delta \log K_C$ will be 0.03 for Na^+; 0.28 for NH_4^+; and 0.53 for Li^+ and $\Delta \log K_A$ will be -0.25 for F^-, -0.87 for Cl^-, and -1.07 for Br^-. The maximum deviation for $\Delta \log K_A$ or $\Delta \log K_C$ calculated for an ion from data concerning different salts containing that ion is less than ± 0.02. The additivity holds less well for I^-, SCN^-, and ClO_4^-, for which $\Delta \log K_A$ is found to be -1.36 ± 0.07, -1.45 ± 0.06, and -1.67 ± 0.06, respectively.

If the negative ions are arranged in order of their $\Delta \log K_A$ values, the series obtained corresponds to the well-known Hofmeister's series. The most extreme charge-depending separatory effect is shown by salts containing chaotropic ions, SCN^- or ClO_4^-, which makes these salts attractive for enhancing the separatory capacity of the biphasic systems. However, such salts should be used with care, since high concentrations of the chaotropic ions may denature proteins.

By using salts for which $\Delta \log K_p^{el} = 0$ the pH-dependent separation effect can be nullified. In the system studied here and at the operating temperature NH_4F as well as K_2SO_4 have this property. Therefore in a system containing one of these salts the partition coefficient of a protein should be independent of the protein net charge and equal to K_p^0. Variations in K_p^0 with pH would indicate structural changes in the protein molecule.

Depending on the salt used, a small yet significant variation in K_p^0 has been noted. It is here assumed that the shift in $\log K_p^0$ is the same for the two proteins studied so that the difference $\Delta \log K_p^0$ is independent of salt. The good agreement between experiment and theory indicates that this is a valid approximation. The relative variation of K_p^0 with salt has been approximated by the change in the means of $\log K_p$ of ovalbumin and lysozyme. The increase in $\log K_p^0$ goes hand in hand with increasing partition coefficient of the salt, given in Table 2. The order of increasing K_p^0 is $F^- < Cl^- < Br^- < I^- < SCN^- < ClO_4^-$ and $K^+ < Na^+ < NH_4^+ < Li^+$. The shifts in $\log K_p^0$ seem to be unique for each ion and additive just as in the case of $\Delta \log K_p^{el}$.

Table 2. Partition coefficients, K_s, of salts in a biphasic system containing 8 % (w/w) dextran and 8 % (w/w) poly(ethylene glycol) at 25 °C.

Salt	Conc. (mM)	$\log K_s$	Salt	Conc. (mM)	$\log K_s$
LiI	20	0.0622	NH_4F	20	−0.0386
NH_4I	20	0.0484	NaF	20	−0.0575
NaI	20	0.0310	KF	20	−0.0640
KI	20	0.0257	LiF	20	−0.0255
LiBr	20	0.0314	KSCN	20	0.0342
NH_4Br	20	0.0224	$KClO_4$	20	0.0362
NaBr	20	0.0043	KNO_3	20	0.0000
KBr	20	0.0009	K_2SO_4	10	−0.0964
LiCl	20	0.0212			
NH_4Cl	20	0.0095			
NaCl	20	−0.0123			
KCl	20	−0.0173			

The zero-potential partition of ions. By combining the partition coefficients for a salt and those for proteins partitioned with excess of this salt, the K_+ and K_- values for the ions of the salt can be calculated. Eqn. (3) yields

$$\log \frac{K_-}{K_+} = \frac{\nu_+ + \nu_-}{\Delta Z} \Delta \log K_p^{el} \times 1.11 \qquad (8)$$

where ΔZ is the difference in net charge of the two proteins. The correction factor 1.11 estimated from Fig. 3 is introduced since Δ log K_p at the salt concentration used is close to, but not identical with, the limiting value obtained with excess of salt. For 1 – 1-electrolytes eqn. (2) can be written as

$$\log (K_+ K_-) = 2 \log K_s \qquad (9)$$

K_+ and K_-, Table 3, column b, have been calculated for alkali, ammonium, and halide ions from (8) and (9) and using Δ log K_p^{el} and K_s from Tables 1 and 2. The charge difference, ΔZ, used in the calculations has been obtained from the titration curves and the isoelectric points. The latter were taken to be 4.75 for ovalbumin and 11.0 for lysozyme.[8]

On this basis, at pH 6.7, Z is −12.0 and 7.5 for ovalbumin and lysozyme, respectively, and therefore $\Delta Z = -19.5$.

A considerable variation is observed in the values of K_+ or K_- for a given ion. In addition, there is also a systematic variation in the values for the various counter ions. The K_+ and K_- have also been calculated *via* eqn. (9) solely from the partition coefficients of salts given in Table 2. This is possible because one of the salts, NH_4F, only gives rise to very small interfacial potential, as can be seen from the data given in Table 1. K_+ and K_- for the ions of this salt must therefore be almost equal to each other and also equal to the partition coefficient of the salt. From these two values, K_+ and K_- for the other ions have been calculated *via* (9) and are recorded in Table 3, column d. These K_+ and K_- differ considerably from the values obtained from Δ log K_p^{el}.

The K_- has also been directly estimated for chloride, bromide, and iodide by partition of salts containing these ions in a system containing K_2SO_4 in large excess. This salt gives only rise to a very low interfacial potential since Δ log K_p^{el} is close to zero, Table 1. These experimental K_- values, Table 3, column a, agree well with the values calculated on the basis of $K_+ = K_-$ for NH_4^+ and F^-, Table 3, column d. K_+ for the cations, Table 3, column e, has been calculated from the experimental values concerning Br^- and Cl^-.

The observed deviation of K_+ and K_- calculated from the Δ log K_p^{el} data when compared with the values obtained experimentally or

Table 3. Logarithms of partition coefficients (K_+ and K_-) of ions when interfacial potential is zero, in a biphasic system containing 8 % (w/w) dextran and 8 % (w/w) poly(ethylene glycol) at 25 °C. Salt concentration, 10 – 25 mM.
a, 10 mM halide in a system containing excess of K_2SO_4, 50 mM.
b, calculated from Δ log K_p^{el} and K_s using eqns. (8) and (9) with $\Delta Z = -19.5$.
c, calculated as in *b* but using $\Delta Z = -14.6$.
d, calculated from K_s *via* eqn. (9), assuming K_+ for ammonium to be equal to K_- for fluoride.
e, K_+ calculated from K_s *via* (9) using K_- for Cl^- and Br^- given under *a*. K_- for F^- is likewise calculated from K_+ so obtained.

Ion	Experimental log K_-	Calculated log K_+ or log K_-			
	a	b	c	d	e
K^+		−0.062 ± 0.016	−0.082 ± 0.004	−0.089	−0.084 ± 0.002
Na^+		−0.056 ± 0.014	−0.073 ± 0.007	−0.076	−0.076 ± 0.002
NH_4^+		−0.025 ± 0.012	−0.037 ± 0.002	−0.039	−0.036 ± 0.003
Li^+		+0.002 ± 0.011	−0.005 ± 0.003	−0.012	−0.015 ± 0.005
I^-	+0.151	+0.107 ± 0.006	+0.130 ± 0.005	+0.136	
Br^-	+0.083	+0.063 ± 0.005	+0.080 ± 0.007	+0.084	
Cl^-	+0.051	+0.038 ± 0.006	+0.051 ± 0.004	+0.058	
F^-		−0.044 ± 0.006	−0.044 ± 0.004	−0.039	−0.040 ± 0.004

via the partition coefficients of the salts indicates that the estimated net charges for ovalbumin and lysozyme are not correct. By using eqn. (3) the net charges have been calculated from the partition data of the two proteins in a system containing KBr and assuming log K_p^0 to be −0.05 and −0.15 for lysozyme and ovalbumin, respectively. The net charge is found to be 7.3 for lysozyme and −7.3 for ovalbumin. The former is in good agreement with the value, 7.4, obtained from the titration curve, while the value for ovalbumin is considerably less than the titrimetrical one, −12.0. If the net charges, calculated *via* (3), are used for calculating K_+ and K_- from Δ log K_p^{el} the result, Table 3, column c, is in excellent agreement with the values obtained from the partition coefficients of the salts, Table 3, columns a, d, and e. The assumption of a smaller Z value for ovalbumin at pH = 6.7 is consistent with the data for this protein in Fig. 2. Ovalbumin shows a deviation from the linear dependence of Z when negatively charged. If, however, the net charge at pH = 6.7 is estimated by extrapolation to the assumed linear relation, one obtains $Z = -7.5$ in the case of KF and $Z = -8.0$ in the case of KBr.

In a biphasic system the ions of a salt would attain the partition coefficients K_+ and K_- if the demand of electroneutrality did not require that their actual partition coefficients are the same. Only in a thin layer around the interface may an unequal distribution of cation and anion exist, a so called Stern layer.[10] K_+ and K_- express the relative affinities of the ions for the two phases. The affinity of ammonium and alkali ions for the dextran-rich lower phase is greater than that for the PEG-rich upper phase; $K_+ < 1$. The affinity for the lower phase increases in the order $Li^+ < NH_4^+ < Na^+ < K^+$. The above finding indicates that these ions, especially K^+ and Na^+, form weak complexes with dextran in water solution. It has further been shown that the IR-spectra of dextran [11] in aqueous solution are influenced by potassium and sodium salts.

The halide ions except F^- have a greater affinity for the upper phase and this affinity increases in the order $Cl^- < Br^- < I^-$. This is in agreement with the electrophoretic mobility of PEG in salt solutions containing these ions.[2]

Effect of salt concentration on K_s. The partition coefficient of the studied salts are found to be independent of the salt concentration in the interval 2 − 100 mM.

Interfacial potential, ψ, was calculated using the following expression (10), obtained by combining (1), (3), and (6).

Table 4. Values of interfacial potential, ψ, in a biphasic system containing 8 % (w/w) dextran, 8 % (w/w) poly(ethylene glycol) and 20 mM alkali or ammonium halide at 25 °C.

Salt	ψ(mV) meas.[a]	ψ(mV) calc.[b]	ψ(mV) calc.[c]
KF	−2.6	−1.1	−1.3
KCl	−4.0	−3.9	−4.0
KBr	−4.5	−4.8	−4.9
KI	−5.6	−6.1	−6.5
NaF	−1.1	−1.0	−1.1
NaCl	−2.9	−3.7	−3.8
NaBr	−3.6	−4.7	−4.7
NaI	−4.2	−5.7	−6.3
NH_4F	−1.4	+0.2	+0.1
NH_4Cl	−3.3	−2.7	−2.6
NH_4Br	−3.8	−3.7	−3.5
NH_4I	−4.6	−5.1	−5.1
LiF	−0.2	+1.3	+0.7
LiCl	−1.5	−1.6	−2.0
LiBr	−2.5	−2.4	−2.9
LiI	−3.0	−3.9	−4.5

[a] Measured relative to KCl and recalculated to absolute values by assuming the potential to be −4.0 mV in the case of KCl. [b] Calculated from Δ log K_p^{el}. [c] Calculated from K_+ and K_-.

$$\psi = \frac{RT}{F}\frac{1}{\Delta Z}\Delta \ln K_p^{el} \text{ (excess of salt)} \quad (10)$$

with $\Delta Z = -14.6$ and introducing the correction factor 1.11 mentioned earlier, in connection with (8), yields

$$\psi = -\frac{59.2}{14.6}\Delta \log K_p^{el} \times 1.11 \quad (11)$$

The interfacial potentials calculated for alkali and ammonium halides are given in Table 4.

The interfacial potential has also been measured relative to a system containing KCl. The results are presented in Table 4. For anions the potential varies in the expected sequence I^-, Br^-, Cl^-, F^-. For cations the sequence expected is K^+, Na^+, NH_4^+, Li^+, but that observed experimentally is K^+, NH_4^+, Na^+, Li^+. This deviation may be due to polarization phenomena at the contact surfaces in the electrical element, the so called junction potentials. These could perhaps be decreased by using another salt in the salt bridge between the two biphasic systems. NH_4NO_3, which has been used here, is ideal in water solution because of the equal transport number of the two ions. The same is not necessarily true in 20 % (w/w) dextran solution, used in these experiments. The experiments show, however, that the interfacial potential is of the predicted order.

Measurements of the interfacial potential in an aqueous biphasic system containing phosphate and chloride has recently been published by Reitherman *et al.*[12] The values of the potentials were of the same order as those here described.

CONCLUSIONS

The effect of electrolytes on the separatory capacity of the aqueous biphasic systems is due to a difference in the affinity of the constituent ions for the two phases.

This leads to the formation of an electrical double-layer and consequently an interfacial potential. When a biphasic system contains an excess of salts the induced interfacial potential depends only on the salt and this will affect the partition of proteins according to their charges.

The separatory capacity of a biphasic system for a given pair of proteins can be split into two parts. One part reflects the solvation of the proteins in the two phases and is almost independent of the presence of salts in the system. The other part is related to the difference in net charges of the two proteins. This charge-depending part of the separatory capacity is characteristically affected by the ions of the salt in the system. The effects of the ions are algebraically additive. By choosing a salt with ions having large effects on the separatory capacity it may be possible to resolve (even complex) protein mixtures by liquid-liquid extraction. Since the separatory capacity induced by a salt is constant throughout a considerably wide range of salt concentrations, separations can be carried out at a suitable ionic strength without any loss in the efficiency of separation.

The experimental data are in excellent agreement with the equations for interfacial potential put forward by Albertsson. They can therefore be used with confidence for quantitative predictions. Thus, partition studies can be used for determination of physico-chemical properties of proteins, *e.g.* net charge. It should also be possible to detect conformational changes, protein-protein associations and binding of low-molecular weight substances to proteins, from partition studies.

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Periodate Oxidation of Phenols. XIV.* Oxidation of *p*-Hydroxybenzyl Alcohol with Periodate and Bismuthate

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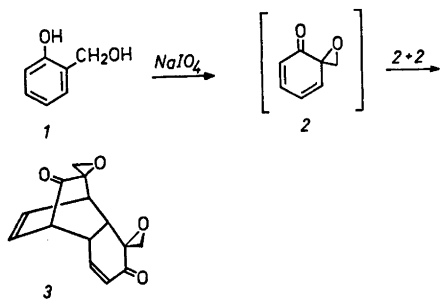
Treatment of *p*-hydroxybenzyl alcohol with sodium metaperiodate in acetic acid-water gives, in addition to amorphous material, *p*-benzoquinone (23 %) and *p*-hydroxybenzaldehyde (4 %). If sodium bismuthate is used as oxidant, the yield of *p*-benzoquinone is only 5 % and a new product, 1-oxaspiro[2.5]octa-4,7-dien-6-one (*5*), is formed in a yield of 20 %.

It was observed several years ago that *p*-hydroxybenzyl alcohols on treatment with aqueous sodium metaperiodate give formaldehyde and the corresponding *p*-benzoquinone.[1] *o*-Hydroxybenzyl alcohols were later found to react with the same oxidant to give the Diels-Alder *endo* dimers of the initially formed spiro(oxirane-2,4-cyclohexadienones).[2,3] For instance, periodate oxidation of salicyl alcohol (*1*) (Scheme 1) afforded a dimer (*3*) of the intermediary 2,4-cyclohexadienone *2* in a yield of 74 %, with only 1 % of salicyl aldehyde being formed simultaneously.[2] Similar oxidation of *o*-hydroxybenzyl alcohols carrying at least one bulky ring substituent gave the corresponding monomeric spiro compounds which, due to steric hindrance, did not dimerize.[4]

The primary aim of the present study was to find conditions under which oxidation of *p*-hydroxybenzyl alcohols leads to the formation of spiro(oxirane-2,5-cyclohexadienones) rather than to the oxidative cleavage to the corresponding *p*-benzoquinone and formaldehyde. It has been possible to perform the first-mentioned reaction with 4-hydroxybenzyl alcohol.



Scheme 1.

From the reaction mixture obtained on treatment of 4-hydroxybenzyl alcohol (*4*) for 30 min with a solution of sodium metaperiodate in acetic acid-water (4:1) only *p*-benzoquinone (yield, 23 %) and *p*-hydroxybenzaldehyde (yield, 4 %) could be isolated. A similar experiment with sodium bismuthate as oxidant gave, in addition to minor amounts of *p*-benzoquinone (5 %) and *p*-hydroxybenzaldehyde (2 %), 20 % of a product $C_7H_6O_2$ (m.p. 51 – 52 °C) which was the desired spiro compound *5*. Its structural assignment is based on the chemical behaviour and the spectral properties described below.

In the reaction mixtures obtained in the periodate and bismuthate oxidation experiments no unreacted 4-hydroxybenzyl alcohol could be detected. The major part of the starting material had been converted into a dark-coloured product which was strongly adsorbed on the silica gel column used in the separation of the reaction products. If the oxidation with periodate was carried out in water or in a 10 %, rather than the above-mentioned 80 %, aqueous acetic acid solution, a dark-brown amorphous material deposited. It is most probable that the

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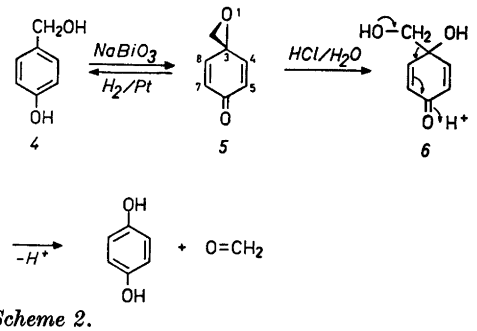
formation of this material is mainly due to *ortho* oxidation of *4*, the resulting *o*-quinone alcohol being unstable under the conditions used, as shown in separate studies.[5] It has been reported earlier that periodate oxidation of 2,4-dimethylphenol, possessing a free *ortho* position, gives 3,5-dimethyl-1,2-benzoquinone [6] among other products.

Catalytic hydrogenation of *5* regenerated 4-hydroxybenzyl alcohol, as could be expected from the similar behaviour of spiro(oxirane-2,5-cyclohexadienones) carrying substituents in the six-membered ring.[7] Dilute aqueous hydrochloric acid caused rapid degradation of *5* to give formaldehyde and hydroquinone. It is assumed that, in this reaction, hydrolysis first gives the *p*-quinol *6* which in a rapid (proton-catalyzed) retro aldol condensation loses its hydroxymethyl group as formaldehyde, concomitant aromatization constituting a driving force (Scheme 2).

Similar loss of a 4-carbinol substituent takes place in 2,5-cyclohexadienone intermediates arising in certain modes of radical coupling involved in the biosynthesis of lignin,[8,9] in the oxidative degradation of *p*-hydroxybenzyl alcohols with dipotassium nitrosodisulfonate,[10] in the formation of oligomers of the polyphenylene oxide type from 3,5-disubstituted 4-hydroxybenzyl alcohols,[11,12] and in the colour reaction of 4-hydroxybenzyl alcohols with *N*-chloroquinone imide.[13]

The 60 MHz NMR spectrum of compound *5* in $CDCl_3$ exhibited two signals at δ 3.36 and 6.49, the ratio of their integrals being 1:2. The former signal can be ascribed to the protons of the oxirane ring. Since the plane of this ring is perpendicular to that of the dienone ring, the two oxirane protons are equivalent and the same is true for the vinyl protons H-4, H-8 and H-5, H-7, respectively. By a fortuitous shift coincidence the two last-mentioned pairs of protons give rise to a single signal.



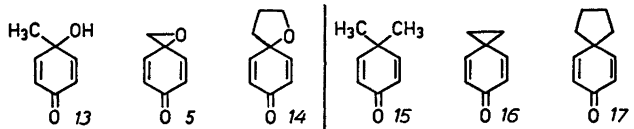
Scheme 2.

The presence of a group of three peaks at 1665, 1635, and 1622 cm^{-1} in the IR spectrum of *5*, arising from the cross-conjugated carbonyl system is in accord with IR spectroscopic characteristics of other 2,5-cyclohexadienones. It may be noted, however, that in the spectrum of *5* these three bands are of high intensity, whereas in the spectra of previously investigated 2,5-cyclohexadienones the band of lowest frequency generally was rather weak.[14,15] A peak of medium strength at 3060 cm^{-1} can be attributed to the CH_2 group of the oxirane ring.[16]

In the UV spectrum of *5* (in ethanol), the maximum of the $\pi \rightarrow \pi^*$ absorption was found at λ 250 nm ($\log \varepsilon = 4.24$) and that of the $n \rightarrow \pi^*$ band at λ 336 nm ($\log \varepsilon = 1.47$). As can be seen from Table 1, the former maximum is located at a considerably higher wavelength than the corresponding maxima of *p*-toluquinol (*13*) [17] and of the spiro(oxolane-2,5-cyclohexadienone) *14*,[18] the positions of the two latter maxima being in accord with that calculated for a β-substituted enone system (227 nm). The deviation from the Woodward rules found for *5* seems to be due to a conjugative effect of the oxirane ring.

For comparison, Table 1 also includes the λ_{max} value of the spiro(cyclopropane-2,5-cyclohexadienone) *16*,[20] as well as those of the dimethyl dienone *15* and the spiro compound *17*.[19] The absorption maximum of *16* is found at a wavelength still higher than that of its oxa analogue *5*. It may further be noted that compound *17* absorbs at a higher wavelength than its oxa analogue *14* and the monocyclic compounds *13* and *15*.

As mentioned above, compound *5* is obtained on oxidation of *4* with bismuthate but seems not to be formed when periodate is used as oxidant. This is remarkable, since the two oxidants are generally considered equivalent as glycol-cleaving agents [21] and have been shown to act similarly in the oxidation of guaiacol to *o*-benzoquinone and methanol.[22] An attempt to rationalize the different action of bismuthate and periodate in the present case has been made in Scheme 3. The oxidation of *4* is believed to be initiated by nucleophilic attack by the phenolic



	13	5	14	15	16	17
λ_{max}, nm	227	250	228	227	274	242
log ε	4.25	4.24	4.08	4.1	4.34	4.20

Table 1. Ultraviolet maxima ($\pi \rightarrow \pi^*$ bands) of some 2,5-cyclohexadienones. Solvents: Ethanol for *13*,[17] *5*, *14* [18] and *17* [19]; methanol for *16* [20]. The λ_{max} value of *15* has been calculated by application of Woodward's rules; the log ε value is that of the 2-methyl derivative.[20]

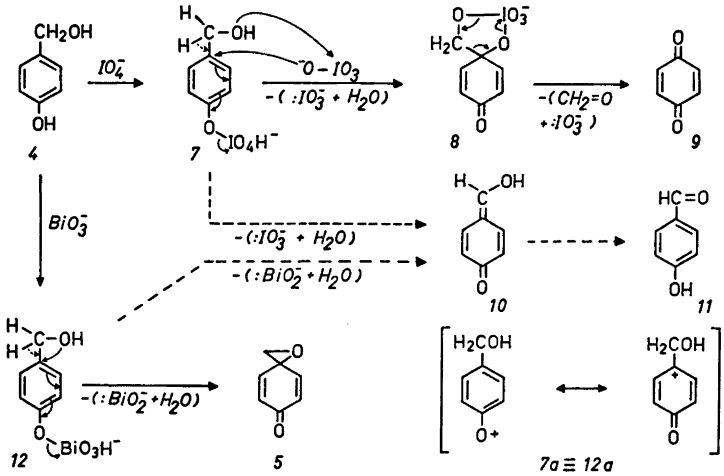
oxygen on the iodine and bismuth atoms of the oxidants, resulting in the formation of aryl esters *7* and *12*, respectively. In the bismuthate system, the formation of *5* is then assumed to proceed by a concerted reaction involving two-electron transfer to the bismuth atom with simultaneous nucleophilic attack by the alcoholic oxygen atom on the aromatic ring. In the periodate system, the oxygen of a second molecule of periodate rather than the alcoholic hydroxyl group may attack the ring, subsequent (or preceding) coordination of the latter group to the iodine atom giving intermediate *8*. This cyclic glycol periodic ester would decompose in the normal way [23] to give formaldehyde and *p*-benzoquinone (*9*).

The different behaviour of esters *7* and *12* can possibly be attributed to the fact that the concentration of bismuthate ions in 80 % acetic acid solution of *4* is low, most of the sodium bismuthate during the reaction being present as a solid, whereas the sodium periodate is completely dissolved.

The formation of small amounts of *p*-hydroxybenzaldehyde (*11*) in both systems may be understood as being due to the loss of a proton from the carbinol carbon atom in *7* and *12* giving the enol *10* which tautomerizes to *11* (see dashed line arrows in Scheme 3).

Instead of the one-step concerted reactions indicated in formulae *7* and *12*, one may as well assume two-step reactions, involving, in a first step, two-electron transfer to the periodate and bismuthate residue with formation of a resonance-stabilized phenoxonium ion (*7a*≡ *12a*), the latter reacting further to give the postulated intermediates *8* and *10*, as well as product *5*.

p-Benzoquinone (*9*) is also formed in the bismuthate oxidation system, although in considerably smaller amounts than in the periodate system, the yields obtained after a reaction time of 30 min being 5 and 23 %, respectively (*cf.* p. 883). Prolonged bismuthate oxidation slowly increased the yield of *9*, whereas the yield of *5* decreased, the total of *9* and *5* remain-



Scheme 3.

ing approximately constant. This indicates that, in the bismuthate system, the *p*-benzoquinone is formed *via* the spiro compound *5*. In fact, treatment of *5* with bismuthate in 80 % aqueous acetic acid for 30 min gave *p*-benzoquinone in a yield of 22 %, in addition to unreacted *5* (65 %).

The possibility of *5* being an intermediate in the formation of *p*-benzoquinone in the periodate system seems to be ruled out by the fact that only 28 % of compound *5* was converted to the quinone when treated with periodate in 80 % aqueous acetic acid for 30 min.

These conversions of *5* to *p*-benzoquinone must be due to hydrolytic opening of the oxirane ring followed by loss of formaldehyde, the resulting hydroquinone (*cf.* the similar hydrolysis by aqueous HCl, Scheme 2) being dehydrogenated by the oxidant present. The hydrolysis of *5* in 80 % aqueous acetic acid, in the absence of oxidant, was followed by UV spectrometry revealing a decrease in absorbance at 250 nm (λ_{max} of *5*) and an increase at 290 nm (λ_{max} of hydroquinone). Expectedly, hydrolysis in this solvent was much slower than that found in aqueous hydrochloric acid (*cf.* p. 884). After 24 h treatment of *5* with 80 % acetic acid, however, hydroquinone could be isolated in a yield of 82 %.

Compound *5* proved to be stable in an aqueous solution of $NaIO_4$, but was rapidly converted to formaldehyde and *p*-benzoquinone in aqueous H_5IO_6, acid hydrolysis of the oxirane ring again being the initial step.

Treatment with aqueous sodium hydrogen carbonate did not appreciably affect compound *5*; however, addition of aqueous NaOH caused immediate conversion into dark brown products. At room temperature, solid *5* slowly deteriorates, whereas it can be stored essentially unchanged for several months at −20 °C.

Some compounds with the basic structure *5* carrying substituents in the dienone ring have been reported earlier. They were prepared by the action of alkali upon 2,5-cyclohexadienones with a halohydrine grouping in the 4-position [24] or by the action of diazomethane on substituted *p*-quinones.[7] A tricyclic analogue of *5* carrying the spirooxirane ring in the 10-position of anthrone has also been described.[25]

EXPERIMENTAL

UV spectra were recorded on a Cary Model 14 spectrophotometer; IR and NMR spectra were obtained using Beckman 9A and Varian A-60 instruments, respectively. Chemical shifts are given in δ (ppm) units, TMS being used as internal standard.

Oxidation of 4-hydroxybenzyl alcohol (4) with sodium metaperiodate. A solution of $NaIO_4$ (0.06 mol) in a mixture of 100 ml of water and 80 ml of acetic acid was added to a solution of *4* (0.03 mol) in 320 ml of acetic acid. After 30 min at room temperature ethylene glycol (5 ml) was added in order to remove excess periodate and the dark red-brown solution was extracted with three 150 ml portions of dichloromethane. The combined extracts were washed twice with aqueous hydrogen carbonate and with water and, after being dried over anhydrous Na_2SO_4, were brought to dryness under vacuum, leaving a brown oil. The latter was chromatographed on a silica gel column (4 × 60 cm) using benzene-ethyl acetate (4:1) as eluent. The first eluted yellow fraction gave p-*benzoquinone* ($R_F = 0.39$) in a yield of 23 %. A following fraction provided 4-*hydroxybenzaldehyde* ($R_F = 0.21$); yield, 4 %. The products were identified by m.p. and mixed m.p. with authentic samples.

Oxidation of 4-hydroxybenzyl alcohol (4) with sodium bismuthate. A solution of *4* (0.03 mol) in 300 ml of an acetic acid-water (4:1) mixture was stirred for 30 min at room temperature with $NaBiO_3$ (0.06 mol). Unconsumed bismuthate was filtered off and the dark brown-red filtrate worked up as described above. Elution of the silica gel column gave:

(*a*) p-*Benzoquinone* ($R_F = 0.39$) in a yield of 5 %.

(*b*) *1-Oxaspiro[2.5]octa-4,7-dien-6-one (5)*, R_F = 0.33, colourless crystals of m.p. 51 – 52 °C after sublimation (40 °C, 1 mmHg); yield, 20 %. (Found: C 69.01; H 5.04. Calc. for $C_7H_6O_2$: C 68.84; H 4.95). UV, IR and NMR spectra, *cf.* p. 884.

(*c*) 4-*Hydroxybenzaldehyde* ($R_F = 0.21$); yield, 2 %.

Catalytic hydrogenation of 5. A solution of *5* (124 mg) in acetone (10 ml) was added to the suspension of prehydrogenated PtO_2 (30 mg) in acetone (5 ml). The mixture was stirred under hydrogen for 30 min after which time 1 mol of H_2 per mol of *5* had been consumed and further hydrogen uptake was slow. Removal of the solvent from the filtered solution and recrystallization of the crystalline residue from ether gave *4-hydroxybenzyl alcohol*, identical by m.p. and mixed m.p. with authentic material. Yield, 80 %.

Hydrolysis of 5 with 2 M aqueous hydrochloric acid. (a) A solution of *5* (110 mg) in 2 M aqueous HCl (25 ml) was kept at room temperature for 3 min and then extracted with three 100 ml portions of ether. The combined extracts were dried over anhydrous Na_2SO_4 and brought to dryness. The crystalline residue was recrystal-

lized from ether and was found to be identical to *hydroquinone* by m.p. (168–170 °C) and mixed m.p. Yield, 74 %. (b) Compound *5* (21.7 mg, 0.18 mmol) was dissolved in 2 M aqueous HCl. After 5 min, the solution was neutralized with aqueous NaOH. A 0.1 M NaOAc-HCl (2:1) buffer solution (300 ml) of pH 4.6 was added, followed by 30 ml of a saturated aqueous solution of dimedone (*cf.* Ref. 26). Filtration after 15 h gave *dimedone-formaldehyde compound*, m.p. 185–187 °C, identical by mixed m.p. with an authentic sample. Yield, 53.2 mg (100 %).

Hydrolysis of 5 with 80 % aqueous acetic acid. A solution of *5* (300 mg) in a 4:1 acetic acid-water mixture (50 ml) was kept at 25 °C for 24 h. The solution was then concentrated under vacuum to one third of its volume and extracted with 3 × 25 ml of chloroform. Removal of the solvent from the combined extracts gave an oily residue which crystallized on addition of a few milliliters of ether. The product after recrystallization from acetone had m.p. 170–171 °C and was identical by mixed m.p. with authentic *hydroquinone*. Yield, 82 %.

Treatment of 5 with $NaIO_4$ and $NaBiO_3$ in 80 % aqueous acetic acid. (a) Solutions of *5* (300 mg) in acetic acid (60 ml) and of $NaIO_4$ (920 mg) in 60 % aqueous acetic acid (60 ml) were mixed and after 30 min the mixture was extracted with chloroform (3 × 40 ml). The residue obtained on evaporation of the combined extracts was chromatographed (silica gel, benzene-ethyl acetate, 4:1) giving p-*benzoquinone* in a yield of 28 % and unreacted *5* (62 %). (b) $NaBiO_3$ (1.2 g) was added to a stirred solution of *5* (300 mg) in 80 % aqueous acetic acid (60 ml). After 30 min, the filtered solution was worked up as described under (*a*), affording p-*benzoquinone* (22 %) in addition to unreacted *5* (65 %).

Treatment of 5 with H_5IO_6 in aqueous solution. Aqueous solutions of *5* (300 mg, 15 ml) and H_5IO_6 (1.1 g, 80 ml) were mixed and the mixture was extracted after 17 h with 3 × 100 ml of chloroform. The combined extracts, after being dried and evaporated, gave a residue which was purified by sublimation (25 °C, 0.05 mmHg) to give p-*benzoquinone* in a yield of 85 %. In a separate experiment, formaldehyde present in the reaction mixture was converted into the dimedone compound; yield, 80 %.

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Periodate Oxidation of Phenols. XV.* Oxidation of 3,5-Dimethyl- and 2,5-Dimethyl-4-hydroxybenzyl Alcohols

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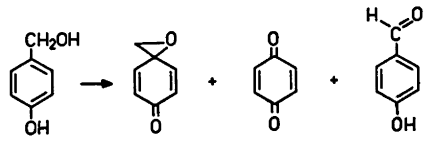
Oxidation of 4-hydroxy-3,5-dimethylbenzyl alcohol (*5*) in aqueous acetic acid with periodate gave 2,6-dimethyl-1,4-benzoquinone (*6*), 4-hydroxy-3,5-dimethylbenzaldehyde (*7*), and 6-acetoxy-4-hydroxymethyl-2,6-dimethyl-2,4-cyclohexadienone (*8*), whereas only *6* and *7* were obtained when bismuthate was used as oxidant. Preferential *para* oxidation by the latter reagent was also observed when mesitol was used as substrate.

When *5* was oxidized with periodate in H_2O/EtOH (60:5), a Diels-Alder adduct (*20*) of *o*-quinol *21* with simultaneously formed *p*-quinone *6* was obtained, in addition to *o*-quinol dimer *22* and aldehyde *7*. Under similar conditions, 4-hydroxy-2,5-dimethylbenzyl alcohol (*25*) yielded the corresponding *p*-benzoquinone *26* and aldehyde *27*, as well as the dimeric *o*-quinol *29* which in the solid state had the structure of a cyclic hemiketal (*29a*).

In a preceding paper,[1] oxidation of 4-hydroxybenzyl alcohol (*1*) with sodium periodate in 80 % aqueous acetic acid was reported to give



1 *2* *3* *4*

p-benzoquinone (*3*) and 4-hydroxybenzaldehyde (*4*), as well as considerable amounts of polymeric material. If sodium bismuthate was used as oxidant, the spiro(oxirane-2,5-cyclohexadienone) *2* was formed in addition to *3* and *4*.

In the present study the behaviour of 4-hydroxy-3,5-dimethylbenzyl alcohol (*5*) towards the two oxidants has been examined using aqueous acetic acid as solvent. Unlike the unsubstituted 4-hydroxybenzyl alcohol, *5* failed to give a spiro compound on treatment with bismuthate, but certain differences in the ratio of *ortho* and *para* oxidation products formed by periodate and bismuthate, respectively, were observed. For comparison, the oxidation of 2,4,6-trimethylphenol (*15*) in the same solvent has been investigated. Furthermore, the oxidation of the 3,5- and 2,5-dimethyl derivatives of *1* with aqueous periodate, in the absence of acetic acid, has been studied.

Oxidation in aqueous acetic acid

4-Hydroxy-3,5-dimethylbenzyl alcohol (5) (Chart 1). Both periodate and bismuthate produced 2,6-dimethylbenzoquinone (*6*) and 4-hydroxy-3,5-dimethylbenzaldehyde (*7*), *i.e.*, the dimethyl homologues of the products (*3*, *4*) formed on similar oxidation of unsubstituted 4-hydroxybenzyl alcohol.[1] With periodate as oxidant a third product, *viz.* the *o*-quinol acetate *8*, was obtained. The total yields of identified products formed after a reaction time of 30 min, which was sufficient for the starting material (*5*) to be completely consumed, were considerably higher than those obtained from *1*, since in the latter case large amounts of amorphous material were formed.[1]

The spiro compound *11* could not be detected in the bismuthate reaction mixture, although

* Part XIV, see Ref. 1.

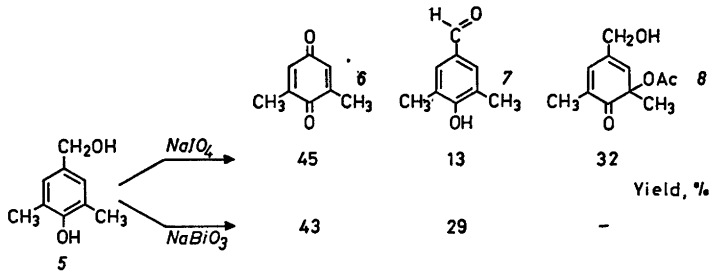


Chart 1.

its formation in the bismuthate system had been expected, since the parent compound *2* had been obtained from *1* in the same system.[1] The failure of the phenol alcohol *5* to give *11* may possibly be understood as shown in Chart 2.

Assuming that the initially formed aryl bismuthate *9* decomposes by two-electron transfer to give the phenoxonium ion *10*, the latter could undergo ring closure to provide the spiro-oxirane *11*. The same result would be obtained if *9* undergoes concerted ring closure and loss of a trivalent bismuth species (dashed line arrow). The unsubstituted analogue *2* of the spiro compound *11* has been found to be slowly hydrolyzed by 80 % aqueous acetic acid, the reaction being complete after about 24 h at room temperature.[1] In the present case, however, the two allylic methyl groups may stabilize the cation *10*, thus favouring reaction *11*→*10* and increasing the rate of hydrolysis. The resulting *p*-quinol *12* would lose formaldehyde to give 2,6-dimethylhydroquinone (*13*) which would be oxidized to the quinone *6* (*cf.* Ref. 1).

The second reaction product (*7*) can be assumed to arise by loss of a proton from the carbinol C-atom of *10* or by a corresponding concerted reaction of *9* (dotted line arrow) followed by rearrangement of the resulting enol *14*.

The fact that *o*-quinol acetate (*8*) is formed in the periodate system but not in the bismuthate system and, furthermore, the comparatively high yield of aldehyde *7* obtained with bismuthate (Chart 1) seemed to indicate that bismuthate preferentially acts as a *para*-oxidizing agent. With the aim of obtaining further support for this view, the following experiments with 2,4,6-trimethylphenol (mesitol) were carried out.

2,4,6-Trimethylphenol (15) (Chart 3). When treated in 80 % aqueous acetic acid with

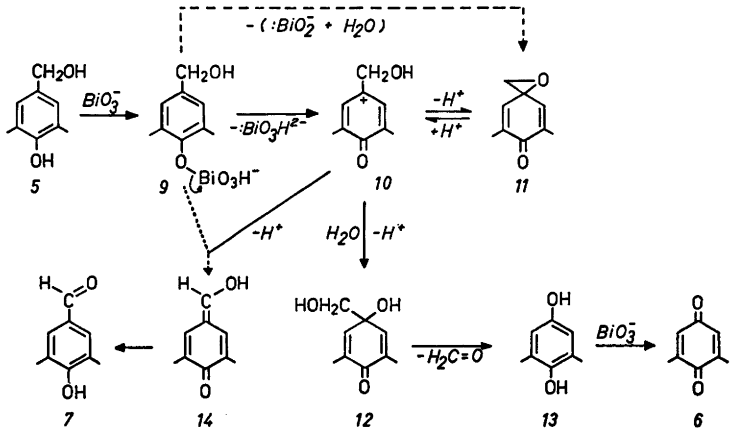


Chart 2.

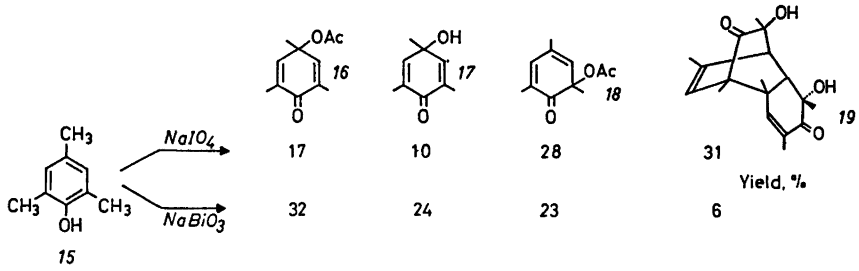


Chart 3.

periodate or bismuthate for 30 min, products *16* – *19* were obtained in either case. However, with periodate as oxidant, the ratio of *ortho*/*para* oxidation products was 59/27, whereas almost the inverse ratio (29/56) was found for the bismuthate system.

Oxidation in aqueous ethanol

In earlier work on the periodate oxidation of 2-methyl- and 2-hydroxymethyl-substituted phenols the reaction had been carried out in aqueous [2-4] or aqueous ethanolic [5] solution and therefore it was of interest to examine the behaviour of the 4-hydroxybenzyl alcohols *5* and *25* under similar conditions. The solvent mixture used in this study contained a small amount of ethanol which was sufficient to keep the starting material, as well as the reaction products, in solution.

4-Hydroxy-3,5-dimethylbenzyl alcohol (5) (Chart 4). Treatment of *5* with periodate gave, in addition to *p*-quinone *6* and aldehyde *7*, which also had been obtained when aqueous acetic acid was used as solvent, two compounds, $C_{17}H_{20}O_5$ and $C_{18}H_{24}O_6$, melting at 129 – 130 °C and 179 – 180 °C, respectively.

The lower-melting yellow compound, $C_{17}H_{20}O_5$, was assigned the structure of a Diels-Alder adduct (*20*) between *o*-quinol *21* and *p*-quinone *6*. This structure is based on the following spectral and chemical evidence.

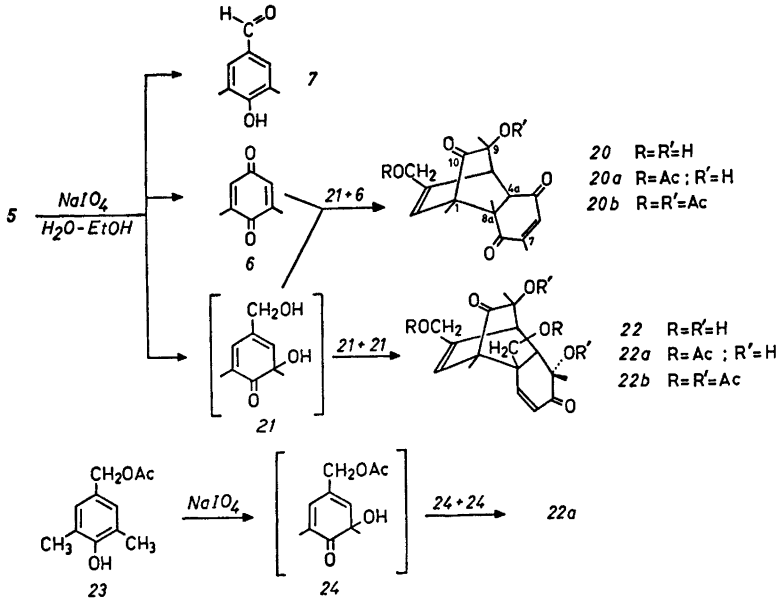


Chart 4. (*Note added in proof*: Formula *22* – *22b* should carry a CH_3 group in position 7.)

The ethanolic solution of *20* showed strong UV absorption with maxima at 215 and 234 nm, as well as low-intensity absorption at 305 nm (sh) and 375 nm. The absorption band at 215 nm can be attributed to transannular charge transfer between the 2,3-ethylenic bond and the C-10 keto group; strong absorption at a similar wavelength has previously been found to be characteristic of the β,γ-conjugated carbonyl system present in dimeric *o*-quinols.[5,6] The maxima at 234, 305, and 375 nm can be ascribed to the enedione system present in the dienophile moiety of the molecule; similar absorption has been reported to be typical of enediones.[7-9] In the IR spectrum of *20* the conjugated CO groups are reflected by an absorption band at 1660 cm^{-1}, whereas the isolated CO group gives rise to a peak at 1723 cm^{-1}.

The NMR spectrum of the compound was in full agreement with structure *20* (see Experimental).

Treatment of *20* with Ac_2O/pyridine, which does not acetylate tertiary hydroxyl groups, gave the monoacetate *20a*, whereas the diacetate *20b* was obtained with $Ac_2O/HClO_4$.[10]

The product $C_{18}H_{24}O_6$, m.p. 179–180 °C, was colourless and had the spectral properties expected for an *o*-quinol dimer of structure *22*. A diacetate (*22a*) was formed with Ac_2O/pyridine and a tetraacetate (*22b*) was obtained from either *22* or *22a* with $Ac_2O/HClO_4$, which is in accord with this structural assignment. Diacetate *22a* was also formed, *via* the intermediary *o*-quinol *24*, on periodate oxidation of 4-hydroxy-3,5-dimethylbenzyl acetate (*23*).

The dimeric *o*-quinol *22* was obtained in a yield of 14 % and the *o*-quinol-*p*-quinone adduct *20* in a yield of 33 %. The formation of these compounds at room temperature illustrates the high diene reactivity of *o*-quinols.

4-Hydroxy-2,5-dimethylbenzyl alcohol (*25*) (Chart 5). Periodate was consumed at a considerably lower rate by *25* than by its isomer *5*, which is in analogy with the exceptionally sluggish reaction of 2,5-dimethylphenol as compared with other phenols, such as 2,6-dimethylphenol.[4] Expectedly, *25* gave the *p*-benzoquinone *26* and the aldehyde *27*. In addition, a colourless compound of m.p. 203–204 °C was isolated. Its composition, $C_{18}H_{24}O_6$, seemed to indicate the compound to be a Diels-Alder dimer (*29*) of the intermediary *o*-quinol *28*. The IR spectrum of the substance (in KBr) revealed the presence of the conjugated keto group at C-6, but, surprisingly, the expected peak due to the keto group at C-10, which in dimeric *o*-quinols is found within the range of 1710–1725 cm^{-1}, was missing. It was supposed, therefore, that the latter keto group was masked by the formation of a hemiketal with the hy-

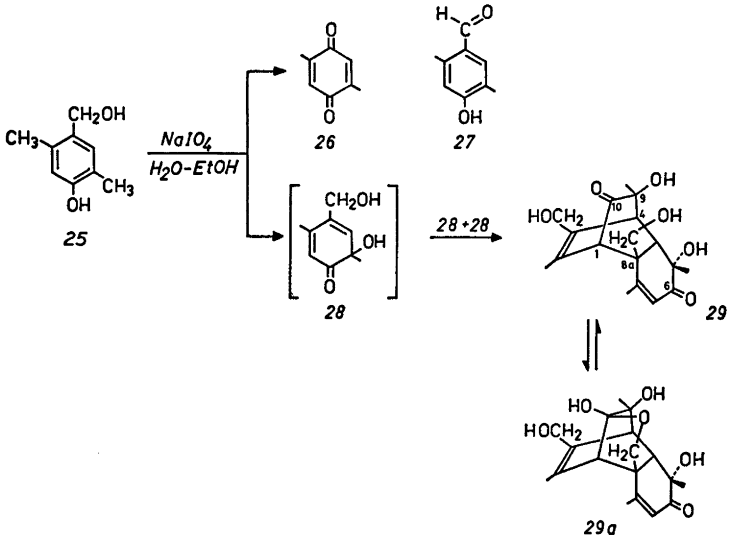


Chart 5.

droxyl group of the substituent at C-8a. In fact, the IR spectrum of the compound dissolved in DMSO, ethanol or dioxan showed peaks due to both the conjugated and the isolated keto groups. Structure *29a* is therefore ascribed to the compound in the solid state and the structure of a dimeric *o*-quinol (*29*) to the substance dissolved in a polar solvent ("ring-chain tautomerism").

No *o*-quinol-*p*-quinone adduct of type *20* was detected in the reaction mixture obtained from phenol *25*.

Structural and stereochemical orientation in dimers *22* and *29* and in adduct *20*

The structural and stereochemical specificity characteristic of the Diels-Alder dimerisation of 2,4-cyclohexadienones [4,6,11] is illustrated again by the fact that only a single *o*-quinol dimer (*22* and *29*, respectively) has been detected in the oxidation of each of the phenols *5* and *25*. Similarly, the Diels-Alder reaction *21*+*6* gives a single adduct (*20*).

The assignment of the structural orientation given in formulae *20* and *22* is based on the NMR spectra of these compounds. They showed the patterns required for coupling between the vicinal protons at C-4 and C-4a as well as between the protons at C-4 and C-2 (for *20*: $J_{4,4a}=2.2$ Hz, $J_{4,2}=2.0$ Hz; for *22*: $J_{4,4a}=2.1$ Hz, $J_{4,2}=2.0$ Hz. See also Experimental).

The dimers *22* and *29*, as well as adduct *20*, were stable towards periodate, in spite of the presence of the 9,10-hydroxyketone grouping. As discussed in Part XII,[6] this can be interpreted to be due to the steric orientation of the 9-OH group as shown in the formulae, in combination with the presence of the CH_2OH or the CH_3 substituent, respectively, at C-8a. Although there is no direct evidence for the configuration at C-5 to be that given in formulae *22* and *29*, its correctness is highly probable since it is favoured if steric approach control is operative in the dimerization and since it actually has been established for other dimeric *o*-quinols.[6,11]

The formation of hemiketal *29a* constitutes proof of the *endo* configuration of this dimer. *Endo* configuration has also been assumed above for dimer *22*, as well as for adduct *20*. This seems justified because the dimerization of *o*-quinols has been shown earlier to follow the *endo* addition rule.[4,11,12]

EXPERIMENTAL

UV spectra were recorded on a Cary Model 14 spectrophotometer; IR and NMR spectra were obtained using Beckman 9 A and Varian A-60 instruments, respectively. Chemical shifts are given in δ (ppm units, TMS being used as internal standard).

4-Hydroxy-3,5-dimethylbenzyl alcohol (*5*), the corresponding benzyl acetate (*23*), and 4-hydroxy-2,5-dimethylbenzyl alcohol (*25*) were prepared according to Refs. 13, 14, and 15, respectively.

Oxidation with periodate in aqueous acetic acid. A solution of phenols *5* or *15* (0.03 mol) in acetic acid (320 ml) was mixed with a solution of $NaIO_4$ (0.06 mol) in a mixture of acetic acid (80 ml) and water (100 ml). After 30 min (room temperature) ethylene glycol (5 ml) was added to remove excess periodate and the mixture was extracted with three 150 ml portions of dichloromethane. The combined extracts were washed with aqueous bicarbonate, dried over Na_2SO_4 and brought to dryness. The resulting residue was chromatographed on a silica gel column (4 × 60 cm) using benzene/ethyl acetate (4:1) as eluent.

Periodate oxidation products obtained from 4-hydroxy-3,5-dimethylbenzyl alcohol (5). The eluate fractions gave:

(a) 2,6-Dimethyl-1,4-benzoquinone (6), $R_F=0.43$; yield, 45 %. M.p., after sublimation, 69–71 °C (Lit.[16] 72–73 °C). The IR spectrum of the compound was in accord with that reported for *6*.[17]

(b) 6-Acetoxy-4-hydroxymethyl-2,6-dimethyl-2,4-cyclohexadienone (8). $R_F=0.38$, yellow oil. Yield, 32 %. (Found: *M*, by mass spectrometry, 210.0902. Calc. for $C_{11}H_{14}O_4$: *M* 210.0892.) IR (KBr): ν_{max}, cm^{-1} 1668 (conj. CO), 1740 (ester CO), 3460 (OH). NMR ($CDCl_3$): δ 1.36 (s, 3 H, CH_3), 1.95 (broad s, 3 H, olefinic CH_3), 2.07 (s, 3 H, CH_3COO), 4.20 (d, 2 H, CH_2), 5.33 (1 H, OH), 6.06 and 6.83 (multiplets, 1 H each, 2 olefinic H).

(c) 4-Hydroxy-3,5-dimethylbenzaldehyde (7), $R_F=0.29$; yield, 13 %. M.p., after recrystallization from benzene, 113–114 °C (Lit.[18] 114–115 °C). The IR spectrum of the compound was in accord with that reported for *7*.[19]

Periodate oxidation products obtained from mesitol (15). The eluate fractions gave:

(a) 6-Acetoxy-2,4,6-trimethyl-2,4-cyclohexadienone (18), $R_F=0.47$; yield, 28 %. Yellow crystals, m.p. 80–82 °C (Lit.[20] 82–84 °C)

(b) 4-Acetoxy-2,4,6-trimethyl-2,5-cyclohexadienone (16), $R_F=0.43$. Pale yellow oil;[20] yield, 17 %. NMR ($CDCl_3$): δ 1.51 (s, 3 H, CH_3-4),

1.89 (s, 6 H, CH_3-2 and CH_3-6), 2.00 (s, 3 H, CH_3COO), 6.69 (s, 2 H, H-3 and H-5).

(*c*) *4-Hydroxy-2,4,6-trimethyl-2,5-cyclohexadienone (17)*, $R_F = 0.14$; yield, 10 %. M.p. 45–46 °C (Lit.[21] 45.5–46 °C). The IR and NMR spectra of the compound were in agreement with those expected for structure *17*.

(*d*) *1,4a,5,8a-Tetrahydro-5,9-dihydroxy-1,3,5,7,8a,9-hexamethyl-1,4-ethanonaphthalene-6,10 (4H)-dione (19)*, $R_F = 0.09$, yield, 31 %. Identical by m.p. and mixed m.p. with an authentic sample.[5]

Oxidation with bismuthate in aqueous acetic acid. Sodium bismuthate ($NaBiO_3$, 0.06 mol) was added to a stirred solution of phenol *5* or *15* (0.03 mol) in 300 ml of acetic acid/water (4:1). After 30 min the mixture was filtered and the filtrate worked up as described for the periodate procedure.

Phenol *5* gave *2,6-dimethyl-1,4-benzoquinone (6)* in a yield of 43 % and *4-hydroxy-3,5-dimethylbenzaldehyde (7)* in a yield of 29 %.

Mesitol (*15*) gave o-*quinol acetate* 18 (23 %); p-*quinol acetate* 16 (32 %), p-*quinol* 17 (24 %) and *dimeric* o-*quinol* 19 (6 %).

Periodate oxidation of 4-hydroxy-3,5-dimethylbenzyl alcohol (5) in aqueous ethanol. A solution of $NaIO_4$ (3.84 g, 18 mmol) in water (150 ml) was added to a solution of *5* (1.80 g, 12 mmol) in 500 ml of ethanol-water (1:10). After 16 h (room temp.) the mixture was extracted with 3 × 200 ml of hexane. The combined extracts gave *2,6-dimethyl-1,4-benzoquinone (6)*; yield, 21 %.

The aqueous phase was extracted with 5 × 400 ml of ethyl acetate. The combined extracts were dried over Na_2SO_4 and evaporated, and the residue obtained was chromatographed on a silica gel column using acetone/hexane (2:1) as eluent. The eluate fractions gave:

(*a*) *4-Hydroxy-3,5-dimethylbenzaldehyde (7)*, $R_F = 0.60$; yield, 6 %.

(*b*) *1,4,4a,8a-Tetrahydro-9-hydroxy-3-hydroxymethyl-1,7,8a,9-tetramethyl-1,4-ethanonaphthalene-5,8,10-trione (20)*, $R_F = 0.53$; yield, 33 %. Yellow crystals, m.p. 129–130 °C (ethyl acetate/hexane). (Found: C 66.7; H 6.5. Calc. for $C_{17}H_{20}O_5$: C 67.1; H 6.6). UV (ethanol): λ_{max}, nm (log ε) 215 (4.05), 234 (4.05), 305 sh (2.70), 375 (2.12). IR (KBr): ν_{max}, cm^{-1} 1635 (C=C), 1660 (conj. CO), 1723 (CO), 3330, 3460 and 3540 (OH). NMR ($CDCl_3$): δ 1.37, 1.40 and 1.42 (singlets, 3 H each, 3 CH_3), 2.10 (d, 3 H, CH_3-7), 2.60 (broad, 2 H, 2 OH, exchangeable with D_2O), 3.31 (t, 1 H, H-4), 3.62 (d, 1 H, H-4a), 4.20 (d, 2 H, CH_2), 5.79 (m, 1 H, H-2), 6.71 (quartet, 1 H, H-6).

(*c*) *1,4a,5,8a-Tetrahydro-5,9-dihydroxy-3,8a-bis(hydroxymethyl)-1,5,7,9-tetramethyl-1,4-ethanonaphthalene-6,10(4H)-dione (22)*, $R_F = 0.42$; yield, 14 %. Colourless crystals, m.p. 179–180 °C (benzene). (Found: C 64.25; H 7.16. Calc. for $C_{18}H_{24}O_6$: C 64.28; H 7.19). UV (ethanol): λ_{max}, nm (log ε) 210 (3.94), 240 sh (3.78), 310 sh (2.48). IR (KBr): ν_{max}, cm^{-1} 1650 (C=C), 1690 (α,β-conj. CO), 1718 (CO), 3400 (broad, OH). NMR (DMSO-d_6): δ 1.09, 1.12 and 1.25 (singlets, 3 H each, 3 CH_3), 1.70 (s, 3 H, CH_3-7), 2.81 (d, 1 H, H-4a), 2.93 (t, 1 H, H-4), 3.50 and 3.83 (broad signals, 2 H each, 2 CH_2), 4.67 and 5.25 (broad signals, 1 H each, 2 prim. OH), 4.82 and 5.41 (singlets, 1 H each, 2 tert. OH), 5.02 (m, 1 H, H-2), 6.19 (broadened s, 1 H, H-8).

Monoacetate 20a. From *20* by treatment with Ac_2O/pyridine. The crude solid obtained was treated with a small amount of diethyl ether and the yellow crystalline product collected. M.p. 106–107 °C (ethyl acetate/hexane). (Found: C 65.67; H 6.53. Calc. for $C_{19}H_{22}O_6$: C 65.88; H 6.40). IR (KBr): ν_{max}, cm^{-1} 1644 (C=C), 1670 (conj. CO), 1722 (CO), 1740 (ester CO), 3450 (OH).

Diacetate 20b. From *20* by treatment with $Ac_2O/HClO_4$.[10] Yellow crystals of m.p. 115–116 °C (ethanol). (Found: C 64.97; H 6.08. Calc. for $C_{21}H_{24}O_7$: C 64.93; H 6.22). IR (KBr): ν_{max}, cm^{-1} 1626 (C=C), 1661 (conj. CO), 1722 (CO), 1740 (ester CO).

Diacetate 22a. (*a*) From *22* by treatment with Ac_2O/pyridine, m.p. 168–169 °C (methanol). (Found: C 62.98, H 6.83. Calc. for $C_{22}H_{28}O_8$: C 62.89; H 6.71). IR (KBr): ν_{max}, cm^{-1} 1638 (C=C), 1673 (α,β-conj. CO), 1728 (CO), 1746 (broad, ester CO), 3470 and 3530 (*tert.* OH).

(*b*) A solution of 4-hydroxy-3,5-dimethylbenzyl acetate (*23*) (2.91 g, 15 mmol) in a mixture of acetic acid (100 ml) and water (300 ml) was mixed with a solution of $NaIO_4$ (4.91 g, 23 mmol) in water (100 ml). After 90 min the solution was extracted with 5 × 75 ml of dichloromethane. The residue obtained from the combined extracts was treated with a few milliliters of ether. The crystalline product formed (yield, 34 %) was identical by m.p. and mixed m.p. with *22a* prepared according to (*a*).

Tetraacetate 22b. From *22* or *22a* by treatment with $Ac_2O/HClO_4$,[10] m.p. 166–167 °C (methanol). (Found: C 61.8; H 6.5. Calc. for $C_{26}H_{32}O_{10}$ C 61.9; H 6.4). IR (KBr): ν_{max}, cm^{-1} 1708 (α,β-conj. CO), 1732 (CO), 1748 (broad, ester CO).

Periodate oxidation of 4-hydroxy-2,5-dimethylbenzyl alcohol (25) in aqueous ethanol. A solution of $NaIO_4$ (10.3 g, 48 mmol) in water (300 ml) was added to a solution of *25* (1.80 g, 12 mmol) in a mixture of ethanol (400 ml) and water (1300 ml). After 72 h (room temp.) the solution was extracted with 3 × 300 ml of hexane. The combined hexane phases gave *2,5-dimethyl-1,4-benzoquinone (26)* in a yield of 22 %. After sublimation (0.05 mmHg, 30 °C) the compound melted at 121–122 °C (Lit.[16] 124–124.5 °C). The IR spectrum of the compound was in accord with that reported for *26*.[17]

The aqueous phase was further extracted with 5 × 400 ml of ethyl acetate. The combined extracts were dried over Na_2SO_4 and evaporated, leaving a dark-coloured oil which was dissolved

in a few milliliters of acetone. A crystalline product deposited, which was recrystallized from acetone to give the *hemiketal* 29a of *1,4a,5,8a-tetrahydro-5,9-dihydroxy-3,8a-bis(hydroxymethyl)-2,5,8,9-tetramethyl-1,4-ethanonaphthalene-6,10(4H)-dione (29)*, m.p. 203–204 °C. Yield, 14 %. (Found: C 64.34; H 7.20. Calc. for $C_{18}H_{24}O_6$: C 64.28; H 7.19). UV (ethanol): λ_{max}, nm (log ε) 210 (3.73), 236 (3.70), 312 sh (2.22). IR (KBr): ν_{max}, cm^{-1} 1630 (C=C); 1680 (α,β-conj. CO), 3400 (broad, OH). IR (DMSO-d_6): ν_{max}, cm^{-1} 1630 (C=C), 1684 (α,β-conj. CO), 1722 (CO). NMR (DMSO-d_6): δ 1.11, 1.40 and 1.43 (singlets, 3 H each, 3 CH_3), 1.96 (broadened s, 3 H, CH_3-8), 2.75 (s, 1 H, H-1), 2.91 and 3.21 (doublets, 1 H each, H-4 and H-4a), 3.62 and 3.74 (broad signals, 2 H each, 2 CH_2), 4.68 and 5.12 (broad signals, 1 H each, 2 prim. OH), 4.83 and 5.48 (singlets, 1 H each, 2 *tert.* OH), 5.84 (s, 1 H, H-7). $J_{4,4a}$ = 2.0 Hz.

The acetone filtrate was chromatographed on a silica gel column using benzene/ethyl acetate (4:1) as eluent. *4-Hydroxy-2,5-dimethylbenzaldehyde (27)*, R_F = 0.31, was isolated in a yield of 5 %. After recrystallization from benzene, m.p. 129–130 °C (Lit.[22] 132–133 °C).

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Non-bonded Oxygen—Oxygen Interactions in 2,4,10-Trioxaadamantane and 1,3,5,7,9-Pentoxecane

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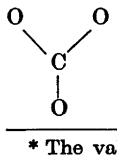
Enthalpies of combustion, utilizing a micro combustion calorimetric technique, and enthalpies of sublimation have been determined for 2,4,10-trioxaadamantane and 1,3,5,7,9-pentoxecane (pentoxane). Standard enthalpies of formation for the compounds in the gaseous state, ΔH_f°(g), have been derived and are $-(499.2 \pm 2.2)$ and $-(779.8 \pm 1.3)$ kJ mol^{-1}, respectively. The calculated stabilization energy due to next-nearest-neighbour oxygen-oxygen interaction in trioxaadamantane, relative to adamantane and aliphatic monoethers, is 53 kJ mol^{-1}. The gaseous enthalpy of formation per $-CH_2O-$ unit in pentoxane is very nearly the same as that in trioxane and tetroxane.

One pair of next-nearest-neighbour oxygen atoms in an aliphatic straight-chain compound gives rise to a stabilization energy as large as 17.$_6$ kJ mol^{-1} relative to monoethers,* whereas the stabilization in an $-O-CH_2-O-CH_2-O-$ arrangement, within experimental uncertainties, is twice that of one pair.[1] Addition of another $-CH_2-O-$ group likewise increases the total stabilization energy to three times that for a single pair.[1] This means that consecutive pairs of next-nearest-neighbour oxygens in straight-chain compounds give rise to the same stabilization energy per pair even though some of the oxygens are simultaneously engaged in two interactions. The orthoester arrangement



in 2,4,10-trioxaadamantane can similarly be looked upon as a three pair oxygen-oxygen next-nearest-neighbour interaction system. Does the fact that the interacting oxygens are all attached to the same carbon atom, and moreover rather rigidly locked into fixed positions, have a marked influence on the resulting stabilization energy in this tricyclic ortho ester?

In s-trioxane (1,3,5-trioxacyclohexane) the stabilization from the three pairs of oxygen atoms appears to be only 28 kJ mol^{-1}, when the aliphatic ether increment is used in the calculations.[1,3] However, if the ether increment is instead evaluated from the enthalpies of formation for gaseous cyclohexane and tetrahydropyran, the calculated stabilization energy in trioxane due to the oxygen-oxygen interactions becomes 42 kJ mol^{-1}. This is a good illustration of the importance of clearly defining the reference system in calculations and discussions of thermochemical stabilization or destabilization (strain) energies. In the study of trioxane and the formaldehyde tetramer tetroxane it was found that the gaseous enthalpies of formation per $-CH_2O-$ unit in the two compounds were not significantly different.[3]

In this paper energy of combustion and enthalpy of sublimation measurements are reported for 2,4,10-trioxaadamantane (2,4,10-trioxatricyclo[3.3.1.1^{3,7}]decane) and pentoxane (1,3,5,7,9-pentoxecane). A recently developed micro-bomb (4.5 cm^3) combustion calorimeter together with an ampoule technique for milligram quantities of material has been used in the combustion studies.[4]

* The value given in Ref. 1 is 16.9 kJ mol^{-1}. A recalculation with parameters for the ether increment taken from Ref. 2 yields 17.6 kJ mol^{-1}.

EXPERIMENTAL

Compounds. 2,4,10-Trioxaadamantane, a gift from Dr. Kjell Olsson, The Royal Agricultural College, Uppsala, Sweden, was synthesized from *cis,cis*-cyclohexane-1,3,5-triol and excess triethylformate in methanolic HCl.[5] The crude product was recrystallized from petroleum ether and the obtained crystals then sublimed at approximately 10 Torr * and 323 K to remove traces of solvent. A concentrated ether solution of purified material was analyzed by GLC on Apiezon, silicone (SE-30), and Carbowax columns. No trace of any impurity could be found. The above described material was used in series A. However, water analysis by a coulometric method[6] indicated 0.07_3 mass per cent of water. A second preparation of trioxaadamantane was therefore sublimed through activated molecular sieves. The dried material was handled in a small plastic glove bag, which had been flushed several times with dry nitrogen and which also housed a large dish with freshly activated molecular sieves. Pellets for the combustion experiments of series B were made inside the glove bag whereas when pellets for series A were prepared no real precautions to avoid moisture had been taken (relative humidity 25 – 30 %). However, the water content of the second preparation was determined to be 0.06_9 mass per cent. Evidently "dry conditions" have to be maintained much more rigorously when one wants to prevent contamination by water while working with milligram quantities of "slightly hygroscopic"[5] materials. A correction for $0.07_0 \pm 0.00_5$ mass per cent of water has been applied to the measured energy of combustion of trioxaadamantane.

The 1,3,5,7,9-pentoxecane (pentoxane) sample was a gift from Dr. Yasuhiko Miyake, Mitsui-Toatsu Chemicals, Inc., Japan. The melting point of the sample, determined on a Perkin-Elmer DSC-1B differential scanning calorimeter, was $335._3$ K; literature value 334 ± 1 K.[7] No impurities were found when a concentrated ether solution of pentoxane was carefully analyzed by GLC on silicone (SE-30) and Carbowax columns. The NMR and mass spectra were in good agreement with those reported in the literature.[8,9] Water analysis,[6] performed on a small sample that had been well exposed to laboratory air, indicated ≤ 0.01 mass per cent of water. No correction for water in pentoxane has been applied.

The Delrin ** (polyoxymethylene) ampoules used in this investigation were not manufactured from the same rod as those referred to in Ref. 4. A redetermination of the energy of combustion of this particular polyoxymethylene material was therefore required. The ampoules were cleaned and conditioned as described earlier.[4]

The benzoic acid used in calibration experiments was National Bureau of Standards SRM 39i.

Combustion calorimetry. The micro combustion calorimeter, the calorimetric and calculational procedures, and the ampoule technique have been described previously.[4] The amount of water initially in the bomb (internal bomb volume 4.50 cm^3) was 20 mm^3. The initial pressure of oxygen, $p^i(O_2)$, varied between 34.6 and 37.1 atm * in the trioxaadamantane experiments; for the pentoxane runs it varied from 35.9 to 37.5 atm. The oxygen was 99.995 % pure and in combination with an efficient flushing procedure, this resulted in negligible corrections for the formation of HNO_3 during combustions.[4] Electrical calibrations were performed after each combustion experiment.

In both series of measurements on trioxaadamantane ($p_{25} \sim 3 \times 10^{-2}$ Torr **) the compound was enclosed in thin-walled polyoxymethylene ampoules.[4] An unusually large number of failures was encountered in the combustions on trioxaadamantane, possibly due to air being trapped in the pellets. Four out of twelve runs were rejected because some trace of soot on the bomb wall indicated incomplete combustion. Five successful runs were performed in series A and three in series B.

Five combustions were performed with pentoxane ($p_{25} \sim 2 \times 10^{-2}$ Torr **) enclosed in ampoules; one was rejected. Four experiments were carried out where somewhat larger pellets of pentoxane were burned without protection. The mass loss of these pellets was determined as a function of time to allow an appropriate correction to be applied for material evaporated during assembly and closing of the bomb. 6 ± 1 μg evaporated in 10 min, while the time for mounting the crucible and closing the bomb was less than four min. In the pentoxane as well as in the trioxaadamantane series all the included successful runs were without any trace of soot.

The Delrin ampoules were weighed on a Sartorius type 4125 electronic micro balance, the linearity of which has been verified. The sensitivity was checked several times during a series of weighings. A Mettler M5 micro balance, carefully calibrated against standard weights,[4] was used when the pelleted samples were weighed either into closed ampoules or in the open crucible. The sensitivity of the Mettler balance was checked before and after each series of weighings.

All weighings have been reduced to masses and molar masses computed from the 1969 table of atomic weights.[10] The energy equiva-

* Torr = (101.325/760) kPa

** "DELRIN acetal resin"; reg. trade name. E. I. du Pont de Nemours & Co. (Inc.), U.S.A.

* atm = 101.325 kPa. ** Estimated from enthalpy of sublimation measurements.

Table 1. Auxiliary quantities used in the calculations.

Compound	ϱ/g cm^{-3}	$(\partial v/\partial T)_p$/mm^3 K^{-1} g^{-1}	c_p/J K^{-1} g^{-1}
2,4,10-Trioxaadamantane	1.1	0.39	1.3
1,3,5,7,9-Pentoxecane	1.48[a]	1.7	1.14[b]
Delrin	1.43	1.0	1.3

[a] Ref. 9. [b] Ref. 7.

Table 2. Summary of typical combustion experiments.[a] Δu_c°(Delrin) = $-(17118._0 \pm 6._4)$ J g^{-1}.[b,c]

	2,4,10-Trioxaadamantane		1,3,5,7,9-Pentoxecane	
	Series A	Series B	In ampoules	"Pellets"
m(comp)/mg	8.526	9.078	9.437	21.492
m(Delrin)/mg	4.341	5.248	4.524	0
ε(calor,b.a.)/J K^{-1}	583.057 $\pm$ 0.093[c]	582.930[d] $\pm$ 0.076[c]	582.908 $\pm$ 0.098[c]	582.908 $\pm$ 0.098[c]
ε^i(cont)/J K^{-1}	0.295	0.288	0.295	0.284
$\Delta\theta$/K	0.49880_5	0.54958_0	0.40607_4	0.62181_3
ΔU_{ign}/J	0.212	0.202	0.189	0.206
ΔU_Σ/J	0.231	0.248	0.201	0.315
$-\Delta u_c^\circ$(comp)/J mg^{-1}	25.3609	25.3624	16.8477	16.8488

[a] For explanation of symbols *cf.* Ref. 11. [b] At 30 % relative humidity. [c] Uncertainties are standard deviations of the mean. [d] Direct calibration with benzoic acid.

Table 3. Results of combustion experiments at 298.15 K. $-\Delta u_c^\circ$/kJ g^{-1}

	2,4,10-Trioxaadamantane		1,3,5,7,9-Pentoxecane	
	Series A	Series B	In ampoules	"Pellets"
	25.3561	25.3624	16.8477	16.8416
	25.3609	25.3313	16.8587	16.8488
	25.3770	25.3481	16.8128	16.8442
	25.3825		16.8228	16.8486
Mean:	25.3691[a]	25.3473[a]	16.8355	16.8458
Standard deviation of the mean:	0.0063	0.0090	0.0107	0.0018
Final overall standard deviation of the mean:	0.0087	0.0105	0.0115	0.0035

[a] Not corrected for $0.07_0 \pm 0.00_5$ mass per cent of water.

lent, ε(calor), of the calorimetric system was calculated from the mean value of pertinent electrical calibrations, values of ε^i(cont) and the experimentally established relation between benzoic acid and electrical calibrations: ε(calor,b.a.) = ε(calor,el.) + (0.060 ± 0.070) J K^{-1}. The corrections to standard states, ΔU_Σ, were calculated using a computer program based on the procedure by Hubbard *et al.*[11] Some auxiliary quantities used in the calculations are listed in Table 1. The final overall precision of the ΔU_c° mean values was estimated as recommended by Rossini.[12] The reference temperature of the combustion experiments is 298.15 K.

Vaporization calorimetry. The enthalpies of sublimation at 298.15 K were measured using

the Morawetz calorimeter.[13] The trioxaadamantane sample was sublimed into the calorimeter. Four experiments were performed following the procedure described in Ref. 13 with around 6 mg evaporated per experiment. A molten sample of pentoxane was dropped from a heated pipette into the evaporation pan of the calorimeter. Seven experiments were performed with on the average 7 mg evaporated per experiment.

RESULTS AND DISCUSSION

In Table 2 detailed results for some typical combustion experiments are given, one from each of the four series of measurements. Values of $-\Delta u_c°$, referring to unit mass, for the individual runs are listed in Table 3. One of the trioxaadamantane values in series A has been discarded on statistical grounds. The calculated mean value for each series together with the standard deviation of the mean and the estimated final overall uncertainty are included in Table 3. No significant difference was found between the results from the two different series of measurements on each compound. Therefore the mean values were weighted proportionately to the inverse squares of their overall uncertainties. The obtained $\Delta u_c°$ values were $-(25.360_1 \pm 0.013_4)$ and $-(16.844_9 \pm 0.006_7)$ kJ g^{-1} for trioxaadamantane and pentoxane, respectively; the uncertainties are twice the final overall standard deviations of the mean. After correction for 0.07_0 mass per cent of water the trioxaadamantane value becomes -25.377_9 kJ g^{-1}. The $\Delta u_c°$ values refer to the idealized combustion reaction in which all reactants and products are in their thermodynamic standard states at 298.15 K. Table 4 gives the standard molar energies, $\Delta U_c°$, and enthalpies, $\Delta H_c°$, of combustion for the compounds in the crystalline state, together with the molar enthalpies of sublimation, ΔH_{subl}, and derived enthalpies of formation, $\Delta H_f°$, for the compounds in the crystalline and gaseous states, all at 298.15 K. The enthalpies of formation at 298.15 K for gaseous carbon dioxide and liquid water used in the calculations are from Ref. 14. No previous determinations of the enthalpies of formation of the compounds studied here have been found in the literature.

Unsubstituted adamantane forms the natural basis for a reference system in the evaluation of the non-bonded oxygen-oxygen interaction energy in trioxaadamantane. Three independent determinations of the energy of combustion of adamantane were published recently.[15–17] Two of these are in close agreement, whereas the third [15] differs by almost 9 kJ mol^{-1} from the others. The reason for this discrepancy has not yet been explained. Taking the average of the three reported determinations of the enthalpies of combustion and sublimation leads to a standard enthalpy of formation for gaseous adamantane equal to -131.6 kJ mol^{-1}. From this value and the aliphatic ether increment for the exchange of a $-CH_2-$ group by an ether oxygen $-O-$ (-104.8 kJ mol^{-1}), the predicted enthalpy of formation of gaseous 2,4,10-trioxaadamantane is -446.0 kJ mol^{-1}. The experimentally obtained value is -499.2 kJ mol^{-1} implying a stabilization energy of 53 kJ mol^{-1} in trioxaadamantane. If on the other hand the cyclohexane-tetrahydropyran based ether increment (-100.0 kJ mol^{-1}) is used, the calculated stabilization energy for the three pair oxygen-oxygen next-nearest-neighbour interaction system is 68 kJ mol^{-1} in the tricyclic ortho ester studied here.

Traditionally, adamantane was considered to be relatively free of strain, but recent calculations show that it is in fact appreciably

Table 4. Results and derived quantities at 298.15 K. The uncertainties given are twice the final overall standard deviation of the mean. cal = 4.184 J.

	2,4,10-Trioxaadamantane	1,3,5,7,9-Pentoxecane
$\Delta U_c°$/kJ mol^{-1}	-3607.60 ± 1.90	-2528.96 ± 1.01
$\Delta H_c°$/kJ mol^{-1}	-3610.08 ± 1.90	-2528.96 ± 1.01
$\Delta H_f°$(c)/kJ mol^{-1}	-573.64 ± 2.12	-867.74 ± 1.22
ΔH_{subl}/kJ mol^{-1}	74.41 ± 0.37	87.94 ± 0.53
$\Delta H_f°$(g)/kJ mol^{-1}	-499.23 ± 2.15	-779.80 ± 1.33
$\Delta H_f°$(g)/kcal mol^{-1}	-119.32 ± 0.51	-186.38 ± 0.32

strained; *cf.* for instance Refs. 18 and 19. The exact nature of the strain, however, still seems to be a matter of some controversy. Schleyer *et al.*[18] account for the strain (~ 27 kJ mol^{-1}) in terms of angle strain and C···C non-bonded repulsions, whereas Allinger *et al.*[19] state that "the strain in adamantane is largely due to an excessive number of H···H repulsions." Since only the overall effect of strain and stabilization in a compound is discernible from thermochemical measurements, a simple calculation of the oxygen-oxygen interaction energy in trioxaadamantane may well be obscured by such changes in the "strain" in adamantane that are not taken care of in the choice of ether increment.

The stabilization due to next-nearest-neighbour oxygens in straight-chain compounds can be rationalized in terms of electrostatic interactions.[20] A simple calculational model of this kind correctly reproduces the additivity found for the stabilizing effect of consecutive pairs of oxygens in $-O-CH_2-O-CH_2-O-$ chains. With parameters calibrated by the 17.$_6$ kJ mol^{-1} interaction energy for a single pair, a value of 58 kJ mol^{-1} is predicted for the stabilization energy due to non-bonded oxygen-oxygen interaction in ortho esters.

Energy of combustion measurements on trimethyl- and triethylorthoformate have been published.[21] From these, and estimates of the enthalpies of vaporization, gaseous enthalpies of formation were derived.[21] The calculated stabilization energy in these compounds is 72 * and 69 kJ mol^{-1}, respectively.**

The gaseous enthalpies of formation per $-CH_2O-$ unit in trioxane, tetroxane, and pentoxane are $-(155.3 \pm 0.2)$, $-(155.1 \pm 0.2)$ and $-(156.0 \pm 0.3)$ kJ mol^{-1}, respectively, which means that the overall stabilization per $-O-CH_2-O-$ interaction is the same in these compounds.

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* The ether increment is slightly different, -91.9 kJ mol^{-1}, when the oxygen atom is attached to a methyl group.[2] This has been taken into account in the calculations.

** The compounds were burned in gelatin capsules. No details are given about the precautions taken to prevent uptake of water when handling these extremely hygroscopic materials. Water, if not accounted for, would tend to "increase" the calculated stabilization energy.

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Homogeneous Electron Exchange in Catalytic Polarographic Reduction

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Electron transfer reactions between an electrolytically generated anion radical $A\cdot^-$ and a more difficultly reducible compound (BX) forming an unstable anion radical has been studied by means of classical polarography. The rate of the reduction of BX by $A\cdot^-$, measured by the catalytic increase of the wave-height of A, is dependent on the difference in the reduction potentials of A and BX; the electron transfer reaction or the decomposition of $BX\cdot^-$ may be rate determining. Comparison of results from direct and indirect electrolytic reduction may throw light on the influence of the electrode on product distribution.

Polarographic "catalytic currents" are well-known (Fig. 1), especially in inorganic polarography, *e.g.* the hydrogen peroxide [1] or hydroxylamine [2] reoxidation of ferrous ions during the polarographic reduction of ferric ions, or the reoxidation of Ti^{3+} by hydroxylamine [3] during polarography of Ti^{4+}. A theory [4-6] for the connection between the ratio of the limiting current to the diffusion controlled current and the rate constant of the catalyzing reaction has been developed.

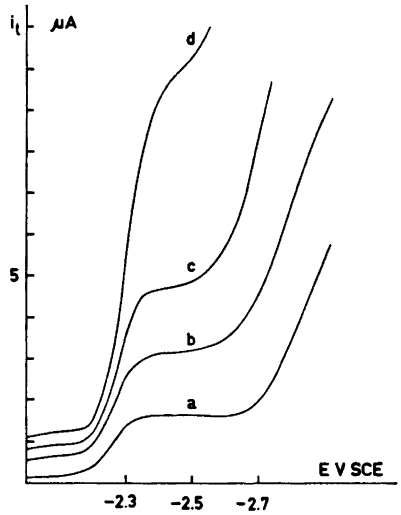

Fig. 1. Polarograms of chrysene (4×10^{-4} m) in DMF containing tetrabutylammonium iodide and (a) 0; (b) 5×10^{-4} m; (c) 1.5×10^{-3} m; (d) 4×10^{-3} m bromobenzene.

A similar increase of a polarographic current due to a regeneration of the depolarisator by electron exchange with another species seems not to have been investigated in detail for organic molecules.[7] The phenomenon seems, however, to be rather general provided certain requirements are met, and below is given a preliminary report on a number of such cases including examples of many different types of compounds. All the examples can be fitted into the general scheme

$$A + e^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A\cdot^- \quad (1)$$

$$A\cdot^- + BX \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} A + [BX]\cdot^- \quad (2)$$

$$[BX]\cdot^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} B\cdot + X^- \quad (3)$$

$$B\cdot + A\cdot^- \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} B^- + A \quad (4)$$

$$B^- + [H^+] \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} BH \quad (5)$$

$$A\cdot^- + A\cdot^- \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} A^{2-} + A \quad (6)$$

$$B\cdot + B\cdot \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} B-B \quad (7)$$

$$B\cdot + A\cdot^- \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} BA^- \quad (8)$$

$$B\cdot + HS \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} BH + S\cdot \quad (9)$$

$$B\cdot + e^- \underset{k_{-10}}{\overset{k_{10}}{\rightleftharpoons}} B^- \quad (10)$$

$$A^{2-} + [H^+] \underset{k_{-11}}{\overset{k_{11}}{\rightleftharpoons}} AH^- \quad (11)$$

Scheme 1.

In this scheme A gives a polarographic wave at a less negative potential than that of BX; the radical anion $A\cdot^-$ produced in (1) should be stable measured on the time scale relevant here. The reoxidation of $A\cdot^-$ to A by BX (2) or by B· (4) gives rise to the observed catalytic current.

BX is a molecule, the anion radical of which is cleaved into the neutral radical B· and the stable ion X^-(3). The radical B· will in most cases, but not all, exchange an electron with $A\cdot^-$ (4), undergo dimerization (7), coupling (8), hydrogen abstraction (9), or direct reduction at the electrode (10) may occur. Usually (2) and (4) together result in the reoxidation of two $A\cdot^-$ for every BX reduced to BH and X^-. It may be noted, that the reoxidation of Fe^{2+} by hydrogen peroxide and hydroxylamine also fits into the scheme with these compounds acting as BX compounds where the first electron uptake is followed by a rapid cleavage to a neutral radical and a stable anion.

As A-compounds have been employed aromatic hydrocarbons, heterocyclic compounds, aromatic ketones, esters, nitriles, and nitro compounds, but other types may work as well. As BX-compounds have been used alcohols, esters, halides, sulphonamides, and azides.

In Table 1 are presented some of the results. In column 1 and 2 are the A and BX compounds, respectively; in the third column is given the difference ΔE between the half-wave potentials of A and BX, and in the fourth one the rate constant k calculated according to the

Table 1. Rate constants of the reaction responsible for the "catalytic" increase in limiting current of a number of aromatic systems (A) in the presence of compounds (BX), which form unstable anion radicals.

A	BX	ΔE V	k
Biphenyl	Chlorobenzene	0.27	2.3×10^4
1-Methylnaphthalene	»	0.28	1.2×10^4
Naphthalene	»	0.32	5×10^3
2-Methylphenanthrene	»	0.37	1.6×10^3
Phenanthrene	»	0.39	1.2×10^3
Terphenyl	»	0.52	1.7×10^1
Benzonitrile	»	0.55	8
Chrysene	Bromobenzene	0.36	4×10^3
Methyl benzoate	»	0.39	1.3×10^2
Biphenyl	Fluorobenzene	0.32	9×10^1
Chinoxaline	1-Bromonaphthalene	0.39	1.5×10^1
Acridine	»	0.45	5
Chinoxaline	9-Bromophenanthrene	0.32	2.7×10^1
Benzophenone	D,L-Stilbene dichloride	0.30	3×10^3
»	*meso*-Stilbene dichloride	0.28	9×10^3
Chrysene	2-Naphthyldimethylcarbinol	0.33	3.3×10^2
Pyrene	*N*,*N*-Dimethyl-*p*-toluenesulphonamide	0.33	4×10^2
Anthracene	Ethyl *p*-toluenesulphonate	0.23	4×10^2
Benzocinnoline	Phenyl azide	0.28	5×10^3
2-Nitroanisole	»	0.48	5×10^1

Koutecký equation.[6]

The results given in Table 1 will be discussed qualitatively on the basis of Scheme 1. The Koutecký equation gives a formal rate constant which could include many of the rate constants in the scheme, but the more significant ones would most likely be k_2 or k_3 or both.

If the most significant factor in k is k_2, and thus (3) and (4) very fast, k would, for a given BX, depend on the potential difference between $E°_A$ and $E°_{BX}$, the rates of the equilibria $A+A\cdot^- \rightleftharpoons A\cdot^- + A$ (12), and the corresponding one for $BX/BX\cdot^-$ (13). The rate constants for a number of equilibria of the type (12) have been measured from the broadening of the lines in the EPR spectra [8-12] and are generally [13] between 10^7 and 10^9 M^{-1} s^{-1}. The instability of the $BX\cdot^-$ makes it difficult to obtain data for (13), but it seems reasonable to assume, that the rate of electron exchange for aromatic BX compounds (13) is of the same magnitude as that of (12). The reported rate constant for (12) includes nitrobenzene [8] (3×10^7 M^{-1} s^{-1}) and benzonitrile [9] (2×10^8 M^{-1} s^{-1}), with benzene,[10] naphthalene,[11] and benzophenone [12] having rate constants between these limits. In this connection the results from the pulse radiolysis on the rate of electron transfer reactions in solution are of interest.[14-16]

From the equation [17] $k_{ABX}=(k_A k_{BX} K_{ABX} f)^{\frac{1}{2}}$, where k_{ABX}, k_A, and k_{BX} are the rate constants of (2), (12), and (13), respectively, K the equilibrium constant of (2), and f a factor which often is close to 1, the dependence of k_{ABX} on $\Delta E°$ can be estimated. The data for k_A [8-12] show only a moderate variation, and regarding k_A as constant is probably not more than a factor 5 off. This approximation would make it possible to predict a decrease in the observed rate constant (2) with a factor 10 for a given BX for an increase in $\Delta E°$ by 0.12 V.

In Fig. 2 is shown as ordinate the logarithm of rate constants k for several A, calculated from Koutecký equation,[5] in the presence of chlorobenzene as the BX-compound, and ΔE as abscissa. Instead of $\Delta E°$ is taken the difference in polarographic half-wave potential between that of A and that of chlorobenzene, as the reversible redox potential for chlorobenzene is difficult to obtain due to the instability of its anion radical. Extrapolation of the experimental values to $\Delta E=0$ suggests $k\sim5\times10^7$; the value,

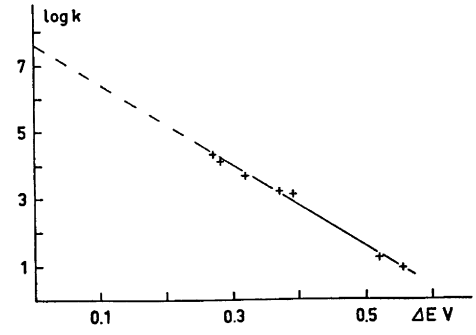

Fig. 2. Dependence on ΔE of the logarithm of the rate constants [5] of the chlorobenzene reoxidation of the following anion radicals (with increasing ΔE): biphenyl, 1-methylnaphthalene, naphthalene, 2-methylphenanthrene, phenanthrene, terphenyl, and benzonitrile.

which a.o. is dependent on how close $E°$ is to $E_{\frac{1}{2}}$ for chlorobenzene, is of the order expected for an electron exchange in solution, and for chlorobenzene the measured rate constant k may well be close to k_2.

When X forms an anion less stable than Cl^- or the reduction potential of BX becomes less negative,[18] the rate of (3) will diminish, and (3) may become the rate determining step, the rate thus being close to $k_3[BX]\cdot^-$. $BX\cdot^-$ could then be regarded as stable in this context and its approximate concentration deduced from a knowledge of the equilibrium constant of (2), which may be estimated from the difference in half-wave potentials between A and BX.[17,19] An increase in ΔE of 0.06 V would diminish K with a factor 10 and as $K<10^{-5}$ in the cases discussed here, $[A]\cdot^-$ and [BX] may be regarded as constant; $[A][BX]\cdot^-$ would then be diminished nearly 10 times and $[BX]\cdot^-$ approximately $10^{\frac{1}{2}}$. A similar dependence of k on ΔE would thus be expected regardless of whether (2) or (3) is the rate-controlling step.

On the basis of the available evidence it seems likely that the rate of the electron exchange between $A\cdot^-$ and BX is the limiting factor when $BX\cdot^-$ decomposes fast, but that the rate of the decomposition of $BX\cdot^-$ becomes more significant when X^- becomes a poorer leaving group. A quantitative analysis involving more data may illuminate these points.

The rate constant k_4 has been assumed to be high compared to k_7 and k_8, and (4) thus

contributing to k with a factor $\frac{1}{2}$. This assumption is valid, when the reduction potential of A is much more negative than that of B·; if this is not the case, k_7 or k_9 may be larger than k_4; k_9 may be larger than k_4 in the indirect reduction of iodobenzene and a number of compounds, where n has been found close to 1; cyclic voltammetric results point in the same direction.[18,20-23]

Electron transfer from a tight ion pair to a molecule is slower than from a loose ion pair or a free radical anion.[15] The rate of electron transfer from anthracene to 2-chloropyridine was nearly the same in DMF containing LiCl or tetrabutylammonium iodide; quinoxaline showed a slower exchange in LiCl than in TBAI; this, however, may be due to a complex formation between the base quinoxaline and the Lewis acid Li^+ prior to reduction so an ion pair is formed directly. In tetrahydrofuran an effect may possibly be found of ion pair formation even for aromatic hydrocarbons.

Sometimes the reduction (amalgam formation) of the supporting electrolyte hinders the reduction of a difficultly reducible substrate. It was found that electron transfer from anthracene anion radical to 2-chloropyridine was possible in the presence of Li^+ although the polarographic wave of Li^+ masks the reduction of 2-chloropyridine. This suggests the possibility of reduction of very difficultly reducible BX compounds, provided $BX\cdot^-$ decomposes faster than $R_4N\cdot$.

An anion radical transports only one electron at a time and delivers it to a molecule outside the electrical double layer where the electrode has no influence on the stereochemistry of the product; thus a possible controversy between a one-electron and a two-electron step and a possible stereochemical influence of the electrode may be resolved. The reduction of D,L and *meso* α,α′-dichlorobibenzyl (stilbene dichloride) to stilbene both by means of anion radicals and directly at the electrode suggests two one-electron steps. The cyclopropane formation by reduction of 1,3-dibromides may follow a similar pattern.

The presence of both a proton donor and a BX compound introduces a competition for the anion radical; addition of phenol to a solution containing naphthalene and chlorobenzene results in a decrease of the catalytic wave. A quantitative treatment of the height of the naphthalene wave in dependence of the concentration of chlorobenzene and phenol would correlate the reaction rates of the anion radical in electron exchange reactions and reactions with proton donors.

EXPERIMENTAL

The polarograph was a Radiometer PO4d Polariter. The *N*,*N*-dimethylformamide (DMF) was dried over molecular sieves A4; further drying with Al_2O_3 (Woelm Al_2O_3W200) directly in the polarographic cell did not change the rate constants found.

A measurement was made as follows: To a solution of tetrabutylammonium iodide in DMF at 25 °C was added the A compound, and the polarogram recorded; an amount of the BX compound was added and the polarogram recorded; more BX was added, a polarogram recorded, and so on until it was apparent from the polarogram that the BX-compound was present in a great excess.

The relative uncertainty of a rate determination is rather large, about 15 – 30 %, mainly due to the following two facts: When ΔE is small, the waves of A and BX become increasingly difficult to separate at higher concentrations of BX which makes the measurements of the wave-height of A less accurate; when ΔE becomes large, the increase in the wave-height of A is small, and the uncertainty in the relative increase is large.

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Non-bonded Oxygen—Oxygen Interaction in Ethoxymethyl Propionate

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The energy of combustion and enthalpy of vaporization at 298.15 K have been measured for ethoxymethyl propionate. Standard enthalpies of formation at 298.15 K have been derived for the compound in the liquid and gaseous states. The results are:

$\Delta H_f°(l) = -(667.2 \pm 1.1)$ kJ mol^{-1}
$\Delta H_f°(g) = -(617.4 \pm 1.1)$ kJ mol^{-1}

Ethoxymethyl propionate is shown to have an additional stabilization energy of 8 kJ mol^{-1} relative to the stabilization energy in ethyl propionate.

Next-nearest-neighbour oxygen atoms in an aliphatic straight-chain compound, $-O-CH_2-O-$, give rise to a stabilization energy of 17.$_6$ kJ mol^{-1}* relative to aliphatic monoethers,[1,3] whereas the next-next-nearest-neighbour arrangement brings about a 10 kJ mol^{-1} destabilization.[1] With three oxygens in "1,3,5"-positions, $-O-CH_2-O-CH_2-O-$, the total stabilization due to non-bonded oxygen-oxygen interaction, within experimental uncertainty, is twice that in the "1,3"-dioxa compound.[1] The stabilizing effect of an $-O-CH_2-O-$ arrangement can be rationalized in terms of electrostatic interactions.[4] The calculational model used so far correctly reproduces the additivity found for consecutive pairs of next-nearest-neighbour oxygens, but does not satisfactorily account for the large destabilizing oxygen-oxygen interaction in 3,6-dioxaoctane.

Gaseous enthalpies of formation for diethyl carbonate and ethyl propionate were used to estimate the stabilization ("resonance") energy of an alkyl carbonate relative to that of an alkyl ester.[5] The calculated stabilization energies were 129 and 80 kJ mol^{-1}, respectively. The possibility of a contribution to the calculated stabilization energy in diethyl carbonate due to oxygen-oxygen interaction of the same nature as that found in "1,3"-diethers is an interesting question which cannot be answered at the present time. However, a knowledge of the $-O-CH_2-O-$ interaction energy, if any, in an alkoxymethyl ester, $-O-CH_2-O-CO-$, might help in attempts to explain the nature and origin of the above mentioned non-bonded oxygen-oxygen interactions. In this paper energy of combustion and enthalpy of vaporization measurements on ethoxymethyl propionate are reported.

EXPERIMENTAL

Compounds. Ethoxymethyl propionate was synthesized essentially as described in Ref. 6. The chloromethyl ethyl ether was prepared from ethanol, s-trioxane and dry HCl, and after removal of water and excess HCl the crude ether product was reacted with (fused) sodium propionate under as moisture-free conditions as possible. The reacted material was distilled twice at reduced pressure through an all-glass column filled with helices, the main fractions of the second distillation boiling at ~339 K/40 Torr.* GLC analysis on this material showed the presence of three impurities, two of which were identified as ethanol and propionic acid, respectively. All of the observed impurities

* The value given in Ref. 1 is 16.9 kJ mol^{-1}. A recalculation with parameters for the aliphatic ether increment taken from Ref. 2 yields 17.6 kJ mol^{-1}.

* Torr = (101.325/760) kPa.

were removed by preparative GLC (Carbowax; 343 K). The identity of the purified compound was confirmed by the NMR and mass spectra. No additional impurities were found when the purified material was tested by GLC on polyethylene glycol (PEG 20M) and diethylene glycol succinate polyester columns. However, GLC analysis on an SE-30 silicone column indicated that about 0.5 % of yet another impurity was present. Unfortunately this particular type of column caused decomposition of the compound when the column and/or injector temperature was increased above ~340 K. Tests on a different silicone (OV-17) column showed no such decomposition, but again the impurity could not be separated from the main component at any of the conditions tried. In an attempt to remove this additional impurity the material was run through the preparative gas chromatograph on an SE-30 column with injector and column temperatures at 333 K. The amount of impurity could, however, only be reduced by about 50 % in this way and therefore its nature and concentration had to be established reasonably well to allow the appropriate correction to be applied to the measured energy of combustion. Mass spectrometric analysis, coupled with GLC, indicated a molecular weight of the impurity of 116. The fragmentation pattern gave several indications of the presence of an ethoxy group as well as strong support for two oxygens in the molecule.[7] The composition $C_6H_{12}O_3$ and the structure of ethoxymethyl ethyl ketone were in accord with the obtained mass spectrum, whereas for instance a structure such as that of ethyl butyrate could be ruled out.[8]

The response of the flame ionization detector, used in the GLC analysis, to 1 mol of impurity was estimated to be very nearly the same as that per mol of ethoxymethyl propionate.[9] From the measured areas under the GLC peaks the amount of impurity was calculated as 0.25 mass per cent with an assigned uncertainty of ±0.03 mass per cent.

Drying the material with molecular sieves before it was transferred to the vacuum line for the ampoule filling procedure caused no detectable change in the purity. The water content, determined by GLC[10] on the sample in one of the combustion ampoules, was 0.002_5 mass per cent. No correction for water has been applied. Densities at 293.15 and 298.15 K were 0.9642_7 and 0.9593_0 g cm^{-3}, respectively. $n_D^{25} = 1.3961$.

The paraffin oil, laboratory designation TKL-66, used as auxiliary material in the combustions, has been described.[11] The benzoic acid, used in the calibration experiments, was National Bureau of Standards SRM 39i.

Combustion calorimetry – apparatus and procedure. The rotating-bomb calorimeter TKL-3 was used with the platinum-lined bomb 3B;[12] internal bomb volume 0.2609 dm^3. All combustions were carried out with 0.79 cm^3 of water initially in the bomb that was flushed with oxygen (99.995 % pure; less than 10 ppm. of nitrogen) for three min before charging to the initial pressure of oxygen $p^i(\text{gas}) = 30.0$ atm* at 298.2 K. Calibrations were carried out under certificate conditions and the other experiments designed to give close to the same temperature rise. Further details of the calorimetric procedure have been given previously.[1] A Hewlett Packard HP-M40-2801A Quartz Thermometer with 2850D probes was used for the temperature measurements. It was used in the 100 s mode (resolution 10^{-5} K) with minimum time between readings. Fore-, main-, and after-periods were all of 20 min duration.

The dried sample was transferred in vacuum to the receiver containing the Pyrex glass ampoules to be filled for the combustion experiments.[13] The ampoule mass varied between 26 and 44 mg. Five combustions were carried out on ethoxymethyl propionate. In all experiments tests were made for carbon monoxide in the final bomb gases. The detection limit of the test is well below 1 ppm of CO in the bomb gas [14] corresponding to a correction of about 4 ppm of the total heat evolved in a combustion. No evidence of CO was found in any of the experiments. The almost negligible amount of nitric acid in the final bomb solution was determined spectrophotometrically [15] at wavelength $\lambda = 202$ nm.

All weighings were reduced to masses and molar masses computed from the 1969 table of atomic weights.[16] The corrected temperature rise, $\Delta\theta$, and the energy equivalent of the standard calorimetric system, ε°(calor), were calculated as outlined by Bjellerup.[17] ε°(calor) was recalculated to air density 1.2 g dm^{-3}, since variations in air density affect the amount of water in the calorimeter.

Vaporization calorimetry. The enthalpy of vaporization at 298.15 K of ethoxymethyl propionate was measured using the Wadsö calorimeter.[18] Five experiments were performed.

RESULTS AND DISCUSSION

All symbols used are those of Hubbard, Scott and Waddington.[19] The Washburn corrections, ΔU_Σ, were calculated using a computer program based on the procedure in Ref. 19. The energy of solution of carbon dioxide in water, $\Delta U_{\text{soln}}(CO_2)$, and the solubility constant, $K(CO_2)$, were taken as −17.09 kJ mol^{-1} and 0.03440 mol dm^{-3} atm^{-1}, respectively, at 298.15 K.[20] The $(\partial v/\partial T)_p$ value for ethoxymethyl propionate was calculated from measured densities, whereas the value used for the

* atm = 101.325 kPa.

specific heat capacity, $c_p = 1.8$ J K^{-1} g^{-1}, is an estimate. The final overall precision of the ΔU_c° mean value was estimated as recommended by Rossini.[21] The enthalpies of formation at 298.15 K for gaseous carbon dioxide and liquid water, used in the calculation of the enthalpy of formation, are from Ref. 22.

Using data from Ref. 2 the energy of combustion of liquid ethoxymethyl ethyl ketone, assumed to be the remaining impurity in the sample, was estimated as -3622 kJ mol^{-1}, equal to -31.18 kJ g^{-1}. An uncertainty of ± 0.5 kJ g^{-1} (58 kJ mol^{-1}) was assigned to this value to account for possible misjudgement of the identity of the impurity. Since it seems very unlikely that the impurity is an ester, the uncertainty does not fully cover the approximately 80 kJ mol^{-1} stabilization energy of an ester group.[5] The net correction to be applied to the measured Δu_c° value due to impurity is (13.5 ± 1.8) J g^{-1}, corresponding to (1.57 ± 0.21) kJ mol^{-1}, where the uncertainty also includes that assigned to the determination of the impurity concentration. This 1.8 J g^{-1} uncertainty has been taken into account in the calculation of the final overall precision of the ΔU_c° mean value.

Details of a typical combustion experiment are given in Table 1. The individual $-\Delta u_c^\circ$ values, referring to unit mass, are listed in Table 2, uncorrected for impurity. The corrected Δu_c° mean value is -25.7649 kJ g^{-1}. The ΔU_c° values refer to the idealized combustion reaction, where reactants and products are in their

Table 1. Summary of a typical combustion experiment.

ε°(calor) = (28677.3 ± 0.7) J K^{-1}[a]
Δu°_c(oil) = (−46041.7 ± 2.0) J g^{-1}[a]
Δu°_c(fuse) = (−16807 ± 4) J g^{-1}[a]

m(comp)/g	0.507371
m(oil)/g	0.166596
m(fuse)/g	0.001185
m(Pt)/g	10.298
$\Delta\theta$/K	0.726561
m^i(cont)/g	22.13
ε^i(cont)/J K^{-1}	13.64
$\Delta U^t_{dec}(HNO_3)$/J	0.17
ΔU_Σ/J	8.81
$-\Delta u_c^\circ$(comp)/kJ g^{-1}	25.7777

[a] The uncertainties are standard deviations of the mean. For explanation of symbols, *cf.* Ref. 19.

Table 2. Results of combustion experiments at 298.15 K.

	$-\Delta u_c^\circ$/kJ g^{-1}
	25.7763
	25.7808
	25.7769
	25.7777
	25.7805
Mean:	25.7784 [a]
Standard deviation of the mean:	0.0009

[a] Not corrected for impurity. See text.

Table 3. Results and derived quantities at 298.15 K. The uncertainties given are twice the final overall standard deviation of the mean.

ΔU_c°/kJ mol^{-1}	−3405.09 ± 0.70
ΔH_c°/kJ mol^{-1}	−3408.81 ± 0.70
ΔH_f°(l)/kJ mol^{-1}	−667.23 ± 1.08
ΔH_v/kJ mol^{-1}	49.88 ± 0.09
ΔH_f°(g)/kJ mol^{-1}	−617.35 ± 1.08
ΔH_f°(g)/kcal mol^{-1}	−147.55 ± 0.26

thermodynamic standard states at 298.15 K. Table 3 gives the standard energy, ΔU_c°, and enthalpy, ΔH_c°, of combustion for the compound in the liquid state, together with the enthalpy of vaporization, ΔH_v, and the derived standard enthalpies of formation, ΔH_f°, for the liquid and gaseous states at 298.15 K.

No enthalpy of formation value for ethoxymethyl propionate or any other alkoxymethyl ester was found in the literature. The calculated gaseous enthalpy of formation increment for insertion of a $-CH_2-O-$ group in an alkane is -145.9 kJ mol^{-1}.[2] From this value and the enthalpy of formation of gaseous ethyl propionate, $-(463.6 \pm 0.7)$ kJ mol^{-1},[5] a value of -609.5 kJ mol^{-1} may be "predicted" for the enthalpy of formation of ethoxymethyl propionate. This "predicted" value rests on the assumption that the total stabilization energy in ethoxymethyl propionate is the same as that in ethyl propionate.* The experimentally derived enthalpy of formation value for the

* The reference system in all the calculations referred to here consists of n-alkanes, and aliphatic monoethers and ketones.

former compound is $-(617.4 \pm 1.1)$ kJ mol^{-1}, implying that there is an extra stabilization energy in ethoxymethyl propionate of (7.9 ± 1.3) kJ mol^{-1}, relative to ethyl propionate, due to interaction between the ether type oxygen and the alkoxy oxygen in the ester group. This is only half of the stabilizing effect, 17.6 kJ mol^{-1}, found for non-bonded interaction between the two oxygens in a "1,3"-dioxa alkane.

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Reactions between Furfurylidenemalonic Esters and Grignard Reagents. III. Diethyl 5-Methylfurfurylidenemalonate. Isolation of a 1,6-Addition Product

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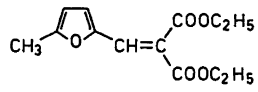
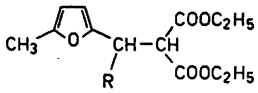
When methylmagnesium iodide and ethylmagnesium bromide react with diethyl 5-methylfurfurylidenemalonate, only 1,4-addition products are formed. Isopropylmagnesium bromide gives a small quantity of reduction product beside the 1,4-addition product. *t*-Butylmagnesium chloride gives reduction product, 1,4-addition product, and two 1,6-addition products. The last-mentioned compounds easily rearrange to diethyl 3-*t*-butyl-5-methylfurfurylmalonate, which was isolated by preparative gas chromatography.

Reactions between diethyl furfurylidenemalonate and different Grignard reagents have previously been studied in this laboratory.[1,2] 1,4-Additions were shown to be the principal reactions. Methylmagnesium iodide and ethylmagnesium bromide did not give any other reactions at all. Isopropylmagnesium bromide caused reduction of a small portion of the unsaturated ester to diethyl furfurylmalonate besides the 1,4-addition. Isobutylmagnesium chloride reacted in the same way but the reduced quantity was larger. *t*-Butylmagnesium chloride gave, in addition to these reactions, also 1,6- and 1,8-additions through extension of the conjugated double bond system to the furan nucleus. Apparently, the product of the last-mentioned reaction immediately rearranged to diethyl 5-*t*-butylfurfurylmalonate when the reaction mixture was brought into contact with water. The two 1,6-addition products were stabler but rearranged prototropically to one and the same product, diethyl 3-*t*-butylfurfurylmalonate, in acid solution.



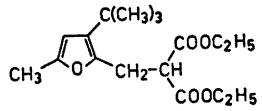
I

The reaction products could not, however, be isolated in the experiments with *t*-butylmagnesium chloride. The structures of the products were therefore deduced mainly from mass spectra taken with a combined gas chromatograph/mass spectrometer. An introduction of an alkyl group in position 5 of diethyl furfurylidenemalonate was expected to reduce the number of reaction products and in this way to facilitate separation and make ordinary structure determinations possible. *t*-Butylmagnesium chloride was therefore allowed to react with diethyl 5-methylfurfurylidenemalonate (I). A gas chromatographic analysis of the reaction products showed that four compounds were present. A fifth compound, probably a dimer, appeared secondarily after standing for some days. Two of the primary reaction products rearranged in acid solution to a new compound while the other two compounds did not react. After the number of products had been reduced in this way, the compounds were easily separated by preparative gas chromatography. The NMR spectra of the fractions obtained showed that the substances



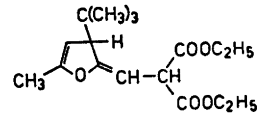
II

were diethyl 5-methylfurfurylmalonate (II; R = H), diethyl 2,2-dimethyl-1-(5′-methyl-2′-furyl)-propylmalonate [II; R = $(CH_3)_3C$], and diethyl 3-*t*-butyl-5-methylfurfurylmalonate (III). Because the last-mentioned compound had formed from two primary reaction products by prototropical rearrangement, it is quite clear that these compounds were the *cis* and *trans* isomers of diethyl 3-*t*-butyl-5-methyl-2,3-dihydro-2-furylidenemethylmalonate (IV).



III

These results show that *t*-butylmagnesium chloride gives reduction and 1,4- and 1,6-additions with diethyl 5-methylfurfurylidenemalonate. The molar ratio of the products of these reactions was 8:58:34 in the present experiments. It is remarkable that the possibilities of the two isomers of diethyl 3-*t*-butyl-5-methyl-2,3-dihydro-2-furylidenemethylmalonate being formed are not equal. The molar ratio of these was 25:9.



IV

A series of experiments was performed with methylmagnesium iodide, ethylmagnesium bromide, and isopropylmagnesium bromide in order to examine whether these Grignard reagents give rise to 1,6-additions beside reduction and 1,4-additions but the results in this respect were as negative as those with diethyl furfurylidenemalonate.[1] The reagents gave reduction and 1,4-addition in the same way as in these experiments. The only difference was that isopropylmagnesium bromide gave a smaller quantity of reduction product and a larger quantity of 1,4-addition product in the experiment with the methyl-substituted ester.

The structures of the reaction products of *t*-butylmagnesium chloride and diethyl furfurylidenemalonate had been deduced mainly from mass spectra. Consequently, the results might be called in question. However, a comparison of these spectra and those now obtained from substances whose structures are unquestionable reveals that the conclusions concerning the structures were correct.

EXPERIMENTAL

Diethyl 5-methylfurfurylidenemalonate (I) was prepared from 5-methylfurfural and diethyl malonate according to the general method for the preparation of α,β-unsaturated esters previously used in this laboratory.[1] The ester, b.p. 177 – 179 °C/12 mmHg, was purified by recrystallisation from ligroin. The yield of pure material, m.p. 56 – 57 °C, was 62 %. (Found: C 62.00; H 6.23. Calc. for $C_{13}H_{16}O_5$: C 61.90; H 6.39.) MS: *m/e*(r.a.) $M^{+\cdot}$ 252(63), calc. 252; $(M+1)^{+\cdot}$ (8.9, calc. 8.9); $(M+2)^{+\cdot}$ (2.0, calc. 1.3); $(M-C_2H_5)^+$ 223(26); $(M-OC_2H_5)^+$ 207 (71); $(M-COOC_2H_5)^+$ 179(100); $CH_3-C_3H_2O-CH=CH-CO^+$ 135(30); unidentified ions at 163(22); 152(15); 110(88). NMR spectrum: the furan proton H-3 at τ 3.41 coupled to H-4 at τ 3.98 ($J = 3.0$ Hz); methyl protons at τ 7.70 long-range coupled to H-4 ($J = 0.9$ Hz); methine proton in position β at τ 2.78 (singlet); methylene protons (ester) at τ 5.75 and 5.80 coupled to the methyl protons at τ 8.67 and 8.70 ($J = 7.1$ Hz).

5-Methylfurfural used in this synthesis was prepared according to Traynelis, Miskel Jr, and Sowa.[3] When a sample of 5-methylfurfural prepared according to Rinkes [4] from sugar was used, the ester obtained was not quite pure.

The reactions of diethyl 5-methylfurfurylidenemalonate with methylmagnesium iodide, ethylmagnesium bromide and isopropylmagnesium bromide were performed on semimicro and macro scales according to the method previously described.[1]

Diethyl 1-(5′-methyl-2′-furyl)ethylmalonate (II; R = CH_3), b.p. 140 – 142 °C/7 mmHg, was the only product in the experiments with methylmagnesium iodide. It was isolated by distillation under reduced pressure. (Found: C 62.46; H 7.30. Calc. for $C_{14}H_{20}O_5$: C 62.67; H 7.51.) The NMR spectrum of the compound shows a rather complex signal at τ 6.4 – 6.80 from the hydrogen atoms at the α and β carbon atoms. The complexity is a consequence of long-range coupling of the hydrogen atoms of the attached methyl group to the hydrogen in the α position. An almost identical signal is found in the spectra of the homologue, diethyl 1-(2′-furyl)ethylmalonate,[1] and α,β-dibromobutyric acid,[5] which contains the same structural element, $CH_3-CH-CH-C=O$, attached to negative atoms and groups. The present spectrum also shows the appropriate signals from the other hydrogen nuclei of the compound.

Diethyl 1-(5′-methyl-2′-furyl)propylmalonate (II; R = C_2H_5), b.p. 144 – 146 °C/18 mmHg, was the only reaction product in the experiment with ethylmagnesium bromide. It was isolated

by distillation under reduced pressure. (Found: C 63.72; H 7.75. Calc. for $C_{15}H_{22}O_5$: C 63.81; H 7.85.) The NMR spectrum shows the presence of an AB spin system with the shifts at τ 6.55 and 6.80 ($J = 9.6$ Hz) due to the hydrogen atoms in the α and β positions. The two signals of the latter atom are split into triplets as a consequence of coupling to the two hydrogen atoms of the attached methylene group. Appropriate signals from the other hydrogen atoms of the compound are found in the spectrum.

Isopropylmagnesium bromide gave diethyl 5-methylfurfurylmalonate (II; R = H; see below) and diethyl 2-methyl-1-(5′-methyl-2′-furyl)propylmalonate (II; R = $(CH_3)_2CH$). The ratio of the reaction products was about 1:100. The latter product was isolated by preparative gas chromatography (column 9 mm × 4.0 m; stationary phase 25 % SE-30 on Chromosorb W; helium flow 200 ml/min; initial temperature about 250 °C, slow programming to 300 °C; injected quantity 0.1 ml each time). (Found: C 64.64; H 7.99. Calc. for $C_{16}H_{24}O_5$: C 64.84; H 8.16.) The NMR spectrum shows the presence of an AB spin system with the shifts at τ 6.37 and 6.72 ($J = 11.0$ Hz) due to the hydrogen atoms in the α and β positions. The two signals from the latter atom are split into doublets as a consequence of coupling to the hydrogen atom of the methine group attached to the β carbon atom. In addition to these signals appropriate signals from the other hydrogen atoms are found in the spectrum.

The reactions of diethyl 5-methylfurfurylidenemalonate with t-*butylmagnesium chloride* were performed according to the method previously described. The reaction products were analysed by gas chromatography (column 3 mm × 1.5 m; stationary phase 3 % SE-30 on Chromosorb W; nitrogen flow about 28 ml/min; initial temperature 100 °C; temperature programming 10 °C/min). Four peaks with the relative retention times 1.00 (compound A), 1.37 (compound B), 1.68 (compound C), and 1.73 (compound D) were obtained when the analysis was carried out immediately after the experiment. When the analysis was repeated the following day, a fifth peak with the relative retention time 2.07 (compound E) appeared. A sample of the reaction products was dissolved in ethanol and the solution was acidified with a small quantity of sulfuric acid. The acid solution was poured into a sodium hydrogen carbonate solution after two days and the mixture was extracted with ether. The gas chromatogram of this ether solution showed four ordinary and two rather small peaks. The relative retention times of the larger peaks were 1.00 (compound A), 1.37 (compound B), 1.59 (compound F), and 2.05 (compound E) and those of the small peaks 1.76 and 1.93. The compounds in the latter reaction mixture were separated by preparative gas chromatography (*cf.* above).

Compound A was diethyl 5-methylfurfurylmalonate (II; R = H). (Found: C 61.69; H 7.05. Calc. for $C_{13}H_{18}O_5$: C 61.41; H 7.14.) The NMR spectrum shows an AB_2 peak system with the shifts at τ 5.56 and 6.99 ($J = 5.8$ Hz) due to the hydrogen atoms at the α and β carbon atoms. In addition to these signals, the spectrum contains the appropriate signals from the rest of the hydrogen atoms of the compound.

Compound B was identified as diethyl 2,2-dimethyl-1-(5′-methyl-2′-furyl)propylmalonate (II; R = $(CH_3)_3C$). (Found: C 66.04; H 8.35. Calc. for $C_{17}H_{26}O_5$: C 65.78; H 8.44.) The NMR spectrum shows a simple AB quartet with the shifts at τ 6.40 and 6.59 ($J = 9.1$ Hz) due to the hydrogen atoms in the α and β positions. The appropriate signals from the remaining hydrogen atoms are found in the spectrum.

Compound F was shown to be diethyl 3-*t*-butyl-5-methylfurfurylmalonate (III). (Found: C 65.54; H 8.24. Calc. for $C_{17}H_{26}O_5$: C 65.78; H 8.44.) The NMR spectrum contains an AB_2 peak system with the shifts at τ 6.41 and 6.86 ($J = 9.1$ Hz) due to the hydrogen atoms in the α and β positions. In addition to these signals and in contrast to the other NMR spectra in this paper, the spectrum of compound F contains only one furan signal, a singlet. The shift is at τ 4.38 and the signal is apparently due to H-4. Appropriate signals from the remaining hydrogen atoms are also found in this case in the spectrum.

The compounds C and D could not be separated by preparative gas chromatography.

Compound E was not isolated in its pure state. Its mass spectrum reveals that it might be a dimer.

The ratio of the peak areas of compounds A, B, C, D, and E is 10:100:42:16:10. The molar ratio of the compounds A, B, C, and D calculated from the above ratio and the structures of the compounds is 8:58:25:9. After treatment with sulfuric acid, the ratio of the compounds, A, B, and F is 8:57:35.

The mass spectra of the reaction products are collected in Table 1. No greater differences are observed between the spectra of the compounds of type II and those of the corresponding compounds without the methyl group in position 5.[1,2] Two fragmentation reactions dominate when R is hydrogen or a small alkyl group: (1) the cleavage of the bond between the α and β carbon atoms which gives rise to the ions $(M-159)^+$ and 2) the loss of a carbethoxy group and a hydrogen atom at the β carbon which results in the ions $(M-74)^{+\cdot}$. These ions finally lose an ethoxy radical, $\cdot OC_2H_5$, and are transformed into the ions $(M-119)^+$. With increasing size and degree of branching of group R, these reactions are suppressed by a third reaction: loss of a carbethoxy group and group R which results in the formation of the ions at *m/e* 180. Even these ions lose an ethoxy radical and are converted into the ions at *m/e* 135, $CH_3-C_4H_2O-CH=CH-CO^+$.

The spectra of the compounds C and D are

Table 1. Abundances of important ions in the mass spectra of the reaction products.

Mass of ion	R in formula II					Compound		
	H	CH_3	C_2H_5	i-C_3H_7	*t*-C_4H_7	C	D	F
M	19	6	9	14	8	3	4	18
M−45	8	1	2	39	4	–	–	–
M−74	40	17	21	13	–	–	–	27
M−89	–	–	–	–	–	–	–	10
M−102	19	6	[a]	–	2	4	7	3
M−119	27	13	11	6	–	–	–	3
M−146	18	10	7	3	2	–	–	4
M−159	100	100	100	100	35	7	3	100
254	[b]	–	–	–	25	27	27	–
253	–	–	3	3	29	25	40	–
180	[c]	–	38	44	43	50	42	4
135	[d]	6	18	98	100	100	100	42
122	–	[e]	7	7	–	2	2	3
95	[f]	–	9	22	26	23	19	7

[a] *cf.* *m/e* 180. [b] *cf.* M. [c] *cf.* M−74. [d] *cf.* M−119. [e] *cf.* M−146. [f] *cf.* M−159.

of the same type as those of the two isomers of diethyl 3-*t*-butyl-2,3-dihydro-2-furylidenemethylmalonate,[2] *i.e.* high abundances of the ions at *m/e* 135, $CH_3-C_4H_2O-CH=CH-CO^+$, and *m/e* 254, $(M-C_4H_8)^{+\cdot}$, and low abundances of the ions $(M-159)^+$. This fact and the rearrangement of the compounds into diethyl 3-*t*-butyl-5-methylfurfurylmalonate show that compounds C and D are the *cis* and *trans* isomers of diethyl 3-*t*-butyl-5-methyl-2,3-dihydro-2-furfurylidenemethylmalonate.

The spectrum of compound F, diethyl 3-*t*-butyl-5-methylfurfurylmalonate agrees well with that of its homologue without the methyl group in position 5.[2] The abundance of the ion $(M-159)^+$ is high and those of the ions $(M-74)^{+\cdot}$ and $(M-89)^+$ are higher than most of the other ions. The relatively high abundance of the ion at *m/e* 135, $CH_3-C_4H_2O-CH=CH-CO^+$, is as difficult to explain as the corresponding ion at *m/e* 121 in the mass spectrum of diethyl 3-*t*-butylfurfurylmalonate.

The elemental analyses were carried out by Mr. F. Sels, Janssen Pharmaceutica, Beerse, Belgium.

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Diels-Alder Reactions of 2,4-Cyclohexadienones.* IV.** Addition of *o*-Quinols to *p*-Benzoquinones

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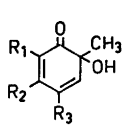
2,6-Dimethylphenol and mesitol were oxidized with aqueous periodate in the presence of an excess of *p*-benzoquinone, toluquinone, 2,6-dimethyl-*p*-benzoquinone or 2,3,5-trimethyl-*p*-benzoquinone. In all cases, the corresponding *o*-quinol-*p*-quinone Diels-Alder adduct was obtained and, in most cases, the *o*-quinol dimer was formed simultaneously. The ratio adduct/dimer was higher with mesitol than with 2,6-dimethylphenol as starting phenol. Furthermore, the yields of adduct increased with decreasing number of methyl substituents in the quinone. No *o*-quinol adducts were obtained with duroquinone.

In each case only one adduct was detected. Spectral and chemical evidence was provided to show that both stereochemical and structural orientation in the adducts were of the same type as previously found in the *o*-quinol dimers.

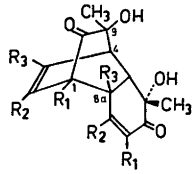
The adducts obtained with *p*-benzoquinone or toluquinone were slowly degraded to 1,4-naphthoquinones by treatment with periodate.

As described earlier,[1-3] periodate oxidation of 2,6-, 2,5-, and 2,4-dimethylphenol, as well as of mesitol, produces the corresponding 6-hydroxy-6-methyl-2,4-cyclohexadienones ("*o*-quinols", *1b – 1e*) which can not be isolated, however, since they undergo rapid self Diels-Alder reaction to give the *o*-quinol dimers *2b – 2e*. Similarly, the parent *o*-quinol *1a*, formed on acid hydrolysis of its acetate, spontaneously dimerizes to compound *2a*.[4]

In all cases, only one dimeric product has been obtained indicating a high degree of both regio- and stereoselectivity of the Diels-Alder dimerizations. All *o*-quinol dimers have the same basic structure involving *endo* configuration and the structural and steric orientation shown in formulae *2a – 2e*.[2,5,6]



1a-f



2a-e

1a, *2a* $R_1=R_2=R_3=H$
1b, *2b* $R_1=CH_3$; $R_2=R_3=H$
1c, *2c* $R_2=CH_3$; $R_1=R_3=H$
1d, *2d* $R_3=CH_3$; $R_1=R_2=H$
1e, *2e* $R_1=R_3=CH_3$; $R_2=H$
1f = *2b*, OAc instead of OH

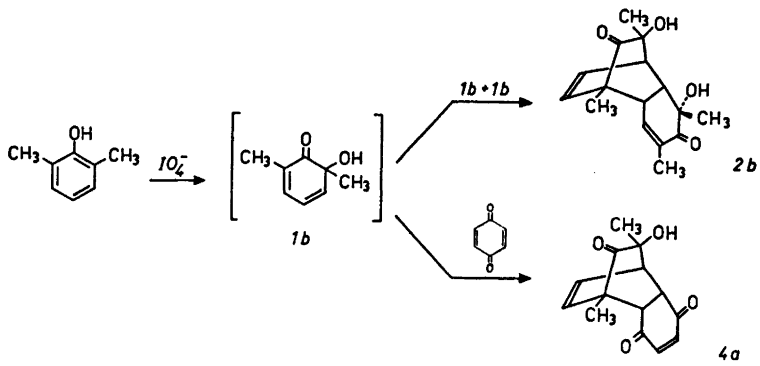
In the present paper we wish to report the trapping of the monomeric *o*-quinols formed on periodate oxidation of 2,6-dimethylphenol and mesitol by *p*-benzoquinones added to the reaction mixture. It was of interest to examine whether the *o*-quinol-*p*-quinone addition reaction involves orientation specificity similar to that encountered in the dimerization of the *o*-quinols.

Formation of *o*-quinol-*p*-quinone adducts

In an attempt to investigate the dienophile character of 2,4-cyclohexadienones, Wessely *et al.*[8] treated an *o*-quinol acetate with typical

* Part XVI in the series "Periodate Oxidation of Phenols". Part XV, see Adler, E., Holmberg, K. and Ryrfors, L.-O. *Acta Chem. Scand. B 28* (1974) 888.

** Part III, see Holmberg, K. *Acta Chem. Scand. B 28* (1974) 857.



Scheme 1.

dienes, *viz.* butadiene and cyclopentadiene. However, in these systems the two last-mentioned compounds added as dienophiles to the *o*-quinol acetate, which acted as diene, indicating a comparatively low dienophile reactivity of the latter compound.

In connection with the present work it may be mentioned that the addition of an *o*-quinol to an *o*-quinone [8] as well as that of another *o*-quinol to a *p*-quinone [9] have been encountered earlier. In both cases the *o*-quinol dimer was formed in addition to the *o*-quinol-quinone adduct. This indicates that the *o*-quinols possessed a dienophile activity comparable to that of the quinones.

In the present work aqueous-ethanolic solutions of 2,6-dimethylphenol or mesitol were added to a solution containing a *p*-benzoquinone (see Table 1) and periodate (molar ratio, 1:1.5:1.1). The competing reactions expected to take place are illustrated in Scheme 1 for the system 2,6-dimethylphenol/*p*-benzoquinone.

The yields of *o*-quinol dimers (*2b*, *2e*) and *o*-quinol-*p*-quinone adducts (*4a*–*h*) formed are listed in Table 1. In each reaction, not more than one product of each type was detected, indicating a high degree of selectivity of both types of Diels-Alder reactions.

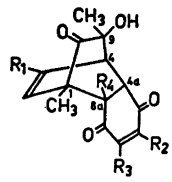
In addition to the dimers and the adducts shown in Table 1, the reactions with 2,6-dimethylphenol gave small amounts of 3,3′,5,5′-tetramethyldiphenoquinone [10] and 2,6-dimethyl-1,4-benzoquinone, whereas those with mesitol yielded minor amounts of 4-hydroxy-2,4,6-trimethyl-2,5-cyclohexadienone (*cf.* also Ref. 1).

The following two conclusions can be drawn from the results presented in Table 1.

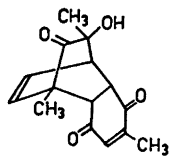
(1) The yield of adduct is higher in the system mesitol/*p*-quinone than in the system 2,6-dimethylphenol/*p*-quinone. This may be understood as follows. Whereas the dienophilic

Table 1. Yields (based on the amount of phenol used) of adducts (*4a*–*4h*) and dimers (*2b*, *2e*) formed on periodate oxidation of 2,6-dimethylphenol and mesitol in the presence of a *p*-benzoquinone.

p-Quinone added	2,6-Dimethylphenol dimer (*2b*)	adduct	Mesitol dimer (*2e*)	adduct
p-Benzoquinone	6	68 (*4a*)	–	78 (*4e*)
Toluquinone	10	73 (*4b*)	–	70 (*4f*)
2,6-Dimethyl-*p*-benzoquinone	66	7 (*4c*)	16	48 (*4g*)
2,3,5-Trimethyl-*p*-benzoquinone	70	2 (*4d*)	18	44 (*4h*)
Duroquinone	40	–	45	–



4a-h, 5



4b'

4a $R_1=R_2=R_3=R_4=H$
4b $R_1=R_2=R_4=H$; $R_3=CH_3$
4c $R_1=R_2=H$; $R_3=R_4=CH_3$
4d $R_1=H$; $R_2=R_3=R_4=CH_3$
4e $R_1=CH_3$; $R_2=R_3=R_4=H$
4f $R_1=R_3=CH_3$; $R_2=R_4=H$
4g $R_1=R_3=R_4=CH_3$; $R_2=H$
4h $R_1=R_2=R_3=R_4=CH_3$
5 = *4a*, OAc instead of OH

double bond, *i.e.* the γ,δ-double bond of the $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl system, is unsubstituted in *o*-quinol *1b*, it possesses a methyl substituent in the γ-position in *o*-quinol *1e*. It is well-known that dienophile reactivity generally decreases with increasing electron density;[7] therefore, due to the electron-releasing effect of the γ-methyl group, *o*-quinol *1e* should be a poorer dienophile than *o*-quinol *1b*. Furthermore, the dimerization of *1e* may be slower than that of *1b* because of steric hindrance exerted by the γ-methyl group present in the former *o*-quinol. Both effects will favour the formation of adducts *4e*–*4h*.

The presence of a γ-methyl substituent has been found to be critical in the dimerization of *o*-quinol acetates. Whereas the *o*-quinol acetates corresponding to *o*-quinols *1a* and *1b* dimerize when heated at 120 °C to give the diacetates of *2a* [4] and *2b*,[1] respectively, the acetates derived from *o*-quinols *1d* and *1e*, possessing a γ-methyl group, proved to be stable even at 160 °C.

(2) The lower the number of methyl substituents in the *p*-benzoquinone, the higher the yield of adduct. This relationship can also be understood to be due to steric and electronic effects, the more heavily methyl substituted *p*-benzoquinone being the poorer dienophile.

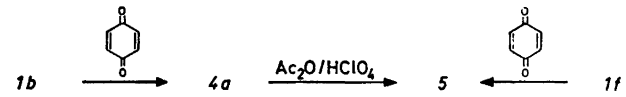
Contrary to the *o*-quinols, their acetates do not undergo Diels-Alder dimerization at room temperature. It was found, however, that a benzene solution of a mixture of *o*-quinol acetate *1f* and *p*-benzoquinone when kept for 10 d at room temperature gave adduct *5* (50 %) as the sole reaction product. Thus *p*-benzoquinone is a better dienophile than the *o*-quinol acetate. Adduct *5* was also obtained by acetylation of the *o*-quinol-*p*-quinone adduct *4a*, indicating analogous structures for the two adducts (Scheme 2).

p-Benzoquinone contains two dienophilic double bonds, and several examples of bis-adducts between *p*-benzoquinone and dienes have been reported.[11] It is known that the formation of the bis-adducts proceeds in two steps, the first diene molecule adding about one hundred times faster than the second one.[11] In the reactions between *p*-benzoquinones and *o*-quinols, however, no bis-adducts have been detected. Furthermore, an attempt to prepare a bis-adduct between *p*-benzoquinone and *o*-quinol *1e* by adding mesitol dropwise to a solution containing a mixture of adduct *4e* and sodium periodate was unsuccessful. The products obtained from the reaction mixture were the *o*-quinol dimer *2e* and unchanged adduct *4e*. Apparently, the adducts of type *4*, for steric reasons, cannot compete as dienophiles with *o*-quinols and *p*-quinones.

The structure of the *o*-quinol-*p*-quinone adducts

The following alternative possibilities regarding the structure of the adducts have to be considered. (*a*) Two structural orientations are conceivable for the adducts *4b*–*4d* and *4f*–*4h*, as exemplified by formulae *4b* and *4b'*. (*b*) The adducts may have *endo* or *exo* configuration. (*c*) The C-9 hydroxyl group may be *anti* and the C-9 methyl group *syn* to the ethylene bridge, or *vice versa*.

The NMR spectra (60 MHz, $CDCl_3$) of the adducts of 2,6-dimethyl- and 2,3,5-trimethyl-



Scheme 2.

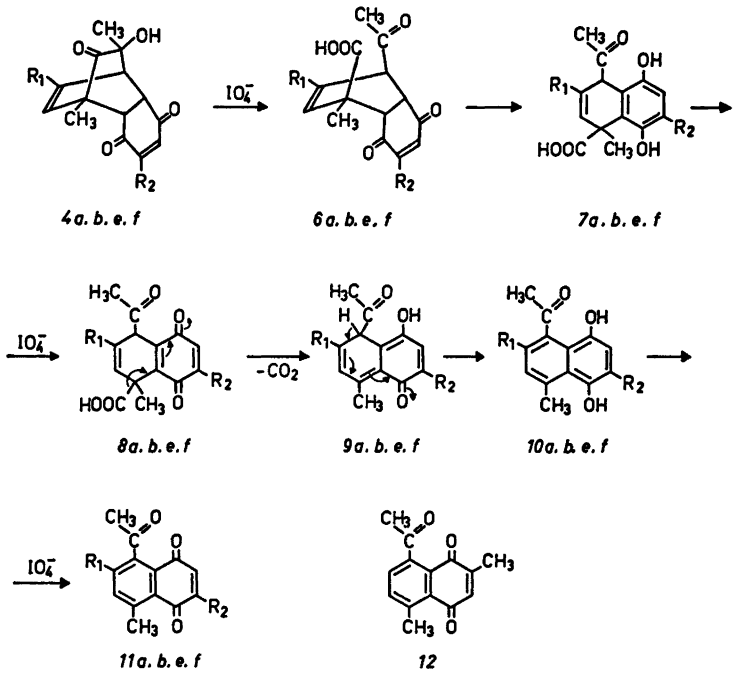
p-benzoquinone clearly showed vicinal coupling between the hydrogen atoms at positions 4 and 4a ($J_{4,4a}=2.0-3.0$ Hz), thus ascertaining the structural orientation of the diene and dienophile moieties to be those shown in the formulae above. In the alternative orientation, represented by formula *4b'*, these adducts would carry a methyl substituent at C-4a.

The structural orientation of the toluquinone adducts could not be clarified by simple NMR analysis. Degradation of the adduct between *o*-quinol *1b* and toluquinone with periodate (see p. 917) gave an acetyl-dimethyl-1,4-naphthoquinone, the m.p. of which (108 – 110 °C) differed from that (150 °C) of a naphthoquinone for which structure *12* has been proposed.[12,13] For this reason, the adduct is assumed to have structure *4b* rather than *4b'*.

By analogy, formula *4f* is assumed for the adduct between toluquinone and *o*-quinol *1e*.

It has been shown that, whereas *o*-quinol dimers *2a – 2c* slowly consume one mol of periodate with cleavage of the 9,10-ketol bridge, dimers *2d* and *2e* are stable towards this oxidant.[6] This has been explained by assuming that the configuration at C-9 is that given in formulae *2*, and that the methyl group at C-8a in dimers *2d* and *2e* prevents the formation of the cyclic periodic ester involving the carbonyl carbon atom at C-10 and the C-9 hydroxyl oxygen atom, which precedes the cleavage reaction. This reasoning, of course, requires *endo* configuration of the dimers.

Periodate oxidation of adducts *4a – 4h* gave the following results. The adducts possessing a methyl group in the 8a-position were stable towards the oxidant, whereas those lacking the angular methyl substituent underwent reaction. The products expected from ketol cleavage, *i.e.* acids *6a*, *6b*, *6e*, and *6f*, however, were



4a. 6a-11a $R_1=R_2=H$
4b. 6b-11b $R_1=H$; $R_2=CH_3$
4e. 6 -11e $R_1=CH_3$; $R_2=H$
4f. 6 -11f $R_1=R_2=CH_3$

Scheme 3.

not obtained from the reactions. Instead, methyl substituted 5-acetyl-1,4-naphthoquinones (*11a*, *11b*, *11e*, and *11f*) were isolated in yields between 52 and 78 % after 2 d reaction time.

A probable pathway for the formation of the naphthoquinones of type *11* is given in Scheme 3. The initial step is believed to be cleavage of the 9,10-ketol bridge of adducts *4* with the formation of carboxylic acids *6*. Aromatization of the enedione ring gives the hydroquinones *7*, which are rapidly oxidized by periodate or iodate to the corresponding *p*-benzoquinones *8*. The latter, being vinylogous β-keto acids, decarboxylate spontaneously to give, via compounds *9*, the 1,4-naphthodiols *10*. These, in turn, are oxidized by periodate or iodate to give the 1,4-naphthoquinones *11*. A similar mechanism has been proposed for the degradation of dimeric *o*-quinones by periodate to give 1,2-naphthoquinones.[14]

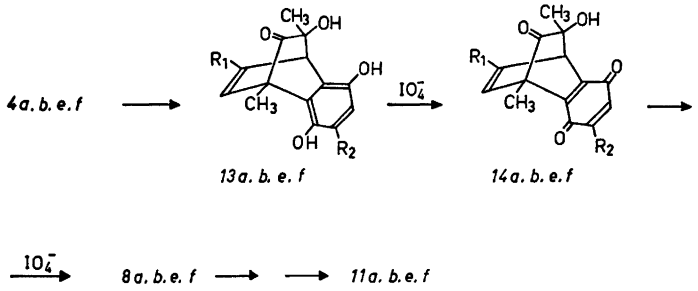
The behaviour of the adducts towards periodate permits the following structural conclusions.

(1) Cleavage of the ketol bridge of the adducts *4a*, *4b*, *4e*, and *4f* by periodate implies that these adducts have *endo* configuration, because an *exo* form of the adducts, similarly to the 8a-methyl substituted *o*-quinol dimers (*2d*, *2e*), would be assumed to be resistant to periodate. By analogy, *endo* configuration is also assigned to the adducts *4c*, *4d*, *4g*, and *4h*, although in these cases experimental evidence is lacking. The exclusive formation of *endo* adducts is in harmony with the behaviour of 2,4-cyclohexadienones in other Diels-Alder reactions.[11]

(2) The resistance to periodate of adducts *4c*, *4d*, *4g*, and *4h*, carrying angular methyl groups at C-8a, indicates that the C-9 hydroxyl group in these adducts is oriented *anti* to the ethylene bridge (compare the analogous behaviour of C-8a substituted *o*-quinol dimers.[6] See also p. 916). This high degree of selectivity regarding the steric orientation at C-9, earlier recognized in the dimerization of *o*-quinols,[6] can be ascribed to steric approach control,[11] the bulky methyl group being directed away from the reaction center. It then seems justified to assume the same steric arrangement at C-9 for adducts *4a*, *4b*, *4e*, and *4f*.

It might be argued that, instead of following pathway *A* presented in Scheme 3, the conversion *4*→*11* may proceed by the alternative route *B* shown in Scheme 4. The initial step of route *B* would be aromatization of the enedione ring of the adducts *4a*, *4b*, *4e*, and *4f* to give the hydroquinones *13*. Rapid oxidation to the *p*-quinones *14*, followed by periodate cleavage of the ketol bridge, would lead to the *p*-quinones *8*, which would be converted into naphthoquinones *11* by the mechanism given in Scheme 3.

If the conversion *4*→*11* proceeds by route *B*, it would not be possible to draw any conclusions regarding *endo* or *exo* configuration of the adducts, since both forms would give rise



Scheme 4.

to the same intermediate hydroquinones *13*.

The following observations, however, speak against pathway *B*.

Firstly, the acetate *5* of adduct *4a* would be expected to be stable towards periodate if ketol cleavage is the primary step (route *A*), but would be transformed into the acetate of *p*-benzoquinone *14a* (*via* the 9-acetyl derivative of hydroquinone *13a*) if *B* is the proper route. Route *A* was supported by the finding that acetate *5* remained unchanged on 2 d treatment with periodate.

Secondly, whereas adduct *4a*, similar to the behaviour of other enediones,[15,16] is smoothly converted into the hydroquinone *13a* by alkali, acid-induced aromatization, which would be involved in the primary step of mechanism *B* (Scheme 4), proceeds less readily. Adduct *4a* proved to be stable in glacial acetic acid and was only slowly transformed into hydroquinone *13a* by 10 % aqueous-ethanolic sulphuric acid, *13a* being obtained in a yield of only 20 % after two days treatment. It is not probable, therefore, that adducts *4* on treatment with aqueous-ethanolic $NaIO_4$ (pH 4 – 5), in a first reaction step, would undergo the aromatization to *13* shown in Scheme 4.

The isolated hydroquinone *13a*, when treated with excess periodate gave the naphthoquinone *11a*. Using a 1:1 ratio of *13a* and periodate, the *p*-benzoquinone *14a* could be isolated, and further oxidation of the latter compound produced the naphthoquinone *11a*. These results support the mechanism for the conversion *13a*→*11a* given in Scheme 4.

It is known that the ethylenic bond of the enedione system of *p*-benzoquinone adducts is easily hydrogenated with zinc/acetic acid.[16,17] The dihydroadduct *15*, obtained in this way from adduct *4a*, on treatment with periodate gave the carboxylic acid *16* (see Scheme 5). This finding lends further support to the assignment of *endo* configuration to adduct *4a*, since the *exo* form of *15* should presumably be resistant towards the oxidizing agent.

There is, however, a possibility for *15* to undergo *exo-endo* interconversion via the dienol. Such an isomerization by base has been reported for a similar adduct.[18] Considering the behaviour of the adducts of type *4* in the presence of acid (see above), however, it seems improbable that isomerization of *15* would take place in acetic acid or aqueous periodate solutions.

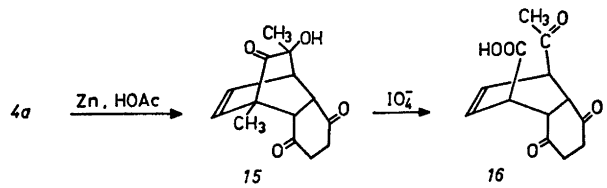
The NMR spectra of adducts *4a* – *4h* were in agreement with the assigned structures. As an example, the NMR signals given by *4a* are reported on p. 919. Similarly, the NMR spectra of the 1,4-naphthoquinones of type *11* have been exemplified by the spectrum of *11a* (p. 920), the spectra of the remaining naphthoquinones also being in accord with the proposed structures.

The UV spectra of the adducts

The β,γ-enone system present in the adducts *4a* – *4h* is expected to give rise to a transannular charge transfer band around 210 nm.[6,19,20] However, such bands were visible above 200 nm only in the spectra of the adducts *4e* – *4h*, obtained from *o*-quinol *1e*.

The three further absorption bands can be ascribed to the enedione system.[16,21] The band around 290 nm (column 2) is enhanced by the overlapping $n\rightarrow\pi^*$ absorption of the β,γ-unsaturated carbonyl system.[1] Although the Woodward rules cannot be applied properly to enediones,[22] the position of the $\pi\rightarrow\pi^*$ bands (column 1) are within the expected range, increasing methyl substitution causing increasing batochromic shift.[15]

The position of the bands listed in columns 2 and 3 are in harmony with data given in the literature for similar enediones.[16,21] Both bands show a hypsochromic shift with increasing methyl substitution of the ethylenic bond. This



Scheme 5.

Table 2. UV maxima in nm and log ε values (in parentheses) of *o*-quinol-*p*-quinone adducts *4a – 4h*. Solvent: ethanol.

Adduct	β,γ-Enone	Enedione 1	2	3
4a		217 (4.08)	290 (2.64)	378 (2.05)
4b		235 (4.11)	284 (2.73) sh	375 (2.07)
4c		236 (3.96)	284 (2.60) sh	375 (2.02)
4d		249 (4.03)	272 (3.42) sh	365 (2.11)
4e	203 (4.70)	222 (4.44) sh	305 (2.77)	383 (2.12)
4f	206 (4.51)	236 (4.22) sh	292 (2.78)	375 (2.16)
4g	213 (4.10) sh	235 (4.11)	288 (2.70) sh	374 (2.07)
4h	213 (4.05)	245 (4.06)	280 (3.20) sh	370 (2.06)

effect becomes clear also from the colours of the adducts, which change from bright yellow (*4a* and *4e*) via light yellow (*4b*, *4c*, *4f*, and *4g*) to almost colourless (*4d* and *4h*).

The transannular charge transfer bands of compounds *4e – 4h* are overlapped to some degree by the adjacent $\pi \rightarrow \pi^*$ bands of the enedione system, which affects their ε values. In the dihydroadduct *15*, where only the former band is present (λ_{max} 204 nm), the log ε value is 3.63, in agreement with values for similar compounds.[19]

EXPERIMENTAL

UV spectra were recorded on a Cary Model 14 spectrophotometer; IR and NMR spectra were obtained using Beckman 9A and Varian A-60 instruments, respectively. Chemical shifts are given in δ (ppm units with TMS used as internal standard).

o-Quinol-p-quinone adducts 4a – 4h. A solution of the phenol (2,6-dimethylphenol or mesitol, 0.010 mol) in ethanol-water 1:1 (20 ml) was added dropwise during a 10 min period to a solution of a mixture of sodium metaperiodate (0.011 mol) and the *p*-benzoquinone (*p*-benzoquinone, toluquinone, 2,6-dimethyl-*p*-benzoquinone, or 2,3,5-trimethyl-*p*-benzoquinone, 0.015 mol) in ethanol-water 1:2 (180 ml). After further 15 min excess periodate was destroyed by 4 ml of ethylene glycol. Red crystals of 3,3′,5,5′-tetramethyldiphenoquinone were filtered off from the oxidation mixtures of 2,6-dimethylphenol. Yield, 1 %; m.p. after recrystallization from chloroform 203 – 210 °C (lit.[19] 207 – 217 °C).

The solution was then extracted with three 40 ml portions of hexane, which removed the major part of the *p*-quinone, and subsequently with dichloromethane, and the latter extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was chromatographed on a silica gel column using benzene-ethyl acetate (4:1). The R_F values of the *o*-quinol-*p*-quinone adducts were between 0.14 and 0.18 and those of the *o*-quinol dimers between 0.08 and 0.11. The products were recrystallized from ethanol. Yields, see Table 1. *o*-Quinol dimers *2b* and *2e* were shown to be identical by mixed m.p. with authentic samples.[1]

1,4,4a,8a-Tetrahydro-9-hydroxy-1,9-dimethyl-1,4-ethanonaphthalene-5,8,10-trione (4a). M.p. 162 – 163 °C. (Found: C 68.53; H 5.66. Calc. for $C_{14}H_{14}O_4$: C 68.28; H 5.73). UV, see Table 2. IR (KBr): ν_{max}, cm^{-1} 1612 (C=C), 1675 (conj. CO), 1730 (CO), 3410 (OH). NMR (DMSO-d_6): δ 1.08 and 1.17 (singlets, 3 H each, 2 CH_3), 2.95 (d, 1 H, H-8a), 3.28 (ddd, 1 H, H-4), 3.67 (dd, 1 H, H-4a), 5.81 (dd, 1 H, H-2), 5.91 (s, 1 H, OH), 6.37 (dd, 1 H, H-3), 6.79 (s, 2 H, H-6 and H-7). $J_{2,3}=8$ Hz, $J_{2,4}=2$ Hz, $J_{3,4}=6$ Hz, $J_{4,4a}=3$ Hz, $J_{4a,8a}=9$ Hz.

1,4,4a,8a-Tetrahydro-9-hydroxy-1,7,9-trimethyl-1,4-ethanonaphthalene-5,8,10-trione (4b). M.p. 171.5 – 172.5 °C. (Found: C 69.23; H 6.20. Calc. for $C_{15}H_{16}O_4$: C 69.21; H 6.20). UV, see Table 2. IR (KBr): ν_{max}, cm^{-1} 1625 (C=C), 1665 (conj. CO), 1715 (CO), 3420 (OH).

1,4,4a,8a-Tetrahydro-9-hydroxy-1,7,8a,9-tetramethyl-1,4-ethanonaphthalene-5,8,10-trione (4c). M.p. 146 – 147 °C. (Found: C 70.0; H 6.7. Calc. for $C_{16}H_{18}O_4$: C 70.1; H 6.6). UV, see Table 2. IR (KBr): ν_{max}, cm^{-1} 1620 (C=C), 1655 (conj. CO) 1730 (CO), 3400 (OH).

1,4,4a,8a-Tetrahydro-9-hydroxy-1,6,7,8a,9-pentamethyl-1,4-ethanonaphthalene-5,8,10-trione (4d). M.p. 148 – 149 °C. (Found: C 70.7; H 7.1. Calc. for $C_{17}H_{20}O_4$: C 70.8; H 7.0). UV, see Table 2. IR (KBr): ν_{max}, cm^{-1} 1630 (C=C), 1660 (conj. CO), 1720 (CO), 3410 (OH).

1,4,4a,8a-Tetrahydro-9-hydroxy-1,3,9-trimethyl-1,4-ethanonaphthalene-5,8,10-trione (4e). M.p. 110 – 111 °C. (Found: C 69.02; H 6.30. Calc. for $C_{15}H_{16}O_4$: C 69.21; H 6.20.) UV, see Table 2. IR (KBr): ν_{max}, cm^{-1} 1610 (C=C), 1665 (conj. CO), 1720 (CO), 3410 (OH).

1,4,4a,8a-Tetrahydro-9-hydroxy-1,3,7,9-tetra-

methyl-1,4-ethanonaphthalene-5,8,10-trione (4f). M.p. 165–166 °C. (Found: C 70.02; H 6.59. Calc. for $C_{16}H_{18}O_4$: C 70.05; H 6.61). UV, see Table 2. IR (KBr): ν_{max}, cm^{-1} 1625 (C=C), 1670 (conj. CO), 1725 (CO), 3380 (OH).

1,4,4a,8a-Tetrahydro-9-hydroxy-1,3,7,8a,9-pentamethyl-1,4-ethanonaphthalene-5,8,10-trione (4g). M.p. 170–171 °C (Found: C 70.70; H 7.01. Calc. for $C_{17}H_{20}O_4$: C 70.81; H 6.99). UV, see Table 2. IR (KBr): ν_{max}, cm^{-1} 1635 (C=C), 1660 (conj. CO), 1715 (CO), 3430 (OH).

1,4,4a,8a-Tetrahydro-9-hydroxy-1,3,6,7,8a,9-hexamethyl-1,4-ethanonaphthalene-5,8,10-trione (4h). M.p. 164–165 °C. (Found: C 71.08; H 7.26. Calc. for $C_{18}H_{22}O_4$: C 71.50; H 7.33). UV, see Table 2. IR (KBr): ν_{max}, cm^{-1} 1630 (C=C), 1670 (conj. CO), 1720 (CO), 3410 (OH).

Oxidation of 2,6-dimethylphenol and mesitol with periodate in the presence of duroquinone: A solution of the phenol (5.0 mmol) in ethanol-water 1:1 (10 ml) was added dropwise during a period of 10 min to a solution of sodium metaperiodate (5.5 mmol) and duroquinone (7.5 mmol) in ethanol-water 3:2 (225 ml). After an additional 15 min, ethylene glycol (2 ml) was added to reduce excess periodate. The reaction mixture was worked up as described above. Thin layer chromatography indicated that no adducts had been formed. The crude reaction products on treatment with acetone/hexane gave the *o*-quinol dimers *2b* and *2e*, respectively, identified by mixed m.p. with authentic samples.[1] Yields, see Table 1.

Acetate 5. (*a*) *By acetylation of adduct* 4a. The adduct was treated with the $Ac_2O/HClO_4$ reagent according to Ref. 23. M.p. 147–149 °C after recrystallization from ethanol. Yield, 72 %. (Found: C 66.3; H 5.5. Calc. for $C_{16}H_{16}O_5$: C 66.66; H 5.60). IR (KBr): ν_{max}, cm^{-1} 1612 (C=C), 1680 (conj. CO), 1740 (CO and ester CO). The NMR spectrum ($CDCl_3$) shows a singlet at δ 2.10 (3 H) due to the ester $-CH_3$.

(*b*) *From* o-*quinol acetate* 1f *and* p-*benzoquinone.* *p*-Benzoquinone (2.0 g) and *o*-quinol acetate *1f*[24] (2.0 g) were dissolved in 20 ml of dry benzene. The mixture was kept for 10 d in the dark at room temperature. The solvent was then removed and the residue treated with 40 ml of ether. The crystals deposited were recrystallized from ethanol. A 50 % yield of acetate *5* was obtained, identical with the product obtained according to (*a*) by mixed m.p. and spectroscopic data.

Naphthoquinones 11a, 11b, 11e, *and* 11f. A solution of 2.5 g of sodium metaperiodate in 100 ml of water was added to a solution of 1.0 g of the adduct (*4a*, *4b*, *4e*, or *4f*) in 750 ml of ethanol-water 1:2. After 2 d at room temperature the solution was extracted with dichloromethane. The extract was dried over anhydrous Na_2SO_4 and brought to dryness under vacuum. The residue, on treatment with ether, gave yellow crystals, which were recrystallized from ethanol.

5-Acetyl-8-methyl-1,4-naphthoquinone (11a). Yield, 78 %. M.p. 142–144 °C. (Found: C 72.59; H 4.73. Calc. for $C_{13}H_{10}O_3$: C 72.89; H 4.71). UV (ethanol): λ_{max}, nm (log ε) 240 (4.31), sh 323 (3.30), 351 (3.42). IR (KBr): ν_{max}, cm^{-1} 1550, 1585 and 1610 (arom. ring and C=C), 1660 and 1705 (CO). NMR ($CDCl_3$): δ 2.48 (s, 3 H, CH_3-8), 2.78 (s, 3 H, CH_3CO), 6.93 (s, 2 H, H-2 and H-3), 7.33 and 7.59 (doublets, 1 H each, H-6 and H-7, $J_{6,7}=8$ Hz).

5-Acetyl-2,8-dimethyl-1,4-naphthoquinone (11b). Yield, 68 %. M.p. 108–110 °C. (Found: C 73.78; H 5.31. Calc. for $C_{14}H_{12}O_3$: C 73.67; H 5.30). UV (ethanol): λ_{max}, nm (log ε) 248 (4.24), 353 (3.42). IR (KBr): ν_{max}, cm^{-1} 1550, 1590 and 1630 (arom. ring and C=C), 1660, 1695 and 1710 (CO).

5-Acetyl-6,8-dimethyl-1,4-naphthoquinone (11e). Yield, 63 %. M.p. 113–114 °C. (Found: C 73.7; H 5.3. Calc. for $C_{14}H_{12}O_3$: C 73.67; H 5.30). UV (ethanol): λ_{max}, nm (log ε) 250 (4.26), 356 (3.46). IR (KBr): ν_{max}, cm^{-1} 1542, 1586 and 1614 (arom. ring and C=C), 1660 and 1702 (CO).

5-Acetyl-2,6,8-trimethyl-1,4-naphthoquinone (11f). Yield, 52 %. M.p. 156–157 °C. (Found: C 74.2; H 5.8. Calc. for $C_{15}H_{14}O_3$: C 74.36; H 5.83). UV (ethanol): λ_{max}, nm (log ε) 253 (4.27), 358 (3.48). IR (KBr): ν_{max}, cm^{-1} 1549, 1591 and 1630 (arom. ring and C=C), 1658 and 1700 (CO).

1,4-Dihydro-5,8,9-trihydroxy-1,9-dimethyl-1,4-ethanonaphthalen-10-one (13a). (*a*) *By alkaline treatment of* 4a. A solution of 2.5 g of adduct *4a* in 150 ml of ethanol was added dropwise, in a nitrogen atmosphere, to ice-cold 1.25 M aqueous NaOH (125 ml). Twenty min after the addition had been completed, the mixture was neutralized with dil. HCl and then extracted with chloroform. The extract was dried over anhydrous Na_2SO_4 and evaporated. Recrystallization of the residue from ethanol gave a 76 % yield of *13a*, m.p. 290–292 °C. (Found: C 68.18; H 5.72. Calc. for $C_{14}H_{14}O_4$: C 68.28; H 5.73). UV (ethanol): λ_{max}, nm (log ε) sh 210 (4.18), sh 235 (3.79), 308 (3.59). IR (KBr): ν_{max}, cm^{-1} 1492, 1600, 1620 (arom. ring), 1708 (CO), 3250 and 3370 (OH). NMR (DMSO-d_6): δ 1.28 (s, 3 H, CH_3-9), 1.73 (s, 3 H, CH_3-1), 4.29 (dd, 1 H, H-4), 5.09 (s, 1 H, OH-9), 6.10 (dd, 1 H, H-2), 6.46 (s, 2 H, H-6 and H-7), 6.61 (dd, 1 H, H-3), 8.60 (s, 2 H, OH-5 and OH-8). $J_{2,3}=7.5$ Hz, $J_{2,4}=2$ Hz, $J_{3,4}=6.5$ Hz.

(*b*) *By acid treatment of* 4a. A solution of adduct *4a* (0.5 g) in 10 % ethanolic sulphuric acid (150 ml) was kept for 2 d at room temperature, then diluted with water and extracted with chloroform. Evaporation of the extract gave *13a* in a 20 % yield, identical with the product obtained according to (*a*) by mixed m.p. and spectroscopic data.

1,4-Dihydro-9-hydroxy-1,9-dimethyl-1,4-ethanonaphthalene-5,8,10-trione (14a). A solution of sodium metaperiodate (0.85 g, 4 mmol) in water (100 ml) was added to hydroquinone

13a (0.98 g, 4 mmol) in ethanol-water 2:5 (700 ml). After 5 min, excess periodate was removed by addition of 5 ml ethylene glycol, and the solution was extracted with chloroform. Removal of the solvent from the combined extracts gave a crystalline residue. Recrystallization from ethanol gave *14a*, m.p. 166–168 °C, in a 64 % yield. (Found: C 68.71; H 4.90. Calc. for $C_{14}H_{12}O_4$: C 68.84; H 4.95). UV (ethanol): λ_{max}, nm (log ε) sh 210 (4.13), 247 (4.19), sh 310 (2.97), 352 (2.95). IR (KBr): ν_{max}, cm^{-1} 1577 and 1605 (C=C), 1655 (conj. CO), 1722 (CO), 3460 (OH). NMR ($CDCl_3$): δ 1.43 (s, 3 H, CH_3-9), 1.85 (s, 3 H, CH_3-1), 2.85 (s, 1 H, OH), 4.53 (dd, 1 H, H-4), 6.14 (dd, 1 H, H-2), 6.52 (t, 1 H, H-3), 6.66 (s, 2 H, H-6 and H-7). $J_{2,3}=J_{3,4}=7$ Hz, $J_{2,4}=2$ Hz.

Naphthoquinone 11a. (*a*) *From* 13a. A solution of 2.0 g of sodium metaperiodate in 100 ml of water was added to 0.50 g of hydroquinone *13a* dissolved in 300 ml ethanol-water 1:2. After 24 h the solution was extracted with chloroform. Evaporation of the extract gave yellow crystals of *11a*, m.p. after recrystallization from ethanol 142–144 °C. Identical by mixed m.p. with the product obtained from periodate oxidation of adduct *4a*.

(*b*) *From* 14a. A mixture of 2.0 g of sodium metaperiodate and 0.50 g of *14a* dissolved in 400 ml of ethanol-water 1:2 was kept at room temperature for 24 h. Extraction with chloroform gave crystals of *11a*, m.p. after recrystallization from ethanol 142–144 °C. Identical by mixed m.p. with the product obtained according to (*a*).

1,4,4a,6,7,8a-Hexahydro-9-hydroxy-1,9-dimethyl-1,4-ethanonaphthalene-5,8,10-trione (15). Zinc powder (2.0 g) was added in portions to a stirred solution of 1.0 g of adduct *4a* in 40 ml acetic acid-water 2:1. After 20 min, the mixture was filtered and the filtrate concentrated to a volume of 10 ml. Water was added and the solution extracted with dichloromethane. The extract was dried over anhydrous Na_2SO_4 and evaporated, yielding almost colourless crystals. Recrystallization from benzene/hexane gave *15*, m.p. 109–110 °C, in a 66 % yield. (Found: C 67.75; H 6.50. Calc. for $C_{14}H_{16}O_4$: C 67.73; H 6.53). UV (ethanol): λ_{max}, nm (log ε) sh 204 (3.63), 306 (2.22). IR (KBr): ν_{max}, cm^{-1} 1655 (C=C), 1697 and 1720 (CO), 3390 (OH).

4-Acetyl-1,4,4a,6,7,8a-hexahydro-1-methyl-5,8-dioxo-1-naphthalenecarboxylic acid (16). A solution of sodium metaperiodate (1.0 g) in 75 ml water was added to a solution of dihydroadduct *15* (0.50 g) in 75 ml ethanol-water 2:3. After 16 h the solution was extracted with chloroform. The extract was dried over anhydrous Na_2SO_4 and concentrated to a volume of 10 ml. The crystals deposited were recrystallized from ethanol to give *16*, m.p. 167–168 °C, in a 80 % yield. (Found: C 63.9; H 6.1. Calc. for $C_{14}H_{16}O_5$: C 63.62; H 6.10). UV (ethanol): λ_{max}, nm (log ε) 283 (2.36). IR (KBr): ν_{max}, cm^{-1} 1710 (CO), 2300–3300 (COOH). The NMR spectrum (DMSO-d_6) shows a singlet at δ 2.22 (3 H) due to the CH_3CO group.

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Resolution of Glutathione-linked Enzymes in Rat Liver and Evaluation of their Contribution to Disulfide Reduction *via* Thiol—Disulfide Interchange

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Glutathione-linked enzymes in the postmicrosomal fraction of rat liver were resolved and the contribution of these enzymes to disulfide reduction *via* thiol-disulfide interchange was evaluated. The mixed disulfide of cysteine and glutathione (CySSG)*, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), and the mixed disulfide of 3-carboxy-4-nitrobenzenethiol and glutathione (ArSSG) were used as disulfide substrates and glutathione (GSH) was the thiol substrate. Thiol-disulfide interchange involving GSH was normally followed by coupling to glutathione reductase (EC 1.6.4.2), but the reaction between DNTB (or ArSSG) and GSH was measured directly without coupling.

CySSG-thioltransferase activity** (EC 1.8.4.1) was separated into two and DTNB-thioltransferase activity into several components. Glutathione reductase and glutathione *S*-aryltransferase (EC 2.5.1.13) behaved as one and two components, respectively. Aged rat liver preparations contained additional, more acidic species of all these activities except the aryltransferase.

NADPH-linked reduction of DTNB coincided with glutathione reductase activity after all purification steps. No significant contribution to the thiol-disulfide interchange activity in rat liver could be ascribed to glutathione reductase or glutathione *S*-aryltransferase.

The aromatic disulfide substrates, DTNB and ArSSG, are not suitable for the study of CySSG-thioltransferase in crude enzyme-preparations unless the effect of interfering activities can be evaluated.

Thiol-disulfide interchange was found to be the major route of DTNB reduction in rat liver. The NADPH-dependent reduction of DTNB described in the literature may be explained by the presence of GSH and glutathione reductase.

In our studies concerning the reduction of low molecular weight disulfides in biological systems the role of glutathione reductase in rat liver cytosol has been evaluated.[1] For disulfides other than glutathione disulfide the main route of enzymatic reduction is composed of thiol-disulfide interchange (eqns. 1, 2) coupled to reduction of GSSG by NADPH and glutathione reductase (eqn. 3).

$$R_1SSR_2 + GSH \rightleftharpoons R_1SSG + R_2SH \quad (1)$$

$$R_1SSG + GSH \rightleftharpoons GSSG + R_1SH \quad (2)$$

$$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+ \quad (3)$$

Glutathione (GSH) is the predominant thiol in living cells and probably the main thiol substrate for thioltransferase-catalyzed reactions *in vivo*. Several low molecular weight disulfides have been reported to be reduced enzymatically by GSH *via* a thiol-disulfide interchange, for instance the naturally occurring homocystine,[2] the mixed disulfide of CoA and GSH,[3–5] cystine,[1,3,6–9] the mixed disulfide of cysteine and GSH,[1,3] and others such as thiamine disulfide derivatives[10] and the nonbio-

* Unusual abbreviations: ArSSG, the mixed disulfide of glutathione and 3-carboxy-4-nitrobenzenethiol; CySSG, the mixed disulfide of glutathione and cysteine; $CySSO_3H$, *S*-sulfocysteine; DTNB, 5,5′-dithiobis(2-nitrobenzoate).

** Thioltransferase has recently been introduced as a name for enzymes catalyzing thiol-disulfide interchange.[12,26] These enzymes have earlier been referred to as transhydrogenases.[3]

logical disulfides 5,5′-dithiobis(2-nitrobenzoate) (DTNB) and the mixed disulfide of 3-carboxy-4-nitrobenzene thiol and GSH (ArSSG).[11] The purpose of using DTNB (denoted ArSSAr in eqn. 4 to emphasize its disulfide character) or ArSSG as the disulfide substrate was to obtain a convenient assay method of the thioltransferase activity based on direct spectrophotometric determination of ArSH released as shown in eqns. (4) and (5)

$$\mathrm{ArSSAr} + \mathrm{GSH} \rightleftharpoons \mathrm{ArSSG} + \mathrm{ArSH} \quad (4)$$

$$\mathrm{ArSSG} + \mathrm{GSH} \rightleftharpoons \mathrm{GSSG} + \mathrm{ArSH} \quad (5)$$

To determine whether DTNB could generally replace cystine derivatives in the determination of thioltransferase activity, the activity profiles with these different substrates obtained after column chromatography and isoelectric focusing have been compared.

It has previously been demonstrated that the essence of the enzymatic thiol-disulfide interchange is thiol transfer.[12] Glutathione reductase and glutathione *S*-aryltransferase also have an element of thiol transfer in their catalytic functions, and the present investigation was undertaken to clarify whether these enzymes contribute to the thioltransferase activity measured in rat liver preparations. The limitations of replacing the glutathione reductase-coupled assay of thioltransferase with direct spectrophotometric measurement using DTNB as substrate as well as the nature of the DTNB-thioltransferase activity are discussed. In addition, DTNB has previously been used to characterize NADPH-dependent reductases with protein disulfides and low molecular weight disulfides as substrates in crude preparations,[9,13–17] but in these studies possible interference by endogenous glutathione reductase, thioltransferase, and GSH, and by the thioredoxin system was not evaluated. It was therefore of interest to see whether NADPH-dependent reduction of DTNB could be explained by a combination of thiol-disulfide interchange according to eqns. (4) and (5) and the reduction of GSSG by NADPH mediated by glutathione reductase.

As regards glutathione *S*-aryltransferase, it has been suggested that this activity is responsible for the enzymatic reaction of GSH with disulfides,[18] and the relation of this enzyme to CySSG-thioltransferase has therefore also been examined. Preliminary results from this investigation have been previously reported.[19,20]

MATERIALS AND METHODS

The mixed disulfide of cysteine and glutathione was synthesized according to Eriksson and Eriksson,[21] *S*-sulfocysteine according to Segel and Johnson,[22] and the mixed disulfide of glutathione and 3-carboxy-4-nitrobenzenethiol according to Mannervik.[23] The homogeneity of the compounds in stock solutions was tested by paper electrophoresis [21] before use in the enzymatic experiments, and in no case were any impurities detected.

Glutathione reductase (yeast), NADPH, GSH, and GSSG were obtained from Sigma; 5,5′-dithiobis(2-nitrobenzoic acid) from Aldrich ;3,4-dichloro-1-nitrobenzene from Schuchardt; Ampholine carrier ampholytes from LKB; Sephadex G-25 Fine from Pharmacia Fine Chemicals; and CM-cellulose CM 32 from Whatman.

Separation of rat liver enzymes. Livers from male Sprague-Dawley rats were homogenized in 4 volumes of 0.25 M sucrose and the resulting homogenate was centrifuged at 105 000 *g* for 60 min. The supernatant was passed through a Sephadex G-25 Fine column which had a volume 5 times the volume of the supernatant. The gel was equilibrated with the eluent required in subsequent experiments. The protein fraction of the effluent was used in the isoelectric focusing and ion-exchange chromatography experiments.

Isoelectric focusing was carried out in a 110 ml column according to the instructions of the manufacturer (LKB). The ampholyte (1 %) covered a pH-range of 3 – 10 (Ampholine No. 8141) in a 0 – 50 % sucrose density gradient. The sample (50 – 100 mg of protein) was desalted on a Sephadex G-25 Fine column (equilibrated with water) and introduced into the middle of the gradient to avoid denaturation by contact with the electrolytes surrounding the electrodes. The isoelectric focusing was run at 4 °C for 36 h at 300 V. The contents of the column were collected in 1.0 – 1.5 ml fractions.

CM-cellulose chromatography was carried out in columns (2 × 10 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.1 (1 mM EDTA). After introduction of the sample, the column was washed with the phosphate buffer used for equilibration until no protein could be detected in the effluent. The elution was continued with a linear concentration gradient of 0 – 0.2 M NaCl (500 ml total volume) in the original buffer and fractions of 7 – 10 ml were collected.

The protein concentration was calculated on the basis of the absorbance at 260 and 280 nm.[24]

Assays of enzymatic activities. Glutathione reductase was assayed spectrophotometrically

by recording NADPH oxidation at 340 nm. The reaction system contained 1 mM GSSG, 0.1 mM NADPH, 0.17 M sodium phosphate buffer (pH 7.6), 1 mM EDTA, and enzyme (10 – 50 μl) in a total volume of 1 ml. The measurements were made on an LKB 8600 Reaction Rate Analyzer, which maintained the reaction mixture at 30 °C. Reductase activity demonstrable with DTNB as the disulfide substrate was determined in the same reaction system with the substitution of 0.5 mM DTNB for GSSG. The formation of 3-carboxy-4-nitrobenzenethiolate was followed at 412 nm with a recording spectrophotometer.

Glutathione *S*-aryltransferase activity was measured by spectrophotometric determination of *S*-2-chloro-4-nitrophenyl-glutathione using 3,4-dichloro-1-nitrobenzene and GSH as substrates (*cf.* Ref. 25). The determination was made on an LKB 8600 Reaction Rate Analyzer at 340 nm, using an extinction coefficient of 10 mM^{-1} cm^{-1}. The reaction system contained 5 mM GSH, 1 mM 3,4-dichloro-1-nitrobenzene (added as a 20 mM solution in ethanol), 0.17 M sodium carbonate buffer (pH 8.0), and enzyme (50 μl) in a final volume of 1 ml.

Assay of CySSG-thioltransferase was based on the use of GSH and CySSG or GSH and $CySSO_3H$ as substrates and determination of the GSSG formed (as shown in eqns. (6) and (7))

$$CySSO_3^- + GSH \rightleftharpoons CySSG + HSO_3^- \quad (6)$$

$$CySSG + GSH \rightleftharpoons GSSG + CySH \quad (7)$$

by coupling to glutathione reductase (eqn. 3). CySSG and $CySSO_3H$ are interchangeable in this assay system; it has previously been demonstrated by column chromatography and isoelectric focusing experiments that these substrates define the same enzymatic activity (as do, for example, *S*-sulfoglutathione and cystine).[19,20,26] $CySSO_3H$ has the advantage over CySSG of giving a three-fold lower non-enzymatic reaction rate when these substrates are used in the concentrations given below, which result in the same enzymatic reaction rate. The activity was determined spectrophotometrically at 340 nm using a Beckman DB-G spectrophotometer. The reaction system contained 0.5 mM GSH, 0.25 mM CySSG, 0.1 mM NADPH, 0.4 unit of yeast glutathione reductase, 0.125 M sodium phosphate buffer (pH 7.6), 1 mM EDTA, and enzyme (50 – 100 μl) in a final volume of 1 ml. The reaction was started by addition of CySSG (or $CySSO_3H$) 3 min after mixing of the other components.[1] The amount of yeast glutathione reductase used was sufficient to eliminate the influence on the thioltransferase assay of endogenous glutathione reductase in the sample tested. $CySSO_3H$ was used in a concentration of 2.5 mM, when substituted for CySSG. A correction for the spontaneous reaction was made by separate determination of thiol-disulfide interchange in the absence of thioltransferase. Boiled enzyme preparations had no catalytic effect on the reaction.

Measurement of thioltransferase activity using ArSSG or DTNB as the disulfide substrate (ArSSG- or DTNB-thioltransferase activity) was based on the formation of 3-carboxy-4-nitrobenzenethiolate, which was monitored spectrophotometrically at 412 nm (*cf.* Ref. 11). The reaction system contained 0.02 mM GSH, 0.1 mM ArSSG or 0.02 mM DTNB, 0.17 M sodium phosphate buffer (pH 5.5), 1 mM EDTA, and enzyme (50 μl). The reaction velocities were corrected for the contribution of the nonenzymatic reaction.

The formation of 1 μmol/min of product was designated as a unit of enzymatic activity.

RESULTS

Isoelectric focusing of thioltransferase from rat liver. To obtain an overall view of all enzymatic activities of interest in this investigation, a post-microsomal supernatant from homogenates of rat liver was subjected to isoelectric focusing in the pH range of 3 – 10. Fig. 1 shows an experiment with a supernatant dialyzed for 30 h against 1 % glycine (pH 7). Four major peaks of thioltransferase activity were demonstrated using $CySSO_3H$ and GSH as substrates. In other experiments following prolonged dialysis (70 h) only one acidic peak was obtained, apparently at the expense of the two basic components. Only the two basic components were observed when the enzyme sample was chromatographed on Sephadex G-25 before the isoelectric focusing.

Two components of glutathione reductase (not shown) and glutathione *S*-aryltransferase which did not coincide with each other or the other activities tested were detected. DTNB-thioltransferase activity gave four peaks (not shown), two of which partially overlapped the two basic peaks of CySSG-thioltransferase activity.

To test whether the activity obtained with DTNB as the disulfide substrate could be resolved further from the other enzyme activities tested, a more extensive separation of the enzyme activities was undertaken using ion exchange chromatography in combination with isoelectric focusing.

Chromatography of the cytosol fraction of rat liver on CM-cellulose. All enzyme activities studied in the cytosol fraction of fresh rat livers were adsorbed onto CM-cellulose equili-

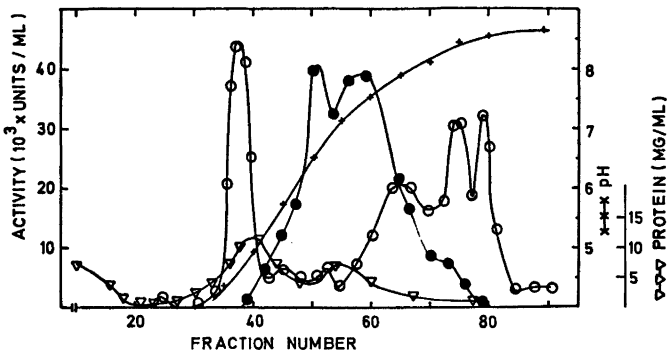

Fig. 1. Isoelectric focusing of the supernatant fraction from rat liver after dialysis for 30 h against 1 % glycine buffer, pH 7. The protein sample, 100 mg, was introduced into the middle of the 0 – 50 % sucrose gradient containing 1 % Ampholine (No. 8141), pH 3 – 10. Isoelectric focusing was carried out at 4 °C for 36 h at 300 V. The contents of the column were collected in 1.0 – 1.5 ml fractions. O, CySSG-thioltransferase; ●, GSH *S*-aryltransferase x 1:20.

brated with 10 mM sodium phosphate buffer, pH 6.1, containing 1 mM EDTA. Elution with a salt gradient resulted in two peaks of CySSG-thioltransferase activity (Fig. 2), the relative sizes of which were dependent on the experimental conditions. This variable elution pattern indicated that the two activities might be interconvertible forms of a single enzyme. This possibility was the subject of a separate investigation in which evidence for a GSH-dependent interconversion of the two forms was obtained [27] (see also Ref. 26). A similar variable elution pattern was not seen for any of the other activities studied. Aged preparations contained additional activities which were not retained by the column and which presumably were identical with the acidic degradation products observed in the isoelectric focusing experiments.

Glutathione reductase appeared as a single component which had its peak in a fraction containing the maximal activities of thioltransferase, measured with ArSSG and DTNB as disulfide substrates. The activities obtained with ArSSG and DTNB were usually distributed in three distinct peaks which essentially coincided for the two substrates. The major peaks of the two activities had shoulders which overlapped with fractions containing the maxima of the CySSG-thioltransferase activity. The glutathione *S*-aryltransferase activity had two peaks which partially coincided with the thioltransferase activity.

Isoelectric focusing of fractions obtained from CM-cellulose chromatography. Fractions 37 and 39 from the CM-cellulose chromatography depicted in Fig. 2 were subjected to isoelectric focusing in an attempt to resolve the different enzymatic activities. The resolution of fraction No. 37 is shown in Fig. 3. Glutathione reductase, glutathione *S*-aryltransferase, and CySSG-thioltransferase activities were clearly resolved. DTNB-thioltransferase activity is separated into three peaks coinciding with the peaks of glutathione reductase, glutathione *S*-aryltransferase, and CySSG-thioltransferase.

Isoelectric focusing of fraction No. 39, containing low CySSG-thioltransferase activity, is illustrated in Fig. 4. CySSG-thioltransferase activity is less stable than the other activities (see Refs. 6, 15, and 28) and could not be detected after the isoelectric focusing. Glutathione reductase appeared as a single component separated from the other activities. The glutathione *S*-aryltransferase activity appeared as one minor and two major components. The enzymatic activity demonstrated with DTNB as disulfide substrate was separated into one major and two minor peaks. The coincidence of glutathione *S*-aryltransferase and DTNB-thioltransferase activities (Figs. 3 and 4) will be considered further in the Discussion.

In a different series of experiments the fraction from the CM-cellulose column containing the second component of CySSG-thioltransferase (corresponding to fraction No. 41 in Fig. 2) was analyzed by isoelectric focusing (Fig. 5).

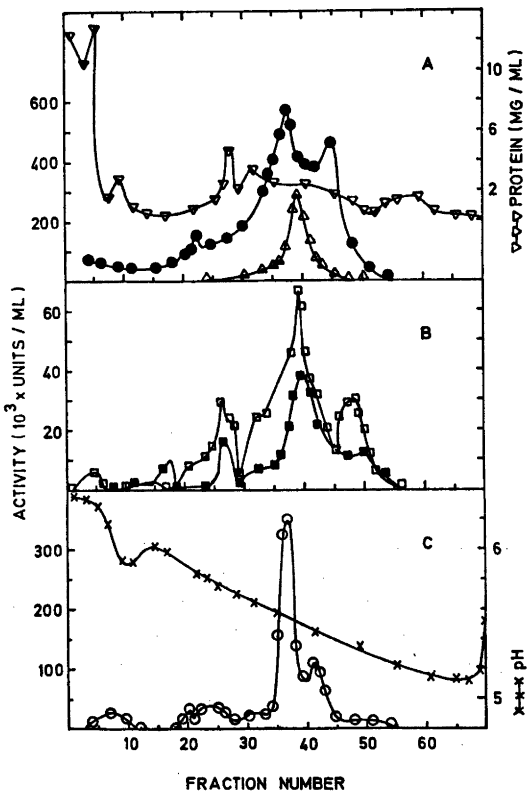

Fig. 2. Fractionation of the supernatant fraction from rat liver by CM-cellulose chromatography. To obtain the protein sample in the same buffer conditions prevailing in the equilibrated CM-cellulose, the supernatant was passed through five volumes of Sephadex G-25 equilibrated with 10 mM sodium phosphate buffer (pH 6.1), 1 mM EDTA. The protein effluent was applied to the CM-cellulose column (2 x 10 cm). The column was washed with the buffer used for equilibration till no protein was eluted. The elution was continued with a linear concentration gradient of 0 – 0.2 M NaCl (500 ml total volume) in the original buffer and fractions of 7 – 10 ml were collected. The enzymatic activities are: (A) ●, GSH *S*-aryltransferase; △, glutathione reductase (activity x 1:5); (B) □, DTNB-thioltransferase activity; ■, ArSSG-thioltransferase activity; (C) ○, CySSG-thioltransferase.

The results were essentially the same as those depicted in Fig. 3, but some of the CySSG-thioltransferase activity was found in the low pH-range of the gradient, indicating degradation due to prolonged dialysis before isoelectric focusing. Furthermore, the reductase activity obtained with DTNB and NADPH (in the absence of added thiols), which was not separated from glutathione reductase by CM-cellulose chromatography, coincided exactly with the glutathione reductase activity.

In addition, we investigated the possibility that thioltransferase activities with DTNB or ArSSG were catalyzed by the same proteins catalyzing the other activities tested. Isoelectric focusing of a fraction corresponding to No. 26 in Fig. 2, the first of the three major peaks containing DTNB-thioltransferase activity, showed high isoelectric points for the components of DTNB-thioltransferase, which were resolved from the other activities tested (Fig. 6). Furthermore, low activity was obtained when ArSSG was used as the substrate in assaying fractions containing high glutathione *S*-aryltransferase and CySSG-thioltransferase activities.

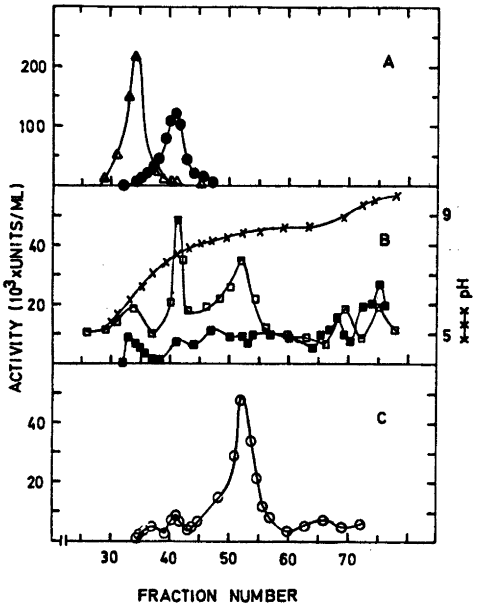

Fig. 3. Isoelectric focusing of fraction No. 37 from the CM-cellulose chromatography depicted in Fig. 2. The symbols for enzymatic activities are explained in the legend to Fig. 2. Further information is supplied in Fig. 1 and Materials and Methods.

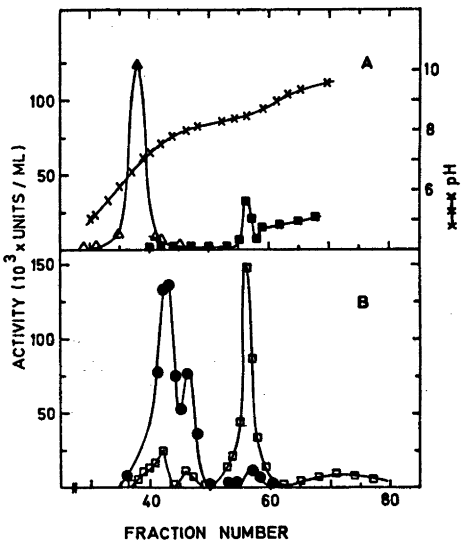

Fig. 4. Isoelectric focusing of fraction No. 39 from the CM-cellulose chromatography depicted in Fig. 2. The symbols are given in Fig. 2. Glutathione reductase (activity × 1:10). Further information is supplied in Fig. 1 and Materials and Methods.

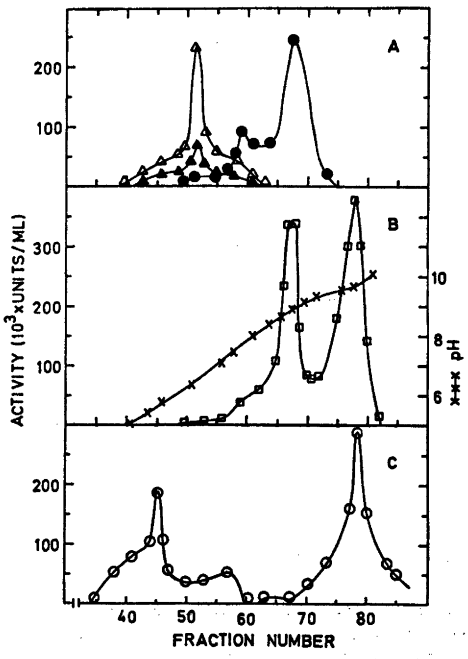

Fig. 5. Isoelectric focusing of a fraction from CM-cellulose chromatography corresponding to fraction No. 41 in Fig. 2. ▲, NADPH-DTNB oxidoreductase; △, glutathione reductase (activity × 1:40), ●, GSH-*S*-aryltransferase (activity × 1:4). The other symbols are given in Fig. 2. Further information is supplied in the legend to Fig. 1 and Materials and Methods.

DISCUSSION

The results of the present investigation demonstrate that the cytosol fraction of rat liver contains at least two forms of thioltransferase active with CySSG (or $CySSO_3H$). These components are distinguishable by chromatography on CM-cellulose columns or by isoelectric focusing. The two activities, after being separated by ion exchange chromatography, are essentially homogeneous in subsequent isoelectric focusing experiments (*cf.* Figs. 3 and 5). Upon aging of the supernatant, additional, more acidic forms appear. (For instance, the experiment demonstrated in Fig. 5 shows activity below pH 8 in the gradient presumably due to degradation of the sample during dialysis before the isoelectric focusing.) It could be established (*cf.* Figs. 3 and 5) that no significant contribution to the CySSG-thioltransferase activity could be ascribed either to glutathione reductase (*cf.*

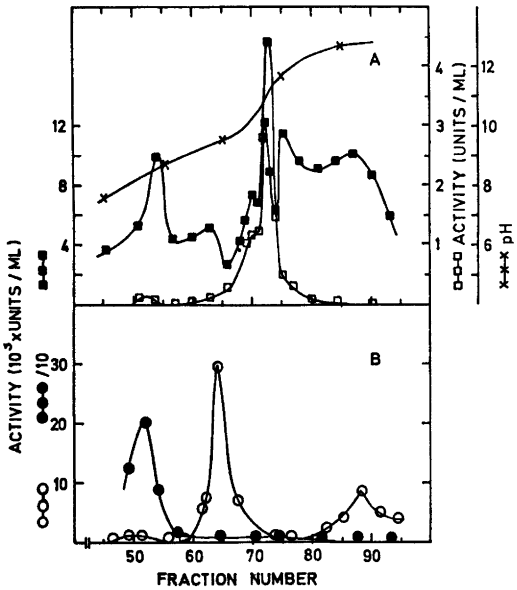

Fig. 6. Isoelectric focusing of a fraction from CM-cellulose chromatography corresponding to the first peak in Fig. 2 B containing fractions Nos. 20 – 29. The substrate concentrations used for measuring DTNB- and ArSSG-thioltransferase activities are: GSH, 50 μM; DTNB, 50 μM; and ArSSG, 135 μM. GSH *S*-aryltransferase (activity × 1:10). The symbols used are explained in the legend to Fig. 2. Further information is supplied in the legend to Fig. 1 and Materials and Methods.

Ref. 11) or to glutathione *S*-aryltransferase, which has been stated to be identical with an enzyme catalyzing the thiol-disulfide interchange involving GSH and thiamine disulfide.[18]

The fact that several different enzymes can use DTNB and GSH as substrates, in combination with the limited resolution of the enzyme activities, explains the unusual shape of the peaks of the DTNB activity profile depicted in Fig. 2.

The third peak of DTNB-dependent activity in Fig. 2 B was not characterized further, but is probably a separate activity, since the activities of all other enzymes examined were low in these fractions from the column. Thus, all three peaks of DTNB activity seemed to contain a component which was distinct from glutathione reductase, glutathione *S*-aryltransferase, and CySSG-thioltransferase. Three major peaks of DTNB-dependent activity have been seen in all experiments, and the first has been found to increase upon aging of the rat liver preparation.

It is obvious from Figs. 2 and 6 that the glutathione reductase-coupled system for determination of thioltransferase activity catalyzing thiol-disulfide interchange of cystine and glutathione derivatives cannot without restrictions be replaced by the assay method based on the thiolysis of DTNB [11] when a crude enzyme preparation is used. In conclusion, a number of protein components in the supernatant fraction from rat liver catalyze the reduction of non-biological aromatic disulfides by GSH.

Glutathione reductase normally appeared as a single component in chromatographic experiments. The reductase activity obtained with DTNB and NADPH as substrates coincided with the glutathione reductase activity in all experiments (*cf.* Fig. 5). This contrasts with the finding of Tietze that the DTNB- and GSSG-reducing activities can be separated by chromatography on DEAE-cellulose.[15]

The glutathione *S*-aryltransferase activity appeared as two components which are separable from the major components of the other activities tested (*cf.* Figs. 1, 2, 4 and Ref. 29). However, isoelectric focusing did not resolve overlapping peaks of glutathione *S*-aryltransferase and DTNB-thioltransferase activity (*cf.* Figs. 3, 4, and 5) or glutathione *S*-aryltransferase and ArSSG-thioltransferase activity (Fig. 6). This finding indicates that glutathione *S*-aryltransferase, which is known to have a broad substrate specificity,[25] can utilize DTNB and ArSSG as substrates. The nature of the reactions with these substrates has not been studied, and although the original assumption was that a thiol-disulfide interchange was measured, it cannot be excluded that arylation of GSH occurred. It is known that DTNB can arylate sulfhydryl groups nonenzymatically.[30]

The results in Fig. 4 also seem to indicate that the component showing the highest thioltransferase activity with DTNB and GSH as substrates is able to catalyze the arylation of GSH with 3,4-dichloro-1-nitrobenzene.

A general conclusion based on the present investigation is that assay of either glutathione reductase, glutathione *S*-aryltransferase, or CySSG-thioltransferase is not interfered with by

the other two enzymes. The glutathione *S*-aryltransferase (*cf.* Refs. 18 and 29) and the CySSG-thioltransferase activities are present in at least two separable forms. The different forms of both of these two enzyme activities seem to be capable of catalyzing the reaction of DTNB and ArSSG with GSH. DTNB-thioltransferase activity is also associated with several protein components distinct from the other enzyme activities measured (Figs. 2, 4, and 6). Before concluding definitely that these activities are thioltransferases, it is desirable to further characterize them, bearing in mind that DTNB is a compound not normally found *in vivo*.

The two components of the CySSG-thioltransferase activity are interconvertible, as is demonstrated in a following paper.[27] This finding indicates that these are different forms of a single enzyme. This activity is responsible for the enzymatic reduction in rat liver of a variety of low molecular weight disulfides (see Introduction), as well as for the reduction of *S*-sulfo-derivatives of, for example, cysteine or GSH.[26] CySSG-thioltransferase is present in the cytosol [1] and is clearly distinguishable from the enzyme ("GSH-insulin transhydrogenase") catalyzing thiol-disulfide interchange between GSH and insulin or other polypeptide disulfides.[31,32] The latter enzyme is also present in rat liver [33,34] and is probably localized in the microsomal fraction.[15,35,36] The conclusion that the two enzymes are different is further supported by the lack of activity of purified CySSG-thioltransferase with 0.25 mM GSH and 0.25 mM insulin or oxytocin in the glutathione reductase-coupled system (S. Eriksson, unpublished experiments). On the other hand, the CySSG-thioltransferase is probably identical with the thiol-disulfide "transhydrogenase" of rat tissues active with low molecular weight substrates.[3,7,15] This latter enzyme was originally described by Racker under the name of glutathione-homocystine transhydrogenase [2] and has subsequently also been purified from yeast.[6]

The enzyme-catalyzed reaction between GSH and DTNB has, with the exception of the activity observed with partially purified CySSG-thioltransferase,[11] not previously been described. It seems possible that the enzymatic activity observed with GSH and thiamine disulfides as substrates may be of a similar nature.[10,18] A partially purified preparation of the latter activity was not active with cystine, cystamine, or homocystine as substrates, which was taken as evidence that this activity is not identical with CySSG-thioltransferase.[10] The thioltransferase activities obtained with DTNB and that described for thiamine disulfides are similar in that some of the components separated by ion-exchange chromatography or isoelectric focusing coincide with peaks of glutathione *S*-aryltransferase activity (Figs. 3, 4, and 5; *cf.* Ref. 18).

It is clear that the high redox potential of DTNB makes it more reactive than alkyl disulfides. Therefore, it is not unexpected that several components of rat liver are able to catalyze the reaction between thiols and DTNB (*cf.* Figs. 3 and 5). Whether the activity demonstrated with DTNB has any physiological significance remains to be seen.

Pyridine nucleotide-dependent reduction of DTNB has previously been observed in several enzymatic systems. In bacteria and bacterial spores a reductase has been identified which is more active with DTNB than with any other disulfide (including GSSG) tested.[13,14] Multicomponent systems,[37,38] probably identical with the thioredoxin system,[39] capable of NADPH-dependent reduction of disulfides are also active with DTNB. A similar reduction of DTNB which may also be partially linked to thioredoxin has been detected in rat liver.[15–17] Furthermore, it is known that glutathione reductase can directly catalyze the reduction of DTNB, although this activity is relatively low.[40,41] In the present investigation the only NADPH-dependent activity detected after isoelectric focusing (Fig. 5) or CM-cellulose chromatography seems to be the latter activity; the thioredoxin system is probably not functional due to separation of thioredoxin and thioredoxin reductase. Finally, it should be observed that the discussion in the literature of the NADPH-dependent reduction of DTNB [9,15–17] has ignored the reactions between DTNB and GSH (eqns. 4, 5), which can be coupled to glutathione reductase (eqn. 3). The present investigation shows that this thiol-disulfide interchange is enzymatic. Failure to properly evaluate the contribution of corresponding thiol-disulfide interchange reactions has previously limited the understanding of the nature of "cystine reductase" [42–44] (*cf.*

Ref. 45), "*S*-sulfoglutathione reductase",[46,47] (*cf.* Ref. 48) and "coenzyme A-glutathione mixed disulfide reductase" [49–52] (*cf.* Refs. 5 and 26).

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Evidence for a Glutathione-dependent Interconversion of Two Forms of a Thioltransferase from Rat Liver Catalyzing Thiol-Disulfide Interchange

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In studies on rat liver two forms of a thioltransferase (EC 1.8.4.1), an enzyme catalyzing thiol-disulfide exchange, were separated by CM-cellulose chromatography and isoelectric focusing. Incubation with glutathione increased the activity of the more acidic form and caused a concomitant loss of the basic form. This effect could be reversed by dithioerythritol. Glutathione disulfide did not effect the conversion of one form of the enzyme into the other. Treatment of the basic component with [^{35}S]-GSH resulted in incorporation of radioactivity which was recovered in the acidic thioltransferase peak found upon repeating the chromatography on CM-cellulose. These experimental findings are consistent with a mechanism of interconversion involving thiol-disulfide interchange of GSH and a disulfide group of the enzyme.

In a previous paper we reported that two forms of a thioltransferase, an enzymatic activity in rat liver catalyzing thiol-disulfide interchange with glutathione (GSH) and the mixed disulfide of cysteine and glutathione (CySSG),* could be separated by CM-cellulose chromatography and isoelectric focusing.[1] These forms were distinct from similar activities observed in aged rat liver preparations. The ratios of the reaction velocities obtained with a variety of glutathione sulfenyl derivatives (such as CySSG, *S*-sulfoglutathione, and the mixed disulfide of coenzyme A and glutathione) as well as with other disulfides and thiosulfate esters were the same for both fractions of the CySSG-thioltransferase activity, indicating that these are different forms of the same enzyme.[2] The distribution of the total CySSG thioltransferase activity between its two different forms varied with experimental conditions in a way which suggested that the variations could be ascribed to dissociation or association phenomena. The present investigation was undertaken to further elucidate the mechanism of interconversion of the two forms of the enzyme.

* Unusual abbreviations: CySSG, the mixed disulfide of cysteine and glutathione; $CySSO_3H$, *S*-sulfocysteine; DTE, dithioerythritol.

MATERIALS AND METHODS

In addition to the materials and methods described in the previous paper,[1] dithioerythritol (DTE) (Sigma); Diaflo ultrafiltration membranes (Amicon); [^{35}S]-GSH (Schwarz/Mann), and Aquasol (NEN) have been used.

Pretreatment of homogenates from rat liver before CM-cellulose chromatography. Livers from male Sprague-Dawley rats (45 g) were homogenized shortly after removal in 4 volumes of 0.25 M sucrose and the resulting homogenate was centrifuged for 1 h at 145 000 *g*. The supernatant (134 ml) was adjusted to pH 8.0 and divided into three equal parts. One part served as a control in the subsequent CM-cellulose chromatography. The remaining two parts were incubated at pH 8.0 for 5 min with 2 mM GSH and one of these was afterwards treated with 1 mM dithioerythritol for another 5 min. The three preparations were then chromatographed on separate Sephadex G-25 columns of equal sizes to remove GSH and dithioerythritol and to change the solvent of the samples to the buffer used for equilibration of the CM-cellulose columns. The sample volume was 20 % of the Sephadex G-25 bed volume.

CM-cellulose chromatography. Fractionation of the three samples on CM-cellulose was carried out simultaneously under identical conditions, and the effluent was collected in 7 – 10

ml fractions. Three CM-cellulose columns (2 × 14 cm) were equilibrated with 10 mM sodium phosphate buffer – 1 mM EDTA (pH 6.2). Each sample was introduced into a separate column, which was then washed with the buffer used for equilibration until the UV absorption of the effluent vanished. Elution was carried out with a linear gradient formed from 250 ml 10 mM sodium phosphate – 1 mM EDTA (pH 6.2) in the mixing chamber and 250 ml 50 mM sodium phosphate – 1 mM EDTA 0.2 M NaCl (pH 6.2) in the reservoir.

[^{35}S]-GSH labelling of thioltransferase collected from CM-cellulose columns. Pooled fractions were concentrated two-fold on a PM 30 Diaflo membrane, adjusted to pH 8.0, and incubated for 10 min with 120 μM [^{35}S]-GSH (spec. act. 5 Ci/mol). The incubation mixture was desalted on Sephadex G-25 according to the procedure described above and subsequently rechromatographed on CM-cellulose to determine whether the basic form of the thioltransferase had incorporated [^{35}S]-GSH to give the acidic form. Radioactivity was determined by liquid scintillation counting of 1 ml samples in 10 ml Aquasol.

Ultrafiltration. The first and second peaks of the thioltransferase activity, obtained after CM-cellulose chromatography, were collected separately and each was subjected to a series of ultrafiltrations on membrane filters of increasing porosities. The collected material (37.5 ml) was first concentrated to a volume of 13.5 ml on a PM 30 membrane. This concentrate was then filtered on an XM 50 membrane until a volume of 4 ml remained in the cell. Finally, this remaining material was concentrated to 1 ml on an XM 100 membrane. Both peaks were treated in the same manner at 4 °C and the enzyme activities in both concentrates and filtrates were measured.

RESULTS AND DISCUSSION

Fig. 1 demonstrates a typical elution profile obtained after chromatography of the supernatant fraction from rat liver on a CM-cellulose column. Gradient elution separated the thioltransferase activity into two components. The distribution of the activity was dependent on the experimental conditions and varied from a distribution in which the first peak contained the major part of the activity (*cf.* Ref. 1, Fig. 2C) to a distribution in which the second peak dominated. The possibility was considered that the appearance of two peaks was an artifact produced by the steep rise in the pH of the effluent which coincided with the appearance of thioltransferase activity (Fig. 1). The initial decrease in the pH of the effluent is caused by

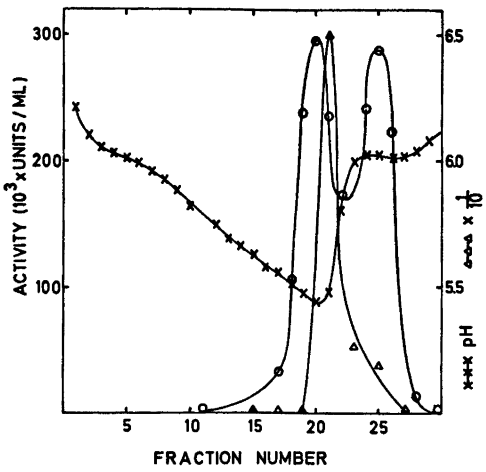

Fig. 1. CM-cellulose chromatography of the supernatant fraction from 30 g of rat liver after chromatography on Sephadex G-25. The sample was eluted with a linear salt gradient. The thioltransferase activity appears in two peaks of similar sizes if the sample volume is about 20 % of the volume of the Sephadex gel bed. (See Materials and methods). The enzymatic activities are: O, thioltransferase measured with $CySSO_3H$ and GSH as substrates; △, glutathione reductase.

substitution of sodium ions of the gradient for hydrogen ions of the ion-exchange matrix and the subsequent rise in pH indicates the establishment of new ionic equilibria. However, glutathione reductase, which appears in the same region of the elution profile, is not split into two components. Furthermore, the two peaks of thioltransferase activity were also obtained when a gradient having less buffering capacity was used, conditions which delay the pH rise (*cf.* Ref. 1, Fig. 2C). Finally, isoelectric focusing also established the existence of two separable components of thioltransferase activity and thus eliminated the possibility that the appearance of the two peaks was an artifact due to a particular separation technique.

It was discovered that the size of the column beds had a marked effect on the distribution of the thioltransferase activity between the two peaks. An increase of the second component at the expense of the more acidic one was correlated with an increase in the column bed size using a constant sample volume. This effect was most clearly observed with changes in the column bed size for gel filtration on Sephadex

G-25, which preceded the CM-cellulose chromatography, but also to a smaller extent in the latter separation. It is well known that Sephadex can retard substances by adsorption due to hydrophobic interactions,[3,4] and the possibility was therefore considered that non-covalently bound lipids could be removed from the enzyme, thereby causing conversion of the more acidic thioltransferase component into the other form.

That lipids are indeed separated from the protein fraction of rat liver supernatant by gel filtration is suggested by the appearance of a very slowly migrating yellowish zone, which causes the fractions it is eluted in to appear turbid. In order to test the possibility that lipids constitute the difference between the two peaks of thioltransferase activity, the supernatant fraction from rat liver was treated, after gel filtration and before ion-exchange chromatography, with the lipid fraction obtained from the Sephadex column. However, no difference between the treated sample and the untreated control could be detected with respect to the distribution of the thioltransferase activity (22 and 78 % of the total activity in the first and second peaks, respectively, for both samples). Treatment of the sample with different mixtures of purified lipids known to be present in rat liver did not change the relative amounts of the two components either.

The influence of the column bed size on the distribution pattern of thioltransferase activity can be explained by assuming that the two components are in a dissociation-association equilibrium. The dissociation could involve removal of a low-molecular weight compound, as in the hypothesis discussed above, or consist of an oligomer-monomer transition by a protein containing subunits. In order to test the latter possibility the two forms of thioltransferase obtained after CM-cellulose chromatography were compared with respect to their molecular weights by means of ultrafiltration on Diaflo filters of different porosities. Ultrafiltration was chosen as the method for molecular weight determination instead of gel filtration, because the latter procedure causes inactivation of thioltransferase activity and is known to remove, for example, GSH from the thioltransferase. A preliminary estimation by gel filtration on Sephadex G-50 of the molecular weight of the basic form of the thioltransferase indicated a value of about 50 000; and the molecular weight of the acidic form was therefore expected to be a multiple of this value, if dissociation and association of subunits would be the mechanism of the interconversion of the two enzyme forms. The results of ultrafiltration of the two forms are shown in Table 1. Care was taken to use membranes with pore sizes such that full and partial retention of the enzyme activity in the ultrafiltration cell were both obtained. This is essential, because both oligomer and monomer may be too large to penetrate the membrane filter or both may be too small to be retained at all. The extent of retention and penetration of the two components was not significantly different on membrane filters of the same porosity, thus excluding major differences in molecular weights. The retention characteristics of the membranes used would have revealed differences between the two components if the molecular weights were 50 000 and a multiple thereof. Consequently, these results seem to

Table 1. Activity distribution in concentrate and filtrate after ultrafiltration of the two thioltransferase components. The most acidic (peak I) and the most basic (peak II) components were separated by CM-cellulose chromatography and were subjected to ultrafiltration as described in Materials and methods.

Filter	Activity (10^3 × units/ml) Peak I Concentrate (C)	Filtrate (F)	$\frac{F}{C}$	Peak II Concentrate (C)	Filtrate (F)	$\frac{F}{C}$
PM 30	99	0	0	1232	0	0
XM 50	332	7.5	0.02	2304	30	0.01
XM 100	504	116	0.23	4192	896	0.21

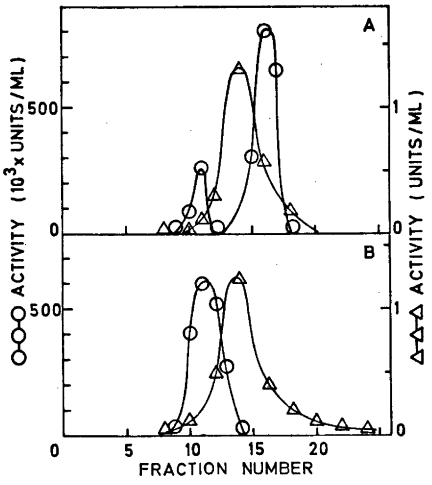

Fig. 2. CM-cellulose chromatography of (A) an untreated control and (B) a GSH-treated rat liver supernatant. The supernatant fraction from 30 g of rat liver was adjusted to pH 8.0 and divided into two parts. One of these was used as a control in the subsequent CM-cellulose chromatography. The second part was treated with 2 mM GSH for 5 min. Both parts were chromatographed simultaneously on separate Sephadex G-25 columns to change the solvent to that used in the subsequent CM-cellulose chromatography. The symbols for enzymatic activities are given in the legend to Fig. 1.

exclude an oligomer-monomer transition as the explanation for the two peaks of thioltransferase activity.

Glutathione, which is present in high concentration in rat liver,[5] is a substrate of the thioltransferase and has been found to activate aged preparations of the enzyme (Ref. 6 and unpublished results). Therefore, another explanation of the appearance of two thioltransferase components could be that the enzyme occurs in both a GSH-containing and a GSH-deficient form. Fig. 2 illustrates an attempt to explore this possibility. It is shown that after incubation with 2 mM GSH (see MATERIALS AND METHODS), the second component, after chromatographing on CM-cellulose had disappeared and the first component had increased. The first component was identified from its position in relation to that of glutathione reductase in the elution profile. The position of glutathione reductase in the elution profile was not affected by pretreatment with GSH, nor could any effect on the positions of the two peaks of aryltransferase be demonstrated (*cf.* Ref. 1). This conclusion was based on measurements of the pH, volume, and conductance of the effluent. Furthermore, the GSH-treated thioltransferase had a total activity corresponding to the sum of the activities of the two peaks obtained from the untreated control sample, which supported the interpretation that the second component was converted into the first one.

To obtain further evidence for this interpretation, the following experiment was carried out. A sample was divided into three equal parts, one of which served as an untreated control for CM-cellulose chromatography. The remaining two parts were treated with 2 mM GSH as in the experiment described above, and one of these was afterwards incubated with 1 mM DTE before the ion-exchange chromatography. The purpose of the experiment was to confirm the previous observation and to find out whether the effect of GSH could be reversed by DTE, which is a strong disulfide-reducing agent. The amount of DTE used was sufficiently small to prevent complete conversion of the first component into the second one. This precaution was taken to eliminate the possibil-

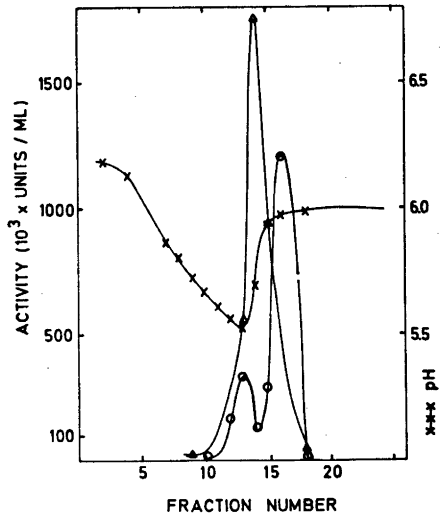

Fig. 3. The reversal by dithioerythritol (DTE) of the effect of GSH-treatment seen in Fig. 2. GSH-treated sample (see Materials and methods and Fig. 2) was incubated with 1 mM DTE for 5 min at pH 8.0 before the subsequent Sephadex G-25 and CM-cellulose chromatography. Thioltransferase (activity x 2). The symbols for enzymatic activities are given in the legend to Fig. 1.

ity that the effect of the DTE-treatment was a change in the relative positions of glutathione reductase and the first thioltransferase peak. The three different preparations were freed by gel filtration from the reagents used in the pretreatment and were chromatographed under identical conditions on CM-cellulose columns of equal sizes. The results of the chromatography of the untreated and GSH-treated samples were similar to those described in Fig. 2; and the result obtained with the DTE-treated preparation demonstrated a reappearance of the second thioltransferase peak (Fig. 3) such that this peak contained an even greater amount of the total thioltransferase activity than the second peak from the untreated control. The chromatographic properties of glutathione reductase were not affected by the DTE-treatment. Thus, this experiment demonstrates that the second component can be converted into the more acidic one by treatment with GSH and that the more acidic component can then be reconverted into the second one by incubation with DTE.

To obtain direct evidence that GSH was bound to the thioltransferase, [^{35}S]-GSH was incubated with different enzyme preparations Treatment of the supernatant fraction of rat liver with [^{35}S]-GSH resulted in only a small incorporation of radioactivity, because the concentration of endogenous GSH in the supernatant was approximately 1 mM, which was in large excess of the [^{35}S]-GSH added. Furthermore, extensive washing of the material adsorbed onto CM-cellulose released [^{35}S]-GSH in a large volume after the effluent containing the unadsorbed protein. This leakage may arise from dissociation of GSH from the enzyme-GSH complex. Consequently, no distinct peak of radioactivity was obtained on subsequent elution with a linear salt gradient.

However, the results were more definitive when the basic form of thioltransferase obtained by CM-cellulose chromatography was treated with radioactive GSH and rechromatographed on CM-cellulose in order to visualize [^{35}S]-GSH binding to thioltransferase. This treatment demonstrated the formation of an acidic thioltransferase which coincided with the peak of radioactivity in the elution profile (Fig. 4). Coincidence between enzyme activity and radioactivity is in itself not conclusive evidence for a

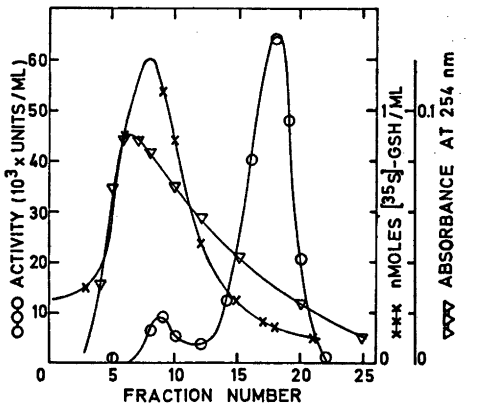

Fig. 4. Incorporation of [^{35}S]-GSH into the basic thioltransferase component. The basic component (22 ml, 1.64 mg protein per ml) obtained after CM-cellulose chromatography was adjusted to pH 8 and incubated with 120 μM [^{35}S]-GSH (spec. act. 5 Ci/mol). The incubation mixture was chromatographed on Sephadex G-25 and then chromatographed on a CM-cellulose column (2 x 4 cm). Unadsorbed proteins and free GSH were washed out before elution of the thioltransferase with a linear salt gradient. The gelfiltration removed 60 % of the original radioactivity, 7 % was recovered in the effluent (50 ml) preceding the gradient, and 0.5 % appeared in the fractions containing the acidic form of the thioltransferase. The symbols are: ∇, absorption at 254 nm; x, [^{35}S]-GSH; O, CySSG-thioltransferase.

GSH-enzyme complex, because the enzyme species in question is not pure enough. However, this evidence for the binding of [^{35}S]-GSH to thioltransferase is strengthened by the fact that incubation of the basic form of thioltransferase with radioactive GSH gives rise to an acidic enzyme form and a coincident peak of radioactivity.

These results support the hypothesis that the enzyme can exist in both a GSH-containing and a GSH-deficient form, as does the finding that the GSH-treatment gives rise to the most acidic thioltransferase component, which is expected due to the acidic properties of GSH. This explanation is also in accord with the results of the ultrafiltration experiments in which no significant difference in the molecular weights of the two components could be demonstrated.

It was also shown by equilibrium dialysis that GSH was bound to a partially purified thioltransferase preparation, but the possibility that the

binding measured was caused by components other than the thioltransferase could not be excluded.

It is assumed that GSH is covalently linked to the enzyme *via* a disulfide bond. If so, DTE-treatment of the native enzyme would be expected to increase the second component at the expense of the first one, a result which was in fact obtained experimentally. The binding of GSH to the enzyme could take place by thiol-disulfide interchange either between GSH and a disulfide group of the enzyme or between GSSG (formed by oxidation of GSH) and a sulfhydryl group of the protein. To test the latter possibility, the enzyme was treated with 1 mM GSSG before chromatography on CM-cellulose. This treatment gave no significant increase of the more acidic component, which lends support to the former mechanism of binding. According to this interpretation, GSH and a disulfide-containing form of the thioltransferase are in equilibrium with a mixed disulfide of GSH and the enzyme (Fig. 5). This scheme explains why the amount of the GSH-containing component decreases with the size of the chromatographic bed in the gel filtration experiments; removal of GSH shifts the equilibrium in favor of the GSH-deficient enzyme form. It should also be pointed out that the binding of GSH according to Fig. 5 may be a partial reaction of the catalytic mechanism of the enzyme.

It is noteworthy that glutathione *S*-epoxide transferase can also occur in multiple forms and that these forms are also interconvertible under the influence of GSH.[7] Furthermore, the GSH concentration influences the relative proportions of the three major components of glucose-6-phosphate dehydrogenase in rat liver.[8] Highly purified enzymes, such as glyceraldehyde-3-phosphate dehydrogenase[9] and acylphosphatase,[10] have been found to contain bound GSH, but in these cases the possibility exists that these enzyme forms are artifacts formed during purification. Data in the literature also suggest that enzymatic activities can be modulated by formation of mixed disulfides with low-molecular-weight compounds (*cf.* Refs. 11–13) and that GSH can be involved in such thiol-disulfide interchange reactions. This postulated physiological function receives support from the finding that a substantial proportion of the cellular GSH is bound to proteins.[14–16]

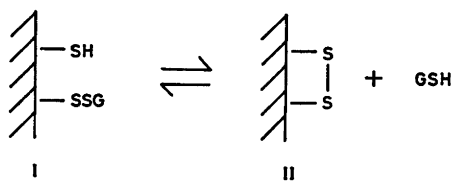

Fig. 5. The proposed equilibrium between GSH and CySSG-thioltransferase. The enzyme forms I and II correspond to the first (acidic) and second (basic) component, respectively, obtained after chromatography on CM-cellulose.

Acknowledgements. This work was supported by grants from Stiftelsen Lars Hiertas Minne and C. F. Lundströms Stiftelse (to S. E.) and from the Swedish Natural Science Research Council and the Swedish Cancer Society (to B. M.).

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Interaction of Quinacrine Mustard with Whole and Partially Deproteinized Calf Thymus Deoxynucleoproteins

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Spectrophotometric and fluorimetric studies were made on the binding of quinacrine mustard to whole and partially deproteinized calf thymus deoxynucleoproteins. Proteins were extracted from the deoxynucleoprotein with 0.2, 0.3, 0.4, 0.6, and 3 M NaCl in the presence of 6 M urea and 0.04 M sodium bisulfite. The proteins were further separated from the remaining deoxynucleoprotein on Bio-Gel A-50 columns. A decrease in the fluorescence intensity of quinacrine mustard was observed as the proteins were removed from the deoxynucleoprotein. The greatest decrease was obtained when most of the proteins (extraction with 0.6 or 3 M NaCl in 6 M urea and 0.04 M sodium bisulfite) were removed from the DNA core. At low dye-to-polymer ratios the removal of all the histones (NaCl concentrations above 0.4 M in 6 M urea and 0.04 M sodium bisulfite) caused a marked change in the fluorescence intensity of quinacrine mustard. Probably the proteins compete for the binding of the dye to deoxynucleoprotein. Natural and artificial deoxynucleoproteins reacted differently with quinacrine mustard. This probably reflects structural differences between the polymers. When quinacrine and quinacrine mustard were mixed with whole deoxynucleoprotein, differences occurred in the absorption and fluorescence properties of the two dyes. This suggests that the two dyes must interact in different ways with the polymer.

Studies on the chemical composition of chromosomes in eucaryotes show that several kinds of proteins, both basic (histones) and acidic (non-histones), as well as RNA, are bound to chromosomal DNA.[1] Various techniques have been used to investigate the structure of these chromosomal components.[2-4] The interaction between small molecules or dyes with macromolecules has also provided information about the molecular organization of chromosomes.

Dyes that have been found useful include acridine derivatives, such as acridine orange,[5-7] proflavine,[8-10] quinacrine and quinacrine mustard.[6,7,11-14] The interaction of other dyes such as azure A,[15] ethidium bromide,[16] and toluidine blue [17] with macromolecules has also been investigated.

A fair amount of evidence indicates that quinacrine and quinacrine mustard bind differentially to the bases in nucleic acids (see reviews above). An enhancement of the fluorescence intensity of these two dyes occurs with adenine residues, whereas guanine residues decrease the fluorescence intensity of the dyes in solution. Weisblum and de Haseth [7] postulated that the fluorescence intensity of bound quinacrine also reflects the pattern of base-repetition in the nucleic acid. The binding of the dye also depends on the strandedness of the nucleic acid.[14,18] Studies on the interaction between quinacrine mustard and proteins (histones) in solution showed that the dye-protein interaction depends on the physical state of the protein.[18]

This paper describes the absorption and fluorescence properties of quinacrine mustard bound to deoxynucleoproteins in solution. Parallel studies were made with the related dye, quinacrine. The data refer to staining of metaphase chromosomes *in situ* with fluorochromes.

EXPERIMENTAL

Deoxynucleoproteins. DNP* from calf thymus was isolated according to the method of Zubay and Doty.[19] The DNP was characterized by a low 320 nm to 260 nm ratio of absorbance (≤ 0.05) and by a protein to DNA ratio of

Table 1. Chemical characterization of DNA, whole DNP and partial DNP's from calf thymus. Extraction performed with urea, $NaHSO_3$, and increasing amounts of NaCl.

Sample	E_{260}[a] (10^{-3})	E_{260}/E_{280}	E_{260}/E_{230}	Protein/DNA ratio	% Protein remaining
DNP (whole)	7.7	1.16	0.71	1.62	100
DNP (extracted)					
0.2 M NaCl	7.1	1.23	0.83	0.68	43
0.3 M NaCl	7.1	1.52	1.13	0.60	32
0.4 M NaCl	7.0	1.53	1.31	0.50	23
0.6 M NaCl	6.8	1.62	1.27	0.37	14
3 M NaCl	6.8	1.62	1.27	0.19	7
DNA[b]	6.8	1.95	1.93	0.00	0

[a] Angerer and Moudrianakis.[16] [b] DNA from calf thymus was isolated as described elsewhere.[20]

approximately 1.6 (Table 1). The whole DNP was stored in 0.7×10^{-3} M potassium phosphate buffer, pH 8.0 at −20 °C.

Partial deoxynucleoproteins. Partial DNP's were prepared by selective dissociation of proteins with NaCl, urea, and sodium bisulfite.[21]

Freshly prepared chromatin was dialyzed against 6 M urea and placed in a buffer solution containing both 6 M urea and 0.04 M sodium bisulfite at pH 7.8 and with increasing amounts of NaCl. The final NaCl concentrations were 0.2, 0.3, 0.4, 0.6, and 3 M. The solutions were stored for 24 h at 4 °C before gel filtration in the same salt concentration. Bio-Gel A-50 (BIO-RAD Laboratories, U.S.A.) columns equilibrated at the same NaCl, urea, and $NaHSO_3$ concentrations were used to separate the dissociated proteins. Samples of 20 ml containing approximately 60 to 80 E_{260} units DNP were placed on these columns, 35 cm in length and 2.5 cm in diameter. The rate of elution at 4 °C was approximately 0.5 ml/min. The DNP peak separated off first followed by the protein peak (Fig. 1). The recovery of the DNP was approximately 75 % and that of the protein 60 to 70 %. The DNP was assayed at 260 nm. It was not possible to assay the proteins at 230 nm since $NaHSO_3$ absorbs strongly at this wavelength. The proteins were therefore assayed by measuring the turbidity at 400 nm after precipitation with trichloroacetic acid.[22] The partial DNP's were stored in 2×10^{-4} M EDTA, pH 6.2 and the proteins in 1 % acetic acid.

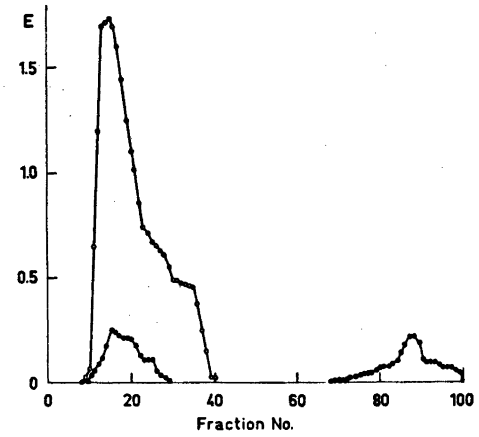
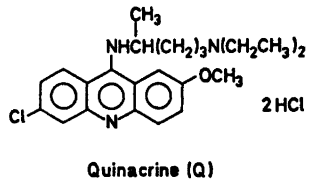
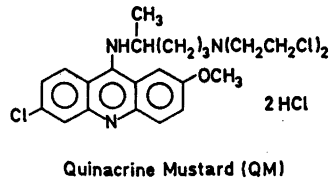


Fig. 1. Chromatography of deoxynucleoprotein on Bio-Gel A-50. The partially deproteinized DNP (*cf.* Experimental) in 0.6 M NaCl, 6 M urea and 0.04 M $NaHSO_3$ was applied to a Bio-Gel A-50 column and step-wise eluted (2.5 ml portions) with the same salt solution. The DNP (○) was estimated at 260 nm and the protein (●) at 400 nm according to Bonner *et al.*[22]

Dyes, The dyes Quinacrine and Quinacrine Mustard* were gifts from Sterling-Winthrop Research Institute, U.S.A. The structures of



Quinacrine (Q)



Quinacrine Mustard (QM)

(I)

* In this publication deoxynucleoprotein = DNP; Quinacrine = Q; Quinacrine Mustard = QM.

the two dyes are shown in (I). Spectral characteristics of QM have been reported earlier.[12] The absorption and fluorescence properties of Q were almost identical with those of QM. The absorption spectra of Q showed peaks at 280, 345, 424, and 435 nm. The molar extinction coefficient at 424 nm was 8.5×10^3.[23] With excitation at 436 nm a fluorescence peak was obtained at 514 nm.[19] The fluorescence intensity of Q was greatest at pH 11 to 12.

Proteins. Calf thymus histone fractions IIA (intermediate histone fraction), III (lysine-rich histones) and IV (arginine-rich histones) were purchased from Sigma Chemical, U.S.A.

Analysis. DNA content was determined by the absorption at 260 nm using the molar extinction coefficients for whole and partial DNP's in Table 1, and cross-checked with the diphenylamine reaction.[24] Proteins were determined by the method of Lowry *et al.*[25] calibrated against bovine serum albumin.

The methods of absorption and fluorescence measurements have been described elsewhere.[12] The fluorescence intensity was measured at 514 nm with excitation at 436 nm. Before any titration measurements the DNP samples were dialyzed against 0.7×10^{-3} M potassium phosphate buffer, pH 8.0 or against 0.05 M Tris solution, pH 7.5 (*cf.* Experimental). In absorption measurements an identical DNP sample without dye was used in the reference cell to eliminate any contribution to the absorption by DNP. DNP did not disturb the fluorescence measurements at 514 nm.

The fluorescence data were expressed as the percentage change (Δ %) relative to the intensity of the fluorescence intensity of the dye alone.

Reconstitution of proteins and DNA. Artificial DNP's were prepared according to the method described by Bonner *et al.*[22] In these experiments histone fractions IIA, III, and IV (Sigma) and calf thymus DNA were used. The histone-to-DNA mass ratios in the artificial nucleohistones were: DNA-IIA 1.24, DNA-III 1.22, and DNA-IV 0.96.

Analytical disc electrophoresis. Free proteins from the Bio-Gel A-50 runs were lyophilized and dissolved in 10 M urea, 0.9 M acetic acid. Histones were removed from whole and partial DNP's by acid extraction (0.2 M H_2SO_4) and dialyzed against 6 M urea, 0.9 M acetic acid. Disc electrophoresis was carried out using the method of Panyim and Chalkley.[26] The samples (50 – 150 μg) were applied on the gels (0.6 cm × 10 cm), which consisted of 15 % polyacrylamide in 2.5 M urea, 0.9 M acetic acid, pH 2.8, and were pre-electrophoresed for 2 h at 200 V. Electrophoreses were carried out at 200 V for 2 h. Both upper and lower buffers were 0.9 M acetic acid. The gels stained in Amido-Black (0.5 % Amido-Black in 50 % ethanol, 7 % acetic acid) for 4 h and destained by electrophoresis in 7 % acetic acid. Bovine serum albumin was added as a marker protein.

RESULTS

Composition of deoxynucleoprotein samples. Fig. 1 depicts a run on a Bio-Gel A-50 column with DNP extracted with 0.6 M NaCl, 6 M urea, and 0.04 M $NaHSO_3$. The DNP separated first (E_{260}) followed by the dissociated proteins (E_{400}).

The DNA and protein content in the various samples were analyzed spectrophotometrically and a summary of the results is shown in Table 1. The electrophoretic patterns for the different protein fractions are shown in Fig. 2. Fig. 2a depicts the pattern obtained when the histone fractions IIA, III, and IV (Sigma) were mixed together.

NaCl concentrations of 0.2 M and 0.3 M (urea, $NaHSO_3$) removed, respectively, 57 % and 68 % of the proteins from the DNA core (Table 1). These proteins were mostly lysine-rich histones, and almost no arginine-rich histones were extracted from the DNP at these low NaCl concentrations (Fig. 2b, c). At 0.4 M NaCl (urea, $NaHSO_3$) some arginine-rich histones could be extracted from the DNP although

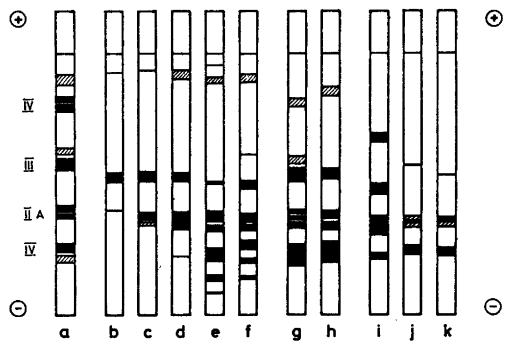


Fig. 2. Polyacrylamide gel electrophoresis. (a) Histones: IIA (intermediate histone fraction), II (lysine-rich), and IV (arginine-rich) from Sigma were dissolved in 10 M urea, 0.9 M acetic acid. (b – f) Bio-Gel A-50 separated free proteins in 10 M urea, 0.9 M acetic acid (*cf.* Experimental) extracted from whole DNP with 6 M urea, 0.04 M $NaHSO_3$ and increasing amounts of NaCl: (b) 0.2 M NaCl, (c) 0.3 M NaCl, (d) 0.4 M NaCl, (e) 0.6 M NaCl and (f) 3 M NaCl. (g – h) Histones extracted with acid from whole DNP in (g) 0.7×10^{-3} M potassium phosphate buffer, pH 8.0 and (h) 6 M urea. (i – k) Histones extracted with acid from partial DNP's, deproteinized with 6 M urea, 0.04 M $NaHSO_3$ and increasing amounts of NaCl: (i) 0.2 M NaCl, (j) 0.3 M NaCl, and (k) 0.4 M NaCl. The slowest moving band was bovine serum albumin.

the fraction still consisted mainly of lysine-rich histones and intermediate histones (Fig. 2d). The extraction with 0.6 M NaCl (urea, $NaHSO_3$) removed all the histones, even the arginine-rich ones (Fig. 2e), leaving 14 % proteins, probably the non-histones, on the DNA core (Table 1). Even after extraction with 3 M NaCl (urea, $NaHSO_3$) some of the proteins (7 %) remain on DNA (Table 1). Fig. 2f depicts the electrophoretic pattern of the histones dissociated at 3 M NaCl (urea, $NaHSO_3$).

The salt-dissociated protein fractions were compared electrophoretically with the histones which remain on partially deproteinized DNP's. The electrophoresis of histones removed by acid extraction from whole freshly prepared chromatin is shown in Fig. 2g. An almost identical electrophoretic pattern was obtained for histones removed from chromatin in 6 M urea (Fig. 2h). From this it is obvious that urea by itself does not remove histones from DNP.[21] The electrophoresis of the histones that remain on the partially deproteinized DNP's shows that an increased amount of histones was removed as the NaCl concentration in urea-$NaHSO_3$ solution was increased (Table 1 and Fig. 2 i-k). Lysine-rich and arginine-rich histones as well as intermediate histones remained on DNA after extraction with 0.2 M NaCl in urea–$NaHSO_3$ (Fig. 2i). An almost complete absence of lysine-rich histones was found on the DNA core after extraction with 0.3 M, respectively 0.4 M, NaCl in urea-$NaHSO_3$ (Fig. 2j and 2k). No histones remained on DNA after extraction with 0.6 or 3 M NaCl in urea-$NaHSO_3$ (not shown in the figure).

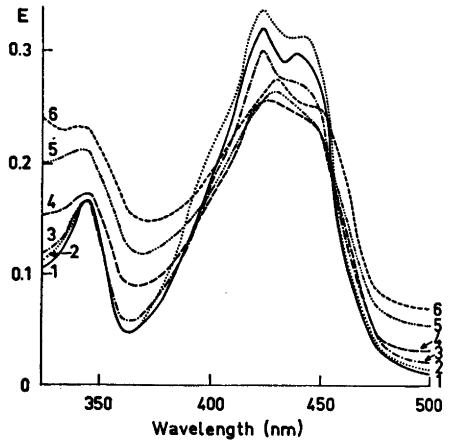


Fig. 3. Absorption spectra of quinacrine mustard mixed with whole deoxynucleoprotein. QM was mixed with different amounts of whole DNP in 0.7×10^{-3} M potassium phosphate buffer, pH 8.0. (1) 0.033 mg/ml QM alone, (2) 0.040 mg/ml DNP, (3) 0.072 mg/ml DNP, (4) 0.115 mg/ml DNP, (5) 0.150 mg/ml DNP and (6) 0.200 mg/ml DNP. (The values of concentration refer to DNA plus proteins).

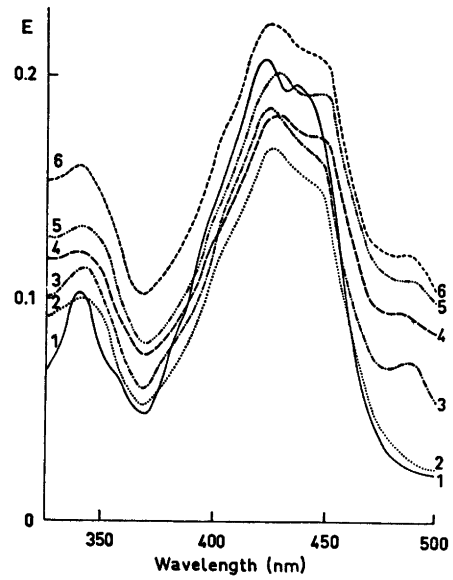


Fig. 4. Absorption spectra of quinacrine mustard bound to selectivity deproteinized deoxynucleoproteins. QM (0.013 mg/ml) was mixed with different partial DNP's (0.025 mg/ml) in 0.05 M Tris solution, pH 7.5. (1) QM alone, (2 – 6) partial DNP's from which the proteins were selectively removed by extraction with 6 M urea, 0.04 M $NaHSO_3$ and decreasing amounts of NaCl: (2) 3 M NaCl, (3) 0.6 M NaCl, (4) 0.4 M NaCl, (5) 0.3 M NaCl and (6) 0.2 M NaCl.

These data clearly show that the removal of the histones is complete at 0.6 M NaCl (urea, $NaHSO_3$) and that almost all lysine-rich histones are removed with 0.4 M NaCl (urea, $NaHSO_3$). These findings are in agreement with the results reported by Kleiman and Huang.[21]

The release of proteins from the DNP's is shown by an increase in the 260 nm to 280 nm ratio as well as in the 260 nm to 230 nm ratio (Table 1).

Absorption studies. The change in the absorption spectra of QM upon the addition of increasing amounts of whole DNP to the dye

solution is shown in Fig. 3. Similar absorption spectra have also been recorded for QM complexed to DNA.[14] At very low DNP-to-dye ratios, however, there was an overall increase in the absorption spectra (Fig. 3, curve 2). A similar enhancement was found in the fluorescence intensity of QM mixed with whole DNP (*cf.* below). The shift of the absorption maximum from 424 nm to higher wavelengths is similar to the shift found with DNA. This shift and a isosbestic point at 455 nm are indicative of the formation of specific complexes of the dye with macromolecules (Fig. 3, curves 3 – 5).[37] The absorption maximum at 345 nm is markedly increased. The fact that the isosbestic point is the same whether QM is bound to DNP or DNA suggests that the dye must interact in the same way with the two polymers. The step-by-step addition of polymers to other dyes in solution leads to similar changes in the absorption spectra of the dye tested.[16,17]

Similar changes occurred in the absorption spectra of QM with addition of partially deproteinized DNP's to the dye solution (Fig. 4). For DNP's from which the proteins have been removed with 0.3 M NaCl in urea-$NaHSO_3$ (mostly the lysine-rich histones, Fig. 2c) the absorption maximum at 424 nm was reduced. However, the DNP fraction extracted with 0.2 M NaCl (urea, $NaHSO_3$) gave an overall enhancement of the absorption spectra of QM

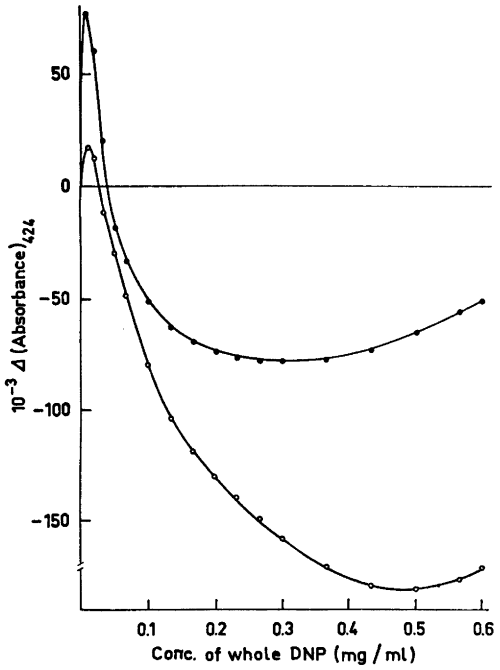


Fig. 6. Absorption properties of quinacrine and quinacrine mustard bound to whole deoxynucleoprotein. To a constant amount of Q and QM (0.033 mg/ml) were added increasing amounts of whole DNP in 0.7×10^{-3} M potassium phosphate buffer, pH 8.0, Differences in the absorption maximum at 424 nm for (O) Q and (●) QM were estimated.

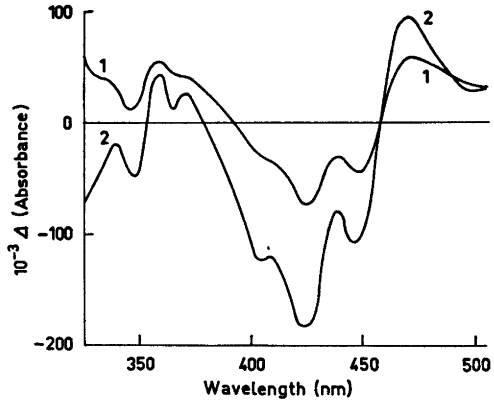


Fig. 5. Difference spectra of quinacrine and quinacrine mustard bound to whole deoxynucleoprotein. Whole DNP (0.115 mg/ml) was mixed with (1) 0.033 mg/ml QM and (2) the same amount of Q in 0.7×10^{-3} M potassium phosphate buffer, pH 8.0.

(Fig. 4, curve 6). The same enhancement was found with whole DNP (Fig. 3, curve 2). The absorption spectra of QM was reduced the most with the DNP fraction extracted with 3 M NaCl in urea-$NaHSO_3$ (all the histones were removed from the DNA core, Fig. 2f). When the amount of the proteins removed from the DNP increased (increased NaCl concentration in urea-$NaHSO_3$), a corresponding increase in the ratio of absorption at 424 and 365 nm was observed. For DNP extracted with 0.2 M NaCl (urea, $NaHSO_3$) the 424 to 365 nm ratio was 2.15 whereas the corresponding value for DNP extracted with 3 M NaCl (urea, $NaHSO_3$) was 3.11.

Whole DNP alters the absorption spectra of Q, too. However, the absorption spectra of bound Q differs from that of bound QM (Fig. 5). When equal amounts of Q and QM was used, the spectra of Q were more reduced upon the addition of whole DNP to the dye solution.

The differences between these two dyes was particularly marked at wavelengths around the absorption maximum at 424 nm (Fig. 5).

The step-by-step addition of whole DNP to Q or QM leads first to an enhancement of the absorption maximum at 424 nm (Fig. 6). However, DNP concentrations above 0.03 mg/ml give a continuous reduction of the absorption maximum at 424 nm. A much higher enhancement of the absorption maximum (424 nm) was recorded in the QM-DNP mixtures than in the Q-DNP mixtures. However, the decrease of the absorption maximum is greater in the Q-DNP system than in the QM-DNP system. A DNP concentration of 0.4 mg/ml was still effective in reducing the absorption maximum of Q. However, only DNP concentrations below 0.2 mg/ml reduce the absorption maximum of QM, and further additions of whole DNP to the dye solution did not change the absorption maximum of bound QM at 424 nm.

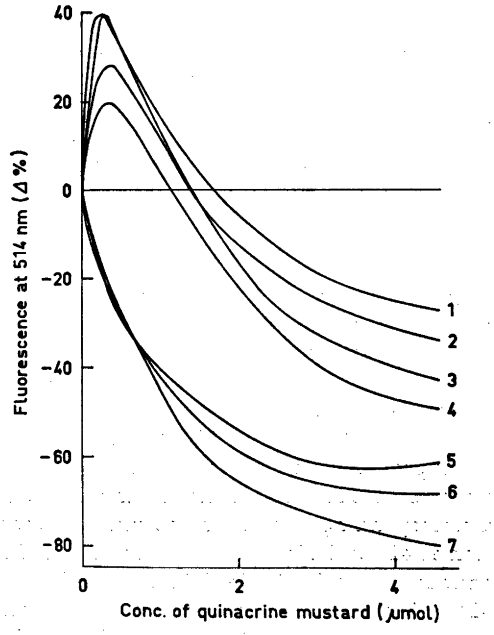


Fig. 7. Effect of DNA, whole and selectively deproteinized deoxynucleoproteins on the fluorescence intensity of quinacrine mustard. To a constant amount (0.025 mg/ml) of (1) whole DNP and partial DNP's extracted with (2) 0.2 M NaCl, (3) 0.3 M NaCl, (4) 0.4 M NaCl, (5) 0.6 M NaCl and (6) 3 M NaCl in 6 M urea, 0.04 M $NaHSO_3$, and (7) DNA, increasing amounts of QM in 0.05 M Tris solution, pH 7.5 were added.

At DNP concentrations above 0.35 mg/ml there was an increase in the absorption maximum of QM. These differences between the two dyes suggest that they must interact in different ways with the DNP. This is in line with the findings reported by Michelson *et al.*[11] They showed differences in the interaction of Q and QM with polynucleotides, particularly with poly(I), poly(dT), and poly(rU).

Fluorescence studies. The changes in the fluorescence intensity of QM bound to DNA, whole DNP, and partial DNP's are shown in Fig. 7. All the samples gave a net quench of the fluorescence intensity of the dye at high dye-to-polymer ratios. From the figure it is obvious that the samples can be divided into two separate groups. At high polymer-to-dye ratios, whole DNP and partial DNP's still containing arginine-rich histones (extraction with 0.2 to 0.4 M NaCl in urea-$NaHSO_3$, Fig. 2) enhanced the fluorescence intensity of the dye, whereas DNA and partial DNP's extracted with $\geq$0.6 M NaCl (urea, $NaHSO_3$) always quenched the fluorescence intensity of QM. It is evident that the proteins bound to DNA are able to

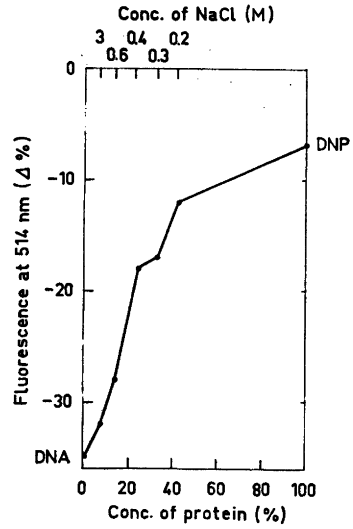


Fig. 8. Effect of selective removal of proteins from deoxynucleoprotein on the fluorescence intensity of quinacrine mustard. To a constant amount of polymer (0.030 mg/ml) was added QM (0.013 mg/ml) in 0.05 M Tris solution, pH 7.5. The proteins were selectively removed with 0.2, 0.3, 0.4, 0.6, and 3 M NaCl in 6 M urea and 0.04 M $NaHSO_3$ (*cf.* Experimental).

prevent the binding of the dye (steric hindrance). The histones are bound to the phosphate groups in DNA,[28] thus also limiting the external ionic binding of the dye to negative phosphate groups. When the dye concentration was increased, the fluorescence intensity of the solution decreased as compared with the solution containing the dye alone. This is probably due to an interaction between proteins and the dye as well as to an increased dye-to-dye interaction.[18,29]

The effect of the quantity of the proteins in the DNP on the fluorescence intensity of QM is shown in Fig. 8. The fluorescence intensity of the dye was directly proportional to the amount of protein in the DNP. The curve was almost rectilinear between 0 and 42 % protein in the DNP. The fluorescence intensity decreased by 75 % when all histones (86 % in the whole DNP, Table 1) were removed from the DNP (extraction with 0.6 M NaCl in urea-$NaHSO_3$). The extraction of the remaining proteins from the DNP (14 % in whole DNP, Table 1) accounts for the further decrease (25 %) of the fluorescence intensity of the dye. Thus the fluorescence intensity of nucleoprotein-bound QM mostly depends on the tightly bound proteins, probably the non-histones, in the DNP. The quenching effect of native DNA on the fluorescence intensity of the dye was 5 times greater than that of whole DNP.

When whole DNP was added step-by-step to QM there was first an enhancement of the fluorescence intensity of the dye (Fig. 9). At higher DNP concentrations (>0.4 mg/ml), however, there was a quenching of the fluorescence intensity of QM until a plateau was obtained (approximately at 0.2 mg/ml DNP in the solution).

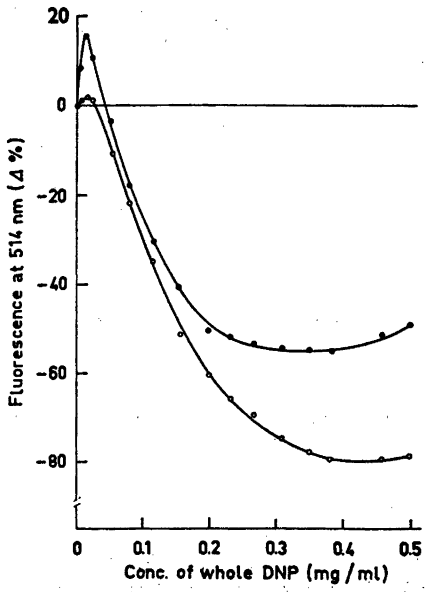


Fig. 9. Effect of whole deoxynucleoprotein on the fluorescence intensity of quinacrine and quinacrine mustard. To a constant amount of (0.033 mg/ml) Q (○) and QM (●) were added increasing amounts of whole DNP in 0.7×10^{-3} M potassium phosphate buffer, pH 8.0.

Whole DNP enhanced and quenched the fluorescence intensity of Q, too (Fig. 9). The

Table 2. Amount of quinacrine mustard bound to DNA, whole DNP and partial DNP's from calf thymus. Extraction performed with urea, $NaHSO_3$, and increasing amounts of NaCl.

Sample	Amount of bound quinacrine mustard at the end of the dialysis.[a] mg dye/mg DNA	%
DNP (whole)	0.12	44
DNP (extracted)		
0.2 M NaCl	0.15	56
0.3 M NaCl	0.17	63
0.4 M NaCl	0.18	67
0.6 M NaCl	0.20	74
3 M NaCl	0.21	78
DNA	0.27	100

[a] Non-equilibrium dialysis was performed in (5 ml) 0.05 M Tris solution, pH 7.5 4 °C for 48 h with 0.020 mg QM/ml and 0.025 mg polymer/ml.

Table 3. Effect of different protein fractions on the fluorescence intensity of quinacrine mustard in solution.[a] Protein fraction obtained by extraction with urea, $NaHSO_3$ and increasing amounts of NaCl.

Conc. of NaCl (M)	Fluorescence at 514 nm (Δ %)	Proteins in the fraction (*cf.* Fig. 2).
0.2	+22	lysine-rich histones
0.3	+25	most lysine-rich and intermediate histones
0.4	+29	lysine-rich, intermediate and some arginine-rich histones
0.6	+35	all histones
3	+22	all histones and some tightly bound proteins (probably non-histones)

[a] Protein fractions (0.025 mg/ml) from partial DNP's were mixed with QM (0.007 mg/ml) in 1 % acetic acid solution.

quenching effect on the fluorescence intensity of Q was more marked than the effect on the fluorescence intensity of QM, particularly at high DNP-to-dye ratios (*cf.* Absorption studies). This probably indicates that more Q is bound to the DNP than QM. Difference in the type of binding between Q and QM have also been shown by Modest and Sengupta.[20] They reported that QM binds approximately 25 times strongly to DNA as does Q, but fewer QM molecules, however, bind per unit length of DNA.

Binding studies. Non-equilibrium dialysis[13] was used to estimate the amount of QM bound to DNA, whole DNP and partial DNP's (Table 2). The removal of the proteins from DNP led to an increase of approximately 50 % in the amount of available binding sites in the DNA. DNP binds 0.12 mg QM/mg DNA, while 1 mg DNA binds 0.27 mg dye. The greatest increase in the binding sites was found after the removal of proteins with 3 M NaCl (urea, $NaHSO_3$) from the DNA core (*cf.* Fig. 8). These findings are unlike the results reported for binding of ethidium bromide to partial DNP's.[16] The greatest effect on the interaction between ethidium bromide and DNP was found when the histone f1 was extracted. Differences in the type of binding between acridine dyes and ethidium bromide have also been shown earlier.[7]

Interaction with proteins. The effect of the selectively dissociated protein fractions on the fluorescence intensity of QM was also tested (Table 3). All the protein fractions enhanced the fluorescence intensity of the dye. The greatest enhancement was obtained with the protein fraction containing all the histones (extracted from DNP with 0.6 M NaCl in urea-$NaHSO_3$). When most of the proteins were extracted (extraction with 3 M NaCl in urea-$NaHSO_3$), the enhancement obtained was identical with that of the protein fraction containing only lysine-rich histones (extraction with 0.2 M NaCl in urea-$NaHSO_3$, Fig. 2). This indicates

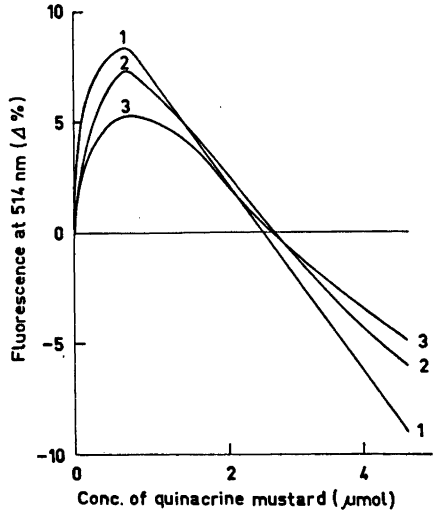


Fig. 10. Effect of artificial partial nucleohistones on the fluorescence intensity of quinacrine mustard. To a constant amount (0.025 mg/ml) of (1) DNA-IIA, (2) DNA-III and (3) DNA-IV were added increasing amounts of QM in 1 M NaCl solution (*cf.* Experimental).

that the proteins which are hard to remove (probably the non-histones) prevent the enhancing effect of the histones on the fluorescence intensity of the dye. The enhancement of the fluorescence intensity of QM with different histone fractions agrees well with previous findings.[13]

Artificial nucleohistones. The effect of artificial DNP's on the fluorescence intensity of QM is shown in Fig. 10. The greatest quench of the fluorescence intensity of the dye was obtained with an artificial DNP composed of DNA and of an intermediate histone fraction (DNA-IIA). A net enhancement of the fluorescence intensity of QM at low dye-to-polymer ratios was obtained with all three artificial DNP's. These curves differ from those obtained for natural DNP's (*cf.* Fig. 7); structural differences between natural and artificial DNP's may account for the differences.

DISCUSSION

Absorption and fluorescence studies. When whole DNP was mixed with Q or QM in solution an enhancement of the absorption maximum as well as of the fluorescence intensity of the two dyes was found. Several interpretations for this phenomenon are possible: (1) the binding mechanism between the dyes and the DNA core in the chromatin; (2) the interactions between the dyes and the proteins in the DNP samples; (3) the limiting of the ionic binding of the dyes by the proteins on the DNA core; (4) the blockade of the base specificity of the dyes by the proteins in the DNP.

The changes in the absorption and fluorescence properties of bound QM appear to be quite similar in DNA, whole DNP, and partial DNP's. This indicates that the binding processes are very similar for these different types of polymers. It is therefore possible that the first binding, at low dye-to-polymer ratios, is an intercalation of aromatic rings of the dye between the bases in the nucleohistone as first suggested with DNA.[31] The second process, at increased dye concentrations in the solution, is an electrostatic interaction between the positive groups in the dye and the negative groups in DNP. The titration curves obtained when different DNP samples were mixed with QM in solution indicate that the fluorescence intensity is determined by the concentration ratio of the dye and DNP. At low dye concentrations the intercalation is dominant and there is an enhancement of the fluorescence intensity of QM, whereas at high dye concentrations the ionic bindings dominate and the fluorescence intensity of QM is quenched.

In this paper it is shown that while all the major classes of chromosomal proteins inhibit the binding of QM to chromatin, the removal of the tightly bound proteins (extraction with 0.6 and 3 M NaCl in urea-$NaHSO_3$) has the greatest effect on the binding of the dye to chromatin. In an earlier paper an interaction between the dye QM and calf thymus histones in solution has been reported.[13] It is therefore likely that the dye also may be able to interact with proteins which cover the DNA core in the chromatin. Likely binding sites on the DNP are the carboxyl groups on the histones, since they are on the surface of the nucleoprotein complex.[28]

The binding sites for QM in whole DNP were reduced as compared to the binding sites for QM in native DNA. An increase of approximately 50 % in the amount of bound QM was found when all the proteins were removed from the DNA core. Clark and Felsenfeld [32] have shown that the proteins are not evenly distributed in the chromatin. They postulated that about half of the DNA is covered with proteins. Their conclusion corroborates other reports of dye-binding to chromatin. Klein and Szirmai [15] found with the dye, azure A, that about the half of the DNA in the chromatin reacted with the dye. A similar dye-binding capacity suggesting that approximately 50 % of the DNA is covered with proteins, has been reported by Angerer and Moudrianakis,[16] Miura and Ohba,[17] Ringertz and Bolund,[33] Bolund,[34] and Itzhaki.[35,36] According to Phillips [28] the negative charge of DNP is about 60 % of that of DNA, *i.e.* about half of the negative groups of DNA are able to combine with the positive groups on cationic dyes in the DNP. The proteins on the DNA core reduce the amount of binding sites for the dye because the positive residues on the histones are bound to the negative groups in DNP, thus limiting the ionic binding of the dye. Since the proteins on the DNA core reduce the ionic binding sites between the dye and DNA, the fluorescence intensity for a QM–DNP

mixture is higher than for a QM–DNA mixture.

The selective removal of proteins from whole DNP increases the quenching of the fluorescence intensity of QM in solution. However, at low dye-to-DNP ratios an enhancement of the fluorescence intensity of the dye was found with whole DNP and partially deproteinized DNP's still containing arginine-rich histones and other proteins (probably non-histones). These results indicate that the arginine-rich histones, which still covered the GC-rich regions in the DNA,[37] prevent the quenching effect of GC-rich regions on the fluorescence intensity of QM.[12] When all the histones were removed from the DNP, a net quench of the fluorescence intensity of the dye was found. The fluorescence intensity of QM as well as of Q is highly depending on the base content of the DNA.[6,7,10–12,14] When histones blockade bases in the DNA they are able to prevent the base specific interactions between the dyes and the bases in the DNA.

However, from the results in this paper it is not possible to say which one of the above given alternatives would explain why the absorption maximum or the fluorescence intensity was enhanced when the two dyes Q and QM were mixed with whole DNP. Probably the phenomenon is caused by the action of all the alternatives together.

Comparison between Q and QM. The main difference of the two dyes Q and QM is in the long aliphatic side chain at C-9. QM has an alkylating mustard group at the end of the side chain and is therefore able to bind chemically to polymers, whereas the binding of Q is only through intercalation or electrostatic (*cf.* the structure of the two dyes).

Differences in the absorption and fluorescence properties were obtained when equal amounts of Q and QM were mixed with whole DNP in solution. The absorption spectra of Q was more reduced and the quenching effect on the fluorescence intensity of Q was more marked upon the addition of whole DNP to the solution as compared with the effect of whole DNP on QM. These differences probably indicate that more Q is bound to whole DNP than QM. This is in agreement with the results reported by Modest and Sengupta.[30] When they compared the two dyes Q and QM they found that fewer QM molecules was bound to DNA.

Natural and artificial nucleohistones. A quite different picture emerged when artificial nucleohistones were mixed with QM in solution. Even arginine-rich complexes (the DNA-IV complex) were able to enhance the fluorescence intensity of the dye at low dye-to-polymer ratios. This reflects structural differences between natural and artificial nucleohistones. These differences might have resulted from the inability of the histones to interact properly with the DNA, *i.e.* the histones had assumed new positions on the DNA core.[38] During the reconstitution a rearrangement of the proteins may occur.[32] Angerer and Moudrianakis [16] reported that the association constant for binding of ethidium bromide to natural DNP differs from the constant for the binding of the dye to artificially reconstituted DNP.

The fluorescence of metaphase chromosomes in situ. With the *in situ* hybridization technique it has been possible to determine the composition of chromosomal DNA in some particular regions in eucaryotic chromosomes. AT-rich satellite DNA was found in the centromeric regions of the mouse chromosomes [38] and in the secondary constrictions of the human chromosomes 1, 9, and 16.[39] Saunders *et al.*[40] were able to show a GC-rich satellite DNA in the secondary constriction in No. 9. Moreover cytochemical experiments indicate that regions staining brightly with Q or QM contain AT-rich DNA, while regions with dull fluorescence contain GC-rich DNA.[41–44] The mouse centromers and the secondary constrictions of human chromosomes 1, 9, and 16, however, show dull fluorescence when stained with quinacrine derivatives. These contradictory results, AT-richness and dull fluorescence, might be explained from the results in this paper.

Methanol-acetic acid fixed metaphase chromosomes contain very little protein.[45] Comings *et al.*[45] showed that the fixative was not able to remove the non-histones. Therefore it can be assumed that the above mentioned chromosomal regions might be covered by non-histones which compete with the dye, thereby giving dull fluorescence. When metaphase slides were subjected to denaturation and reassociation and thereafter stained with QM, the centromeric regions of the mouse chromosomes as well as of the secondary constrictions in human chromosomes 1, 9, and 16 showed a bright fluorescence.[46]

This phenomenon can be explained by a possible extraction of proteins (non-histones) during the treatment in sodium hydroxide (denaturation) and the incubation in salt solution (reassociation).[45] Protein dissociation might uncover the AT-rich regions, resulting in an enhancement of the fluorescence. At all the dye-to-polymer ratios tested, a quenching of the fluorescence intensity of QM was seen with DNP from which only the histones were removed (*cf.* Results). The other proteins, probably the non-histones, therefore may be able to prevent the effect of the bases in DNA on the fluorescence intensity of the dye.

Comings *et al.*[45] proposed that the non-histone-DNA complexes contribute to the banding after C- and G-banding procedures.[47] On the other hand recent findings of Zirkin [48] showed an almost complete absence of non-histones in the heterochromatin in metaphase chromosomes from bovine kidney. It is therefore questionable whether bands reflect only the presence of non-histones. Secondary and tertiary configurations in the chromosomal macromolecules may also contribute to the bands. Moreover, the degree and pattern of the base repetition apperently contribute to the occurrence of brighter and duller regions along the metaphase chromosomes.[18]

In this paper it is shown that tightly bound proteins, probably the non-histones in the deoxynucleoprotein markedly affect the fluorescence intensity of QM in solution. The study of the effect of isolated non-histones in solution is underway in this laboratory.

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Algal Carotenoids. XIII.** Chemical Reactions of Allenic Carotenoids

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The conversion of the allenic end group I to acetylenic (II) and chlorinated (presumably V) end groups on treatment with chloroformic hydrogen chloride is demonstrated.

Treatment with phosphorus oxychloride in pyridine of carotenoids with the allenic end group I also caused transformation to acetylenic products, in addition to previously reported allenic anhydro-products.

Facile dehydration and chlorine substitution of the secondary hydroxy group of peridinin (*1*) were observed.

Chlorinated carotenoids, identified by mass spectrometry, appear to be readily formed.

On the basis of electronic spectra and R_F-values alone Egger *et al.*[1] and Nitsche *et al.*[2,3] have claimed the conversion of carotenoids with the allenic end group I to carotenoids with the acetylenic end group II besides end group IV on treatment with chloroformic hydrogen chloride, Scheme 1B. More recently [3a] IR-evidence for the conversion of I (unacetylated) to II (unacetylated) has been published.

Such conversion was not observed on treating peridinin (*1*) with methanolic hydrogen chloride, and only products with the allenic end group intact were reported.[4,5]

Also lithium aluminium hydride treatment of carotenoids with end group I has been reported to give conversion to the acetylenic (ν_{max} 2150 cm^{-1}) end group II.[5a]

We now report further evidence on the reactions of carotenoids containing the allenic end group I in chloroformic hydrogen chloride and on treatment with phosphorus oxychloride, resulting in the formation of acetylenic and chlorinated derivatives.

RESULTS AND DISCUSSION

Carotenoids containing the allenic end group I were on treatment with chloroformic hydrogen chloride converted to products with acetylenic (II) and chlorinated (probably V) end groups, whereas products with end groups III, IV, and VI were not observed, Scheme 1. Further evidence for the position of the chloro substituent in end group V will be presented.[5b]

Thus peridinin (*1*, Scheme 2) on such treatment provided besides three minor products (*2**, *3**, and *5**) a major presumably mixed product (*4a*, *b*; Scheme 2) with highest mass number ion at *m/e* 648.3216 (calculated 648.3218 for $C_{39}H_{49}O_6{}^{35}Cl$) and with no allene, but a very weak acetylenic (2170 cm^{-1}) IR absorption. Asterisks indicate that the structural formulae are not given; numbers refer to compounds described in the Experimental part.

Likewise treatment of peridinin acetate (*6*) gave two major products (*7** and *8**) of similar polarity. The least polar product *7** had highest mass number ion at *m/e* 630 (consistent with $C_{39}H_{47}O_5{}^{35}Cl$, supported by ^{37}Cl isotope peak at *m/e* 632) compatible with the formation of end group V and elimination of acetic acid from one of the acetoxy groups. The more polar product *8** had molecular ion at *m/e* 690 and ^{37}Cl isotope peak at *m/e* 692 compatible with $C_{41}H_{51}O_7Cl$, again consistent with the formation of end group V.

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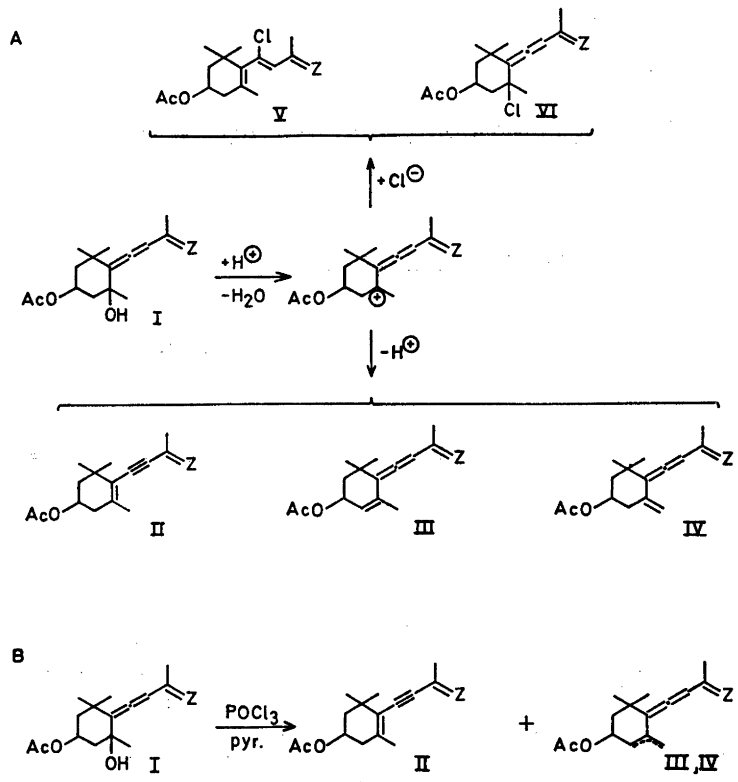
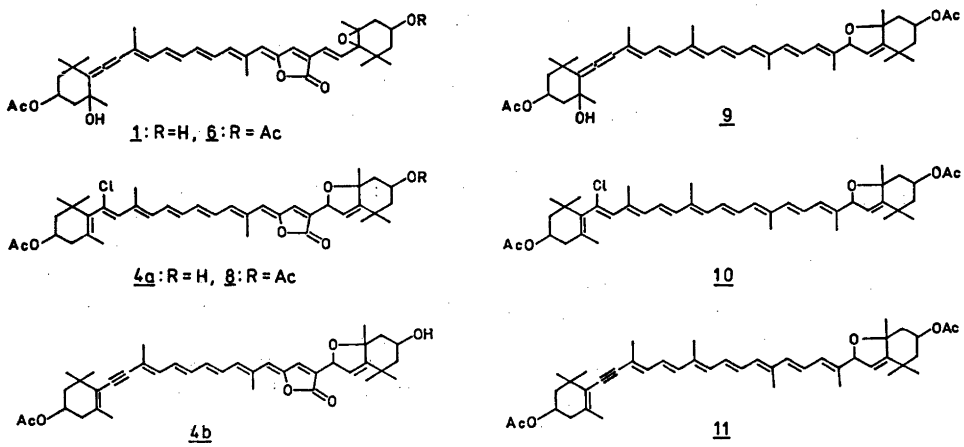


Scheme 1.



Scheme 2.

Small amounts of dinochrome acetate (*9* = neochrome diacetate [6]) were available. Treatment with chloroformic hydrogen chloride gave a product (*10*) with molecular ion at *m/e* 702 and ^{37}Cl isotope peak at *m/e* 704 (consistent with $C_{44}H_{59}O_5Cl$) and with a 4 nm bathochromic shift in the visible spectrum in acetone solution relative to *9*. The product was unseparable from diadinochrome diacetate (*11*) with the acetylenic end group II on kieselguhr paper.[1]

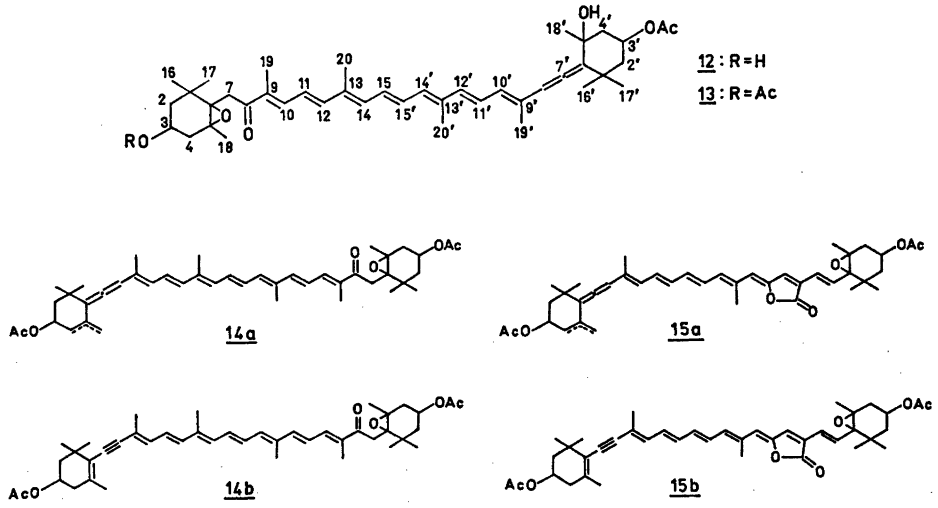
The mass spectra of the chlorinated products *2**, *4*, *5**, *7**, *8**, and *10* all showed strong M − 36 ions. This might be due to loss of HCl from the molecular ion, to the presence of carotenoids with end group II, or both of these possibilities.

Failure to effect chromatographic separation (TLC or paper) of the actylenic and chlorinated derivatives may partly be due to accompanying epoxide-furanoid rearrangement of the second end group in the carotenoids (*1* and *6*) studied, theoretically resulting in two epimeric furanoxides in each case.

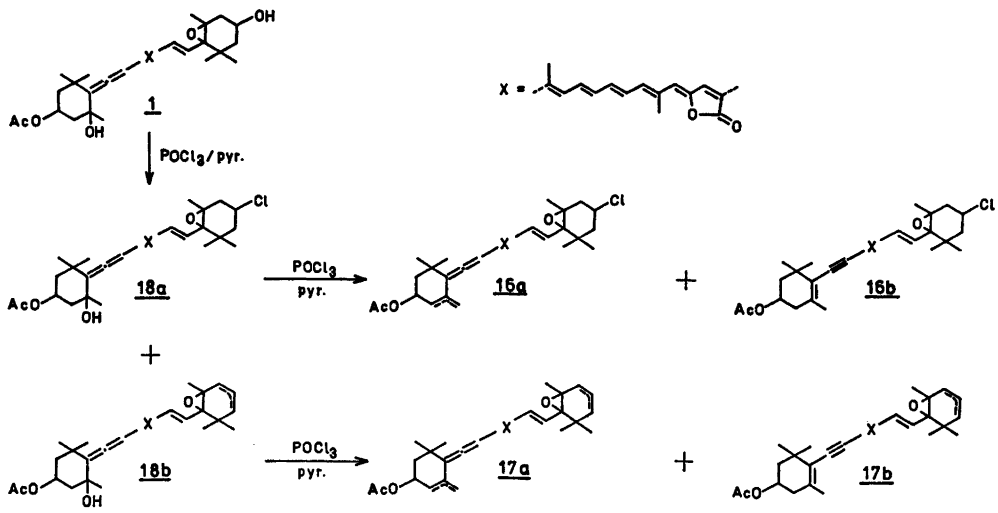
We further report the conversion of the allenic end group I to the acetylenic end group II on treatment with phosphorus oxychloride in pyridine, in addition to the transformation of I to III/IV previously reported by Bonnett *et al.*,[7] Scheme 1B.

Thus fucoxanthin acetate (*13*, prepared from fucoxanthin (*12*, Scheme 3) when treated with phosphorus oxychloride gave the mixed anhydro-products (*14a, b*) judged by visible-, IR-, NMR-, and mass spectra. Absorption in the IR region at 1920 and 2170 cm^{-1} were assigned to the allenic group in *14a* and the acetylenic group in *14b*, respectively. No absorption due to terminal methylene was observed in the IR- or NMR spectra of the mixed products (*14a, b*). Separation of *14a* and *14b* was not effected by TLC, but achieved by circular kieselguhr paper chromatography on the micro scale. The visible spectrum in hexane solution of the tentatively identified acetylenic *14b* showed less fine-structure and had absorption maximum at 1 nm longer wavelength than that of the tentatively allenic *14a*. The acetylenic product (*14b*) may previously have been overlooked.[7]

Likewise peridinin acetate (*6*) on similar treatment with phosphorus oxychloride gave the allenic anhydro-peridinin acetate (*15a*) and the acetylenic pyrrhoxanthin acetate (*15b*). Anhydro-peridinin acetate (*15a*) had absorption maximum in visible light at 1.5 nm longer wavelength than that of peridinin (*1*) in hexane solution, but exhibited the same spectral fine structure, whereas pyrrhoxanthin acetate (*15b*) had less fine structure and absorption maximum at 3 nm longer wavelength than that of peridinin (*1*). No absorption characteristic of terminal methylene was observed. Pyrrhoxanthin acetate (*15b*) was inseparable by co-



Scheme 3.



Scheme 4.

chromatography from authentic *15b* [6] on circular kieselguhr paper.

Peridinin (*1*) gave in the same reaction three main products [8] by TLC (*16*, *17*, and *18*, Scheme 4), each considered to represent mixtures. Product *16* had molecular ion at *m/e* 630.3109 (calculated 630.3112 for $C_{39}H_{47}O_5{}^{35}Cl$) in addition to ions at *m/e* 199.0883 (calculated 199.0890 for $C_{11}H_{16}O^{35}Cl$) and 201.0865 (calculated 201.0860 for $C_{11}H_{16}O^{35}Cl$), compatible with pyrylium ions [9] revealing that the 3′-hydroxy group was substituted by chlorine. Product *17* had molecular ion at *m/e* 594 and a fragment ion at *m/e* 163, demonstrating that the 3′-hydroxy group had been eliminated as water. Products *16* and *17* were each mixtures containing both the allenic (*16a*, *17a*) and acetylenic (*16b*, *17b*) end group as dicussed above for the dehydration of fucoxanthin acetate (*13*). Product *18* exhibited molecular ions at *m/e* 648 (*18a*) and 612 (*18b*). Fragment ions at *m/e* 223, 212, and 197 indicated that the allenic end group was intact.[5,10] Product *18* thus represented a mixture of products where the 3′-hydroxy group partly was substituted by chlorine (*18a*) and partly eliminated as water (*18b*).

In conclusion further evidence for the conversion of the allenic (I) to acetylenic end groups in carotenoids is here presented, in chloroformic hydrogen chloride as well as in phosphorus oxychloride/pyridine.

Under both conditions stable, chlorinated products were also observed. Without mass spectrometry such halogenated carotenoids would not be identified.

The facile dehydration and chlorine substitution of the secondary hydroxy group in peridinin (*1*) in phosphorus oxychloride/pyridine was unexpected.

EXPERIMENTAL

Materials and methods were as described elsewere.[11]

Acid treatment of allenic carotenoids

Peridinin (*1*, 15.7 mg, available from a previous study [6]) was kept in 0.02 M HCl–$CHCl_3$ (100 ml) for 4 h at room temp. The mixture was diluted with petroleum ether, the organic extract washed with water, solvents removed, and the residue dried under vacuum. Chromatography on kieselgel G (25 % acetone in petroleum ether = 25 % APE) gave several bands. The four major products (*2–5*, representing 36 % of starting material) were further investigated.

Product *2*, yield 0.08 mg (1 %), had R_F = 0.75 (Schleicher and Schüll paper No. 287 = SS287, 5 % APE); λ_{max}(hexane) *430.5* and 458.5 nm (the main maximum is given in italics); *m/e* 510.2531 (M, 48 %, calc. 510.2537 for $C_{31}H_{39}O_4{}^{35}Cl$), 474 (M–36, 17 %), 439 (8 %), 383 (11 %), 257 (12 %), 236 (16 %), 211 (10 %), 207 (13 %), 183 (23 %), 57 (100 %) and no *m/e* 181.

Product *3*, yield 1.2 mg (22 %), had R_F= 0.41 (broad, SS287, 5 % APE); λ_{max}(hexane) *437.5* and 464 nm; *m/e* 552.3237 (M?, 3 %, calc. 552.3240 for $C_{37}H_{44}O_4$) and 181 (53 %); ν_{max} (KBr) 3425 (OH), 3012–2870 (CH), 2170 (very weak, –C≡C–), 1730 (C=O), 1640, 1530, 1453 (CH_2), 1366 (CH_3), 1241, 1164, 1100, 1038 (C–O), 988 and *697* (*trans* –CH=CH–) and 783 cm^{-1}.

Product *4*, yield 4.0 mg (71 %), had R_F= 0.18 (broad, SS287, 5 % APE); λ_{max}(hexane) *435* and 462.5 nm; *m/e* 648.3216 (M, 46 %, calc. 648.3218 for $C_{39}H_{49}O_4{}^{35}Cl$), 612 (M–36, 29 %), 588 (M–60, 0.9 %), 570 (M–78, 1.0 %), 568 (M–80, 1.9 %), 556 (M–92, 2.2 %), 552 (M–60–36, 10 %), 460 (M–92–60–36, 8.2 %), 234 (58 %) and 181 (100 %); δ ($CDCl_3$) 0.93 (imp.), 1.07, 1.17, 1.21, 1.27 (imp.), 1.35, 1.67, 1.70, 1.83, 2.09 and 2.21. Product *4* was crystallized from acetone-petroleum ether, yield 1.8 mg; λ_{max} (hexane) 323, *435.5* and 462.5 nm; ν_{max}(KBr) 3420 (OH), 3013–2870 (CH), 2170 (weak, acetylene), 1740 (C=O), 1629, 1598, 1531, 1446 (CH_2), 1386, 1367, 1352 (CH_3), 1243, 1214, 1180, 1145, 1100, 1040 (C–O), 987, 974, 947, 908, 864, 837, 801, 782 (Cl?), 729 and 665 cm^{-1}.

Product *5*, yield 0.34 mg (6 %), had R_F= 0.78 (SS287, 20 % APE); λ_{max}(hexane) *436* and 459.5 nm; *m/e* 648 (M, 3.3 %), 612 (M–36, 2.5 %), 570 (M–78, 3.4 %), 552 (M–60–36, 3.5 %), 430 (70 %), 412 (34 %) and 181 (70 %).

Peridinin acetate (*6*, 1.5 mg) treated as above gave after chromatography on kieselgel G several products; the two major ones *7* and *8* were further investigated.

Product *7*, yield 0.12 mg (8 % of starting material) had λ_{max}(acetone) *440* and (458) nm; *m/e* 630 (M, 1.8 %, ^{37}Cl isotope peak at *m/e* 632), 594 (M–36, 13 %), 223 (18 %), 216 (12 %) and 181 (24 %).

Product *8*, yield 1.07 mg (70 % of starting material) had λ_{max}(acetone) *437.5* and (456) nm; *m/e* 690 (M, 22 %, ^{37}Cl isotope peak at *m/e* 692, 654 (M–36, 13 %), 630 (M–60, 0.8 %), 612 (M–78, 1.3 %), 610 (M–80, 2.0 %), 598 (M–92, 3.0 %), 594 (M–60–36, 9.8 %), 502 (7.5 %), 303 (5.3 %), 223 (22 %), 216 (30 %) and 163 (43 %).

Dinochrome acetate (*9*, 0.22 mg) treated as above for 2 h gave after chromatography on kieselgel G one main product (*10*, representing 70 % of reaction mixture); λ_{max}(acetone) 407, *428* and 454.5 nm; *m/e* 702 (M, 3.2 %, ^{37}Cl isotope at *m/e* 704), 666 (M–36, 3.7 %), 622 (M–80, 4.3 %, ^{37}Cl isotope at *m/e* 624), 223 (8.1 %) and 183 (11 %). Co-chromatography with diadinochrome diacetate (*11*) gave no separation on SS288 paper (R_F= 0.65, 10 % APE).

$POCl_3$/pyridine treatment of allenic carotenoids

Fucoxanthin acetate (*13*, 34.3 mg prepared from fucoxanthin (*12*) treated with $POCl_3$ (20 drops) in dry pyridine (5 ml) for 6 h at 40°C gave recovered fucoxanthin acetate (*13*, 1.3 mg, 4 % of starting material) and dehydrated fucoxanthin acetate (*14a, b*, 18.0 mg, 53 % of starting material). The mixed products *14a, b* had λ_{max}(acetone) *446* and 466 nm; ν_{max}(KBr) 3420 (H_2O), 3030 2958, 2920 and 2860 (CH), 2167 (–C≡C–), 1912 (>C=C=C<), 1735 (C=O), 1658, 1609, 1575, 1530, 1468 and 1452 (CH_2), 1364 (CH_3), 1242, 1200, 1154, 1130, 1030, (C–O), 968 (*trans* –CH=CH–), 919, 891, 836 (>C=CH–), 805, 735, 658 and 645 cm^{-1}; δ($CDCl_3$, for numbering see fucoxanthin acetate (*13*) in Scheme 3) 0.90 and 0.93 (imp.), 0.98 s (CH_3–1), 1.07 s (CH_3–1), 1.13, 1.17, 1.19 and 1.22 (CH_3–5,1′, 1′), 1.26 (imp.), 1.77 (CH_3–5′ in *14a*?), 1.85 (CH_3–9′ in *14a*?), 1.97 (CH_3–9 and CH_3–5′ in *14b*?), 1.99, 2.01, 2.04 and 2.06 (CH_3–9 in *14b*?, CH_3–13,13′ and CH_3– in OAc), 2.61 d(H–7, J=18 Hz), 3.71 (H–7, J= 18 Hz), 4.23 (H–4, J=6 Hz), *ca.* 4.89 (H–3) and 5.56 (H–3 in *14a*); *m/e* 682 (M, 16 %), 664 (M–18, 0.9 %), 638 (M–44, 0.1 %), 622 (M–60, 11 %), 604 (M–78, 4.1 %), 590 (M–92, 2.2 %) and 43 (100 %).

The mixed products (*14a, b*) gave three zones on paper chromatography (SS287, 2 % APE): Unassigned: R_F= 0.41 (5 %); λ_{max} (hexane) 263, 329, (425), *446.5* and 475 nm; % III/II = 28;[12] *14a* (tentatively): R_F= 0.38 (55 %); λ_{max}(hexane) 263, 329,427, *448.5* and 477.5 nm; % III/II = 35 [12] and *14b* (tentatively) R_F= 0.29 (40 %); λ_{max}(hexane) 277, 340, *449.5* and 477 nm; % III/II = 9.[12]

Peridinin acetate (*6*, 4.6 mg) treated with $POCl_3$ in dry pyridine as above gave after purification by TLC recovered peridinin acetate (*6*, 1.8 mg, 38 % of starting material) and dehydrated peridinin acetate (*15a, b*, 1.5 mg, 32 % of starting material). The mixed product *15a, b*, had λ_{max} (acetone) *459.5* and (475) nm; λ_{max} (KBr) 3020, 2958, 2960 and 2855 (CH), 2167 (–C≡C–), 1908 (>C=C=C<), 1754 and 1739 (C=O), 1640, 1524, 1449 (CH_2), 1364 (CH_3), 1243, 1181, 1166, 1126, 1032 (C–O), 985 (*trans* –CH=CH–), 943, 905, 819 (>C=CH–), 795, 768, 726 and 643 cm^{-1}; *m/e* 654 (M, 20 %), 594 (M–60, 24 %), 579 (M–75, 2.3 %), 574 (M–80, 0.6 %), 562 (M–92, 1.5 %), 478 (9.6 %), 443 (1.2 %), 431 (1.5 %), 343 (2.2 %), 285 (4.3 %) and 223 (19 %). Two zones were obtained by paper chromatography (SS287, 5 % APE): Anhydro-peridinin acetate (*15b*, tentatively); R_F= 0.50 (60 %); λ_{max} (hexane) 327, *456* and 486.5 nm: % D_B/D_{II} = 17;[12] % III/II = 72 [12] and pyrrhoxanthin acetate (*15a*, tentatively); R_F= 0.41 (40 %); λ_{max} (hexane) *458.5* and 488 nm; % D_B/D_{II}= 12,[12] % III/II = 44.[12] These were not inter-convertible by iodine catalyzed stereomutation.

Peridinin (1, 21.8 mg) treated as above gave 9.5 mg (44 %) recovery. Chromatography on kieselgel G ($CHCl_3$) gave three major products: *16*, *17* and *18* numbered from the solvent front.[8]

Product *16a*, *b*, yield 0.28 mg (4 %), had λ_{max} (hexane) *457* and 487 nm; *m/e* 630.3109 (M, 30 %, calc. 630.3112 for $C_{39}H_{47}O_5{}^{35}Cl$), 594 (M − 36, 0.3 %), 570 (M − 60, 40 %, ^{37}Cl isotope peak at *m/e* 572), 534 (M − 60 − 36, 10 %), 373 (14 %), 223 (20 %), 201.0865 (12 %, calc. 201.0860 for $C_{11}H_{16}O^{37}Cl$), 199.0883 (34 %, calc. 199.0890 for $C_{11}H_{16}O^{35}Cl$) and 183 (14 %). Product *16* gave two zones by paper chromatography (SS287, 5 % APE): *16a*; R_F = 0.77 (60 %); λ_{max}(hexane) 326, *458* and 488 nm; % III/II = 75,[12] % D_B/D_{II} = 22 [12] and *16b*; R_F = 0.64 (40 %; λ_{max} (hexane) (335), *461* and 490 nm; % III/II [12] = 28; % D_B/D_{II} [12] = 16.

Product *17a*, *b*, yield 2.5 mg (35 %), had λ_{max} (hexane) *455* and 484 nm; ν_{max} (KBr) 3022 − 2855 (CH), 2167 (−C≡C−), 1912 (>C=C=C<), 1740 (C=O), 1638, 1570, 1460 (CH_2), 1374 and 1360 (CH_3), 1240, 1180, 1144, 1124, 1074, 1047, 1023, 986, 944, 905, 870, 818, 769, 718, and 634 cm^{-1}; *m/e* 594 (M, 11 %), 534 (26 %) and 163 (26 %) and no *m/e* 181. Product *17a*, *b*, gave two zones by paper chromatography (SS287, 10 % APE): *17a*: R_F = 0.77 (60 %); λ_{max} (hexane) (307), 320.5, 334.5, *455* and 484.5 nm; % III/II = 72, [12] % D_B/D_{II} = 37 and *17b*: R_F = 0.64 (40 %); λ_{max} (hexane) (307), 320, 335.5, *458* and 487 nm; % III/II = 41; % D_B/D_{II} = 26.

Product *18a*, *b*, yield 4.0 mg (59 %), had λ_{max} (hexane) *455* and 484.5 nm; ν_{max} (KBr) 3400 (OH), 3022 − 2855 (CH), 1928 (allene), 1730 (C=O), 1640, 1520, 1455 (CH_2), 1366 (CH_3), 1247, 1182, 1162, 1124, 1071, 1025, 984, 956, 924, 903, 859, 820, 768, 718, and 633 cm^{-1}; *m/e* 648 (M_1, 5.1 %, ^{37}Cl isotope peak at *m/e* 650, 612 (M_2, 16 %), 594 (M_1 − 36, M_2 − 18, 50 %), 570 (M_1 − 60, 44 %), 534 (M_1 − 60 − 36, 28 %), 520 (M_2 − 92, 73 %), 223 (32 %), 212 (50 %), 201 (18 %), 199 (28 %), 197 (99 %), and 163 (100 %) and no *m/e* 181.

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Separation of Sugars into Anomers by Partition Chromatography on Ion Exchange Resins

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Various mono- and disaccharides were separated into anomers by partition chromatography on an anion exchange resin (SO_4^{2-}) in aqueous ethanol at −10 °C. With most sugars two peaks representing the α- and β-pyranoses were recorded. For some saccharides anomerization interfered with the separation. The conformation of the sugars exerts a predominant influence on their chromatographic behaviour.

Reducing sugars can be well separated into α- and β-anomers by gas chromatography of their trimethylsilyl derivatives.[1] Separate peaks corresponding to the furanoid and pyranoid forms are often obtained. Efficient separations of methyl glycosides can be achieved by ion exchange chromatography on strongly basic anion exchange resins in their hydroxide form [2] and by partition chromatography on ion exchange resins in aqueous ethanol.[3] In separation of sugars by paper chromatography at room temperature only one spot is obtained for each sugar although two or more forms of each sugar are present in most systems.[4] In thin-layer chromatography single spots are obtained at 20 − 30 °C in contrast to the appearance of elongated or double spots at −18 °C.[5] Evidently, a partial separation of different forms occurs in favourable systems.

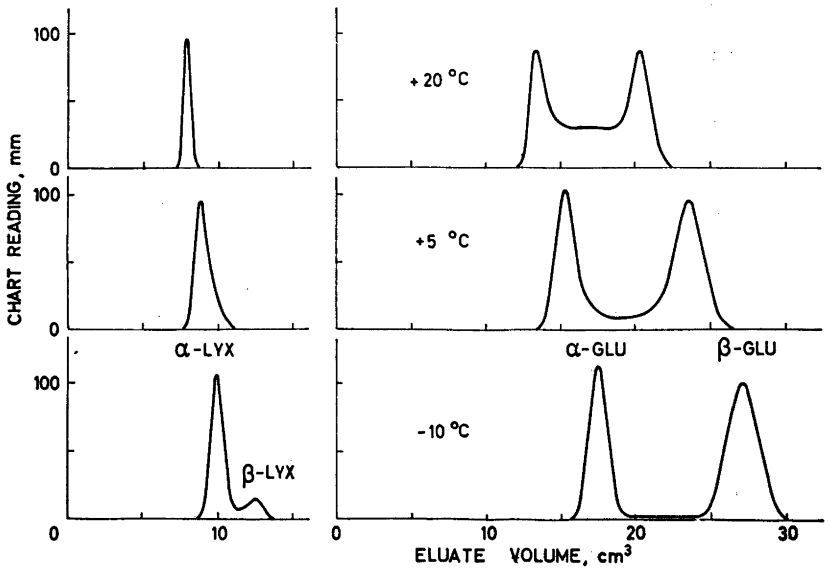

Fig. 1. Separation into anomers of D-lyxose, 20 μg (left diagrams), and D-glucose, 75 μg (right diagrams), at various temperatures.

We now report on the separation of sugars into anomers by partition chromatography on ion exchange resins in aqueous ethanol at low temperature. Separations at elevated temperature have previously been applied in analyses of complex mixtures of sugars.[6]

RESULTS AND DISCUSSION

When a mixture of α- and β-D-glucose dissolved in 75 % ethanol was eluted at 75 °C a sharp single peak was obtained. At 20 °C the mixture was resolved into two overlapping peaks [Fig. 1]. When the temperature was lowered, both peaks were eluted later which is in agreement with the earlier observation that the distribution coefficients of sugars increase with decreasing temperature.[7] At −10 °C the curve between the two peaks approached the baseline. Separate experiments with fresh solutions of α- and β-D-glucose gave sharp single peaks and showed that the first peak contained the α-anomer and the second peak the β-anomer. The retention time of the β-anomer at −10 °C was 6 h. The anomerization of the compounds was negligible at this temperature whereas at +5 °C the anomerization interfered with the separation. Evidently, the α- and β-forms can be separated by partition chromatography on ion exchange resins at low temperatures. Favourable aspects in this connection are that the mutarotation is slower in ethanol than in water [8] and that the low freezing point of the eluent permits separations at low temperature. A prerequisite for an efficient separation is that fine resin particles be used so that the sorption equilibrium can be approached during the chromatographic run. The volume distribution coefficients, D_v, of the anomers are given in Table 1.

Table 1. Volume distribution coefficients (D_v) of mono- and disaccharides in 75 % ethanol at various temperatures on an anion exchange resin (SO_4^{2-}).

Saccharide	−10 °C	+5 °C	+20 °C
D-Ribose I	4.6	–	–
D-Ribose II	5.3	4.2	3.6
α-D-Arabinose	9.1	7.1	6.3
β-D-Arabinose	4.9	4.1	4.1
α-D-Xylose	5.5	5.0	4.5
β-D-Xylose	9.4	8.2	7.0
α-D-Lyxose	5.6	4.9	4.4
β-D-Lyxose	7.0	–	–
α-D-Allose	6.2	–	–
β-D-Allose	10.8	–	–
α-D-Glucose	9.7	8.5	7.4
β-D-Glucose	15.1	13.1	11.3
α-D-Mannose	6.5	5.9	5.4
β-D-Mannose	10.9	9.3	7.8
α-D-Gulose	6.6	–	–
β-D-Gulose	10.6	–	–
α-D-Galactose	8.2	7.3	6.4
β-D-Galactose	15.1	13.2	11.1
α-D-Talose	4.7	–	–
β-D-Talose	7.1	–	–
β-D-Fructose	6.7	5.9	5.2
D-Fructose II	7.5		
α-L-Sorbose	9.2	7.3	6.7
α-Cellobiose	22.5	20.4	15.6
β-Cellobiose	34.7	30.6	22.2
α-Lactose	19.9	17.8	14.1
β-Lactose	30.4	25.5	20.6

Equally good resolution into two compounds was obtained at −10 °C with solutions of D-allose, D-mannose, and D-xylose. Aqueous ethanol solutions were kept at room temperature so that equilibrium was approached. One of the peaks was identified from the position of the single peak recorded in a separate run with a fresh solution of an authentic crystalline sample. The proportion of furanose forms of D-glucose, D-mannose, and D-xylose in equilibria in aqueous solutions is less than 1 % [9] which means that the two peaks recorded must represent the pyranose forms. The relative amounts of the anomers determined from the peak areas, were in good agreement with literature data.[9] The proportion of furanose of D-allose in aqueous solution is about 10 %,[9] but for unknown reasons no indication of its presence was obtained in the chromatograms.

D-Galactose as well as D- and L-arabinose gave rise to two well separated main peaks which were identified as α- and β-pyranose by the same technique. With both sugars a minor, seriously overlapping compound appeared after the first main peak. The experiments with D-galactose were repeated on an overloaded column to make it possible to isolate this compound. The eluate was analysed by both the orcinol and the periodate-formaldehyde methods. The results given in Fig. 2 show that the compound present in the distorted tail of the first peak gave a much stronger relative response in the periodate-formaldehyde channel

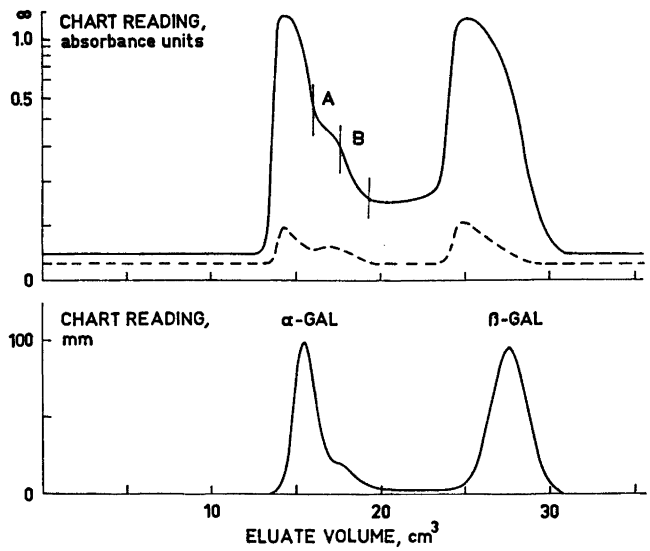

Fig. 2. Separation into anomers of D-galactose at −10 °C. Analysis by the orcinol method (full line) and periodate-formaldehyde method (broken line). Loading: 3500 μg (upper diagrams); 75 μg (lower diagram).

than the major compounds. This suggests that the compound is either a furanose or a ketohexose. D-Tagatose, which can be formed from D-galactose by isomerization, is eluted as a single peak at −10 °C at about the same position as the unknown compound. Since the elution volume of D-tagatose would probably be affected by the presence of large amounts of D-galactose, two fractions, A and B, were cut as indicated in the figure and rechromatographed on analytical columns at −10 °C and 75 °C. The chromatograms from the experiments at −10 °C could not be distinguished from that recorded with the original galactose sample [Fig. 2]. The eluate from the runs at 75 °C was analysed by the orcinol method at high amplification. The separation of D-tagatose from D-galactose is very favourable at this temperature. In addition to D-galactose only minor amounts of D-tagatose (totally in both fractions 0.5 % of the starting material) were detected. The concentration of D-tagatose in fraction B was about 5 times greater than in fraction A, which means that the unknown compound cannot be D-tagatose. The results indicate that one or both furanose forms of D-galactose were present in fraction A.

With D-gulose two well separated peaks were recorded. No crystals were available and an equilibrium mixture in aqueous ethanol was therefore analysed. The relative areas of the peaks obtained at −10 °C were in agreement with literature data.[9] The smaller peak was therefore ascribed to the α-pyranose form and the larger one to the β-pyranose form. No other compounds were recorded.

D-Talose, which is known to exist as both anomers of the furanose and pyranose forms and to give an extremely rapid mutarotation,[10] gave only two distinct peaks. The first of these was identified as the α-pyranose form by the study of an authentic sample. A large amount of the sugar was eluted between the peaks. This confirms that a rapid interconversion of various forms occurs with D-talose.

The chromatograms from the run with D-lyxose [Fig. 1] show tailing single peaks at +20 °C and +5 °C and two partially separated peaks at −10 °C. Authentic samples were available for both α-L- and β-D-lyxose and identification of the peaks was obtained as with D-glucose. In agreement with mutarotation data[10] the chromatograms showed that the anomers were rapidly interconverted.

With D-ribose which exhibits a very rapid mutarotation, appreciable amounts of the furanose forms are present together with the pyranoses.[9] Only two seriously overlapping

peaks were recorded at −10 °C. The resolution was much lower for solutions equilibrated at +80 °C and −20 °C after mixing with ethanol than in the experiments with D-ribose dissolved in 75 % ethanol (−20 °C) or first in water (+2 °C) followed by addition of ethanol (−20 °C) and chromatographed as soon as the sugar was dissolved. These results as well as the shape of the elution curves show that at least three forms were present at equilibrium.

Among the ketoses studied, D-fructose gave rise to two peaks which exhibited a severe overlapping at −10 °C. At +5 °C and +20 °C only one peak was recorded. L-Sorbose was the only sugar which gave a sharp single peak even at the low temperature. This was expected since it had previously been observed that L-sorbose exists almost exclusively in the α-pyranose form.[10]

The two disaccharides studied, cellobiose and lactose, can exist only in the pyranose forms and as expected, two sharp peaks were recorded at low temperature with each sugar. Negligible amounts were recorded between the peaks. The mutarotation was so slow that rough estimates of the distribution coefficients could be obtained even at +20 °C.

The results presented above show that the method is well suited to the separation at −10 °C of anomeric pyranoses both for analytical and preparative purposes. Separation at low temperature can also be of interest for identification of single sugars or simple mixtures when only trace amounts are available. With some sugars the interconversion of different anomers is so rapid at −10 °C that the resolution is poor.

A previous study of methyl pyranosides of D-glucose and D-xylose by partition chromatography on a sulfate resin at high temperature showed that the α- and β-forms were well separated and appeared in that order.[3] The results given in Table 1 show that for those sugars for which it was possible to determine the elution order of the anomeric pyranose forms, the α-anomer was eluted before the β-anomer, *i.e.* the elution order was the same as with the derivatives. This holds true for all disaccharides and monosaccharides with the exception of D-arabinose. The fact that D-arabinose constitutes an exception is not unexpected in regard to results from conformational studies.[11] They show that the $^1C_4$ conformation is favoured for α-D-arabinopyranose and to a lesser extent for β-D-arabinopyranose whereas with the other monosaccharides studied the $^4C_1$ conformer is favoured. A study of ethyl-α- and β-D-arabinopyranoside on a sulfate resin [92.4 % (w/w) ethanol] at 75 °C showed that for the α-form the D_v value was equal to 2.0 whereas for the β-form it was 1.5. Hence, the arabinosides were eluted in the same order as the arabinose anomers. The results show that the conformation of the sugars and sugar derivatives have a predominant influence on their chromatographic behaviour.

EXPERIMENTAL

The chromatographic equipment was the same as described earlier.[6] The eluate was automatically analysed by the orcinol method [12] and by the periodate-formaldehyde method at pH 2.[13] A strongly basic anion exchanger in its sulfate form (Technicon T5C, 10 − 17 μm) was used. The eluent (75 % aqueous ethanol, w/w) was cooled before entering the column. The volume of the resin bed decreased with decreasing temperature. The volume (143 × 4 mm) measured at −10 °C was used in the calculation of all D_v-values. Because of high pressure in the column, the flow rate was low, 0.6 cm min^{-1} (−10 °C), 1.1 cm min^{-1} (+5 °C) and 1.7 cm min^{-1} (+20 °C). The ethanol concentration in all sugar solutions was 75 %, w/w. All sugars were commercial samples except D-allose and D-gulose, which were kindly supplied by Dr. O. Theander (Stockholm) and Dr. W. Meyer zu Reckendorf (Münster).

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Short Communications

The Role of H_2O_2 in the Reversible Inhibition of RNA Synthesis by Thiols in *E. coli*

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Cysteine has been shown by Roberts *et al.*[1] to inhibit the growth of *E. coli*. Vergroesen *et al.*[2] reported that thiols with $pK_a < 10$ were toxic at concentrations between 0.1 and 2.0 mM in human kidney tissue culture. Nagy *et al.*[3,4] demonstrated that cysteine had an inhibitory action on RNA and protein synthesis in *E. coli*. According to Kari *et al.*[5] 0.4 mM cysteine inhibits inducible synthesis of β-galactosidase, and at high concentrations cysteine as well as cysteamine give rise to energy depletion and inhibition of RNA synthesis by inhibiting membrane bound respiratory enzymes.

In the present communication we report some experiments carried out in order to clarify the mechanism of the RNA synthesis inhibiting action of thiols in cells of *E. coli*. Thiols (*e.g.* mercaptoethanol) are known not to affect RNA-polymerase *in vitro*. Therefore the inhibition *in vivo* appears to be caused by some reaction products of thiols. Possibly, cytotoxicity of thiols is due to the same products.

Materials and methods. Chemicals. (5-^{3}H)Uracil was purchased from Radiochemical Centre, Amersham, inorganic chemicals for analytical purpose were obtained from Merck, Darmstadt, and all other chemicals and biochemicals from Sigma Chemical Co.

Medium I. Phosphate buffer, pH 6.8, 60 mM; $MgSO_4$, 0.8 mM; 19 amino acids, 0.1 mM of each (except cysteine); cysteine, 0.05 mM; thymine, 0.015 mM; uracil, 0.02 mM; glycerol, 22.0 mM.

Medium II. Phosphate buffer, pH 6.8, 75 mM; $MgSO_4$, 1.0 mM; 19 amino acids, 0.125 mM of each, (except cysteine); thymine 0.01 mM; glycerol, 5 mM; (5-^{3}H)uracil, 2.5 μCi per ml, 500 mC per mol.

Washing medium. Phosphate buffer, pH 6.8, 60 mM; NaCl, 120 mM; $MgSO_4$, 0.5 mM.

RNA-SDS solution. 50 mg yeast RNA (Sigma, grade VI) and 5 g SDS dissolved in 100 ml distilled water and, to eliminate RNase contamination, incubated with 0.6 ml diethyl pyrocarbonate at 90 °C for 30 min.[6]

Cell suspension A. E. coli TAU⁻ CP 107 (rel+) cells (kindly provided by Dr. L. Alföldi, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary) from an overnight culture on nutrient agar were suspended in saline to yield a cell suspension with a density equal to OD = 0.2, measured at 520 nm, in 1 cm cuvettes, with a Unicam model SP 600 spectrophotometer.

Cell suspension B. 0.5 ml Cell Suspension A was diluted 10 times with Medium I and the culture was aerated for about 3 h by shaking at 37 °C until a cell density equal to OD = 0.4 – 0.5 was reached. The cell suspension was cooled to 0 °C, the bacteria were collected and washed with Washing medium at 0 °C on Sartorius membrane filter (pore size 0.45 μm) and then suspended in Washing medium to yield a cell suspension with a cell density equal to OD = 0.2.

Assay for RNA synthesis. 0.1 ml Cell Suspension B was added to 0.4 ml prewarmed Medium II and the suspension was incubated by shaking at 37 °C. At given times 0.1 ml RNA-SDS solution was added and after 5 min incubation at 37 °C the lyzed cells were chilled and mixed with 5 ml 5 % cold trichloroacetic acid. The precipitate was collected on Sartorius membrane filter (pore size 0.45 μm) and washed with cold 5 % TCA. The filters were placed into scintillation vials and nucleic acids were hydrolyzed with 0.2 ml 5 % TCA at 60 °C for 30 min. The filters were then dissolved in 5 ml methyl cellosolve, and the radioactivity was measured after the addition of 10 ml scintillation liquid (0.5 % PPO and 0.005 % POPOP in toluene) in a Liquid Scintillation Counter Intertechnique SL 30 with an efficiency of 29 %.

Results and discussion. The action of cysteine, cysteamine, and 2-mercaptoethanol on the RNA synthesis is summarized in Fig. 1. The time curve of RNA synthesis in control and in cysteine-inhibited cells is shown in Fig. 2. Catalase or a peptide, called Fraction V (FV) isolated from Red Kidney beans,[7,8] completely counteracted the inhibitory effect of cysteine (heat inactivated catalase was inactive). Inhibition of RNA synthesis by cysteine in *E. coli* was reversible by catalase, as demonstrated by adding catalase after preincubation with cysteine for 20 min (Fig. 2). RNA synthesis started 10 min after the addition of catalase and proceeded with the same rate as in the control cells.

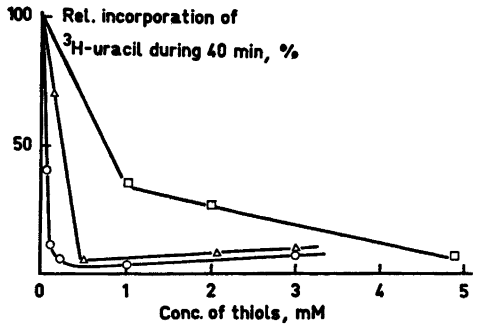

Fig. 1. The effect of cysteine (○), cysteamine (△) and 2-mercaptoethanol (□) on the RNA synthesis. (100 % corresponds to 3.3×10^5 cpm incorporated into *E. coli* TAU⁻ CP 107 (rel⁺) cells during 40 min incubation in the absence of thiols, *cf.* control in Figs. 2 and 3).

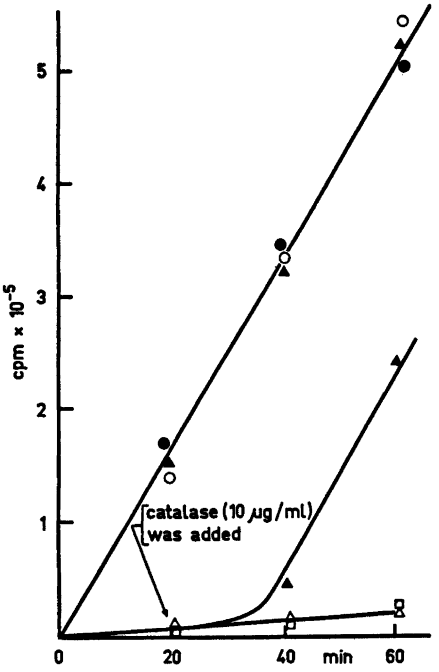

Fig. 2. The effect of catalase and FV on the inhibition of RNA synthesis caused by cysteine. Control (○); cysteine, 0.1 mM (△); cysteine, 0.1 mM plus catalase, 10 μg/ml (▲); cysteine, 0.1 mM plus heat inactivated catalase, 10 μg/ml (□); cysteine, 0.1 mM plus FV, 50 μg/ml (●), Catalase (Sigma C-100 40 000 units/mg) was added either at 0 or at 20 min.

Catalase did not reduce the concentration of cysteine.[9] From the fact that in the presence of catalase RNA synthesis was found to be unaffected by thiols, we conclude that peroxide formed during the oxidation of thiols rather than thiols as such was responsible for the inhibition of RNA synthesis. Indeed, we found that H_2O_2 inhibited RNA synthesis in *E. coli* cells and that this inhibition could be reversed by catalase in the same way as the inhibition caused by thiols (Fig. 3).

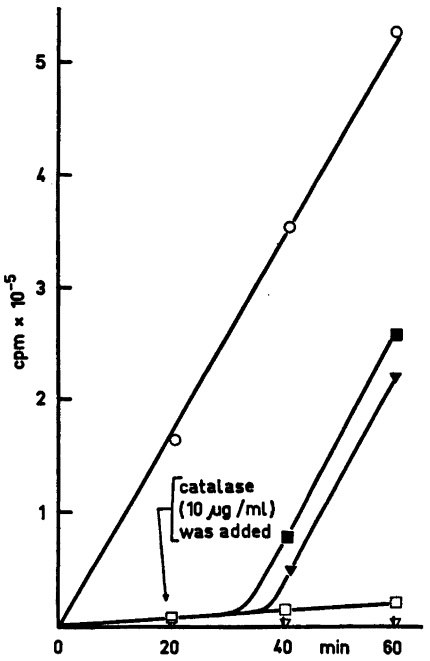

Fig. 3. The effect of H_2O_2 and catalase on the RNA synthesis. Control (○); H_2O_2 0.1 mM (□); H_2O_2, 1.0 mM (▽); H_2O_2, 0.1 mM plus catalase, 10 μg/ml (■); H_2O_2, 1.0 mM plus catalase, 10 μg/ml, (▼). Catalase (Sigma C-100 40 000 units/mg) was added at 20 min.

The concentration of H_2O_2 in aerobic systems containing thiols seems to be low, because H_2O_2 generated according to eqn. (1) is continuously eliminated according to eqn. (2).[10]

$$2RSH + O_2 \rightarrow RSSR + H_2O_2 \quad (1)$$

$$H_2O_2 + 2RSH \rightarrow 2H_2O + RSSR \quad (2)$$

From rate constants determined for reactions (1) and (2) the steady concentration of H_2O_2 in a 1 mM aerated cysteamine solution was estimated to be 0.01 – 0.1 mM.[9] In this concentration range H_2O_2 clearly inhibits RNA

synthesis. Additional experiments show that the cytotoxic action of thiols is also due to peroxide.[9] Evidently, some cell constituent(s) are highly sensitive towards H_2O_2. Therefore, H_2O_2 should always be considered as a potential cytotoxic agent and/or as an efficient inhibitor of RNA synthesis in aerobic biochemical and biological systems containing thiols or other autoxidable materials. These effects can be prevented by catalase or by peptide FV. Catalase rapidly decomposes H_2O_2 formed in reaction (1). FV has no H_2O_2 decomposing activity [9] but prevents the formation of H_2O_2 by inhibiting reaction (1). Reaction (1) requires trace amounts of metal catalyst. FV was found to bind metal ions, especially copper, and by doing so to inhibit generation of H_2O_2 and aerobic oxidation of thiols.[9] This peptide has also been shown to counteract the inhibition of RNA synthesis caused by thiols in lymphocytes.[11]

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The Absolute Configuration of α-(Benzotriazolyl-1)propionic Acid. Synthesis of α-(4,5,6,7-Tetrahydrohydrobenzotriazolyl-1)propionic Acid

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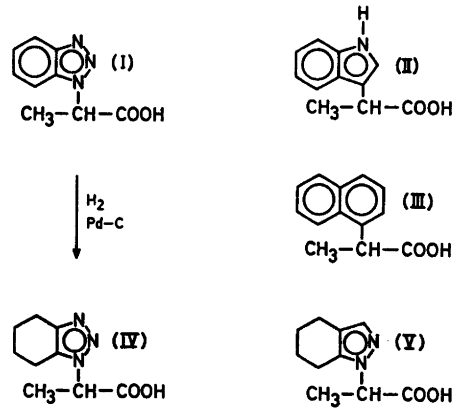
α-(Benzotriazolyl-1)propionic acid (I) has been resolved by Fredga and Lindgren.[1] Its absolute configuration has now been determined by X-ray powder photogram studies and CD measurements.

Attempts were first made to correlate I with α-(indolyl-3)propionic acid (II) of known configuration [2] by the quasi-racemate method.[3] However, neither melting-point diagrams nor X-ray powder photograms gave any indication of formation of quasi-racemates or solid solutions.

The acid I was then tested against α-(naphthyl-1)propionic acid (III).[4] Here the great difference in melting-point (~120 °C) is not favourable for thermal analysis. X-Ray powder photograms showed no quasi-racemate, but (+)-I and *S*-(+)-III gave solid solutions indicating the same absolute configuration. Thus (+)-I has *R*-configuration.

(−)-I was also hydrogenated yielding (−)-α-(4,5,6,7-tetrahydrobenzotriazolyl-1)propionic acid (−IV) (Scheme 1). The CD-spectrum of



Scheme 1.

(−)-IV was compared with that of *S*-(+)-α-(4,5,6,7-tetrahydroindazolyl-1)propionic acid (+V), whose absolute configuration is known.[5] (−)-IV and *S*-(+)-V give the same type of CD-curves (Fig. 1). This is also the case for (+)-I

synthesis. Additional experiments show that the cytotoxic action of thiols is also due to peroxide.[9] Evidently, some cell constituent(s) are highly sensitive towards H_2O_2. Therefore, H_2O_2 should always be considered as a potential cytotoxic agent and/or as an efficient inhibitor of RNA synthesis in aerobic biochemical and biological systems containing thiols or other autoxidable materials. These effects can be prevented by catalase or by peptide FV. Catalase rapidly decomposes H_2O_2 formed in reaction (1). FV has no H_2O_2 decomposing activity [9] but prevents the formation of H_2O_2 by inhibiting reaction (1). Reaction (1) requires trace amounts of metal catalyst. FV was found to bind metal ions, especially copper, and by doing so to inhibit generation of H_2O_2 and aerobic oxidation of thiols.[9] This peptide has also been shown to counteract the inhibition of RNA synthesis caused by thiols in lymphocytes.[11]

Acknowledgement. The investigation was supported by the Swedish Natural Science Research Council and the Gesellschaft für Strahlen- und Umweltforschung, FRG. We thank Mrs. Marie-Louise Hanngren and Miss Inga-Lise Kinell for skilful assistance.

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The Absolute Configuration of α-(Benzotriazolyl-1)propionic Acid. Synthesis of α-(4,5,6,7-Tetrahydrohydrobenzotriazolyl-1)propionic Acid

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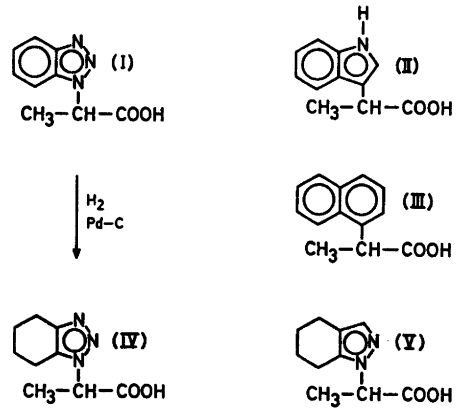
α-(Benzotriazolyl-1)propionic acid (I) has been resolved by Fredga and Lindgren.[1] Its absolute configuration has now been determined by X-ray powder photogram studies and CD measurements.

Attempts were first made to correlate I with α-(indolyl-3)propionic acid (II) of known configuration [2] by the quasi-racemate method.[3] However, neither melting-point diagrams nor X-ray powder photograms gave any indication of formation of quasi-racemates or solid solutions.

The acid I was then tested against α-(naphthyl-1)propionic acid (III).[4] Here the great difference in melting-point (~120 °C) is not favourable for thermal analysis. X-Ray powder photograms showed no quasi-racemate, but (+)-I and *S*-(+)-III gave solid solutions indicating the same absolute configuration. Thus (+)-I has *R*-configuration.

(−)-I was also hydrogenated yielding (−)-α-(4,5,6,7-tetrahydrobenzotriazolyl-1)propionic acid (−IV) (Scheme 1). The CD-spectrum of



Scheme 1.

(−)-IV was compared with that of *S*-(+)-α-(4,5,6,7-tetrahydroindazolyl-1)propionic acid (+V), whose absolute configuration is known.[5] (−)-IV and *S*-(+)-V give the same type of CD-curves (Fig. 1). This is also the case for (+)-I

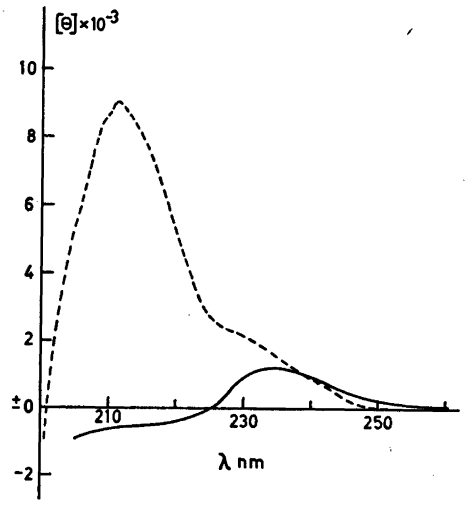


Fig. 1. CD spectra of *S*-(−)-IV (− − −, $c = 3.26 \times 10^{-2}$ g/100 ml) and *S*-(+)-V (———, $= 4.04 \times 10^{-2}$ g/100 ml) in methanol.

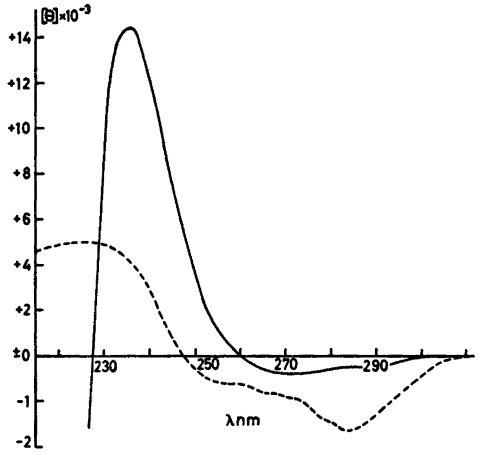


Fig. 2. CD spectra of *R*-(+)-I (− − −, $c = 3.52 \times 10^{-2}$ g/100 ml) and *S*-(+)-II (———, $c = 3.30 \times 10^{-2}$ g/100 ml) in methanol.

and *S*-(+)-II (Fig. 2). We have here additional evidence for the *R*-configuration of (+)-I.

Experimental. The UV spectra were recorded on a Bausch-Lomb Spectronic 505 spectrophotometer and the IR spectra on a Perkin-Elmer 157 spectrophotometer in KBr pellets. The ^{1}H NMR spectra were recorded on a Varian A-60 instrument using sample solutions of about 10 % and tetramethylsilane as internal standard. The optical activity was measured with a Perkin-Elmer 141 spectropolarimeter in micro cells of 10 cm length. The CD curves were recorded in 1 ml cells in methanol solutions with a Cary 60 spectropolarimeter equipped with a CD accessory and the mass spectra recorded at 70 eV with an LKB 9000 instrument. The X-ray powder photograms were taken with a Guinier-Hägg camera using Cr$K\alpha_1$ radiation.

The thin layer chromatograms were run in 20 % acetic acid − benzene on non-activated plates (E. Merck) coated with silica gel F 254 with a nominal thickness of 0.25 mm. The chromatograms were examined under UV light or developed with iodine vapour.

The melting points were determined with a hot stage microscope and are uncorrected. The micro analyses were carried out in the analytical department of the institute.

R,S-α-*(4,5,6,7-Tetrahydrobenzotriazolyl-1)propionic acid (IV).* 0.40 g *R,S*-I was dissolved in 4.0 ml 96 % ethanol and 0.80 g 10 % palladium-on-charcoal was added. Hydrogenation was carried out in an autoclave at a hydrogen gas pressure of 90 atm for 22 h at 75 °C with shaking. The catalyst was then filtered off and washed with a small amount of 96 % ethanol. The solvent was evaporated and the residue tested by thin layer chromatography. In addition to IV ($R_F = 0.53$) a small amount of I ($R_F = 0.63$) was indicated. A small spot at $R_F = 0.75$ was probably the ester of IV.

The product was dissolved in a small amount of warm 96 % ethanol and warm ligroin (b.p. 60 − 71 °C) was added. After cooling 0.045 g pure IV was obtained. M.p. 197 − 198 °C. ^{1}H NMR (DMSO-d_6): δ 1.70 (m and d, $-CH_2-$ and CH_3-), δ 2.52 (complex m, $-CH_2-\underset{|}{C}=$), δ 5.28 (q, α-H). λ_{CO} 5.8 μm. UV: λ_{max} 230 nm (log ε 3.67).

[Found (195.2, MS): C 55.31; H 6.73; N 21.29. Calc. for $C_9H_{13}N_3O_2$ (195.2): C 55.37; H 6.71; N 21.53.]

S-(−)α-(4,5,6,7-Tetrahydrobenzotriazolyl-1)-propionic acid (−IV). 0.20 g (−)-I was dissolved in 4.0 ml 96 % ethanol and hydrogenated in the same way as described above with 0.40 g 10 % palladium-on-charcoal as catalyst. 0.053 g (−)-IV was obtained after two recrystallizations. M.p. 187.5 − 188 °C. $[\alpha]_D^{25} = -19.9°$, $[\alpha]_{365}^{25} = -56.7°$ (*c* 0.1410, 96 % ethanol). The acid was chromatographically pure.

Acknowledgements. The authors are greatly indebted to Professor S. Abrahamsson for help and advice with the X-ray work.

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Mass Spectrometric Studies of α-(1,3-Dithiol-2-ylidene)-thioketones and Thioaldehydes

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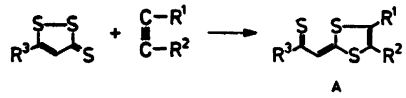
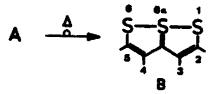
The reactions of 1,2-dithiol-3-thiones with activated acetylenes such as phenylacetylene, benzoylacetylene, acetylene dicarboxylic acid ester, and phenylacetylene carboxylic acid esters result in compounds which can be formulated as α-(1,3-dithiol-2-ylidene)thioketones and thiolaldehydes (A).[1-3]



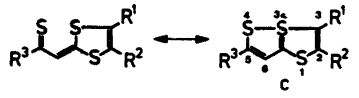
A

The compounds readily rearrange at high temperature in the presence of sulfur containing compounds such as phosphorus pentasulfide, thioacetamide and sulfur to the isomeric 1,6,6aλ^4-trithiapentalenes (B).[1,4,5]



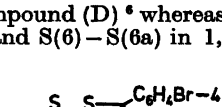
B

The close relationship to the 1,6,6aλ^4-trithiapentalenes has resulted in the formulation of analogous bicyclic canonical forms such as (C) for these compounds, (C) is a 1,3aλ^4,4-trithiapentalene. However, X-ray investigations



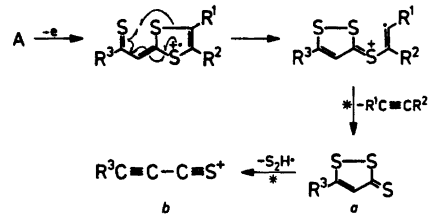
C

have shown, that the distance S(3a)–S(4) is 2.91 Å in compound (D) [6] whereas the distances S(1)–S(6a) and S(6)–S(6a) in 1,6,6aλ^4-trithiapentalene fall in the range 2.23–2.51 Å.[7] This seems to indicate that these compounds are most correctly described by structure (A); this assumptions is in accordance with the electron impact induced fragmentation of the compounds. The loss of a hydrogen atom from the aromatic bicyclic 1,6,6aλ^4-trithiapentalenes and loss of substituents from the aryl substituted 1,6,6aλ^4-trithiapentalenes were found to be a characteristic feature in the mass spectra of the latter,[8] whereas the corresponding processes are insignificant here.



D

The mass spectra of VI and IX have recently been published by one of us.[9] In both cases the predominant fragmentation was loss of phenylacetylene from the molecular ions probably under formation of 5-substituted-1,2-dithiol-3-thiones. We now report this fragmentation to be a general process for compounds of this type *cf.* Table 1. A probable mechanism is suggested in scheme 1.



Scheme 1.

This fragmentation mode accounts for most of the significant ions. One exception is the $[C_6H_5CO]^+$ ion found in the spectra of IX to XII (this ion gives rise to the base peak in XII). Another ion which was found in some abundance when both R^1 and R^2 are aromatic substituents is the $[R^1C{\equiv}CR^2]^{+\cdot}$ ion. The peak corresponding to $[R^3CS]^+$ is remarkably small when compared with $[C_6H_5CS]^+$ in the mass spectra of 5-phenyl substituted 1,6,6aλ^4-trithiapentalenes.[8] The same ion is present in the mass spectra of phenyl substituted 1,2-dithiol-3-thiones [10] in which case it appears in approximately the same abundance as in I to XII. However, it cannot be excluded that the $[R^3CS]^+$ ion in these cases also is formed directly from the molecular ion. The mass spectrum of VIII (Fig. 1) indicates that also a $[R^1CS]^+$ ion is formed (*m/e* 121) (in this case in the same abundance as $[R^3CS]^+$, *m/e* 151). The ion at *m/e* 132 corresponds to $[R^3C{\equiv}CH]^{+\cdot}$ which is a typical fragment in the spectra of 1,2-dithiol-3-thiones.[10]

Small peaks from doubly-charged ions corresponding to M^{2+} are present in all cases and to $[M-CS]^{2+}$ in the spectra of II to VIII. In the spectra of III and VIII the latter ion appears in relatively high abundance (*cf.* Fig. 1). If R^3 is a *p*-methoxy-phenyl group the methoxy

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Table 1.

Compound A										
	R^1	R^2	R^3	M^+ %	$[M-R^1C{\equiv}CR^2]$ a	$[a-S_2H]$ b	R^3CS	COPh 105	$R^1C{\equiv}CR^2$	C_6H_5 77
I	$COOCH_3$	H	H	54	100	18	8	—	—	—
II	$COOCH_3$	H	C_6H_5	55	100	47	14	—	—	8
III	$COOCH_3$	H	4-$C_6H_4OCH_3$	68	100	49	9	—	—	—
IV	$COOCH_3$	$COOCH_3$	C_6H_5	36	100	43	17	—	—	14
V	$COOCH_3$	C_6H_5	C_6H_5	35	100	44	11	—	—	6
VI	C_6H_5	H	CH_3	48	100	38	10	—	9	4
VII	C_6H_5	H	C_6H_5	61	100	48	14	—	13	6
VIII	C_6H_5	H	4-$C_6H_4OCH_3$	28	100	58	8	—	4	17
IX	COC_6H_5	H	C_6H_5	40	100	41	10	11	1	22
X	COC_6H_5	H	4-$C_6H_4OCH_3$	43	100	40	6	11	6	17
XI	C_6H_5	COC_6H_5	C_6H_5	23	100	95	35	18	1	58
XII	COC_6H_5	COC_6H_5	C_6H_5	18	62	25	8	100	1	52

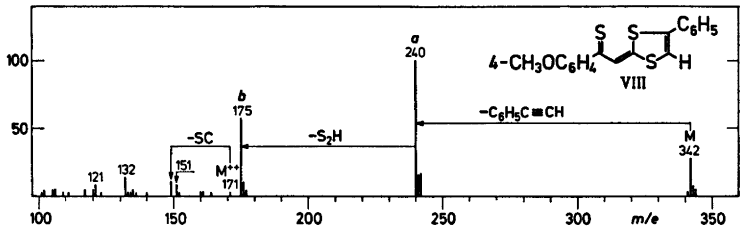

Fig. 1.

group may allow further stabilization of $[M-CS]^{2+}$ as important factors for stabilization of doubly charged ions are separation of charges and a possible formation of a conjugated system in the ion.[11] Loss of CS from the singly charged molecular ions in no cases gave rise to discernible ions.

Experimental. Mass spectra were obtained on a MS 902 spectrometer using the direct sample insertion system and the lowest feasible ion source temperature. 70 eV electrons were used. Peaks corresponding to doubly-charged ions appearing at half mass numbers and peaks of abundance lower than 2 % were omitted. When necessary the elemental composition of an ion was determined by accurate mass measurements (± 10 ppm).

α-(1,3-Dithiol-2-ylidene)thioketones and thioaldehydes were prepared by reacting the appropriate 1,2-dithiole-3-thiones with acetylenes.[12]

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A Method of Studying the Competitive Binding of Small Molecules to Macromolecules in the Gel State by High Resolution ^{1}H NMR Spectroscopy. Solvent Effects in Polyuronide Gels

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It has been known for some time that it is possible to prepare stable, rigid, voluminous, and highly transparent gels of calcium alginate in organic solvents.[1,2] The state of solvation of the macromolecules in these gel systems, as well as of macromolecular gels in general, is far from completely understood. The main object here is to describe a method by which high resolution ^{1}H NMR-spectroscopy can be used for studying the solvation of macromolecules bound in gel matrices.

Gels were prepared by dialysing solutions of sodium alginate (*Laminaria hyperborea*, stipe, M/G = 0.57) contained in standard 5 mm thin wall NMR-tubes against 0.1 M aqueous $Ca(NO_3)_2$, and then against a large volume of water to remove excess $Ca(NO_3)_2$. The gels formed had a regular cylindrical shape with a diameter about 3 mm and could be removed from the NMR-tube and placed in large volumes of different liquids for solvent exchange, and then reinserted into a heavy wall NMR-tube (inner diameter ~3 mm) for recording of spectra (VARIAN A-60A at about 39 °C).

The exchange of H_2O for EtOH occurred with a permanent slight contraction of the gels, which for a 5 % w/v water gel was found to be 8 % (v/v) and 13 % at 95 % and ~100 % EtOH, respectively, after 2 d of dialysis, after which time no further volume contraction occurred. The significant shrinkage observed at very low water content suggests that the macromolecules are preferably associated with water molecules.

The ^{1}H NMR spectra of ethanol in the gels were found to be highly dependent on the content of water in the ethanol. The spectra from gels containing 95 % EtOH had almost as narrow lines as in pure ethanol solution, while the spectra from gels containing ~100 % EtOH had very broad lines, suggesting more interaction between ethanol and the polyuronide chains in the latter case. Since the small amount of water in 95 % EtOH had such an immense effect on the ethanol spectrum, it was thought that a more systematic perturbation of the ethanol spectrum by dialyzing the gels against EtOH containing different solutes might yield information on their solvation relative to ethanol.

In order to determine the time needed for a complete exchange of solvent in each case some information on the mobility of the solvent in the gels was needed. The mobility of solvents in macromolecular gels is in general fairly high as evidence by self-diffusion coefficient measurements.[3-5] The time for the EtOH/H_2O solvent exchange in the gels used here was found to be about 3 min for hydroxyl and water protons and about 6 min for ethanol molecules as reflected by ^{1}H NMR line intensities in the external solution. The exchange times were almost independent of the alginate concentration in the range up to 10 % and comparable to the self-diffusion times in free solvents.[6] This is also to be expected because of the relative wide pores in alginate gels (mean diameter 200 – 500 Å as judged from electron micrographs).[7-8] Based upon these experiments a standard time of 10 min was used for equilibration of the gels against large excess of solvent mixtures. Attempts were also made to determine the corresponding equilibrium composition in the gels for the EtOH/H_2O system by measuring the release of water by dialysis against ~100 % EtOH. It was found that the gels in general contained more water than the external solution. A gel equilibrated aginst 98 % EtOH had for example about 20 % higher H_2O/EtOH-ratio than the external solution. Due to the

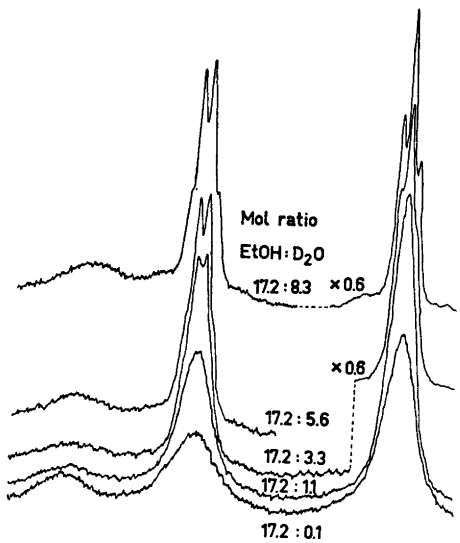

Fig. 1. ^{1}H NMR spectra of EtOH in a 10 % w/v calcium alginate gel at ~39 °C with successive addition of D_2O. The "100 % EtOH" used in the experiment was found by analysis by gas chromatography to contain less than 0.2 % water.

small volume of the gels, *ca.* 0.15 cm³, this ratio could not be determined with high accuracy, and we will at this stage relate the spectra to the composition of the external solutions.

The striking effect of water in narrowing the ethyl lines of EtOH in a 10 % alginate gel is demonstrated in Fig. 1. The behaviour can be interpreted in terms of a two-phase model for the binding of ethanol to alginate.[9] In pure ethanol a minor fraction of the solvent molecules may be restricted in their movements by interaction with the macromolecules leading to increased relaxation rates and broad ^{1}H NMR lines for their protons. If a rapid molecular exchange occurs between the interacting molecules and the bulk solvent, population-weighted average line widths are obtained. In support of this model is the observations that in pure ethanol the line widths increase with increasing concentration of alginate in the gels, and an observed difference in the line width of the CH_2 and the CH_3 lines in the ethanol-signal. In a 10 % gel in pure EtOH the CH_2 lines were 35 Hz wide and the CH_3 lines about 20 Hz wide. This difference is expected if some ethanol molecules are bound by hydrogen bonds to the alginate chains, because the CH_3-group then will be less restricted in its movement through rotation around one extra bond. The high resolution spectra obtained after addition of relative small amounts of water (corresponding to about 4 water molecules per monomer residue) indicate that no appreciable solvation of EtOH occurs under these conditions. This result strongly indicate that the water molecules bind more strongly to the alginate chains, and thereby hinder their solvation by ethanol.

In Fig. 2 the effect of water is compared with that of methanol, acetone, and cyclohexane. By assuming that the spin-spin coupling in the $-CH_2-CH_3$ group was unaffected by the broadening mechanism, the full line width at half maximum could be obtained by computer simulation, and the width of the CH_3 lines is plotted against the mol fraction of ethanol in the external solution. The results indicate a very marked difference in the solvation of the four solutes relative to ethanol with decreasing solvation in the order water > methanol > ethanol > acetone ≥ cyclohexane. This was also supported by the relative line widths of the solutes at a mol fraction of 0.3. They decreased in the order water > MeOH > acetone (10 Hz) > cyclohexane (4 Hz). The series are expected except for the lacking effect of acetone in perturbating the ethanol spectrum. Acetone could be thought to bind to the hydroxyl groups of the alginate, and it is most interesting that this seems not to be the case. This result suggests that alginate is more effective in forming hydrogen bonds as a hydrogen acceptor than as a hydrogen donor, which agrees with current ideas concerning the solvation of carbohydrates in general.[10]

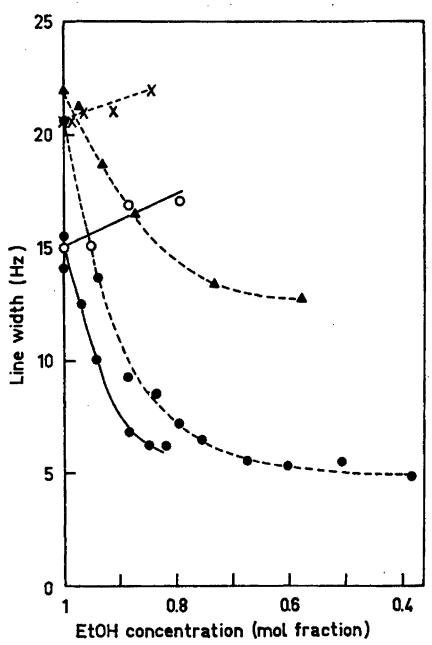

Fig. 2. The width of EtOH methyl ^{1}H NMR lines in calcium alginate gels at ~39 °C as a function of mol fraction of EtOH in the solvent. – – –, 10 % gel; ——— 5 % gel; ●, EtOH/H_2O; ▲, EtOH/MeOH; ○, EtOH/cyclohexane; ×, EtOH/acetone.

Whatever the molecular mechanism for the differences are, the effects shown in Fig. 2 are large and easy to detect, indicating that the technique may prove valuable in solvation studies on macromolecular gels. A more detailed study involving more model solutes will now be undertaken in our laboratories.

Another interesting aspect of the method is that the solvent binding capacity of the macromolecules must depend on the degree of junction formation of the chains. Crosslinking will probably reduce the macromolecular surface available for solvent interaction. Evidence for this is seen in Fig. 2 where the CH_3 lines in dry EtOH gels increase somewhat less than proportional to the concentration of alginate. This is also the case for the H_2O line in pure water gels. A study involving alginate gels of different composition and concentration may therefore shed more light on the chain structure of these gels.

Acknowledgement. Dr. T. Painter is thanked for valuable discussions.

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Studies on the Possible Effects of Adsorption on the Product Distribution of Organic Electrode Reactions. IV. Anodic Acetoxylation of 2-Methylindan, 2,2-Dimethylindan, 5,6-Dimethoxy-2-methylindan and Neopentylbenzene

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2-Methylindan, 2,2-dimethylindan, 5,6-dimethoxy-2-methylindan and neopentylbenzene have been anodically oxidized in an acetic acid/sodium acetate medium with different anode materials. The stereochemistry of the products was analyzed with special attention to the possible steric influence of the electrode surface on product distribution. Further, the influence of pulsing the anode potential has been investigated.

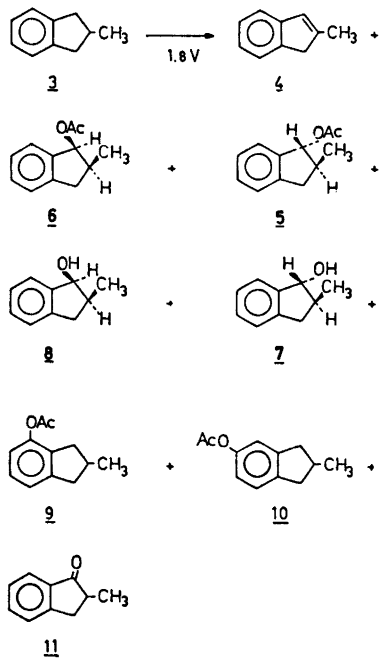
Previous papers in this series have dealt with the possible stereochemical consequences of adsorption in the anodic oxidation of 2-*t*-butylindan (*1*) and 1-*t*-butylacenaphthene (*2*) in HOAc/NaOAc.[1] By introducing a sterically demanding substituent on one side of the plane defined by the ring plane it was hoped that adsorption with the less hindered side toward the electrode surface would result in an increased *cis-trans* ratio in the side-chain substitution product. To some extent this hypothesis was substantiated: the *cis-trans* ratio of the α-acetates from *1* was 16:84, as compared to < 2:98 in a related homogeneous reaction. On the other hand, the behaviour of *2* did not show any unambiguous preference for the formation of the *cis* isomer (*cis-trans* ratio 3:97; no related homogeneous reaction could be studied due to experimental difficulties.)

The substrates *1* and *2* might, however, not be quite ideal for studying the problem at hand. NMR data for several 1,2-disubstituted *t*-butylindan derivatives indicated that the *t*-butyl group does not exert the kind of clearcut steric hindrance of one side of the molecule that might be expected from studies of molecular models. On the contrary, there is evidence that the *t*-butyl group points out in an "equatorial" manner from the saturated ring, due to hydrogen-π-orbital repulsion.[2] This means that the tertiary carbon atom of the *t*-butyl group is situated almost in the plane of the aromatic ring and thus presents steric hindrance on both sides of the molecule, even if not to the same extent. By instead using 2-methylindan as a substrate, this possibly exaggerated effect should be eliminated. The ratio of the diameters of the rotation bodies swept out by the van der Waals radii of the two substituents is approximately 2:1 as measured perpendicular to the C_2-R bond. Other investigators have reported chemically non-equivalent aromatic faces in homogeneous solution, due to alkyl-aromatic π-electron interaction.[3]

This paper reports the stereochemical outcome of the anodic oxidation of 2-methylindan in glacial acetic acid/anhydrous sodium acetate. In addition 2,2-dimethylindan, 5,6-dimethoxy-2-methylindan, and neopentylbenzene were examined under the same conditions.

RESULTS

Products formed in the anodic oxidation of 2-methylindan (*3*) in HOAc/1 M NaOAc are 4 – 11 (Scheme 1).



Scheme 1.

The major products were the side-chain acetates *5* and *6* which made up 80 % of the total product mixture. The *cis-trans* ratio was 51:49 as measured from the NMR integrals of the proton α to the acetoxy group; for *5* δ 5.84 ppm, *J* 4.3 Hz; and for *6* δ 6.06, *J* 5.6 Hz. For details of the product distribution see Table 1.

The products were identified in the following manner. The side-chain alcohols *7* and *8* were known from previous work (*trans* isomer, m.p. 88 – 89 °C; *cis* isomer, m.p. 50 – 51 °C).[4] In the present study, the trans isomer *7* was prepared by hydration of *4* according to a literature method using lithium aluminium hydride and boron trifluoride etherate in diethyl ether,[5] m.p. 88 °C (NMR for α-H of *7*: δ 4.51, *J* 6.8 Hz) after recrystallization from petroleum ether. The crude product contained about 5 % of the *cis* isomer. Lithium aluminium hydride, sodium borohydride, and aluminium isopropoxide reduction of *11* according to standard procedures gave mixtures of *8*:*7* in the ratios of 33:67, 20:80, and 15:85, respectively. From these mixtures, the necessary spectral characteristics (NMR, mass spectrum) of *8* could be obtained (NMR for α-H: δ 4.78, *J* 6.0 Hz). The mixture of side-chain alcohols *7* and *8* was treated with acetic anhydride and pyridine, which gave acetates *5* and *6* in the same ratio (NMR: *5* δ 5.85, *J* 4.3 Hz, *6* δ 6.06, *J* 5.6 Hz).

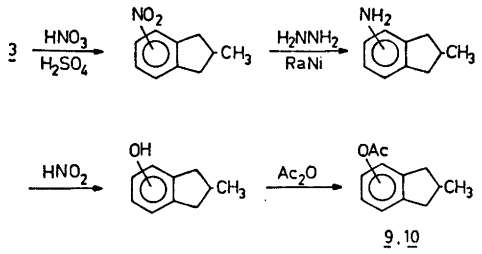
The nuclear acetates were prepared according to the reaction sequence in Scheme 2. The isomers *9* and *10* were formed in the ratio of 45:55, and *10* was considered to be the major product on the basis of earlier studies of the indan system.[6] 2-Methylindene was prepared

Table 1. Product distribution in the anodic oxidation of 2-methylindan in acetic acid/1 M sodium acetate. Anode potential 1.8 V *vs.* SCE.

Anode material and additive	Yield of products (in mol % of total products) *4*	*5+6*	*7+8*	*9+10*	*11*	Current yield, %	*6:5*
Pt	1.3	79.6	4.2	7.3	7.6	70	51:49
C	1.1	54.0	18.9	4.5	21.5	33	44:56
PbO_2	1.0	73.1	10.5	5.7	9.7	62	42:58
Pt, 5 % H_2O	1.9	54.8	28.5	7.1	7.7		51:49
Pt, 10 % H_2O	2.7	45.2	42.3	3.4	6.4	49	52:48
Pt, Bu_4NBF_4[a]	1.1	74.4	8.8	–	15.7		48:52
$Co(OAc)_3$ oxidation	traces	51.4	17.2	–	31.4		37:63
Solvolysis							43:57

[a] In this case no sodium acetate was present.

from the mixture of *7* and *8* by acid-catalyzed elimination.



Scheme 2.

As seen by inspection of Table 1, the products from the anodic oxidation of *3* using Bu_4NBF_4 as supporting electrolyte instead of sodium acetate are the same as above, except for the nuclear acetates *9* and *10* which are not expected to be formed in this case.[7] Two runs were made with water added, 5 and 10 %, respectively. This increased the amount of *7* and *8* formed (*cis-trans* ratio 34:66) so that with 10 % water added it almost equalled the amount of side-chain acetates. This is in accordance with the accepted reaction mechanism.[8] The *cis-trans* ratio for the acetates *5* and *6* was in this case 52:48.

No significant influence of the electrode material on the ratio between *5* and *6* could be detected. Table 1 shows the *cis-trans* ratio for the electrode materials used. Cobalt(III) acetate oxidation of 2-methylindan and solvolysis of *trans*-1-(*p*-nitrobenzoyloxy)-2-methylindan were used as homogeneous reference reactions. The mechanism of cobalt(III) acetate oxidation is known to be of the electron transfer type and in the last few years studies have been reported of the oxidation of a number of hydrocarbons,[9] where one of the main reaction modes has been acetoxylation in benzylic positions. The cobalt(III) oxidation of *3* gave *5* and *6* as the main products with a *cis-trans* ratio of 37:63.

For the solvolysis experiment the pure *trans* isomer of 1-(*p*-nitrobenzoyloxy)-2-methylindan was prepared by treating the 85:15 mixture of *7* and *8* with *p*-nitrobenzoyl chloride and recrystallization of the product from ethanol. No trace of the α proton from *cis*-1-(*p*-nitrobenzoyloxy)-2-methylindan was evident in the NMR spectrum of the *trans* form, not even when the signal from the α proton of the *trans* isomer (δ 6.04, J 4.0 Hz) was several times off scale. Solvolysis for 24 h in HOAc/1 M NaOAc at 75 °C produced the side-chain acetates *5* and *6* in a *cis-trans* ratio of 43:57.

If two oxidizable substrates with different adsorption properties are present in the electrolyte, the competition factor between them can be changed by pulsing the electrode potential.[10] For 2-substituted indans the two possible modes of adsorption (with the substituent pointing towards and away from the electrode surface, respectively) could be regarded in the same way as two different substrates. As before, the orientation with the substituent pointing away from the electrode surface is expected to be energetically favoured. Pulsing the anode potential should then change the ratio between *5* and *6*. As is seen from the results in Table 2, no difference in the *cis-trans* ratio for the side-chain acetates between pulse and constant potential electrolysis can be detected.

Another possibility to change the *cis-trans* ratio of the side-chain acetates, was tried with 2-*t*-butylindan as a substrate, and the results are shown at the last entry of Table 2. It is known that adsorption on electrodes reaches a maximum value at the potential of

Table 2. Results from pulse experiments with 2-methylindan and 2-*t*-butylindan in acetic acid/1 M sodium acetate.

Substrate	Duration of A pulse	B pulse	*cis-trans* Ratio for side-chain acetates
2-Methylindan	2.5 ms/0.2 V	7.5 ms/1.8 V	46:54
»	0.25 ms/0.2 V	0.75 ms/1.8 V	48:52
»	0.75 s/0.2 V	0.25 s/1.8 V	48:52
2-*t*-Butylindan	3 s/0.5 V	0.3 s/1.8 V	16:84

Table 3. Product distribution in the anodic oxidation of 2,2-dimethylindan *13* in acetic acid/1 M sodium acetate and neopentylbenzene *12* in acetic acid/0.1 M tetrabutylammonium tetrafluoroborate, anode potential 1.8 V *vs.* SCE, in both cases.

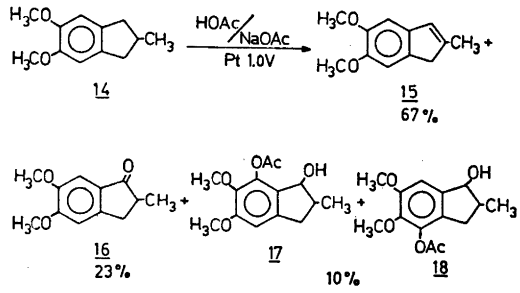
Compound	Anode material, *etc.*	Yield of products (in mol % of total products) Side-chain acetate	Side-chain alcohol	Nuclear acetate	Ketone	Unknown	Current yield, %
13	Pt	95.4	1.5	1.3	0.4	1.3 [a]	69
13	C	75.5	4.4	13.2	5.1	1.8 [a]	15
13	PbO_2	78.7	1.1	12.3	2.3	5.6 [a]	57
13	$Co(OAc)_3$ ox.[c]	39.4	–	–	44.5	16.1 [b]	–
12	Pt	73.4	–	–	26.6	–	–
12	$Co(OAc)_3$ ox.	24.6	–	–	75.4	–	–

[a] See Table 6. [b] This compound has a mass spectrum *m/e* (%) 218(0.6) 176(8) 161(8) 133(10) 118 (100) 90(30) 43(8). [c] Carried out as for 2-methylindan but at 90 °C.

zero charge and diminishes as the electrode potential is changed toward both negative and positive potentials.[11] In this experiment the anode was kept at a potential where no current passed through the cell, 0.50 V *vs.* SCE, for three seconds to allow an adsorption equilibrium to be established, and then the adsorbed material was oxidized by a short (0.3 s at 1.8 V) pulse. The *cis-trans* ratio, 15:85, for 1-acetoxy-2-*t*-butylindan, does not differ from the value obtained in constant potential electrolysis.

The oxidation of neopentylbenzene (*12*) in HOAc/1 M NaOAc gave a somewhat unexpected result. No products were formed (product integral in GLC less than 0.5 % of recovered starting material). Changing the anode material did not alter this behaviour. On the other hand, with Bu_4NBF_4 as a supporting electrolyte side-chain acetoxylation took place as expected (Table 3). In order to see if the anomalous oxidation behaviour of neopentylbenzene was shown also by its "back-bonded" analogue 2,2-dimethylindan (*13*), this compound was prepared and oxidized. Here the side-chain acetate was formed predominantly. For details of the product distribution, see Table 3. Product identification from neopentylbenzene oxidation was carried out by comparison of mass spectra and GLC retention times with authentic samples. For 2,2-dimethylindan the products were identified by mass spectral analysis.

In order to find out whether the presence of polarizable groups in the molecule would enhance adsorption and thus influence product distribution, 5,6-dimethoxy-2-methylindan (*14*) was oxidized in HOAc/1 M NaOAc. The preparative run (to 25 % of a 2e transfer) was made at 1.0 V *vs.* SCE. GLC analysis showed four major product peaks corresponding to the elimination product *15*, ketone *16* and side-chain alcohol acetoxylated in the nucleus, *17* and *18*. The product distribution (mol % of total products) was as given in Scheme 3.



Scheme 3.

The products were identified on the basis of their mass spectra and, in addition, for compound *16*, the GLC retention time.

DISCUSSION

The stereochemistry of the anodic oxidation of 2-methylindan in glacial acetic acid/sodium acetate does not give any evidence for a preferential adsorption mode of the substrate. Thus the expectation that there would be a

more distinct difference between the two faces of this molecule than in the case of 2-*t*-butylindan has not been fulfilled.

2-*t*-Butylindan shows a significant difference between anodic and comparable homogeneous reactions [1] which is not the case for 2-methylindan, as can be seen from Table 1. This study is based on the idea of different reactivities of the two sides of the molecule defined by the plane of the aromatic ring. This in turn invokes a kind of selection process where molecules with the "right" side toward the electrode are preferred for reaction. For 2-*t*-butylindan this selection process appears to be operating to some extent. For 2-methylindan the result might be explained by the assumption that the energy difference between the two modes of adsorption, with the methyl group pointing toward and away from the electrode surface, respectively, is so small that it is not sufficient to cause any preferred orientation at the electrode surface once the molecule has arrived there. (This would require an extra desorption-absorption sequence.)

Here it is interesting to note the special stereochemical features of neopentylbenzene. The side-chain of this molecule has rotational freedom around the two bonds connecting the *t*-butyl group with the aromatic nucleus. This makes it possible for the side-chain to orient itself out from the electrode surface at the same time as the molecule is adsorbed at the aromatic ring. This would explain why no α substitution product is formed from neopentylbenzene. In 2,2-dimethylindan, which can be considered as a neopentylbenzene without free rotation, normal product formation takes place. One objection against this kind of reasoning is clearly that 2,2-dimethylindan formally is a dialkylsubstituted aromatic hydrocarbon and consequently should be easier to oxidize than a monosubstituted one.[12]

The differing ease of oxidation of 2-methyl- and 2,2-dimethylindan on the one hand and neopentylbenzene on the other is evident from the voltammograms of the compounds (Figs. 1 – 4). These have been recorded at the beginning of the experiments. The current-potential curve for the solvent-supporting electrolyte was recorded first, measuring the current at each tenth of volt of anode potential in the range 0 to +3 V *vs.* SCE. The substrate was

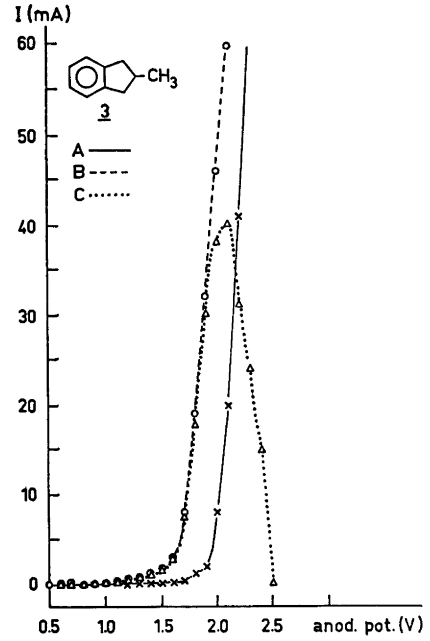


Fig. 1. Voltammogram for compound *3* in HOAc/1 M NaOAc. Curve A: Background current. Curve B: Current with 5 mmol of substrate added. Curve C: Shows the difference between B and A.

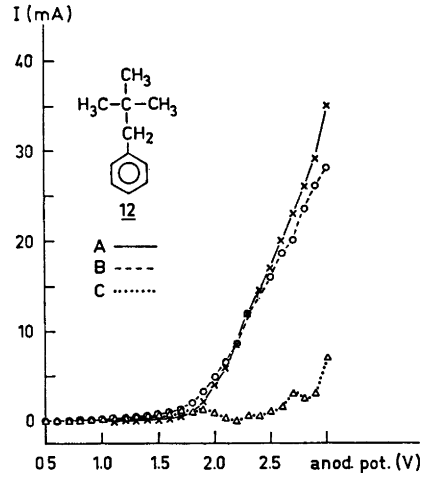


Fig. 2. Voltammogram for compound *12* in HOAc/1 M NaOAc. Curve A: Background current. Curve B: Current with 5 mmol of substrate added. Curve C: Shows the difference between B and A.

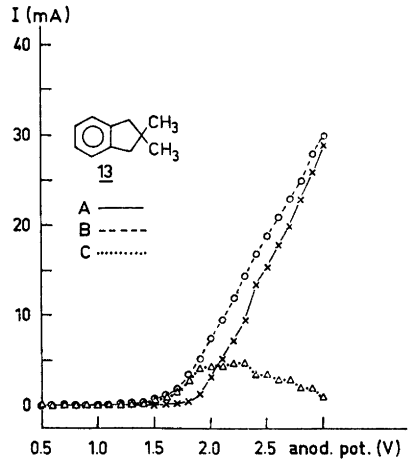


Fig. 3. Voltammogram for compound *13* in HOAc/1 M NaOAc. Curve A: Background current. Curve B: Current with 5 mmol of substrate added. Curve C: Shows the difference between B and A.

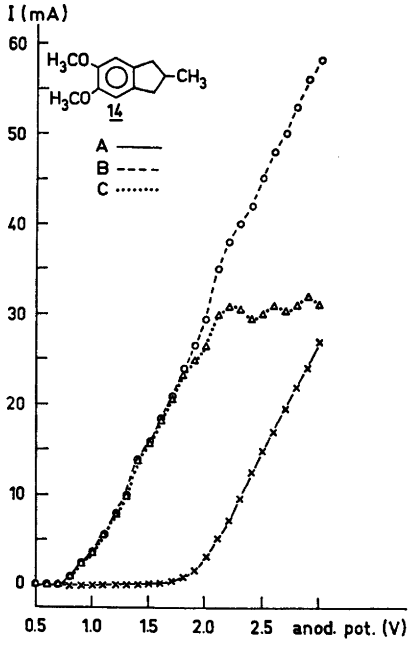


Fig. 4. Voltammogram for compound *14* in HOAc/1 M NaOAc. Curve A: Background current. Curve B: Current with 5 mmol of substrate added. Curve C: Shows the difference between B and A.

then added and the procedure repeated. Thus voltammetric curves and product analyses relate to the same single experiment.

In the pulse experiments, the goal has been to maximize adsorption. At mercury electrodes, it has been shown that substrate adsorption reaches a maximum value at the potential of zero charge. The same behaviour is exhibited by solid electrodes, even if data are relatively scarce in such systems.[11,13] It is considered that the potential of zero charge (p.z.c.) at platinum lies in the range of 0.20 – 0.5 V *vs.* SCE. Accordingly, the possibility of revealing any preferential orientation in adsorption should be most favourable at p.z.c. However, neither varying the pulse frequency nor keeping the anode at p.z.c. and using short oxidation pulses, give any results in support of such a behaviour of the substrates used here (2-methylindan and 2-*t*-butylindan). These facts cannot be explained at the present time. In one pulse experiment an attempt was made to increase the yield of the elimination product, 2-methylindene, by expelling the intermediate carbonium ion from the electrode surface. Experimentally this was done by applying a positive pulse at + 1.8 V *vs.* SCE for two seconds and a negative pulse at − 1.8 V for one second. However, the amount of *4* did not increase. It is interesting to note that the side-chain acetates *5* and *6* made up 88 % of the products in this experiment, the highest value obtained in any experiment with *3*. This may have synthetic implications, since the pulse sequence used corresponds to changing polarity over the cell.

In a recent investigation on oxidative coupling of an isoquinoline derivative, the authors found stereospecific behaviour of an electrode process but not of an analogous homogeneous reaction.[14] They explained this behaviour with adsorption of the substrate at the electrode surface. Since the isoquinoline substrate used contained polarizable groups, 5,6-dimethoxy-2-methylindan was oxidized to trace if the presence of such groups in the systems pertinent to this work would enhance adsorption. Unfortunately no side-chain acetates were formed and thus no information regarding this aspect was obtained.

In some experiments using 2-methylindan as a substrate water was added to the electrolysis solution (see Table 1). This increased the

amount of side-chain alcohols formed. The addition of water might have given stereochemical information, since if acetate ions are preferentially adsorbed at the electrode surface, as is known from studies on the Kolbe reaction,[15] a substrate molecule which reacts not on, but in close vicinity of the electrode surface, should have different concentrations of the two nucleophiles on each side of the symmetry plane. If the substrate shows any preferred orientation this should be revealed in the *8:7* ratio. The result (*8:7* = 34:66) is, however, almost equal to the *cis-trans* ratio obtained in the homogeneous Co(III) oxidation.

EXPERIMENTAL

Materials. Analytical grade acetic acid (Merck AG, Germany) was frozen out twice. Anhydrous sodium acetate (analytical grade) was used as purchased (Merck AG, Germany). Tetrabutylammonium tetrafluoroborate was prepared as described previously.[16]

Electrolysis experiments. The electrolyses were carried out in a water-jacketed vessel, volume 55 ml. The upper part was ground planar to fit a "Quickfit" cover; catalogue number MAFo/50, with five B 10 necks. A saturated calomel electrode (Radiometer K 401 or K 4016) was used as reference electrode.

Electrode arrangement. In the case of platinum two parallel foils, 20 by 30 mm and 0.1 mm thick, at a distance of 15 mm from each other were freely immersed in the solution. When using carbon or lead dioxide anodes, one platinum foil was exchanged for a graphite plate, 20 by 30 mm and 5 mm thick, or a lead dioxide plated (see below) graphite plate of the same dimensions. The electrode distance was the same as above. The porous tip of the reference electrode was placed between the electrodes as close to the anode as possible.

Efficient stirring was achieved with a magnetic stirrer. In all electrolysis experiments the temperature was kept at 25 ± 0.5 °C. The potentiostat was the TR.70/2 A type, equipped with an RB 1 type waveform generator, both from Chemical Electronics Co., Newcastle upon Tyne, England. The time calibration of the pulse length was performed with a Philips PM 3220 0 – 10 MHz oscilloscope. The amount of charge passed through the cell was measured by a 60 mV electronic integrator built by the electronic workshop, Chemical Center, Lund. The integrator was fed over a variable resistor.

Procedure for plating lead dioxide upon graphite. An acidic lead nitrate (200 g $Pb(NO_3)_2$/l, 10 g $Cu(NO_3)_2$/l, 4 g HNO_3/l, 1 g NaF/l) solution was placed in a two-compartment cell with a liquid junction. A copper wire was placed in the cathode compartment. The piece of graphite to be plated was then rotated in the anode compartment with a speed of half a revolution per second. Sand was added to the anode compartment and stirred by a magnetic stirrer to puncture gas bubbles formed during plating in order to prevent the formation of holes in the lead dioxide layer. The current density was kept at 40 mA/cm^2.

Analytical procedures. GLC analysis was carried out using a Perkin-Elmer 880 gas chromatograph, equipped with a Perkin-Elmer Model D-26 Integrator. A 4 m 3 % OV-25 on Chromosorb W column was used for all analyses. Mass spectra were recorded by an LKB 9 000 mass spectrometer and NMR spectra by a Varian A-60 NMR spectrometer. Deuteriochloroform was used as solvent.

2-Methylindan was prepared by catalytic hydrogenation of 2-methylindanone-1.[17] 2-Methylindanone-1 (31 g) in acetic acid (93 ml) was shaken with 10 % palladium on carbon (1.5 g) in a Parr low pressure hydrogenation apparatus. The pressure had dropped to a constant value after 2 h. The solution was poured into water (400 ml). Potassium hydroxide (98 g) was used to neutralize the acid. After extraction three times with ether, the extracts were washed twice with water. After drying with anhydrous $MgSO_4$, the ether was evaporated and the residue distilled through a 20 cm Vigreux column, giving 19.9 g (72 %) of 2-methylindan, b.p. 66 – 68 °C/11 mmHg. NMR: δ 1.07 (d, J 5.5 Hz, 3, methyl protons), 2.16 – 3.16 (m, 5, aliphatic side-chain protons), 7.03 (s, 4, aromatic protons).

4- and 5-Nitro-2-methylindan. 2-Methylindan (6.6 g) was cooled in an ice-bath. A cold (< 10 °C) solution of 5 ml concentrated nitric acid (68 %) in 6 ml of concentrated sulphuric acid was added dropwise with stirring. The temperature was kept at 5 – 10 °C. After the addition of acid the reaction mixture was stirred for another 20 min with cooling and then the temperature was allowed to rise to ambient for 3 h. The reaction mixture was poured into ice water (200 ml) and stirred for 15 min. After extraction three times with ether the ether solution was washed with water, $NaHCO_3$ solution and water again. Anhydrous $MgSO_4$ was used for drying the ethereal solution. The solvent was evaporated and the residue, 7.2 g, was distilled *in vacuo* through a 5 cm Vigreux column giving 5.45 g (62 %) of 4- and 5-nitro-2-methylindan, b.p. 94 – 98 °C/0.3 mmHg. The isomers were formed in a 37:63 ratio, as determined by GLC, the major product being assigned the 5-structure [6] MS, see Table 4). NMR: δ 1.13 (d, J 6 Hz, 3, methyl protons) 2.30 – 3.70 (m, 5, aliphatic protons), 7.05 – 7.55 and 7.66 – 8.02 (m, 3, aromatic protons).

4- and 5-Amino-2-methylindan. The above mixture of 4- and 5-nitro-2-methylindan (5.2 g) was dissolved in ethanol (50 ml). Hydrazine hydrate, (100 %, 3.7 g) was added and the

Table 4. Retention times and mass spectral data for 2-methylindan and derivatives. Analysis carried out on a 3 % OV-25, on Chromosorb W column (4 m × 0.3 mm), temperature 80 – 250 °C, 6 °C/min, initial period 6 min.

Compound	Retention time, min	Mass spectrum, *m/e* (% of base peak)
3	7.2	133(7) 132(66) 131(19) 117(100) 115(23) 91(19)
4	12.3	131(9) 130(100) 129(69) 128(37) 127(13) 115(74) 64(13) 51(12)
7+8	17.1	149(8) 148(89) 147(100) 133(33) 130(69) 129(65) 128(20) 127(9) 115(57) 105(35) 91(50)
11	18.7	147(13) 146(90) 145(22) 132(20) 131(100) 117(34) 115(28) 103(33) 51(22)
5+6	19.7	148(13) 147(12) 131(22) 130(100) 129(25) 115(21) 91(13) 43(26)
9	20.9	190(9) 149(9) 148(100) 147(21) 133(51) 43(11)
10	22.0	see above
4-Nitro-2-methylindan	22.6	178(3) 177(35) 160(100) 130(30) 129(24) 116(17) 115(53) 91(18)
5-Nitro-2-methylindan	23.6	178(10) 177(100) 160(27) 131(34) 130(32) 116(41) 115(48) 91(45)
4- and 5-Amino-2-methylindan	20.6	148(10) 147(100) 146(43) 133(12) 132(86) 131(17) 130(17)
4- and 5-Hydroxy-2-methylindan	20.4	149(6) 148(67) 147(16) 133(100) 131(16) 105(17)

solution warmed to 40 °C. Freshly prepared Raney nickel (0.25 g), suspended in a few ml of ethanol, was added in portions. When no further gas was evolved on adding the catalyst, the reaction was refluxed for one hour. The catalyst was filtered off. After evaporating the solvent *in vacuo*, the residue was distilled through a 5 cm Vigreux column giving 2.68 g (62 %) of 4- and 5-amino-2-methylindan, b.p. 72 – 74 °C/0.3 mmHg. The two isomers could not be separated by GLC, (MS, see Table 4). NMR: δ 1.10 (d, J 5 Hz, 3, methyl protons) 2.0 – 3.2 (m, 5, broadened aliphatic side-chain and amine protons) and 6.22 – 7.10 (m, 3, aromatic protons).

4- and 5-Hydroxy-2-methylindan.[18] To the above mixture of 4- and 5-amino-2-methylindan (2.36 g) was added concentrated sulfuric acid (4.7 g), water (2.7 g) and acetic acid (5 ml). Sodium nitrite (1.11 g) in water (6.5 ml) was then added dropwise, the temperature of the reaction mixture being kept below 5 °C. The reaction solution was then stirred at 0 °C for 30 min and at 100 °C for another 30 min. The ether solution was dried with $MgSO_4$ and the solvent removed in a rotating film evaporator. The residue was distilled yielding 1.04 g (44 %) of 4- and 5-hydroxy-2-methylindan, b.p. 72 – 74 °C/0.2 mmHg (MS, see Table 4). NMR: δ 1.13 (d, J 5.5 Hz, 3, methyl protons), 2.13 – 3.25 (m, 5, aliphatic side-chain protons), 5.18 (s, broadened, 1, hydroxylic proton), 6.43 – 7.23 (six discrete bands, with a strong singlet at δ 6.63, 3, aromatic protons).

4- and 5-Acetoxy-2-methylindan. The above mixture of 4- and 5-hydroxy-2-methylindan (0.9 g) was dissolved in acetic anhydride (30 ml). Two drops of pyridine were added and the solution refluxed overnight. After cooling, water (60 ml) was added. The mixture was allowed to stand with occasional shaking for 4 h. The water phase was extracted with ether. After drying with $MgSO_4$, the ether was removed in a rotating film evaporator. GLC of the residue showed two components in the ratio 44:56, (MS, see Table 4). NMR: δ 1.12 (d, J 6 Hz, 3, methyl protons in 2-position), 2.07 – 3.35 (m, 5, aliphatic side-chain), 2.22 (s, 3, methyl protons in acetoxy group), 6.63 – 7.20 (m, 3, aromatic protons, strong bands at 6.83 and 7.02).

Hydration of 2-methylindane.[5] Lithium aluminium hydride (0.2 g) in dry ether (15 ml), boron trifluoride etherate (0.9 g) and 2-methylindene (1.5 g) in dry ether (25 ml) were reacted according to Ref. 5. *trans*-2-Methylindanol-1 (1.49 g, 87 %) was isolated as crude product. NMR analysis showed traces of the *cis* form (appr. 5 %). Recrystallization from petroleum ether gave a sample melting at 88 °C. For NMR and MS, see below.

Reduction of 2-methylindanone-1 by aluminium isopropoxide. Aluminium isopropoxide (0.13 mol) in dry isopropyl alcohol (67 ml) was heated to reflux in a distilling flask connected. to a column of 7 – 8 theoretical plates 2-Methylindanone-1 (19.3 g) dissolved in dry isopropyl alcohol (40 ml) was added dropwise. Simul-

taneously with the addition of ketone, distillate was taken out from the still head at a rate of four drops a minute. When the addition of ketone was completed the distillation was continued until the temperature in the still head was 82.5 °C. The solution was allowed to cool and then poured onto ice (400 g). Hydrochloric acid (6 M) was added until acidic reaction was shown on indicator paper. The organic product was extracted with ether and the solution then washed with water and $NaHCO_3$ solution. After drying with $MgSO_4$ the ether was evaporated. Distillation of the white solid residue gave 15.7 g (80 %) of 1-hydroxy-2-methylindan, b.p. 116 – 117 °C/ 11 mmHg (MS, see Table 4). NMR: δ 1.16 (d, J 6 Hz, 3, $-CH_3$) 2.0 – 3.30 (m, 4, –OH proton at 2.55), 4.51 (d, J 6.8 Hz, α-H in *trans* form), 4.78 (d, J 6.0 Hz, α-H in *cis* form, *cis- trans* = 15:85) and 6.97 (m, 4 aromatic protons).

Reduction of 2-methylindanone-1 by lithium aluminium hydride. 2-Methylindanone-1 (2.92 g) dissolved in dry ether (10 ml) was added dropwise to lithium aluminium hydride (0.84 g) in dry ether (25 ml). After the addition of ketone, the solution was refluxed for 20 min. The excess of hydride was destroyed by water. After acidification, the water phase was extracted with ether. The ether solution was dried with $MgSO_4$. Evaporation of the ether gave a white solid residue of 1-hydroxy-2-methylindan (2.30 g, 78 %). NMR: as for reduction by aluminium isopropoxide, *cis-trans* = 33:66.

Reduction of 2-methylindanone-1 by sodium borohydride. 2-Methylindanone-1 (25 g) was dissolved in ethanol (99.5 %, 250 ml). Sodium borohydride (25 g) was added. The solution was stirred for 2 h. Additional sodium borohydride was added (12.5 g) and the solution stirred overnight. After addition of water (1 l) the reaction was extracted with ether. The ether solution was washed with water and dried with $MgSO_4$. Evaporation of the ether gave 11.2 g of 2-methylindanol-1 (44 %). GLC analysis showed no trace of the starting material. NMR analysis gave a *cis-trans* ratio of 20:80. For details of NMR spectrum, see above.

cis- and trans-*1-Acetoxy-2-methylindan.* A sample of 1-hydroxy-2-methylindan (0.4 g) (*cis-trans* ratio 15:85) was dissolved in acetic anhydride (5 ml) with a drop of pyridine added. The solution was refluxed overnight. The work-up procedure was the same as for the nuclear acetatets. NMR: δ 1.15 (d, J 6.5 Hz, 3, methyl protons), 2.05 (s, 3, methyl protons of the acetoxy group), *cis* and *trans* isomers not resolved due to the low intensity of the signal due to the *cis* isomer), 2.15 – 3.50 (m, 3, aliphatic protons in the side-chain), 5.84 (d, J 4.3 Hz, α-H to acetoxy group in the *trans* isomer), 6.06 (d, J 5.6 Hz, α-H to the acetoxy group in the *cis* isomer. The integral over the doublets corresponded to 1 H; *cis-trans* ratio = 15:85), 7.06 – 7.44 (m, 4 H, aromatic protons).

2-Methylindene.[19] 2-Methylindanol-1 (3.0 g, 85 % *trans* isomer) and phosphoric acid (1.5 g) were warmed slowly under reduced pressure to 130 – 140 °C. A lime coloured fluid (0.86 g) distilled over at a temperature of 80 °C in the still head. The distillate was taken up in ether, treated with $NaHCO_3$ solution and dried. The ether evaporated. NMR: δ 2.02 (s, 3, methyl protons), 3.10 (s. 2, $-CH_2-$ in aliphatic ring), 6.33 (unresolved quartet, 1, vinylic proton), 6.86 – 7.39 (m, 4, aromatic protons).

trans-1-(p-*Nitrobenzoyloxy)-2-methylindan.* 1-Hydroxy-2-methylindan (3 g, 85 % *trans* isomer) was dissolved in pyridine (35 ml). *p*-Nitrobenzoyl chloride (12 g) was added with cooling in an ice-bath. Thereafter, the reaction mixture was heated on a water-bath at 100 °C for 30 min. The reaction mixture was cooled to room temperature, poured on ice and acidified with concentrated hydrochloric acid. After suction and washing of the precipitate it was stirred in 0.05 M sodium hydroxide solution with addition of solid sodium hydroxide until the water remained alkaline to remove excess *p*-nitrobenzoic acid. After filtering, the product was air dried, giving 6.8 g of crude material. Recrystallization from ethanol, gave 2.4 g of *trans*-1-(*p*-nitrobenzoyloxy)2-methylindan, m.p. 81 – 82 °C. NMR: δ 1.23 (d, J = 6.8 Hz, 3, methyl protons), 2.26 – 3.65 (m, 3, aliphatic protons in side-chain), 6.04 (d, J = 4.0 Hz, 1, α-H to acyloxy group). No trace of the signal from the α proton of the *cis* isomer could be seen even when increasing the amplitude ten times, 7.25 (s, 4, aromatic protons in indan ring), 8.11 (s, 4, aromatic protons in *p*-nitrophenyl ring).

Solvolysis of trans-*1-*(p-*nitrobenzoyloxy)-2-methylindan.* The ester (1.0 g) was dissolved in glacial acetic acid (20 ml), 1 M in anhydrous sodium acetate and the solution kept at 75 ± 1 °C for 24 h. The acetic acid was neutralized in a $NaHCO_3$ slurry and the organic material extracted with ether. After washing and drying, the ether was evaporated. The NMR spectrum for the residue showed that the solvolysis had only run to approximately 60 %. The *cis-trans* ratio for side-chain acetates was 43:57 as measured from the integral of the α protons corrected for remaining *p*-nitrobenzoate. No significant amount of the elimination product, 2-methylindene, was detected. In an attempt to complete the solvolysis, the reaction time was extended to 3 d. The solvolysis still did not go to completion. Besides 12 % starting material, elimination now accounted for 22 % of the product.

Oxidation of 2-methylindan by cobalt(III) acetate. 2-Methylindan (645 mg) was dissolved in glacial acetic acid (50 ml) and cobaltic acetate [290 mg, 20 % in Co(III)][20] was added. The dark green homogeneous solution was kept at 70 ± 1 °C for 20 h. The work-up procedure was the same as for the electrolysis

Table 5. Retention times and mass spectral data for neopentylbenzene and derivatives. Analysis carried out on a 3 % OV-25 on Chromosorb W column (4 m × 0.3 mm), temperature 80 – 250 °C, 6 °C/min, initial period 6 min.

Compound	Retention time, min	Mass spectrum, *m/e* (% of base peak)
1-Phenyl-2,2-dimethyl-propane	5.0	148(9) 133(8) 92(85) 91(45) 57(100) 41(25)
1-Phenyl-2,2-dimethyl-propanone-1	14.6	162(4) 105(100) 77(18) 57(8)
1-Phenyl-2,2-dimethyl-propanol-1	14.6	164(2) 149(2) 108(7) 107(100) 105(6) 79(38) 77(12) 57(10)
1-Phenyl-1-acetoxy-2,2-dimethylpropane	17.2	206(5) 149(45) 131(5) 108(22) 107(100) 105(7) 57(20) 43(89)

Table 6. Retention times and mass spectral data for 2,2-dimethylindan and derivatives. Analysis carried out on a 3 % OV-25 on Chromosorb W column (4 m × 0.3 mm), temperature 80 – 250 °C, 6 °C/min, initial period 6 min.

Compound	Retention time, min	Mass spectrum, *m/e* (% of base peak)
2,2-Dimethylindan	7.5	146(49) 132(9) 131(100) 115(11) 91(25)
2,2-Dimethylindanol-1	17.0	162(70) 161(25) 131(20) 129(100) 120(27) 119(70) 91(40) 59(27)
2,2-Dimethylindanone-1	17.8	160(50) 145(100) 131(40) 74(44) 59(62) 45(46)
1-Acetoxy-2,2-dimethyl-indan	19.2	204(trace) 162(18) 161(10) 145(20) 144(100) 143(17) 129(64) 128(15) 91(13) 43(28)
4-Acetoxy-2,2-dimethyl-indan	20.4	204(11) 163(11) 162(100) 147(83) 131(9) 59(11) 43(20)
5-Acetoxy-2,2-dimethyl-indan	21.5	204(8) 163(10) 162(100) 161(7) 148(6) 147(62) 131(6) 147(62) 131(6) 91(7) 43(14)
Unknown [a]	28.2	262(1) 202(12) 178(10) 177(11) 161(41) 160(100) 145(26) 43(61)

[a] Compound whose mass spectrum corresponds to 1-acetoxy-2,2-dimethylindan, acetoxylated in the nucleus.

Table 7. Retention times and mass spectral data for 5,6-dimethoxy-2-methylindan and derivatives. Analysis carried out on a 3 % OV-25 on Chromosorb W column (4 m × 0.3 mm), temperature 120 °C, 4 °C min.

Compound	Retention time, min	Mass spectrum, *m/e* (% of base peak)
5,6-Dimethoxy-2-methyl-indan	12.8	193(12) 192(100) 177(48) 161(9) 117(8) 107(37) 91(13)
5,6-Dimethoxy-2-methyl-indene	15.8	191(11) 190(100) 175(64) 147(19) 132(15) 115(12) 103(9)
5,6-Dimethoxy-2-methyl-indanone-1	23.8	207(10) 206(88) 192(11) 191(100) 163(15) 91(13) 89(12)
5,6-Dimethoxy-2-methyl-indanol-1 acetoxylated in the nucleus	25.2	266(13) 224(38) 206(12) 167(100) 154(16) 131(14) 69(13) 43(21)
»	26.2	266(20) 224(42) 206(20) 205(15) 191(16) 167(100) 154(14) 43(29)

experiments. NMR analysis showed a *cis-trans* ratio of 37:63. GLC, see Table 1.

Neopentylbenzene was prepared according to a literature method,[21] yield 43 % (lit. 30 %), b.p. 63–67 °C/14 mmHg (MS, see Table 5). NMR: δ 0.89 (s, 9, *t*-butyl protons), 2.47 (s, 2, $-CH_2-$) and 7.13 (s, 5, aromatic protons).

1-Phenyl-2,2-dimethylpropanol-1. Pivalophenone (2.5 g), dissolved in dry ether (10 ml), was dropped onto lithium aluminium hydride (340 mg) in dry ether (25 ml) and the mixture stirred under reflux for 4 h. Water was then added dropwise. When no more hydrogen evolved, sulphuric acid (10 %, 40 ml) was added to dissolve the aluminium hydroxide. The organic material was collected by extraction with ether. The combined ether extracts were washed with $NaHCO_3$ solution and water, and then dried with $MgSO_4$. After evaporation of the solvent, distillation gave 0.67 g of product, b.p. 92–94 °C/12 mmHg (MS, see Table 5). NMR: δ 0.88 (s, 9, *t*-butyl), 2.16 (s, 1, $-OH$), 4.30 (s, 1, $-CH-$) and 7.22 (s, 5, aromatic protons).

1-Phenyl-2,2-dimethyl-1-propyl acetate. 1-Phenyl-2,2-dimethylpropanol-1 (0.48 g) was dissolved in acetic anhydride (5 ml), one drop of pyridine was added and the solution refluxed overnight. Working-up as for 4- and 5-acetoxy-2-methylindan gave a sample for NMR and mass spectral analysis (MS, see Table 5). NMR: δ 0.91 (s, 9, *t*-butyl), 2.00 (s, 3, $-OC-CH_3$), 5.47 (s, 1, $-CH-$ proton α to acetoxy group) and 7.20 (s, 5, aromatic protons).

Oxidation of neopentylbenzene with cobalt(III) acetate. Neopentylbenzene (776 mg) was dissolved in glacial acetic acid (50 ml). Cobaltic acetate [20 % $Co(OAc)_3$, 307 mg], was added and the solution kept at 110 °C for 20 h, after which the colour of the solution had turned pink. For product distribution, see Table 3.

2,2-Dimethylindanone-1.[22] 2-Methylindanone-1 (7.3 g) and methyl iodide (17.8 g) was dissolved in dry ether (125 ml). Potassium *t*-butoxide (14 g) was added in portions with stirring. After half a minute a vivid reaction started. The remaining base was added at such a rate that the solvent kept boiling. Addition completed, the reaction mixture was stirred under reflux for 2 h. Water (150 ml) was added and the phases separated. The water phase was extracted with ether. The combined ether solutions were washed with $NaHCO_3$ solution and very diluted sulphuric acid was added dropwise until gas evolution started. After drying and evaporation as earlier 8.3 g of 2,2-dimethylindan remained. GLC showed only one peak and the product was used without further purification (MS, see Table 5). NMR: δ 1.22 (s, 6, methyl protons), 2.98 (s, 2, methylene protons) and 7.23–7.85 (m, 4, aromatic protons).

2,2-Dimethylindan.[22] 2,2-Dimethylindanone-1 (8.3 g) was dissolved in glacial acetic acid (20 ml). Palladium on carbon (10 %, 0.8 g) was added as a catalyst. Hydrogenation and work-up procedure as for 2-methylindan. Distillation gave 2,2-dimethylindan (2.53 g), b.p. 65–66 °C/11 mmHg. NMR: δ 1.13 (s, 6, methyl protons), 2.68 (s, 4, methylene protons) and 7.07 (s, 4, aromatic protons).

Electrolysis experiments. The solvent-supporting electrolyte (50 ml) was poured into the electrolysis vessel. A background voltammogram was taken up measuring a stable current at each 0.1 V anode potential from 0 to +3 V *vs.* SCE. Then approximately 5 mmol of substrate was added. A new voltammogram was taken up and thereafter a preparative run was made until 25 % of the calculated charge for a 2e transfer process had passed. With platinum electrodes the anode potential was pulsed when necessary in order to diminish passivation of the electrodes which otherwise drastically lowered the current (2.9 s at working potential, 0.1 s at 0 V).

Work-up procedure. A sample of 5 ml was withdrawn from the electrolysis solution for determining current yield. Naphthalene (30–50 mg) was added as an internal standard and this solution was taken up in methylene chloride (25 ml). This solution was shaken with water (25 ml) and then with $NaHCO_3$ solution. After drying with anhydrous $MgSO_4$ the analysis was made by GLC. The remaining 45 ml of the electrolysis solution was diluted with methylene chloride (100 ml) and shaken with water (150 ml). The water phase was shaken with further methylene chloride (25 ml) and the combined organic extracts were then shaken with $NaHCO_3$ solution until no more gas evolved. After separation of the phases, the organic layer was dried and the solvent evaporated. The residue was used for product analysis, NMR and MS.

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On the Reaction of 2,5-Dihalothiophenes with Tetracyanoethylene Oxide

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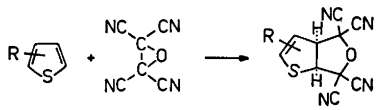
The reaction between 2,5-dihalothiophenes or 2,5-dihaloselenophenes and tetracyanoethylene oxide leads to 2,5-bis(dicyanomethylene)-2,5-dihydrothiophene (IIIa) and 2,5-bis(dicyanomethylene)-2,5-dihydroselenophenes (IIId), respectively. Possible reaction paths are discussed.

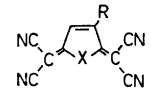
The reaction of 2,5-bis(dicyanomethylene)-3-bromo-2,5-dihydrothiophene (IIIb), obtained from 2,3,5-tribromothiophene and tetracyanoethylene oxide, with thiophenol gave 2,5-bis-(dicyanomethylene)-3-phenylthio-2,5-dihydrothiophene (VII).

Linn and coworkers [1,2] have shown that tetracyanoethylene oxide (I) adds to olefins and also to aromatic systems like benzene and thiophene in a stereospecific manner to give derivatives of tetracyanotetrahydrofurans. In connection with our general study of this cycloaddition reaction in the thiophene series, we made attempts to rearomatize the 1,1,3,3-tetracyano-1,3,3a,6a-tetrahydrothieno [2,3-c]-furans (II) formed and in this way open a new route to 2,3-disubstituted thiophenes.



I II

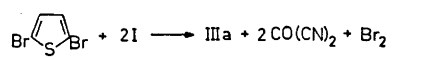
Our rearomatization attempts have hitherto failed. One of our hopes was that this could be achieved by adding I to 2,4- or 2,5-dibromothiophene followed by elimination of hydrogen bromide. 2,4-Dibromothiophene did not react. When 2,5-dibromothiophene was heated with I in ethylene bromide solution a red liquid started to reflux after a few hours. This liquid was distilled off and boiled at 60 – 65 °C and was identified as carbonyl cyanide by its reaction with *N*,*N*-dimethylaniline, yielding bis(*p*-dimethylaminophenyl)dicyanomethane.[3] From the reaction mixture, which contained 2,3,5-tribromothiophene and tetrabromothiophene (identified by combined VPC-MS analyses), an intense yellow compound could be isolated which by elemental analysis and mass spectrometry was shown to have the composition $C_{10}H_2N_4S$. The IR spectrum of this compound was unusually simple, showing only a few peaks, but indicated the presence of sp^2-hybridized C – H bonds and of nitrile groups. The ^1H NMR spectrum showed only one sharp peak at δ 7.33. The structure of this product was determined by X-ray crystallography and shown to be 2,5-bis(dicyanomethylene)-2,5-dihydrothiophene (IIIa).[4]



III

a X = S, R = H
b X = S, R = Br
c X = S, R = Cl
d X = Se, R = H
e X = Se, R = Br

From the products formed in this reaction, the following stoichiometry seems reasonable:



* Taken in part from the Ph.D. thesis of Bengt Uppström, University of Lund, 1973.

The molecular bromine which is formed reacts immediately with the excess of 2,5-dibromothiophene to give 2,3,5-tribromothiophene and tetrabromothiophene. We checked of course that these compounds were not formed upon refluxing 2,5-dibromothiophene in ethylene bromide. Nor could we detect any appreciable amounts of carbonyl cyanide upon refluxing only I in ethylene dibromide. Based upon the above mentioned stoichiometry, the yield of IIIa calculated on I was as high as 70 %.

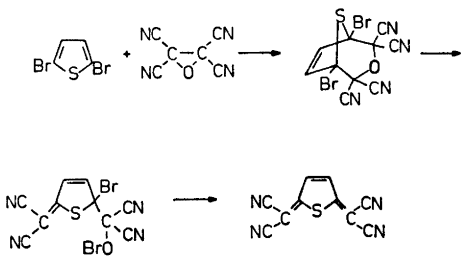
As we could find no precedent for this reaction in the literature, we were interested in studying its scope. We found that 2,3,5-tribromothiophene reacted similarly, yielding a compound $C_{10}HBrN_4S$ (IIIb), which was hydrogenolysed to IIIa over Pd on carbon. Also 2,3,5-trichlorothiophene, 2,5-dibromo- and 2,3,5-tribromoselenophene reacted similarly to give 3-chloro-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene (IIIc), 2,5-bis(dicyanomethylene)-2,5-dihydroselenophene (IIId), and 3-bromo-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene (IIIe). The yields, however, were in these cases much lower: 16, 26 and 38 %, respectively. Removal of carbonyl cyanide during the reaction by distillation, which in the reaction between 2,5-dibromothiophene and I doubled the yield, had no effect in these cases.

The reaction most probably also occurred with 2,3,5-triiodothiophene, but due to separation difficulties the product was not obtained pure. In the reaction of 2,5-dibromo-3-methyl thiophene with I only traces of the expected product were obtained.

In spite of several changes in the experimental conditions the yields could unfortunately not be increased. The long initiation period before carbonyl cyanide appeared in the reaction mixture led us to believe that the reaction was perhaps radical in nature. However, the addition of benzoyl peroxide had no effect on the reaction rate.

The mechanism of the reaction must be rather complex. The formation of carbonyl cyanide obviously makes the simple mechanism indicated in Scheme 1 less likely, if it is assumed that IIIa and carbonyl cyanide are formed in the same reactions.

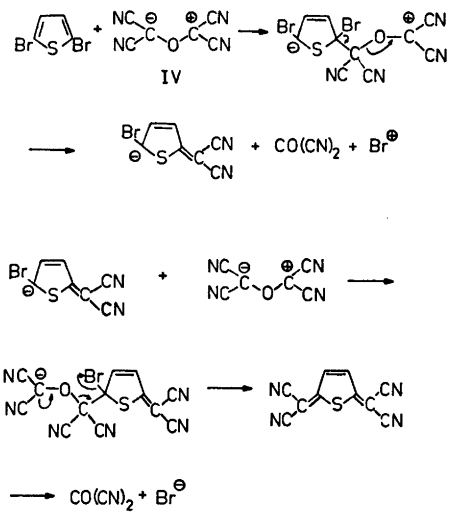
In this mechanism, it is assumed that I adds over the 2,5-position instead of the normal cycloaddition over the 2,3-bond. The bicyclic



Scheme 1.

adducts open and after complex elimination of Br_2O, IIIa is obtained. However, this path does not explain the formation of carbonyl cyanide.

In the normal cycloadditions of I to olefins and aromatics a 1,3-dipolar form of I (IV) has been assumed to be the reactive intermediate.[5] The following complex mechanism, shown in Scheme 2, can therefore be envisaged.

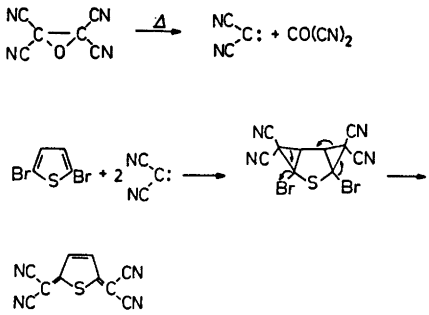


Scheme 2.

It is assumed that the 1,3-dipolar form of I first reacts as a nucleophile and the second molecule as an electrophile.

The opposite orders of attack are of course also possible, but appear even less likely than the reaction path indicated in Scheme 2. An important objection to this mechanism is the sluggishness with which non-activated halothiophenes undergo nucleophilic aromatic substitution.

Linn and coworkers [6,7] found that I was very susceptible to nucleophilic attack, which formally led to cleavage into the elements of carbonyl cyanide and dicyanomethylene. It is therefore not unlikely that by thermal dissociation or induced by nucleophilic species small amounts of dicyanomethylene are in equilibrium with I and that IIIa is formed as indicated in Scheme 3.



Scheme 3.

Schemes 2 and 3 are both in accordance with the products obtained.

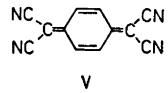
We tried to obtain support for the mechanism in Scheme 3 by reacting 2,5-dibromothiophene with dicyanomethylene in the absence of carbonyl cyanide. Streith and Cassel,[8] through photolysis of pyridinium dicyanomethylide,[7] generated dicyanomethylene which added to benzene.

However, both photolytic and thermal attempts to obtain IIIa from 2,5-dibromothiophene and pyridinium dicyanomethylide failed. Dicyanomethylene has also been obtained from dicyanodiazomethane,[9] but due to the explosive nature of this precursor no experiments were undertaken with it.

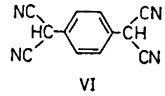
A weak support for the mechanism indicated in Scheme 3 stems from the fact that in a few experiments tetracyanoethylene was observed as a minor by-product. This could possibly have been formed through dimerization of dicyanomethylene. The mechanism indicated in Scheme 1 could be disproven (or proven) by the use of ^{13}C labelled I.

The mass spectra of III show very little fragmentation. The total intensity of all other ions is 15 % of that of the molecular ion, indicating the stability of these compounds. The two unsubstituted derivatives primarily lose HCN from the molecular ion, and this is also true for the 3-methyl derivative. The halogen-substituted compounds first lose the halogen atom and the HCN.

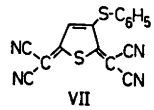
Compounds III are analogues of the interesting tetracyanoquinodimethane (V). This compound can easily be reduced to *p*-phenylenedimalononitrile (VI) by reagents such as thiophenol, mercaptoacetic acid or hydroiodic acid.[10] Compound IIIa was, however, decomposed by hydroiodic acid and with thiophenol the starting material was recovered. IIIb on the other hand yielded with thiophenol in acetic acid a beautifully red, difficultly soluble compound, m.p. 215 – 216 °C, which according to mass spectrum and elemental analyses had the composition $C_{16}H_6N_4S_2$. Its NMR spectrum, which due to its difficult solubility had to be recorded in trifluoroacetic acid, shows two peaks at δ 7.08 and δ 6.64 with the relative intensities 5:1. These facts indicate that the compound is 3-phenylthio-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene (VII) and that nucleophilic substitution of the olefinic bromine had occurred.



V



VI



VII

EXPERIMENTAL

2,5-Bis(dicyanomethylene)-2,5-dihydrothiophene (IIIa). A solution of 24.0 g (0.10 mol) of 2,5-dibromothiophene and 5.0 g (0.035 mol) of tetracyanoethylene oxide [11] in 50 ml of 1,2-dibromoethane was heated with stirring in an oil-bath at 160 °C, in an apparatus arranged for distillation. After 3 h carbonyl cyanide started to distill of, b.p. 60 – 65 °C. After heating for an additional 2 h, 1.8 g (65 %) of carbonyl cyanide was obtained, characterized through reaction with *N,N*-dimethylaniline, yielding bis(*p*-dimethylaminophenyl)dicyanomethane, m.p. 193 – 194 °C; literature value,[3] m.p. 192 – 193 °C. The reaction mixture was cooled, carbon tetrachloride added, the precipitate filtered off and recrystallized, yielding 2.5 g (70 %) of the

title compound, m.p. 182–183 °C. The first filtrate was distilled and the high-boiling distillation residue (1.8 g) consisted according to VPC (column OV-17 (3 %) on gaschrom Q, 3 m × 3 mm) of 70 % 2,3,5-tribromothiophene and 30 % of tetrabromothiophene. NMR (CD_3COCD_3): δ 7.33. IR (KBr): 3120, 3100, 3050, 2220, 1550, 810, and 700 cm^{-1}. Mass spectrum (*m/e*, %): 212, 7; 211, 14; 210, 100; 185, 5; 183, 6; 121, 8; 118, 6; 108, 13; 94, 15; 83, 5; 82, 10; 76, 8; 75, 15; 70, 11; 69, 13; 64, 6; 63, 6; 62, 6; 57, 8; 55, 7; 45, 15; 43, 12; 41, 6; 40, 5; 38, 8; 37, 5; 32, 5; 28, 5; 27, 5. [Found: C 58.02; H 1.11; N 25.72; S 15.41. Calc. for $C_{10}H_2N_4S$: C 57.14; H 0.96; N 26.65; S 15.25.]

3-Bromo-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene (IIIb). A solution of 10.0 g (0.070 mol) of tetracyanoethylene oxide and 64.0 g (0.20 mol) of 2,3,5-tribromothiophene in 100 ml of 1,2-dibromoethane was refluxed for 24 h and filtered hot. After 2 d a black tarry product was isolated which was extracted with benzene in a Soxhlet apparatus. Evaporation yielded 2.8 g (28 %) of the title compound, m.p. 173–175 °C after recrystallization from benzene or chloroform. NMR (CD_3COCD_3): δ 8.22. IR (KBr): 3090, 2220, 1545, 1520, and 810 cm^{-1}. Mass spectrum (*m/e*, %): 292, 7; 291, 15; 290, 100; 289, 14; 288, 99; 265, 5; 263, 5; 210, 17; 209, 17; 199, 6; 165, 8; 157, 6; 145, 10; 144, 6; 138, 5; 124, 10; 121, 5; 120, 10; 119, 5; 118, 15; 113, 6; 108, 15; 106, 10; 101, 13; 100, 15; 98, 5; 94, 15; 93, 5; 92, 5; 89, 10; 88, 5; 87, 7; 86, 12; 82, 15; 81, 5; 80, 5; 79, 8; 76, 10; 75, 15; 74, 5; 70, 15; 69, 15; 68, 5; 64, 12; 63, 18; 62, 15; 61, 5; 60, 5; 49, 10; 45, 13; 44, 12; 38, 13; 37, 12; 32, 10. [Found: C 41.6; H 0.52; Br 27,7; N 19.1; S 10.9. Calc. for $C_{10}HBrN_4S$: C 41.5; H 0.35; Br 27.6; N 19.4; S 11.1.]

Hydrogenolysis of 3-bromo-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene. In a Parr hydrogenation apparatus, 200 ml of peroxide-free dioxane, and 1.16 g of 3-bromo-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene were placed. The mixture was hydrogenated at 5 atm. for 24 h, the catalyst filtered off and the dioxane removed *in vacuo*. The tarry residue was extracted with benzene, the benzene solution evaporated to a small volume, and then chromatographed on a silica gel column using benzene as eluent. 0.3 g (36 %) of 2,5-bis(dicyanomethylene)-2,5-dihydrothiophene, having the spectral properties as the sample described above was obtained.

3-Chloro-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene (IIIc). A solution of 18.7 g (0.10 mol) of 2,3,5-trichlorothiophene, 7.0 g (0.049 mol) of tetracyanoethylene oxide in 50 ml of 1,2-dibromoethane was refluxed for 48 h. The solution was filtered hot and evaporated to a volume of about 10 ml. The tarry product was filtered off and recrystallized from benzene (charcoal) to give 0.90 g (16 %) of the title compound, m.p. 180–181 °C. NMR (CD_3COCD_3) δ 8.15. IR (KBr): 3090, 2220, 1550, 920, and 865 cm^{-1}. Mass spectrum (*m/e*, %): 247, 5; 246, 40; 245, 15; 244, 100; 209, 8; 118, 8; 94, 10. [Found: C 49.1; H 0.55; Cl 14.35; N 22.95; S 13.00. Calc. for $C_{10}HClN_4S$ (244.7): C 49.09; H 0.41; Cl 14.49; N 22.90; S 13.11.]

2,5-Bis(dicyanomethylene)-2,5-dihydroselenophene (IIId). A solution of 28.9 g (0.10 mol) of 2,5-dibromoselenophene,[12] 5.0 g (0.035 mol) of tetracyanoethylene oxide in 50 ml of 1,2-dibromoethane was refluxed for 48 h. After filtration, evaporation and recrystallization from benzene, 1.2 g (26 %) of the title compound, m.p. 199–201 °C, was obtained. NMR (CD_3COCD_3): δ 7.93. IR (KBr): 3120, 3050, 2220, 1545, and 810 cm^{-1}. Mass spectrum (*m/e*, %): 259, 12; 258, 100; 257, 8; 256, 50; 255, 16; 231, 6; 178, 5; 142, 11; 140, 5; 125, 6; 124, 5; 118, 5; 101, 5; 100, 5; 93, 5; 80, 16; 78, 8; 76, 6; 75, 12; 63, 5; 62, 5. [Found: C 47.56; H 1.20; N 21.48 Calc. for $C_{10}H_2SeN_4$ (257.1): C 46.71; H 0.79; N 21.79.]

3-Bromo-2,5-bis(dicyanomethylene)-2,5-dihydroselenophene (IIIe). A solution of 36.8 g (0.10 mol) of 2,3,5-tribromoselenophene[12] and 5.0 g (0.035 mol) of tetracyanoethylene oxide in 50 ml of 1,2-dibromoethane was refluxed for 48 h. After filtration and evaporation, the residue was chromatographed on silica gel using benzene as eluent, yielding 2.2 g (38 %) of the title compound, m.p. 165–166 °C. NMR (CD_3COCD_3): δ 8.28. IR (KBr): 3080, 2215, 1535, and 750 cm^{-1}. Mass spectrum (*m/e*, %): 340, 12; 339, 10; 338, 78; 337, 12; 336, 100; 335, 16; 334, 45; 333, 12; 332, 14; 330, 11; 258, 5; 257, 16; 255, 6; 168, 11; 166, 10; 161, 12; 157, 6; 150, 8; 141, 12; 140, 5; 130, 5; 124, 16; 101, 16; 100, 12; 98, 5; 86, 7; 80, 21; 78, 6; 75, 18; 62, 7. [Found: C 35.64; H 0.56; Br 23.73; N 16.64; Se 23.40. Calc. for $C_{10}HBrN_4Se$ (336.0): C 35.75; H 0.30; Br 23.78; N 16.67; Se 23.50.]

3-Phenylthio-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene (VII). To a solution of 1.0 g (0.0035 mol) of 3-bromo-2,5-bis(dicyanomethylene)-2,5-dihydrothiophene in 100 ml of acetic acid, 0.55 g (0.0050 mol) of thiophenol was added and the acetic acid evaporated over night. The solid residue was washed with a little ether and chromatographed on silica gel using chloroform-ether (4:1) as eluent, yielding 0.60 g (54 %) of the title compound, m.p. 215–216 °C. NMR (CF_3COOH): $\delta_{C_6H_5}$ 7.08, δ_4 6.64. IR (KBr): 3070, 2230, 2210, 1538, 1490, 830, and 740 cm^{-1}. Mass spectrum (*m/e*, %): 320, 5; 318, 100; 293, 5; 292, 5; 291, 77; 261, 5; 260, 5; 259, 5; 254, 5; 253, 5; 233, 5; 210, 5; 110, 5; 109, 5; 108, 5; 94, 5; 89, 5; 84, 5; 83, 5; 77, 43; 75, 5; 69, 5; 66, 5; 65, 5; 51, 52; 50, 5; 45, 5; 39, 5. [Found: C 59.58; H 2.09; N 16.79; S 20.53. Calc. for $C_{16}H_6N_4S_2$ (318.4): C 60.30; H 1.90; N 17.60; S 20.14.]

IR spectra were recorded on a Perkin-Elmer 257 Grating Infrared Spectrophotometer, NMR spectra on a Varian A-60 spectrometer and mass spectra on an LKB 9000 mass spectrometer. The gas chromatographic analyses were

performed with a Perkin-Elmer 900 gas chromatograph.

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Ionisation Potentials in Structure Analysis of Gaseous Hydroxypyridine 1-Oxides*

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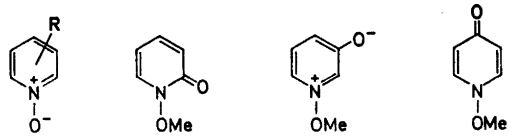
The 3- and 4-isomers of the tautomeric hydroxypyridine 1-oxides exist predominantly in the hydroxy form in the gaseous state in the mass spectrometer. Internal hydrogen bonding in a pseudo-ring structure is suggested for the 2-isomer. The conclusions are based on comparisons of ionisation potential data.

Extensive physical measurements on the tautomerism of hydroxypyridine 1-oxides in solution and solid state have been carried out.[2] We wish to report studies of hydroxypyridine 1-oxides in the gaseous state by the determination of ionisation potentials (IP). IP determinations have recently been found very useful in structure analysis of gaseous molecules;[3-4] such data for the tautomeric hydroxy and amino pyridines have been interpreted in favour of the hydroxy and amino forms.[3,4]

Interpretation of the IP data for the hydroxypyridine 1-oxides (*1*—*3*) required corresponding data for the phenolic ethers (*4*—*6*) and the 1-methoxy analogues (*7*—*9*). These derivatives correspond to fixing the hydroxypyridine 1-oxides in either tautomeric form. The IP values were obtained by the semi-log plot method with xenon as reference gas.[6]

The influence of a substituent on the ease of ionisation depends on the nature of other substituents as well as on the ring system and relative position;[4,7] generally an electron releasing substituent decreases the IP. In the methoxypyridine 1-oxides the IP value (*4*—*6*) is increased by 0.2 eV from the 2- to the 3-isomer, but decreased by 0.4 eV from the 3- to the 4-isomer. The IP decrease of a methyl group relative to that of a hydrogen atom on a phenolic oxygen in simple systems is of the order 0.2—0.3 eV. This corresponds to the observed difference (0.2 eV) between the 3-methoxy isomer (*5*) and the 3-hydroxy isomer (*2*). The latter can be assumed to be present in the gas phase as such rather than as its tautomeric 1-hydroxy zwitterion. The charge separation introduced in the zwitterionic form will reduce the volatility compared to that of the non-charged 3-hydroxy form; furthermore, structures with charge separation should be disfavoured in the gas phase because at 10^{-6} Torr the charges cannot be dispersed by solvation or other intermolecular interactions. The IP for the 4-methoxy isomer (*6*) is also about

Table 1. Ionisation potentials.



1—6 7 8 9

Compound	R	IP (eV)
1	2-OH	8.90
2	3-OH	8.60
3	4-OH	8.18
4	2-OMe	8.21
5	3-OMe	8.40
6	4-OMe	7.98
7		8.32
8		(8.3)
9		8.49

* Part XXVII in our series on "Mass Spectrometry of Onium Compounds".[1]

Table 2. Characteristic ion intensities in the mass spectra.

Fragment	*1*	*2*	*3*	*4*[b]	*5*	*6*	*7*[c]	*9*
[M]	75	100	100	29	100	100	84	100
[M−15]			25[a]	2	5	44	–	21
[M−16]	100	24	79	28	72	81	2	8
[M−17]	–	–	8	62	2	8	2	–
[M−30]				13	3	16	58	9
[M−31]				–	11	5	3	1

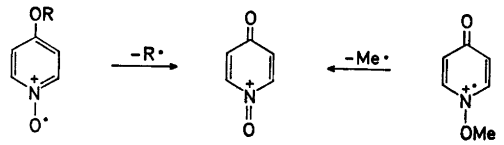
[a] [M−1]; [b] *m/e* 78 base peak; [c] *m/e* 39 base peak.

0.2 eV lower than for the 4-hydroxy isomer (*3*) which supports the hydroxy formulation for the latter. The difference (0.6 − 0.7 eV) between the 2-isomers (*1* and *4*), however, is too large to be inherent in the methyl group. Nor can the difference be explained by the molecule being in the form of 1-hydroxypyrid-2-one as IP of the model 1-methoxy derivative (*7*) is about 0.6 eV below that of (*1*); the effect of the *O*-methyl group is assumed to be of the same order as discussed above. It is well established, however, that *1* forms strong intramolecular hydrogen bonding.[2,8] A major part of the observed IP difference in the 2-series is therefore attributed to ground state destabilisation on *O*-alkylation which prevents intramolecular hydrogen bonding. Presumably the 2-hydroxy isomer, therefore, is present in the gas phase as a strongly hydrogen bonded molecule with structure in between those of the two tautomeric forms.

A further comparison of the IP data for the 4-isomers (*6* and *9*) supports the hydroxy formulation for *3*. Assuming similar effect from the methyl groups (0.2 eV) the estimated IP's for 4-hydroxypyridine 1-oxide and 1-hydroxypyrid-4-one are 8.2 and 8.7 eV, respectively; the former is in agreement with the observed value (8.18 eV).

The reproducibility of IP for the betaine (*8*) was poor; the value also appears higher than expected for a pyridinium betaine, and $[M-O]^+$ is a major fragment in its spectrum. The data are consistent with a large degree of transmethylation to the 3-methoxy derivative (*5*) before evaporation.

To verify that the gaseous molecules studied have not undergone structural rearrangements their characteristic primary fragmentations are summarised in Table 2. The mass spectra of heteroaromatic *N*-oxides are characterised by the $[M-O]^+$ and $[M-OH]^+$ fragments; the latter is the more important if a hydrogen can readily be abstracted from an α-substituent.[9,10] In the present series the molecular ion is the base peak except for the 2-isomers. The near absence of $[M-OH]^+$ in the spectrum of *2*, which has $[M-O]^+$ as base peak, shows that hydrogen abstraction is unfavourable when the hydrogen is situated on an electronegative atom such as oxygen. The $[M-OH]^+$ fragment is important, however, for the 2-methoxy derivative (*4*), presumably because of hydrogen abstraction from the *O*-methyl group. The isomeric 1-methoxypyrid-2-one (*7*) is characterised by the $[M-CH_2O]^{+\cdot}$ fragment. An interesting feature for the 4-isomers is loss of the substituent on the oxygen atom (Scheme 1). The driving force may be preferable charge stabilisation on the nitrogen atom with formation of a quinonoid structure.



3 R = H
6 R = Me

Scheme 1.

EXPERIMENTAL

The mass spectra were recorded on an AEI MS-902 mass spectrometer attached to an AEI DS30 data system. The electron energy was 70 eV and the ionising current 100 μA. During recording of the ionisation efficiency curves the repeller was at cage potential and the ionising

current 20 μA. Xenon was the reference compound. The IE-curves were interpreted by the semi-log plot method. The recorded IP values are the average of three determinations, the deviation being ± 0.05 eV except for compound *8* as discussed above. The compounds *1*, *7* and *4*, *5*, *9* were introduced by the indirect insertion system at 100 and 150 °C, respectively, and the remaining compounds by the direct insertion probe. The source temperature was 220 °C.

The compounds used were prepared according to the literature;[8,11,12] the reactive betaine (*8*) was isolated from its $MeHSO_4$-salt by freeze-drying of the eluate from an Amberlite IRA-400 (OH^-) column.

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The Crystal Structure of 7-Hydroxy-6-methyl-7,6-borazarothieno[3,2-*c*]pyridine, $C_6H_7N_2BSO$

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The crystal and molecular structure of 7-hydroxy-6-methyl-7,6-borazarothieno[3,2-*c*]-pyridine has been studied by the aid of three-dimensional X-ray integrated film data. The symmetry is monoclinic, space group $P2_1/c$. The unit cell contains 4 molecules $C_6H_7N_2BSO$ and the cell dimensions are $a=7.732(4)$, $b=10.059(4)$, $c=11.946(6)$ Å, $\beta=124.56(6)°$. The structure has been refined to an R-value of 0.080 (1135 independent reflections). The fused rings, C_5H_2BS, are approximately planar. Neglecting the substituents, the largest deviation from the best plane is 0.03 Å. The average of the various distances within the pyridine-like skeleton C_3N_2B is close to 1.40 Å.

Gronowitz and coworkers [1] have investigated several organoboron compounds from physicochemical and synthetic points of view. The aim of the present study was to investigate the planarity of the borazaropyridine ring which is isoelectronic with the pyridine ring, and also to determine its bond lengths. The substance chosen for this investigation was the compound 7-hydroxy-6-methyl-7,6-borazarothieno[3,2-*c*]pyridine. A study of the crystal structure of a related monocyclic compound, *e.g.* 5-ethyl-3-hydroxy-3,2-borazaropyridine,[2] would have been more appropriate for our purposes, but we have hitherto not succeeded in protecting these single crystals from deteriorating during the X-ray work.

7-Hydroxy-6-methyl-7,6-borazarothieno[3,2-*c*]pyridine, $C_6H_7N_2BSO$, was first synthesized by Gronowitz and Namtvedt.[3] Single crystals suitable for this study were kindly supplied by Dr. A. Maltesson. X-Ray powder diffraction photographs were recorded in a Guinier-Hägg focusing camera with Cu$K\alpha_1$ radiation and potassium chloride ($a=6.2909$ Å) added as an internal standard. The following lattice parameters were obtained with the aid of least-squares calculations: $a=7.732(4)$, $b=10.059(4)$, $c=11.946(6)$ Å, $\beta=124.56(6)°$, $V=768$ Å^3. The density observed by flotation methods was 1.40 g cm^{-3}. Assuming 4 formula units $C_6H_7N_2BSO$ per unit cell, the calculated density is 1.43 g cm^{-3}. The needle-shaped single crystals used for the X-ray work were enclosed in a Lindemann capillary over a saturated solution of the substance in water. The reason is that the substance decomposes in air of normal relative humidity. The capillary was mounted on an integrating Weissenberg camera, in such a way that the needle axis [001] was parallel to the rotation axis. The layers $hk0$ to $hk10$ were registered with Cu$K\alpha$ radiation. The multiple film technique was used, and the intensities of the reflections were measured with the aid of a Nonius Mark II micro densitometer. The following conditions limiting possible reflections were found: hkl, no conditions; $h0l$, $l=2n$; $0k0$, $k=2n$. These extinctions are characteristic of the space group $P2_1/c$ (No. 14). The intensities were corrected for Lorentz and polarization effects, but not for the absorption effects ($\mu=31.3$ cm^{-1}).

The crystal structure was solved by means of symbolic addition methods, using the program GAASA.[4] The program worked automatically, and peaks corresponding to the 11 non-hydrogen atoms in the asymmetric part of the unit cell could be recognized in the first E-map. The positions thus obtained were refined by means of least-squares calculations

using anisotropic temperature factors for all atoms. Difference Fourier maps revealed the positions of all hydrogen atoms except the one attached to the oxygen atom. A final refinement including all atoms but one hydrogen atom gave a conventional R value of 0.080 for all observed 1135 reflections. The R value for the 1121 reflections actually used in the refinement was 0.068. The reflections given non-zero weight in the refinement had $|F_o|$ values according to $0.5 \leq |F_o|/|F_c| \leq 2.0$. Cruickshank's weighting scheme with $A=1.5$, $C=0.17$ and $D=0$ was used. The S value (goodness of fit) was 0.17, and thus deviated heavily from 1, which is not unusual using film data. The final weighting scheme gave for ten $|F_o|$ intervals values of $\overline{w\Delta^2}$ between 0.6 and 1.6. The final positional and thermal parameters of the atoms are given in Table 1. Lists of observed and calculated $|F|$ values can be obtained on request from the Division of Inorganic Chemistry 2, Lund.

Table 1. Final positional and thermal parameters for 7-hydroxy-6-methyl-7,6-borazarothieno[3,2-c] pyridine. Standard deviations are given in parentheses. The anisotropic thermal parameters are based on the expression: exp $[-(h^2\beta_{11}+k^2\beta_{22}+l^2\beta_{33}+2hk\beta_{12}+2hl\beta_{13}+2kl\beta_{23})]$.

Atom	x	y	z	B(Å^2)
S(1)	0.66581(14)	0.05643(10)	0.17299(9)	For β_{ij},
C(2)	0.5365(6)	−0.0695(4)	0.1926(4)	see below
C(3)	0.5707(6)	−0.0684(4)	0.3173(5)	
C(4)	0.7715(6)	0.0737(4)	0.5335(4)	
N(5)	0.8941(4)	0.1720(3)	0.6025(3)	
N(6)	0.9701(4)	0.2480(3)	0.5442(3)	
B(7)	0.9218(6)	0.2304(4)	0.4102(4)	
C(8)	0.7695(5)	0.1162(3)	0.3330(3)	
C(9)	0.7018(5)	0.0394(3)	0.3972(3)	
C(10)	1.1121(7)	0.3527(4)	0.6355(4)	
O	1.0158(4)	0.3131(3)	0.3707(3)	
H(21)	0.448(7)	−0.134(4)	0.117(4)	2.3(8)
H(31)	0.516(8)	−0.126(5)	0.346(5)	3.3(10)
H(41)	0.729(6)	0.022(4)	0.582(4)	1.6(7)
H(101)	1.046(13)	0.425(8)	0.662(9)	8.7(22)
H(102)	1.177(8)	0.400(5)	0.594(5)	3.2(10)
H(103)	1.217(8)	0.312(6)	0.732(5)	3.9(11)

Atom	β_{11}	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
S(1)	0.02703(28)	0.01074(11)	0.00885(13)	−0.00142(12)	0.00712(14)	−0.00154(7)
C(2)	0.0260(10)	0.0119(5)	0.0118(5)	−0.0020(5)	0.0071(5)	−0.0021(3)
C(3)	0.0238(9)	0.0099(4)	0.0144(6)	−0.0020(5)	0.0096(6)	−0.0009(3)
C(4)	0.0274(9)	0.0099(4)	0.0115(4)	0.0002(4)	0.0112(5)	0.0006(3)
N(5)	0.0274(8)	0.0094(3)	0.0098(3)	0.0004(4)	0.0097(4)	0.0002(2)
N(6)	0.0247(7)	0.0079(3)	0.0099(3)	0.0000(3)	0.0086(4)	−0.0006(2)
B(7)	0.0228(9)	0.0081(4)	0.0095(4)	0.0020(4)	0.0077(5)	0.0002(3)
C(8)	0.0203(7)	0.0078(3)	0.0092(4)	0.0017(4)	0.0067(4)	0.0006(2)
C(9)	0.0218(8)	0.0089(3)	0.0092(4)	0.0003(4)	0.0081(4)	−0.0003(2)
C(10)	0.0344(11)	0.0091(4)	0.0113(5)	−0.0024(5)	0.0099(6)	−0.0020(3)
O	0.0379(9)	0.0103(3)	0.0123(3)	−0.0047(4)	0.0138(5)	−0.0011(2)

DISCUSSION OF THE STRUCTURE

Intramolecular distances. One molecule of 7-hydroxy-6-methyl-7,6-borazarothieno[3,2-c]-pyridine is projected on its best plane in Figs. 1a – 1c, where distances, angles, and deviations from the best plane are given. *E.s.d.*'s for distances between non-hydrogen atoms are 0.004 Å for carbon-sulfur interactions and

0.005 – 0.006 Å for other distances. *E.s.d.*'s for carbon-ring hydrogen bond lengths are 0.04 – 0.05 Å, and for carbon-methyl hydrogen distances 0.05 – 0.09 Å. *E.s.d.*'s for angles between non-hydrogen atoms are 0.2 – 0.3° and for angles involving only one hydrogen atom 3 – 5°.

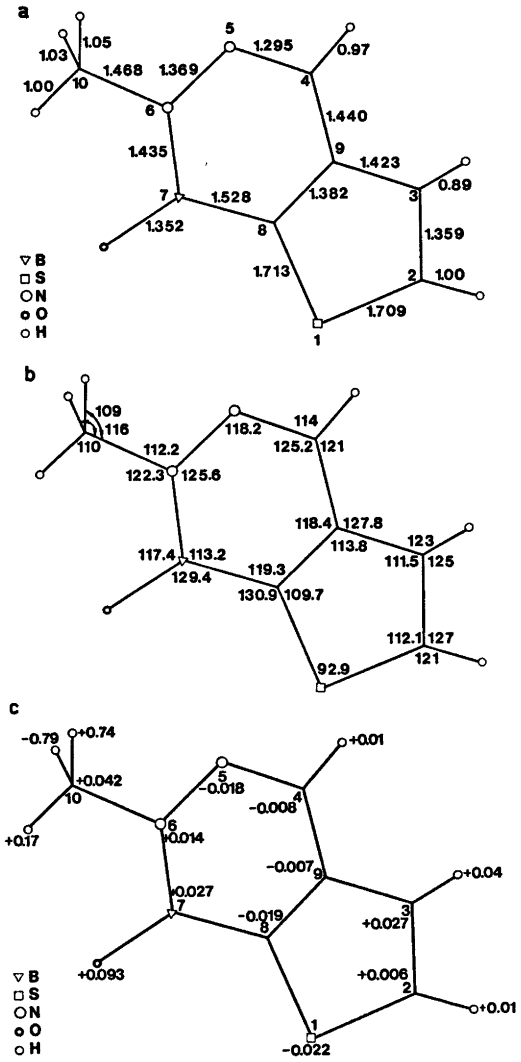

Fig. 1. Projection of one molecule of 7-hydroxy-6-methyl-7,6-borazarothieno[3,2-*c*]pyridine on its best plane. Fig. 1a shows the numbering of the atoms together with the intermolecular distances, Fig. 1b gives pertinent angles within the molecule, and Fig. 1c gives the deviations (Å) of the various atoms from a best plane calculated using only the nine ring atoms.

The various distances within the pyridine-like skeleton C_3N_2B differ significantly, but as expected their average is close to 1.40 Å. The C(4) – N(5) distance of 1.295(5) Å corresponds to a partial double bond and according to the curve given by Donohue, Lavine and Rollett [5] the π bond order is 0.67. The N(5) – N(6) distance of 1.369(4) Å is shorter than a single bond N – N and its length corresponds to π bond order of 0.45 according to calculations made by Sabesan and Ventkatesan.[6] The B(7) – N(6) distance of 1.435(5) Å is about the same as the B – N distance 1.424(1) Å found in hexachloroborazine in a recent refinement.[7] The B(7) – C(8) distance of 1.528(5) Å may be compared to the B – C distances in derivatives of the pyridine-like compound borin, C_5H_5B. Thus Huttner, Krieg and Gartzke,[8] in their paper on π complexes between cobolt(0) and two substituted borins, found an average B – C (ring) distance of 1.519(5) Å. The B – OH distance in the present compound is 1.352(5) Å. It is appreciably shorter than the value 1.395(7) Å given for a boron-ether oxygen distance in Ref. 8 but agrees fairly well with the value of 1.366 Å given for a formal single bond between three-coordinated boron and oxygen in a number of borates.[9] One noteworthy thing about the angles in the pyridine-like skeleton C_3BN_2 is that those around boron deviate appreciably from 120°. The distances and angles within the thiophene ring are normal. The fused rings C_5N_2BS are approximately planar. Neglecting the substituents, the best plane was calculated for the nine atoms of the ring skeleton. The largest deviations of the ring atoms from the plane occurred for the B(7) and C(3) atoms and amounted to +0.03 Å in both cases. The substituents O and C(10) were at distances of +0.09 and +0.04 Å, respectively, from the best plane (*cf.* Fig. 1c). The dihedral angle between the thiophene ring and the borazaropyridine ring is 178(6)° and does thus not deviate significantly from zero. The respective least-squares planes were calculated using only ring atoms. It may also be noted that one of the methyl hydrogen atoms is situated nearly in the ring plane, whereas the other two hydrogen atoms of the same group are situated at equal distances above and below the best plane.

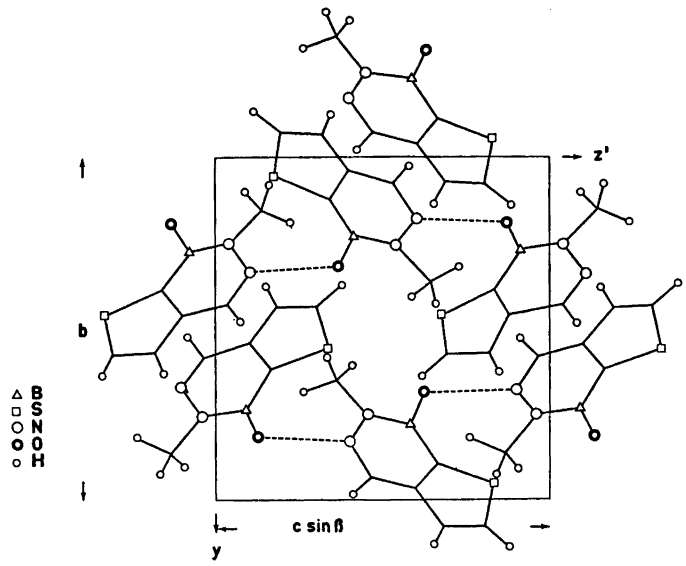

Fig. 2. Projection of the structure on a plane perpendicular to the *a*-axis.

Intermolecular distances. Fig. 2 shows the projection of the crystal structure on a plane perpendicular to the *a* axis. The molecules are joined by O–H···N hydrogen bonds to two chains, identical by centrosymmetry. The corresponding O–N distances are 2.789(4) Å.

A final difference map showed a peak corresponding to 0.24 of the height of a hydrogen atom at the expected position of the H(OH) atom. However, the map also revealed peaks and pits, which measured in the same scale amounted to 0.3 and 0.6 units, respectively.

Except for the hydrogen bond just mentioned no other intermolecular distances shorter than the corresponding sums of the van der Waals radii occur.

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A New Preparative Method for *trans* Alkenols*

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Four 4-alken-1-ols (*3b–f*) have been prepared in a new stereoselective mode, which involves treatment of 5-(tetrahydro-2-pyranyloxy)-3-penten-1-ols and the corresponding 5-alkoxy derivatives (*2b–f*) with lithium aluminium hydride in refluxing dioxane. The method is an extension of a reported analogous reaction of 4-alkoxy-2-buten-1-ols, which gives rise to 3-alken-1-ols. 3-Penten-1-ol is prepared here using this latter method. Disubstituted olefinic alcohols are formed in a minimum *trans:cis* ratio of 97:3. The reaction is proposed to have a transition state with strong carbanion character.

According to our recent publication,[1] 3-alken-1-ols can be prepared in good yields using readily available acetylenes as key intermediates (*cf.* Scheme 1, $n=0$). Furthermore, this reaction is stereoselective as shown by the formation of 3-hepten-1-ol in a *trans:cis* ratio of 97:3 and ~100 % pure 2-methyl-*trans*-4-octen-2-ol. In the present paper we have included the preparation of *trans*-3-penten-1-ol, to further verify the high stereoselectivity in the formation of 3-alken-1-ols. Trisubstituted olefins, however, are formed with poor stereoselectivity, the ratio of *trans:cis* beeing 3:2 in the typical case.

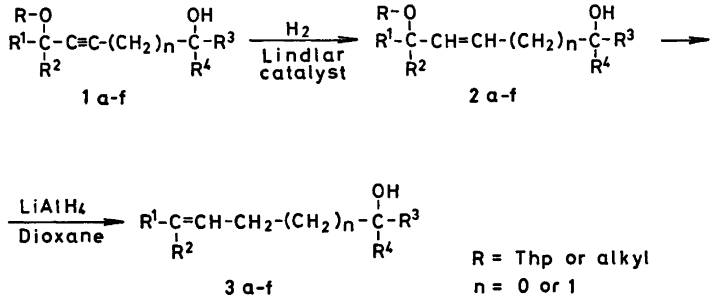
We here wish to report on an extension of this new synthetic method to the stereoselective preparation of *trans*-4-alken-1-ols starting with the acetylenic derivatives *1b–f* (*cf.* Scheme 1, $n=1$, and Table 1).

RESULTS

The starting acetylenes *1a–f* were obtained using standard procedures. Their preparation has been described elsewhere.[8,23]

The preparations of *trans*-3-penten-1-ol (*3a*) and the 4-alkenols *3b–f* from the acetylenes *1a–f* were carried out as described for 3-alkenols in our previous work [1] (*cf.* Scheme 1 and Table 1). Partial hydrogenation of *1a–f* in hexane gave the corresponding alkoxy- or Thp-oxy-alkenols (*2a–f*) which without prior

* Allenes and Acetylenes VII. Part VI: Ref. 8.



Scheme 1

Table 1. Starting acetylenes and products in the LAH reductions of *1a–f* according to Scheme 1.

Acetylenic substrate	Product	Reaction time (h)	Yield[a] GLC (%)	Isolated yield (%)	*trans*	*cis*
Thp–O | $CH_3-CH-C\equiv C-CH_2OH$ *1a*[23]	$CH_3-CH=CH-CH_2-CH_2OH$ *3a*[3]	2.5	95	–	97	3
Thp–O | $CH_3-CH-C\equiv C-CH_2-CH_2OH$ *1b*[23]	$CH_3-CH=CH-(CH_2)_3OH$ *3b*[2]	6	93	71	~100	<0.2
Thp–O | $C_3H_7-CH-C\equiv C-CH_2-CH_2OH$ *1c*[3]	$C_3H_7-CH=CH-(CH_2)_3OH$ *3c*[2]	38	65	–	>98	<2
t-But–O | $C_3H_7-CH-C\equiv C-CH_2-OH_2OH$ *1d*[3]	$C_3H_7-CH=CH-(CH_2)_3OH$ *3c*[2]	28	60	48	>98	<2
CH_3-O OH | | $C_2H_5-CH-C\equiv C-CH_2-C-CH_3$ | CH_3 *1e*[3]	OH | $C_2H_5-CH=CH-CH_2-CH_2-C-CH_3$ | CH_3 *3e*	13	95	–	100[b]	0[b]
CH_3-O OH | | $C_2H_5-C-C\equiv C-CH_2-CH-CH_3$ | CH_3 *1f*[3]	OH | $C_2H_5-C=CH-CH_2-CH_2-CH-CH_3$ | CH_3 *3f*	15	85	62	~60	~40

[a] From rel. areas of peaks. Not corrected for detector response. [b] *Cf.* text.

purification were allowed to react with lithium aluminium hydride (LAH) in refluxing dioxane.

The *cis:trans* ratios of the so obtained alkenols *3a–e* were determined on GLC using 3 or 6 m long columns containing 20 % Carbowax 20 M or SE-30. In all cases, except that of *3e*, the corresponding *cis* alkenols were prepared independently to allow a correct assignment of the *cis:trans* ratios. *cis*-4-Hexen-1-ol,[2] *cis*-3-penten-1-ol,[3] and *cis*-4-octen-1-ol were obtained through partial hydrogenation of the corresponding 4-alkyn-1-ols.[2-4]

The *cis* and *trans*-4-hexen-1-ols separated on a 6 m Carbowax 20 M column with a ratio of retention times *cis:trans* = 1.09 at 25 min. Other authors have reported clean separation on PEG-1500.[5] In contrast, the isomers of 4-octen-1-ol could not be completely separated on a 6 m Carbowax column and there was no separation on a 3 m SE-30 column. The ratio of retention times for the *cis* and *trans* isomers was 1.06 at 25 min on Carbowax. However, the separation attained allowed an estimation accurate enough to establish the maximum content of the *cis* isomer indicated in the Table 1. No difference in the *cis:trans* ratios of 4-octen-1-ol from different starting materials (*1c* and *1d*) could be detected.

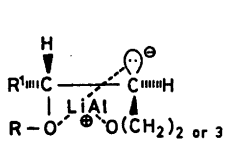
The tertiary alcohol *3e* was homogeneous on all the above columns even at retention times of about 1 h.

3-Penten-1-ol (*3a*) contained 3 % of the *cis* isomer, which is the same percentage, that we have reported [1] for 3-hepten-1-ol prepared in the same way. (R_t *cis*: R_t *trans* = 1.14 at 22 min on a 3 m Carbowax column).

We have made use of the different chemical shifts in NMR of *cis* and *trans* methyl groups in trisubstituted olefins [6] in determining the isomer composition of some 4-methyl-3-alken-1-ols.[1] From the 100 MHz NMR spectrum of *3f*, it could be estimated that the *trans:cis* ratio was 3:2, in spite of some interference from methylene protons at δ 1.50.

DISCUSSION

The above formations of 3- and 4-alkenols formally constitute a type of allylic rearrangement (S_N2' reaction). However, mechanistically the question arises as to what degree the attack by hydride on the double bond and the expulsion of alkoxide are concerted.



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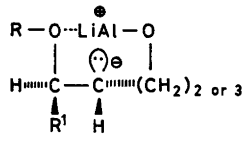

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There are essentially two main reaction mechanisms. (*i*) The first is a normal concerted reaction with a more or less synchronous attack by hydride and allylic rearrangement in the transition state. (*ii*) The reaction may also, in the extreme case, proceed in two steps *via* carbanions, that might be pictured as *4* or *5*, both of them affording *trans* olefins upon decomposition.

When considering the most likely mechanism one should have in mind that the double bond of allylic alcohols having a carbanion stabilizing group at C-3 is easily reduced by LAH with formation of an organometallic bond at this position. The cinnamyl alcohol system is particularly well investigated.[7]

Therefore, in the present case it seems reasonable to assume that attack by hydride on the double bond is well ahead of the expulsion of the alkoxide group, leading to a transition state having strong carbanion character. The reaction might possibly proceed *via* organometallic intermediates, which then is especially conceivable, when the poorly leaving *t*-butoxy group is substituted.

We have not been able to detect a hydrolysis product derived from carbanions such as *4* and *5*, which is not surprising in view of their expected instability. In an analogous reaction we have allowed acetylenic derivatives of type *1b–f* to react with LAH in tetrahydrofurane.[8] In contrast, this reaction proceeds *via* clearly detectable vinylic organometallic intermediates to β-allenic alcohols.

For a hypothetical organometallic intermediate there should be two possible modes of elimination, which are depicted as *4* and *5* and which are suggestive of the *anti* and *syn* modes of β-elimination [9-12] respectively, terms which, however, refer to the relative positions of groups, when they are eliminated from a stable molecule.

Drawings, such as *4* and *5*, also give a clear indication why the reaction is stereoselective, though they cannot explain the reason why the formation of 4-alken-1-ols is more stereoselective than that of 3-alken-1-ols (*cf.* *3a* and *3b*).

When speculating about the mechanism it is interesting to note the resemblance of the above new *trans* alkenol synthesis to some other more or less stereoselective formations of these products, which also proceed *via* elimination of an alkoxy group from an organometallic intermediate or *via* carbanionic transition states.[2,13–22]

One example of the reaction dealt with in this paper has actually been reported by other authors but was not applied to stereoselective synthesis. LAH reduction of 3,4-diphenyl-4-methoxy-2-cyclopenten-1-one gave 3,4-diphenyl-3-cyclopenten-1-ol in good yield.[22]

As indicated in Table 1 the yields of 4-alken-1-ols in the above reactions of 5-alkoxy-3-penten-1-ols with LAH are variable and they are also poorly reproducible (±15 % yield). However, because of the high stereoselectivity of the reaction it can be concluded that it should in some cases be useful for preparing *trans* 4-alken-1-ols.

EXPERIMENTAL

Infrared spectra were recorded on a Perkin-Elmer 15G spectrophotometer as a film between NaCl discs. NMR spectra were taken on solutions in $CDCl_3$ with tetramethylsilane as an internal standard, using a Perkin-Elmer R 12 B or a Varian HA 100 D spectrometer.

All reactions with Grignard reagents and $LiAlH_4$ were performed under nitrogen.

4-Alken-1-ols (3b–f) and 3-penten-1-ol (3a). General procedure. The acetylenes (*1a–f*) (0.02 mol) were hydrogenated in hexane over Lindlar catalyst (Fluka) at atmospheric pressure until the calculated volumes of hydrogen were adsorbed(1 – 3h) to give the corresponding olefinic alcohols (*2a–f*). The catalyst was filtered off and the solvent evaporated. The residue was dissolved in 15 ml of dioxane and slowly added dropwise to a stirred suspension of $LiAlH_4$ (0.03 mol) in dioxane. *Note:* It is important to allow complete alcoholate formation between $LiAlH_4$ and the alcohol before heating is started as otherwise a violent reaction may occur. After refluxing the reaction mixture was cautiously poured on ice and ether. The alcohol was taken up in ether, which was washed once with a saturated ammonium chloride solution and dried over potassium carbonate. Microdistillation or preparative GLC yielded the title compounds in states of purity exceeding 96 % on GLC.

2-Methyl-trans-5-octen-2-ol (3e) was isolated by preparative GLC (Carbowax 20 M). IR: 965 cm^{-1} (HC=CH, *trans*). NMR: δ 5.60 – 5.40 (m, 2 H), 2.30 – 1.75 (m, 5 H), 1.50 (t, 2 H), 1.20 (s, 6 H), 0.97 (t, 3 H). (Found: C 75.7; H 12.6. Calc. for $C_9H_{18}O$: C 76.02; H 12.76).

6-Methyl-5-octen-2-ol (3f) was isolated by microdistillation at an oil-bath temperature of 95 °C and a pressure of 11 mmHg. NMR: δ 5.35 – 5.05 (m, 1 H), 3.80 (sextet, 1 H), 2.80 (s, 1 H), 2.30 – 1.75 (m, 4 H), 1.65 (m, 3 H), 1.50 (q, 2 H), 1.20 (d, 3 H) and 0.96 (t, 3 H). The multiplet at δ 1.65 could be resolved at 100 MHz into a downfield multiplet and an upfield singlet, the latter interfering with the methylene quartet. Rel. areas ~2:3. (Found: C 75.9; H 12.7. Calc. for $C_9H_{18}O$: C 76.02; H 12.76).

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X-Ray Crystallographic Studies on Cycloheptadithiophene Compounds and Similar Systems. II. The Crystal Structure of Bis(4-dithieno[3,2;2′,3′-*f*]borepinyl) Ether, $(C_{10}H_6BS_2)_2O$

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The crystal and molecular structure of bis(4-dithieno[3,2;2′,3′-*f*]borepinyl) ether has been studied by the aid of three-dimensional X-ray diffraction data. The symmetry is monoclinic, space group $C2/c$. The unit cell contains 4 molecules $(C_{10}H_6BS_2)_2O$ and the cell dimensions are: $a = 23.101(9)$ $b = 6.819(2)$, $c = 11.991(4)$ Å and $\beta = 97.79(3)°$. The structure has been refined to an R-value of 0.059 for 1896 reflections. Each $C_{10}H_6BS_2$ part is approximately planar. The largest deviation from the best plane occurs for the boron atom and amounts to 0.09 Å. A twofold axis passes through the ether oxygen atom and the whole molecule has a propeller form. The two non-equivalent B – C distances have equal lengths, 1.533(3) Å.

The present investigation is part of a more general study on the aromaticity of a number of cycloheptadithiophene compounds and dithienoborepines by Gronowitz and coworkers.[1,2] The crystal structure of dithieno[2,1-*b*;4,5-*b*′]-tropylium perchlorate[3] has previously been discussed by Gronowitz *et al.*,[4] who pointed out that the tropylium cations in the compound are essentially planar, the largest deviations from the planes being about 0.05 Å. Besides the approximate planarity of the molecule, the crystal structure determination[3] gave as a result that the sulfur to tropylium carbon atom distances were slightly longer than the purely thiophenic sulfur-carbon distances.

X-RAY WORK

The compound bis(4-dithieno[3,2;2′,3′-*f*]-borepinyl) ether, $(C_{10}H_6BS_2)_2O$, was first synthesized by Gronowitz, Gassne and Yom-Tov.[1] Later on Jeffries[5] described another route to the synthesis of the compound. Single crystals for the present work were kindly supplied by Dr Jeffries. X-Ray powder diffraction photographs were recorded in a Guinier-Hägg focusing camera with Cu$K\alpha_1$ radiation and potassium chloride (a = 6.2909 Å) added as an internal standard. The following lattice parameters were obtained with the aid of least-squares calculations: a = 23.101(9) Å, b = 6.819(2) Å, c = 11.991(4) Å, β = 97.79(3)°, V = 1871 Å^3. The density 1.47 g cm^{-3} was obtained by flotation methods. Assuming 4 formula units $(C_{10}H_6BS_2)_2O$ per unit cell, the calculated density is 1.48 g cm^{-3}. The single crystal used for the structure determination had the form of a nearly rhombic prism with the basal lengths 0.21 mm and the height 0.074 mm. The acute angle of the rhomb was 75°. The monoclinic b axis extended along the short basal diagonal and the c axis approximately along the long one. The a axis was perpendicular to the basal plane. Intensity data (graphite-monochromatized Cu$K\alpha_1$ radiation) were collected for 1896 reflections with an Enraf-Nonius single crystal diffractometer using the $\omega - 2\theta$ scan technique with a scan interval $\Delta\omega = (0.90 + 0.50 \tan\theta)$. The background was measured by extending the scan interval by ¼ at each end. Out of the 1896 reflections 127 are not above the background by giving net counts less than 10 in a fast prescan of approximately 13 s. On data reduction another 320 reflections, although considered

Table 1. Positional and thermal parameters of the atoms of $(C_{10}H_6BS_2)_2O$. Estimated standard deviations are given in parentheses. The anisotropic thermal parameters are based on the expression: $\exp[-(h^2\beta_{11}+k^2\beta_{22}+l^2\beta_{33}+2hk\beta_{12}+2hl\beta_{13}+2kl\beta_{23})]$.

	x	y	z	β_{11}	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
S(1)	0.18309(3)	−0.09547(11)	0.46017(6)	0.00243(2)	0.02534(17)	0.00780(5)	0.00237(4)	−0.00081(2)	0.00196(7)
C(2)	0.11630(14)	−0.2021(4)	0.4491(2)	0.00339(7)	0.0217(6)	0.0083(2)	0.0008(2)	0.00083(10)	0.0027(3)
C(3)	0.07545(11)	−0.0953(3)	0.3851(2)	0.00205(5)	0.0211(5)	0.0080(2)	−0.0004(1)	0.00094(8)	0.0003(3)
B(4)	0.05760(9)	0.2241(3)	0.2685(2)	0.00109(3)	0.0174(5)	0.0059(2)	0.0002(1)	0.00043(6)	−0.0020(2)
C(5)	0.04330(9)	0.5311(3)	0.1384(2)	0.00165(4)	0.0240(5)	0.0064(1)	0.0013(1)	0.00052(6)	0.0013(2)
C(6)	0.07122(12)	0.6832(4)	0.0985(2)	0.00236(5)	0.0278(6)	0.0077(2)	0.0022(2)	0.00135(8)	0.0047(3)
S(7)	0.14310(3)	0.68680(10)	0.15009(6)	0.00211(1)	0.02303(16)	0.00936(6)	−0.0050(4)	0.00176(2)	0.00368(7)
C(8)	0.18938(9)	0.4068(4)	0.2923(2)	0.00127(4)	0.0282(6)	0.0083(2)	−0.0013(1)	0.00044(7)	−0.0003(3)
C(9)	0.19737(8)	0.2463(4)	0.3558(2)	0.00103(3)	0.0303(6)	0.0076(2)	0.0004(1)	−0.00000(6)	−0.0009(3)
C(2′)	0.15639(9)	0.0990(3)	0.3768(2)	0.00148(4)	0.0209(5)	0.0056(1)	0.0011(1)	−0.00002(6)	−0.0005(2)
C(3′)	0.09703(8)	0.0807(3)	0.3421(2)	0.00139(4)	0.0174(4)	0.0056(1)	0.0003(1)	0.00066(6)	−0.0009(2)
C(5′)	0.07987(8)	0.4066(3)	0.2130(2)	0.00131(3)	0.0182(4)	0.0052(1)	0.0006(1)	0.00044(5)	−0.0007(2)
C(6′)	0.13698(8)	0.4759(3)	0.2273(2)	0.00155(4)	0.0196(5)	0.0061(1)	−0.0004(1)	0.00083(6)	0.0000(2)
O	0(0)	0.1770(3)	¼(0)	0.00110(4)	0.0210(5)	0.0132(3)	0(0)	0.00020(8)	0(0)

	x	y	z	B(Å^2)
H(2)	0.1115(14)	−0.332(5)	0.477(3)	3.6(7)
H(3)	0.0349(12)	−0.130(3)	0.371(2)	1.8(5)
H(5)	0.0018(11)	0.505(4)	0.123(2)	1.4(4)
H(6)	0.0556(16)	0.786(5)	0.050(3)	4.6(8)
H(8)	0.2235(11)	0.492(4)	0.292(2)	2.1(5)
H(9)	0.2367(11)	0.221(4)	0.390(2)	2.0(5)

above the background during the prescan, were found to be weaker than $3\sigma(I)$, where I is the intensity and $\sigma(I)$ its standard deviation. The intensities were corrected for Lorentz, polarization and absorption effects. The linear absorption coefficient used for the crystal was 44.6 cm^{-1}. Systematically absent reflections were hkl with $h+k=2n+1$ and $h0l$ with $l=2n+1$, indicating either the space group Cc (No. 9) or $C2/c$ (No. 15). As a reasonable structure was obtained assuming space group $C2/c$, the space group Cc was not further considered.

The positions of the sulfur atoms were derived from the three-dimensional Patterson function and the positions of all other atoms from successive difference Fourier maps. The positions of all atoms were refined using a full-matrix least-squares computer program. At this refinement all non-zero 1769 reflections were used, but reflections outside the limits $0.5 \leq |F_o|/|F_c| \leq 2.0$ were given zero weight. The number of zero-weighted reflections was 97, and all but one had initial I values less than $3\sigma(I)$. The weight factor used was $w_i = (\sigma^2(F_o) + 0.002 \cdot F_o^2 + 0.067)^{-1}$. Anisotropic temperature factors were used for the non-hydrogen and isotropic ones for the hydrogen atoms. The final R_1 values, $\sum|\Delta F|/\sum|F_o|$, were 0.0592 for all 1896 reflections and 0.0469 for 1672 reflections. The weighted factor $R_2 = (\sum w_i(\Delta F)^2/\sum w_i(F_o)^2)^{1/2}$ was 0.0592 for the 1672 reflections. The value of S (goodness of fit) was 1.00. The final weighting scheme gave the following $\overline{w_i\Delta^2}$ values for the ten $|F_o|$ intervals: 0.34 for the $|F_o|$ interval 0.0 – 1.8 and 0.91 to 1.17 for the rest of the intervals. The isotropic formal equivalents of the anisotropic temperature factors for the non-hydrogen atoms varied between 2.88 and 5.07 Å^2. Final positional and thermal parameters of the atoms are given in Table 1. Lists of observed and calculated structure factors are available on request from the Division of Inorganic Chemistry 2, Lund.

DISCUSSION OF THE STRUCTURE

Intramolecular distances and angles. Pertinent distances and angles for the present compound are given in Fig. 1. *E.s.d.*'s for distances between non-hydrogen atoms vary between 0.002 and 0.004 Å, and corresponding values for angles between 0.1 and 0.2°. *E.s.d.*'s for

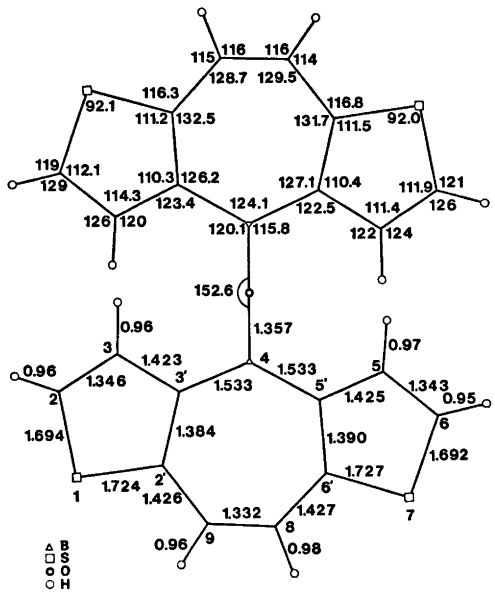

Fig. 1. Pertinent distances and angles in one $(C_{10}H_6BS_2)_2O$ molecule. Each $C_{10}H_6BS_2$ part is projected on its best plane. The carbon atoms are numbered but not marked.

carbon-hydrogen distances vary between 0.03 and 0.04 Å, and are about 2° for the S – C – H and C – C – H angles.

A twofold axis passes through the ether oxygen atom, and the whole molecule has a propeller form (Fig. 2). The two dithienoborepinyl parts form an acute angle of 63.2(7)°. As seen from Fig. 1, each $C_{10}H_6BS_2$ part of the molecule has an approximative symmetry plane passing through the boron atom B(4) and the midpoint of the line C(8) – C(9). The borepinyl ring is, however, not quite symmetrical; the corresponding angles within the ring differ at levels $(3.0 \pm 0.3)\sigma$. The difference between the external angles O – B(4) – C(3′) and O – B(4) – C(5′) is strongly significant, $\Delta/\sigma(\Delta)$ being 16. The two thiophene groups of the asymmetric part of the unit cell do not differ significantly with respect to the distances and angles. Each of the sulfur-borepinyl carbon distances [S(1) – C(2′); S(7) – C(6′)] is significantly longer than the purely thiophenic sulfur-carbon distances [S(1) – C(2); S(7) – C(6)], $\Delta/\sigma(\Delta)$ being 8 and 10 in the two cases. The same tendency was found for the sulfur-carbon distances in dithienotropylium perchlorate,[3]

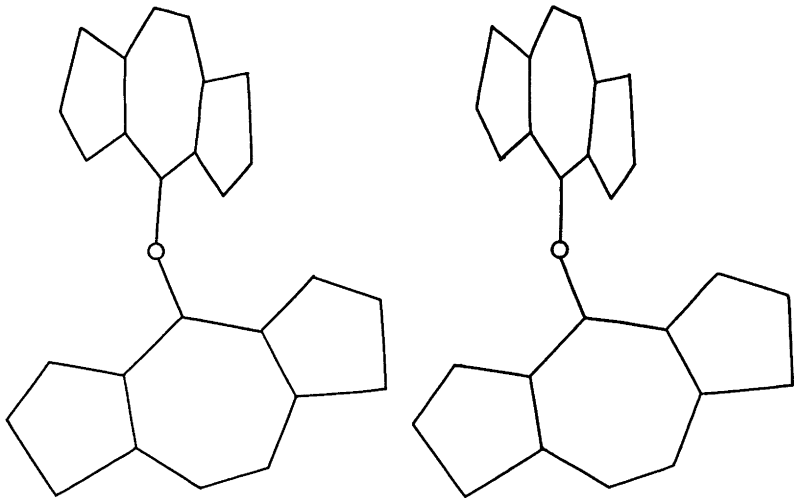

Fig. 2. A stereoscopic view of one $(C_{10}H_6BS_2)_2O$ molecule. Only non-hydrogen atoms are shown. The crystallographic y axis points towards the reader. The ether oxygen atom is marked by a small ring.

and the corresponding sulfur-carbon distances compare quite well in the two compounds. Similar disparities of C–S bonds in thiophene derivatives have been discussed by, *e.g.*, Goldberg and Shmueli,[6] who suggest that the longer sulfur-carbon bond is the one connecting sulfur to the carbon atom more actively engaged in the π delocalization (*e.g.* the borepinyl carbon atoms in the present compound). The fused double bonds C(2′)–C(3′) and C(5′)–C(6′) (Fig. 1) in the thiophene groups of the present borepinyl compound are significantly longer than the thiophenic ones, $\Delta/\sigma(\Delta)$ being 8 and 9, respectively.

The same situation was indicated in the crystal structure of dithienotropylium perchlorate.[3] The differences in the C–S and C–C distances may tend to support the view that the borepinyl ring, and probably also the tropylium ring, have aromatic properties.

The boron atoms are three-coordinated to two carbon atoms and one oxygen atom in a planar arrangement. The B–C distances are 1.533(3) Å. These distances are slightly longer, or perhaps equal to, the bond length 1.519(5) Å obtained for the B–C distances in the borin (C_5H_5B) skeleton.[7] It may also be mentioned that similar short B–C distances, 1.528(5) Å, have also been found in 7-hydroxy-6-methyl-7,6-borazarothieno(3,2-*c*) pyridine.[8] The short B–C distances now obtained seem reasonable since the boron atom takes part in a borepinyl ring. Another consequence of this participation is that the C(3′)–B(4)–C(5′) angle is larger than 120°. The boron-ether oxygen distance, 1.357(2), is shorter than the B–O distance of 1.395(7) Å found by Huttner, Krieg and Gartzke[7] in bis(1-methoxyborinato)cobolt(0). It is also shorter than the mean value of the B–O distances, 1.372 Å, obtained for the crystal structure of B_2O_3 *I*, where boron is three-coordinated,[9] although individual bond lengths in this compound are as short as 1.34(2) Å. Thus the boron and oxygen atoms in the present compound are partially double bonded. However, the B–O distance, 1.357Å, compares well to the B–OH distance of 1.352(5) Å found in the borazaro compound,[8] discussed above. As mentioned previously, the boron atom has a planar coordination in the present structure. The angles around boron deviate significantly from 120°. The same situation is encountered in other compounds with three-coordinated boron, *e.g.* $Na_2B_4O_7$.[10] The B–O–B angle of 152.6(3)° (Fig. 1) is essentially larger than is usually found for boron-oxygen compounds. According to Krogh-Moe,[11] values of 123–138° are normal for intergroup B–O–B

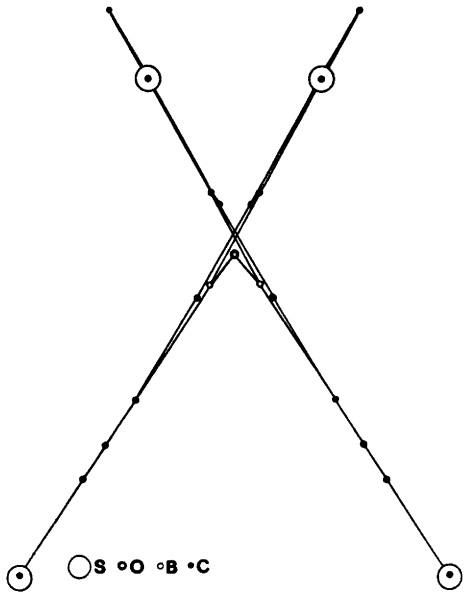

Fig. 3. Projection of one molecule of $(C_{10}H_6BS_2)_2O$ on a plane perpendicular to the line of intersection between the best planes of the $C_{10}H_6BS_2$ parts. Only non-hydrogen atoms are shown. Carbon atoms overlap sulfur atoms in four cases in the projection.

angles in complex borates.

Planarity of the different rings. The best plane through the $C_{10}H_6BS_2$ part was calculated using only the positions of the 13 non-hydrogen atoms, giving them all unit weight. The largest deviation from the plane occurred for the boron atom, amounting to 0.09 Å, whereas the deviations of the sulfur atoms were 0.04–0.05 Å. As mentioned in Ref. 3, the same calculations were made for the dithienotropylium cation, where deviations of 0.04 – 0.05 Å were found for the sulfur atoms, while all other atoms were closer to the best plane. Similar least-squares calculations were performed for the borepinyl ring only, using the positions of the 7 non-hydrogen atoms, and giving them unit weight. The largest departure from planarity, 0.038 Å, was found for the boron atom and the smallest one, 0.015 Å, for C(8). Calculations of the same kind for the tropylium ring[3] showed that no atom was more distant from the plane than 0.015 Å. Considering the *e.s.d.*'s of the atomic positions, it is concluded that neither the dithienoborepinyl parts, $C_{10}H_6BS_2$, nor the borepinyl parts, C_6B, of the present compound $(C_{10}H_6BS_2)_2O$, are planar from a statistical point of view. It also seems probable that the 7- and 13-membered rings of the borepinyl compound are less planar than the corresponding rings in the dithienotropylium cation.[3]

A projection of one molecule $(C_{10}H_6BS_2)_2O$ on a plane perpendicular to the common line to the best planes of the $C_{10}H_6BS_2$ parts is given in Fig. 3, which shows that the dithienoborepinyl parts have boat forms. However, the best planes were calculated for the two thiophene rings separately and the angle between the normals to these planes was found to be 4(2)°. Thus the boat form has no statistical significance.

Intermolecular distances. No intermolecular distances occurring in the crystal structure are shorter than the respective sums of the van der Waals radii. The shortest hydrogen-hydrogen interaction is 2.6 Å.

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Studies on the Heterogeneity of Commercial Pronase-P Based on a Preparative Separation Method Using Sephadex G-75 Superfine

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A highly improved separation method for Pronase-P was developed by gel chromatography on a specially selected more uniform fraction of the commercial superfine Sephadex G-75 beads under suitable packing and running conditions. A 10 × 90 cm bed capable of separating 1300 mg of Pronase was packed for preparative purposes and found to have better resolving efficiency than smaller columns. The enzymatically active material in Pronase was by this chromatography method separated into seven fractions with molecular weights ranging from 15 000 to 50 000 dalton. Thus Pronase components are far more heterogeneous in molecular size than had earlier been appreciated. Complete resolution was obtained between components of an estimated difference in molecular weight as small as 2000 – 3000 dalton. The possibility of intrinsic proteolysis causing specific digestion of some of the Pronase components was also demonstrated using the gel chromatography method. The results confirmed the extensive heterogeneity of Pronase earlier found using polyacrylamide gel electrophoresis, thus indicating the presence of at least 14 different enzymatically active components.

Pronase was first isolated by Nomoto and Narahashi [1] from the culture broth of *Streptomyces griseus* strain K1, and characterized as a homogeneous proteolytic enzyme with a broad substrate specificity. [2,3] Further characterization [4,5] revealed the presence of several proteolytically active components in Pronase. The most extensive analysis of the heterogeneity of Pronase to date was performed by Löfqvist and Sjöberg [6] using polyacrylamide gel electrophoresis. By this technique 14 different bands were resolved, of which 13 showed enzymatic activity using casein or LNA* as substrates. On the other hand Jurášek *et al.* [7] were able to separate only 6 different components using CM-Sephadex at pH 5 and a Ca^{2+} free medium. Since the electrophoretic separation method [6] permitted separation only on an analytical scale and further characterization of the heterogeneity of Pronase and the Pronase components required larger amounts of material, a preparative separation method had to be developed.

Already Nomoto *et al.*, [3] however, observed that the presence of Ca^{2+} ions was crucial to stabilize Pronase against autodigestion. In proceeding investigations [8] it has also been shown that different buffer-anions as well as pH have a marked influence on the stability of the Pronase components. Because of these restrictions in composition of the medium the number of suitable preparative separation techniques available was limited. In preliminary investigations the use of preparative polyacrylamide gel electrophoresis [9] was examined, but the resolution obtained was inferior to the separation of Pronase on analytical gels under similar conditions.

Since the resolution of gel chromatography is independent of the composition of the elution buffer, this technique was also considered suitable. The separation medium could thus be selected due to the stability demands of the Pronase components. Earlier reports [10,11] indi-

* *Non-standard abbreviations.* *N*-Benzoyl-L-arginine methyl ester.HCl, BAME; L-leucine-β-naphthylamide.HCl, LNA; Pronase-P lot No. 511324, Pronase I: Pronase-P lot No. 592045, Pronase IV.

cated that the molecular weights of four of the Pronase components were between 15 000 and 27 000 dalton. A preparative separation, using Sephadex G-75, therefore seemed possible. The present report describes the optimization of such a method based on a specially prepared Sephadex G-75 Superfine, and the application of this method to the examination of the heterogeneity and stability of Pronase-P.

MATERIALS AND METHODS

Pronase-P was purchased from Kaken Chemical Company, Tokyo, (lot No. 511324 and 592045, abbreviated Pronase I and IV). In the present investigation Pronase IV was used, while Pronase I was used only as a reference to the work of Löfqvist and Sjöberg.[6]

Sephadex G-75 (particle diameter in the dry state 40 – 120 μ), and Sephadex G-50, G-75, and G-100 Superfine (particle diameter in the dry state 10 – 40 μ) were obtained from Pharmacia Fine Chemicals. Several lots of G-75 Superfine, differing in resolving capacity, were used.

For the analysis of enzymatic activity were used L-leucine-β-naphthylamide.HCl, *N*-benzoyl-L-arginine methyl ester.HCl, ammonium sulfonate, N(1-naphthyl)-ethylenediamine dihydrochloride purchased from Sigma Chem. Co., and casein of Hammarsten grade from Merck AG. Acrylamide, *N,N'*-methylene-bisacrylamide and *N,N,N',N'*-tetramethylene diamine were obtained from Eastman Organic Chem. The staining of analytical gels was performed with Coomassie Blue (Sigma Chem. Co.).

Highly purified, crystallized samples of bovine serum albumin, soybean trypsin inhibitor, and lysozyme from Sigma Chem. Co., ovalbumin, β-lactoglobulin, and chymotrypsinogen from Calbiochem AG, and whale myoglobin from Schwartz were used for the estimation of the molecular weights of the Pronase components. All other reagents were of analytical grade.

Column chromatography. The columns used were, if nothing else is indicated, of the hydrodynamic precision type K 25/45 (bed size 2.5 × 40 cm), K 25/100 (bed size 2.5 × 90 cm), and K 100/100 (bed size 10 × 90 cm). (Pharmacia Fine Chemicals). UV-monitor and fraction collector for the eluate was from LKB-Products.

Gel swelling. The Sephadex beads were swelled for 24 h in standard buffer (30 mM boric acid – NaOH, pH 7.5; 30 mM $CaCl_2$; and 3 mM NaN_3). The same buffer was used for the separations, which were performed at 25 ± 0.5 °C.

Gel sieving. To obtain more defined beads the swelled gels were sieved on nylon nets allowing particles of maximum 40 μ to pass. During this procedure the small beads were washed through the net by buffer. The distribution in particle size in the obtained fractions was examined in a light microscope. As illustrated in Fig. 1, the sieving procedure gave a sharp cut in particle size between the beads, which stayed on the net, and those, which were washed through. In one case the beads that passed the 40 μ-net were also sieved on a 28 μ-net. The diameter of the beads, which were removed through the 40 μ- and 28 μ-net corresponded to 20 and 14 μ, respectively, in dry state.

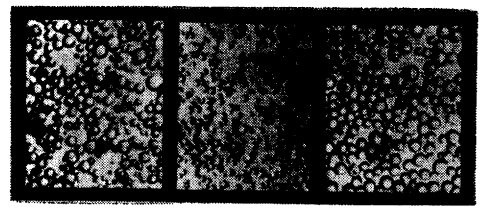

Fig. 1. The distribution in particle size of: (a) the beads of commercial Sephadex G-75 Superfine: (b) the beads removed by wet sieving on a 40 μ nylon net: (c) the residual beads after sieving.

Gel packing and sample application. In order to pack the Sephadex Superfine beads into a separating bed with good flow characteristics a special packing procedure had to be developed. A cylindrical perspex extention tube with the same inner diameter as the column was mounted on top of the column. The column was filled with buffer to a height of 5 cm above the bottom net (28 μ). The swelled gel was diluted to 1.5 – 1.6 bed volumes and filled into the center of the column along a glass rod. The lower end of the glass rod was kept below the liquid surface in the column. A 40 cm hydrostatic pressure was maintained during the packing procedure. Two or three bed volumes (V_t) of buffer were passed through the column before its flow properties were tested. The column K 100/100 had to be packed by a special procedure which will be reported separately as well as further details concerning optimal conditions for gel packing.

A solution (2 mg/ml) of Blue Dextran 2000 (Pharmacia Fine Chemicals) was used for control of the flow characteristics of the packed bed, and for determination of the void volume (V_0).

Pronase-P was dissolved in standard buffer at a concentration of 80 mg/ml. Samples were applied to columns within 2 min in order to reduce the intrinsic proteolysis of the Pronase components. The sample load was usually kept at 16 mg per cm^2 gel area. For all columns, except for K 100/100, the sample was applied by means of a syringe beneath the buffer surface on the top of the column.

Elution. The operating pressure was kept at

about 40 cm and regulated by a Mariotte flask to get a constant flow rate. The obtained flow rates for most beds were from 2 to 3 ml/cm² h. The smallest beads (diameter 14 – 20 μ in the dry state), and the K 100/100 column produced flow rates of 1.6 – 2.0 ml/cm² h. The collected fractions were stored at +4 °C.

Enzyme assays. Caseinolytic activity at pH 7.5 and 10 was measured according to the method of Kunitz, [12] activity towards BAME was determined using the method of Schwert *et al.* [13] and leucine aminopeptidase activity was measured using LNA by the method of Goldbarg and Ruthenburg, [14] all modified according to Löfqvist and Sjöberg.[6]

Analytical polyacrylamide gel electrophoresis was performed [6] in order to explore the heterogeneity of the gel chromatography peaks. Samples of either 20 or 50 μl of the column eluates were applied to the gels without prior concentration. The buffer used was 0.38 M Boric acid – 0.01 M CaO, pH 6.8.

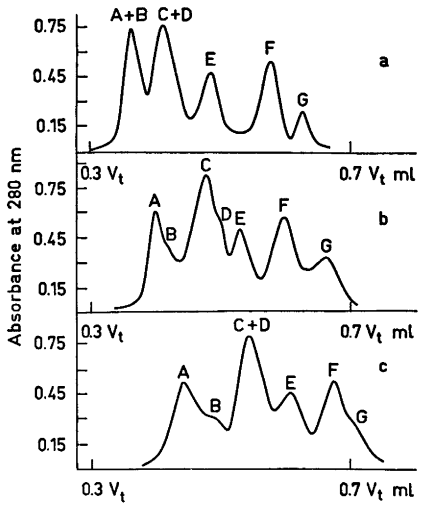

Fig. 3. The high molecular weight components of Pronase IV separated on a 2.5 × 40 cm bed of: (a) Sephadex G-50 Superfine: (b) Sephadex G-75 Superfine: (c) Sephadex G-100 Superfine. The flow rates were 2 – 3 ml/cm² h and the amount of sample applied was 16 mg/cm².

RESULTS

Conditions for preparative chromatography on Sephadex G-75 Superfine

The molecular weights determined for the Pronase components characterized so far [10,11] indicated that the pore size of Sephadex G-75 ought to be suitable for separation of Pronase. A 1.5 × 60 cm bed of Sephadex G-75 (particle diameter 40 – 120 μ in the dry state) was packed in a column of simple construction and examined for its resolving capacity on Pronase. A result similar to that of Narahashi *et al.*[15] was obtained (Fig. 2a). Two broad regions of A_{280}-absorbing material were eluted. The first region was poorly resolved into three fractions, which showed protease and peptidase activity using casein and LNA as substrates. The other region emerged close to V_t and contained components with a molecular weight below 5 000 dalton and without enzymatic activity. In order to examine whether a better resolution of the Pronase components could be obtained the column was repacked with Sephadex G-75 Superfine, particle size 10 – 40 μ in the dry state. (Usually these small beads are difficult to pack into separating beds with good flow characteristics and are therefore seldom used in column chromatography.) When Pronase was chromatographed on this bed (Fig. 2b) a better resolution than with G-75 was obtained of both the high and low molecular weight material. Five peaks could be distinguished in the high molecular weight region, and three peaks were indicated in the low molecular weight region. In order to further improve the separation results the packing procedure described in Materials and Methods was introduced along with the use of hydrodynamically more optimal columns (Pharmacia K 25/45). At the same time the separation on Sephadex G-50, G-75, and G-100 (all Superfine) were compared (Fig. 3) to establish the optimal pore size for

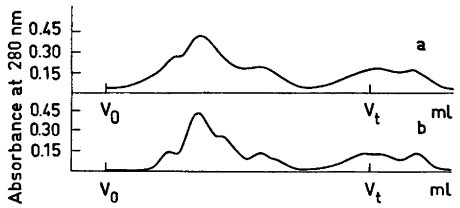

Fig. 2. The effect of bead size on the resolution of Pronase IV on a 1.5 × 60 cm gel chromatography bed of: (a) Sephadex G-75; (b) Sephadex G-75 Superfine. The rates of elution were 2.5 ml/cm² h and the amount of sample applied was 12 mg/cm².

the resolution of the Pronase components. Using Sephadex G-75 Superfine a remarkable improvement of the separation was noted on this bed compared to the results obtained on the earlier 1.5 × 60 cm bed (Fig. 2b). Thus seven different high molecular weight peaks (labelled A – G) were discerned, and five of these peaks were well separated from the other material. Using Sephadex G-50 Superfine a better resolution of F and G was achieved than with G-75 Superfine (Fig. 3a), while there was a poorer separation in the A – D region. As expected, the reverse was true, when Sephadex G-100 Superfine was used (Fig. 3c). With this material a better resolution of peaks A and B was obtained, while F and G separated poorly. Thus Sephadex G-75 Superfine was the most suitable for studies of the heterogeneity of Pronase, when an optimal separation of all the Pronase components was desired.

In order to improve the resolution of the Pronase components in peaks A – G a 2.5 × 90 cm bed was packed. For that purpose a new lot of Sephadex had to be used. The packing performance as well as the resolution of this lot of Sephadex G-75 Superfine was, however, not as good as expected. Therefore a number of different lots were tested. A considerable variation in the resolution power between the lots was found. The best lot was selected and packed in a K 25/100 column. The elution profile for Pronase fractions from this column is shown in Fig. 4a. The resolution was, however, still not complete between any of the peaks, and each peak eluted in a rather large volume. It was assumed that the variations in performance between different batches of Sephadex were due to inhomogeneities in particle size. Therefore, in an attempt to further improve the separation properties of the gel, the swelled Sephadex G-75 Superfine particles were sieved on a nylon net. About 10 % of the gel particles were in wet state smaller than 40 μ and passed through the net and were removed. The separation of Pronase-P on a bed (2.5 × 90 cm) packed with the sieved and more homogeneous gel material (Fig. 4b) resulted in an improved resolution compared to the unsieved gel (Fig. 4a). No new peaks were resolved but the elution volume for each peak was markedly reduced, clearly indicated by comparison of the elution volume of peak E in the two elution diagrams,

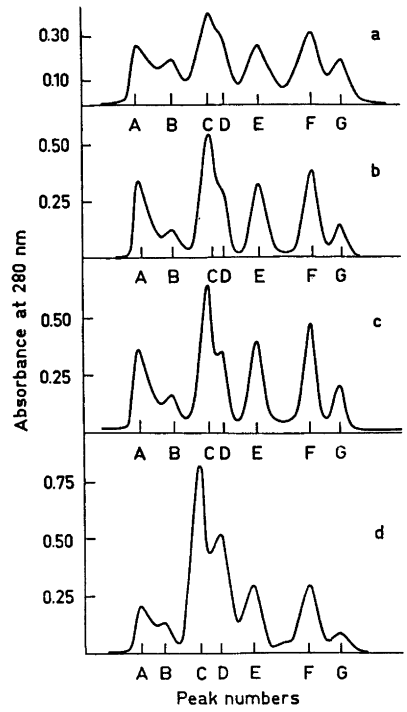

Fig. 4. The elution diagrams of the high molecular weight components in Pronase IV separated: (a) on a 2.5 × 90 cm bed of Sephadex G-75 Superfine (particle size 10 – 40 μ in the dry state): (b) on a 2.5 × 90 cm bed of sieved Sephadex G-75 Superfine (particle size 20 – 40 μ in the dry state): (c) on a 10 × 90 cm bed of sieved Sephadex G-75 Superfine (particle size 20 – 40 μ in the dry state). In (d) is shown the elution diagram of Pronase I on the same bed as used in (b). The flow rates were 1.6 – 3 ml/cm² h. The amount of sample applied was 16 mg/cm².

and a more complete separation of the peaks was thus obtained.

The same sieving procedure was also applied to batches with poor original resolving power in order to examine if their quality could be improved. This was, however, only possible to a limited extent, and it can for these batches be concluded that other factors than inhomogeneities in particle size are responsible for the poor separation characteristics. In accordance with the theory of gel chromatography [11] the smallest beads, which in wet state passed the 40 μ sieving net, should give a further improved resolution of the Pronase components. To facilitate the packing of this material it was

sieved further, and the smallest particles, which passed a 28 μ net, were removed. In spite of this fractionation it was not possible to pack the residual beads (particle diameter 14 – 20 μ in the dry state) into a separating bed with acceptable flow characteristics. Nevertheless a remarkably small zone spreading could be observed on the best packed bed, indicating that a further resolution could be achieved, provided a bed with good flow characteristics could be packed.

The applied amount of Pronase on the 2.5 × 90 cm bed (Fig. 4b) was 80 mg. With increased amounts of protein applied the resolution between the peaks declined considerably. Only a few mg of each component could therefore be obtained in each run. Consequently it was desirable to scale up the separation. A K 100/100 column was therefore packed, which with the same load per area would allow 16 times as much material to be separated. The separation obtained (Fig. 4c), where thus 1300 mg Pronase was chromatographed, was even better than that obtained when the K 25/100 column was used.

Investigation of Pronase heterogeneity

Qualitative and quantitative differences in the composition of different Pronase preparations. In all the separations presented so far Pronase IV was used. The elution diagram of Pronase I, the preparation used in earlier investigations,[6] is presented in Fig. 4d. A comparison of the K_{av} values for the peaks in Fig 4b and 4d (Table 2) indicated only small differences in the qualitative composition of the two Pronase preparations. Certain divergences were, however noted. Thus the K_{av} for peak A and D was substantially lower for Pronase IV than for Pronase I. The separation between peak D and E was also incomplete in the elution diagram of Pronase I. These differences were significant and reproducible, and indicate that the two analyzed Pronase preparations might contain different components.

The quantitative differences between the two Pronase preparations were more pronounced judged from the amount of A_{280}-absorbing material found in different peaks (Table 1). Thus peak A is proportionally larger in Pronase IV, while in Pronase I peaks C and D are more predominant. The amount of enzymatically inactive low-molecular A_{280}-absorbing material is about 30 % in both the Pronase batches tested.

Molecular weights of the Pronase components. The bed used for chromatography in Fig. 4b and 4d was calibrated with a number of proteins of known molecular weights. When the logarithm of their molecular weights were plotted against the determined V_e/V_0 (Fig. 5) a straight line was obtained. The V_e/V_0 values for the peaks of Pronase IV are also indicated in Fig. 5. and show that the approximate molecular weights of the Pronase components vary between 15 000 and 50 000 dalton. Thus the Pronase components exhibit a much larger variation in molecular size than was suggested by the ultracentrifugation studies of Narahashi *et al.*,[15] where a single peak corresponding to a molecular weight of 20 000 dalton was obtained. The

Table 1. K_{av} and amount of A_{280}-absorbing material of the gel chromatography peaks A – G.

Peak	K_{av} of the gel chromatography) peaks		Amount of A_{280}-absorbing material, %		% Staining [c]
	Pronase IV[a]	Pronase I[b]	Pronase IV[a]	Pronase I[b]	
A	0.165	0.196	18	8.5	9
B	0.241	0.248	7	5	7
C	0.322	0.326	30.5	30	27
D	0.352	0.367		23	21
E	0.437	0.439	18	14	15
F	0.557	0.560	20	15	13
G	0.621	0.624	6.5	4.5	8

[a] Elution diagram 4b. [b] Elution diagram 4d. [c] Staining in the corresponding electrophoretic bands as measured by Löfqvist and Sjöberg in Pronase I.[6]

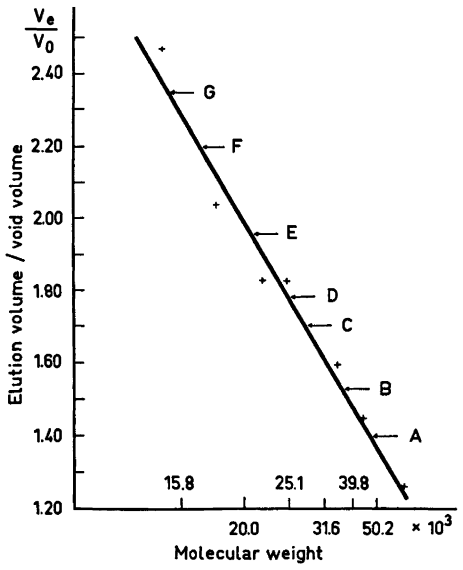

Fig. 5. The elution volume/void volume (V_e/V_0) of a series of standard proteins (see Materials and Methods) *versus* their molecular weights. V_e/V_0 values for the chromatographically separated peaks A – G are indicated by arrows.

molecular weight of peak G (15 000 dalton) corresponds well to the value (17 750 dalton) calculated by Bauer and Löfqvist [16] from the amino acid composition. On the other hand peak A with an apparent molecular weight of 50 000 dalton shows a molecular weight twice as high as previously reported for any Pronase component.[10,11]

Electrophoretic analysis of the different gel chromatography peaks. In order to identify the Pronase components present in the different peaks in terms of the electrophoretic bands identified by Löfqvist and Sjöberg [6] analytical polyacrylamide gel electrophoreses were run on each of the seven peaks in the elution diagrams presented in Fig. 4b and 4d. The content of electrophoretic bands in the two Pronase preparations are given in Table 2. All the major bands of Pronase I,[6] except band 11, which could not be examined in the buffer system used, were identified in the chromatography peaks. This further establishes that the heterogeneity of Pronase is actually as extensive as earlier shown [6] and not caused by intrinsic proteolysis during the separation. Most of the major electrophoretic bands of Pronase I were also identified in the corresponding peaks of Pronase IV (Table 2). A few interesting differences were, however, observed. Thus Pronase IV contained two components (one electrophoretic band 9 in peak A and one electrophoretic band 10 in peak CD), which were earlier not found in Pronase I.[6] On the other hand the major bands 2 and 5 occurring in peak F and the tailing edge of peak D of Pronase I were hardly detected or lacking in Pronase IV.

In addition to the major components minor bands appeared in most of the peaks of the elution diagrams. They only occupied a small fraction (less than 10 %) of the material in each peak and have either been regarded as

Table 2. The heterogeneity of the chromatography peaks A – G in terms of components found by analytical polyacrylamide gel electrophoresis.

Peak	Major electrophoretic bands[a] present in Both Pronase I and IV	Pronase IV only	Pronase I only	Minor electrophoretic bands present in Pronase I or IV
A	8	9	13 – 14[b]	–
B	6, 7	–	13 – 14[b]	8
C	12, 13 – 14[b]	–	–	4, 10, 9
D	13 – 14b, 10, 9	10[c]	5	4, 7, 12
E	4	–	–	7
F	1	–	2	3, 2
G	3	–	–	1

[a] The numbering of the bands refer to the earlier results by Löfqvist and Sjöberg.[6] [b] Band 13 and 14 were not separated in the system used for the electrophoretic analysis. [c] Band 10 appeared as a double band in Pronase IV.

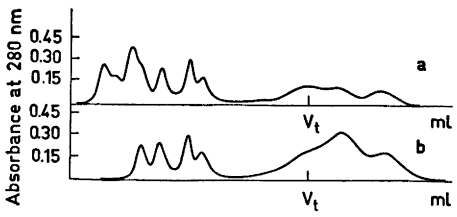

Fig. 6. The elution diagrams of Pronase IV in 30 mM ammonium acetate buffer, pH 4.8 (a) in the presence of 30 mM $CaCl_2$, (b) without $CaCl_2$ on a 2.5 × 90 cm bed of Sephadex G-75 Superfine. The flow rates were 5 ml/cm² h, and the sample loads were 16 mg/cm².

contaminants from a neighbouring peak or as impurities of less importance at this stage of investigation.

Stability of the Pronase components. The potential risk for intrinsic proteolysis of the Pronase components during the separation was considered as a critical factor in this investigation of the heterogeneity of Pronase. Since the resolving power of a Sephadex bed, however, is not influenced by the composition of the eluting medium used, the stability of the separated components in various media could be studied by comparing the elution profiles obtained in different buffers. This advantage of gel chromatography was used to investigate the stability of the Pronase components under the conditions used by Jurášek *et al.*[7] These investigators were able to identify 6 Pronase components after dialysis of Pronase against Ca^{2+} -free acetate buffer pH 5 and separation on a CM-Sephadex column. They thus concluded that "Pronase appears to be a less complex mixture of proteolytic enzymes than had previously been appreciated". When we separated Pronase on a 2.5 × 90 cm Sephadex G-75 Superfine bed equilibrated with a Ca^{2+}-free acetate buffer at pH 4.8 (Fig. 6b), peaks A, B and C were found to be completely digested into low molecular weight fragments. The protective effect of 30 mM Ca^{2+} under in other respects similar conditions is shown in Fig. 6a. The stability of peaks A, B, and C is remarkably increased but still not as good as that at pH 7.5 as judged by the amount of enzymatically inactive low molecular weight components produced. At pH 4.8 these occupy 40 % instead of the at pH 7.5 normally found 30 % of the A_{280}-absorbing material. This sign of increased intrinsic proteolysis was, however, not correlated with the disappearance of any particular peak in the elution diagram.

The stability of Pronase IV was further examined by analysis of the recovery of enzymatic activity after gel chromatography at pH 7.5 in the presence of Ca^{2+}. The residual enzymatic activities for a parallel sample of unfractionated Pronase IV stored beneath the Sephadex column (80 mg/ml) (Table 3) were also measured. A substantial loss in activity towards casein when analyzed at pH 7.5 and a small loss in activity towards BAME occurred both in the chromatographed Pronase sample and in the sample stored unfractionated. The activities towards casein at pH 10 and LNA were largely unaltered.

Table 3. Residual activity after separation of Pronase IV on Sephadex G-75 Superfine or preincubation at different concentrations in standard buffer.

Pronase IV dissolved in standard buffer at a concentration of	% Residual activity Casein at pH 7.5	Casein at pH 10	LNA	BAME
1.5 mg/ml	100	100	100	100
80 mg/ml, chromatographed and pooled before analysis	65	96	98	86
80 mg/ml, stored 36 h before analysis	61	90	97	87
80 mg/ml, diluted to 1.6 mg/ml after 2 min and analyzed	86	101	99	100

From Table 3 it can also be seen that the loss in activity is highly dependent on the concentration of Pronase in the solution. After 2 min of incubation a 15 % loss in enzymatic activity towards casein analyzed at pH 7.5 was observed at a Pronase concentration of 80 mg/ml. It thus appears that the presence of calcium ions was particularly important for the stability of the components that exhibited only neutral protease activity.

DISCUSSION

The resolution obtained by gel chromatography is highly dependent on the amount of sample applied. This fact is not always considered in practical separation work. Thus overloading especially in the case of complex samples often leads to a poor resolution. In the work of Narahashi *et al.* [15] and Awad *et al.*[10] 4 – 10 times as much Pronase was applied to Sephadex G-75 or G-100 columns as was used in the present report. Consequently this might be one reason, why these investigators were only able to partially separate two fractions and get weak indications of one additional component.

There are, however, several other parameters that have to be regarded in order to achieve good separation results on gel chromatography. The theory of gel chromatography gives the relation of these parameters to each other as well as their contribution to the separation efficiency.[17] The applicability of the theory in practical separational work is, however, limited by restrictions like packing performance of the gel, uniformity of the beads and obtainable flow properties of a packed bed, and these factors have to be examined and optimized in experimental work.

The effect of packing procedure but also to some extent column design on the separation result is illustrated by comparison of Figs. 2b and 3b. The superfine beads were found to be more difficult to pack and thus the in theory predicted advantage of using small beads was limited by the poorer packability of these beads. By modification of the packing procedure it was, however, possible to obtain a separating bed giving account for the larger resolving capacity expected from superfine beads. Further improvement of the separation results was also achieved, when a more uniform preparation of superfine beads (particle diameter in the dry state 20 – 40 μ instead of the normal 10 – 40 μ) was used. This was most likely due to improved packing performance of the gel with the more uniform particle distribution. The differences in resolving efficiency from one batch of Sephadex G-75 Superfine to another also indicated the presence of other hitherto not described variations in the uniformity of the Sephadex beads. Investigations in progress show that the separation efficiency can be further improved by partial elimination of such variations. The improved gel chromatography method on Sephadex G-75 Superfine columns, as described in the present paper showed, however, that the Pronase components were far more heterogeneous in molecular size than had earlier been appreciated. Thus Pronase was separated into seven peaks with molecular weights ranging from 15 000 to 50 000 dalton and with complete resolution between components of an estimated difference in molecular weight as small as 2000 – 3000 dalton.

Through analysis of each of the gel chromatography peaks by polyacrylamide gel electrophoresis the qualitative nature of the Pronase heterogeneity was investigated. From the result thereby obtained and the enzymatic activities observed for the different electrophoretic bands [6] it can be concluded that chromatography peaks A – D contain neutral proteases as well as leucine aminopeptidases. In the absence of Ca^{2+} ions at pH 4.8 these components are found to be highly susceptible to autodigestion and peaks A, B, and C are completely digested already during the course of separation. These results explain the findings of Jurášek *et al.* [7] that "Pronase appears to be a less complex mixture of proteolytic enzymes than had previously been appreciated", and that it does not contain any neutral proteases. The conditions used by Jurášek *et al.* [7] simply caused a total digestion of several of the Pronase components, and these investigators were only able to detect the most stable ones. A certain loss in neutral protease activity also occurred under the conditions used for the separations presented in this paper. This was probably caused by intrinsic proteolysis. The nature of the resulting products, however, has not been investigated, and the possibility that any of

the components originally present in Pronase are completely digested also under the conditions applied to this investigation cannot be excluded. However, this seems unlikely and is not supported by any results obtained by analysis of Pronase in various media on either gel chromatography or gel electrophoresis,[6] and we thus believe that the found heterogeneity gives a complete picture of the heterogeneity present in the investigated Pronase preparations.

Since the most pronounced heterogeneity is found in the region (A–D), where the neutral proteases are eluted and since both qualitative and quantitative variations (Tables 1 and 2) in the heterogeneity between different Pronase preparations also are found in this region, it can be questioned to which extent the found heterogeneity represents enzymes originally produced by the microorganism and to which extent the heterogeneity is caused by intrinsic proteolysis of the originally produced enzymes occurring during the fermentation and isolation processes. Further attention to the question of the originality of the identified Pronase components will be given in a separate paper.[18] At this stage, however, the fate of two of the Pronase components in the A–D region will be discussed in some detail.

Firstly peak D of Pronase I was found to contain electrophoretic band 5, not visible in Pronase IV. Peak D in Pronase I also showed a higher K_{av} than that of peak D in Pronase IV, causing a poor resolution between peak D and E. The component equivalent to electrophoretic band 5 thus seems to be eluted as the smallest component in peak D. In the investigation by Löfqvist and Sjöberg [6] band 5 was the only component, which was not active towards any of the substrates tested. Later studies have shown that this component is active towards *N*-carbobenzoxy-L-glycyl-L-leucine and thus corresponds to the carboxypeptidase in Pronase,[15] The molecular weight can be estimated at around 23 000 dalton.

Secondly Pronase IV was found to contain two components with the same electrophoretic mobility. Thus both peak A and D produced a major band in position 9. In Pronase I, however, band 9 was only found in peak D. In the earlier investigation [6] this band also showed activity towards casein at pH 10. The molecular weight of the components in peak D was found to be 25 000 dalton. Band 9 in peak D thus seems to correspond well with the subtilisin-like alkaline protease with a molecular weight of 28 000 dalton isolated by Gertler and Trop [11] and Awad *et al.*[10] The other band 9, which was only found in peak A of Pronase IV, seems to be responsible for the lower K_{av} obtained for that peak in Pronase IV and would thus have the largest molecular weight, 50 000 dalton, of the Pronase components.

The heterogeneity of the remaining peaks E, F, and G is less complex. In the case of Pronase IV only one main component is found in each peak. The K_{av} values as well as the amounts of A_{280}-absorbing material of the two Pronase preparations also agreed well, thus indicating a higher stability of these components. From the activities earlier observed for the electrophoretic bands [6] it can be concluded that peak E contains the trypsin-like enzyme in Pronase, further characterized by Jurášek and Smillie [19] and peak F the elastase-like enzyme III, described by Gertler and Trop. [11] Peak G finally contains an enzyme with broad substrate specificity, showing similarities to both elastase and chymotrypsin.[16,20]

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Zinc Enzymes in Commercial Pronase-P. Further Characterization of the Heterogeneity of the Proteolytic Enzymes of the K-1 Strain of *Streptomyces griseus*

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Pronase has been shown to contain both zinc endopeptidases and zinc aminopeptidases. The zinc atom could be reversibly removed by pentaethylenehexamine in presence of 30 mM Ca^{2+}. A considerable heterogeneity was found within both groups of enzymes by separation on gel electrophoresis and gel chromatography, and it was concluded that limited proteolysis had occurred during the fermentation or isolation procedures of Pronase. Consequently it is uncertain to which extent the zinc enzymes present in Pronase are identical to the ones produced by the actinomycete. A minimum number of genuine enzymes, which could account for the observed heterogeneity, was suggested. This included two zinc endopeptidases and two zinc exopeptidases. In addition to these enzymes it was concluded that the K1 Strain of *Streptomyces* also produced one carboxypeptidase and four different serine endopeptidases. The catalytic mechanism of the carboxypeptidase was not conclusively elucidated. It was, however, proposed to be a metallo enzyme with a most firmly bound prosthetic ion.

Pronase, which was first isolated and characterized as a homogeneous extracellular enzyme produced by *Streptomyces griseus* Strain K1,[1] has been shown to be an extremely complex mixture of proteolytically active components.[2-4] Due to substrate analysis evidence has also been presented indicating that Pronase contains various groups of proteinases, namely neutral and alkaline proteases, aminopeptidases, and carboxypeptidases.[2,4-6] The heterogeneity of the alkaline proteinases is well established and four different enzymes have been identified. One of these is described as subtilisin-like,[7] while two show similarities to the mammalian proteinases from pancreas.[7,8] These three alkaline proteinases are thus found to be serine enzymes. The fourth component with alkaline proteinase activity has so far only been found in one particular batch of Pronase-P [4] and has not been further characterized. Further one serine proteinase, described as trypsin-like,[8,9] is present in Pronase, which hence is found to contain four or maybe five serine proteinases.

The other groups of proteinases in Pronase are less well-known and have so far not been subject to any extensive characterization. Narahashi *et al.*[10] observed that EDTA inhibits the amino- and carboxypeptidase activity as well as 65 % of the neutral proteinase activity. Although Ca^{2+} ions are known to be essential for the stability of Pronase,[11] it is not clear whether the inactivation caused by EDTA is due to destabilization of the tertiary structures of Pronase components by removal of the Ca^{2+} ions [12] or to inhibition of enzymatic activity of metallo enzymes by removal of prosthetic metal ions.

In a preceding paper [13] Löfqvist and Klevhag described the separation of Pronase into seven enzymatically active peaks by chromatography on Sephadex G-75 Superfine, as well as the heterogeneity of each peak on polyacrylamide gel electrophoresis. In this paper these different Pronase peaks are characterized further with respect to their metal ion requirement and enzymatic activity in order to elucidate the nature of the observed heterogeneity of Pronase.

EXPERIMENTAL

Materials. Enzyme: Pronase-P (lot No. 592 045, in the preceding paper[13] abbreviated Pronase IV) was obtained from the Kaken Chemical Company, Tokyo.

Inhibitors: Diisopropylfluorophosphate, pentaethylenehexamine, and 1,10-phenantrolin were products of Fluka AG.

Enzyme substrates: Casein of Hammarsten grade and glutaryl-L-phenylalanine-*p*-nitroanilide were obtained from Merck AG, L-leucine-β-naphthylamide.HCl and *N*-benzoyl-L-arginine methyl ester.HCl from Sigma, *N*-carbobenzoxy-L-glycyl-L-leucine and 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-L-glycyl-L-prolyl-D arginine.$2H_2O$ from Fluka AG. All other low molecular weight substrates were purchased from Cyclo Chemical Co.

Separation methods. Preparative gel chromatography was performed on a 2.5 × 90 cm bed of Sephadex G-75 Superfine prepared according to Löfqvist and Klevhag.[13] The protein fractions were stored at 4 °C.

Preparative polyacrylamide gel electrophoresis: 1.4 mg Pronase or 100 μl of the eluate in peak F from the gel chromatography separation was applied in sucrose stabilized 0.38 M boric acid − 0.01 M CaO buffer, pH 6.8, to preflushed 1 × 10 cm gels and analyzed for enzymatic activity according to Löfqvist and Sjöberg.[4]

Analytical gel chromatography: A 2.5 + 90 cm Sephadex G-75 Superfine bed was used for analytical purposes by applying only 1 mg Pronase. An improved resolution was thereby obtained, but due to the low amount of A_{280} nm absorbing material the eluted Pronase components had to be identified by their enzymatic activity.

Determination of metal content. The metal content in Pronase was determined by atomic absorption spectrometry of suitable amounts dissolved in nitric and perchloric acid. Zinc was also determined directly in the eluted fractions from the gel chromatography column. Corrections for blanc values were made.

Inhibition procedures. Inhibition by both DFP* and penten was, unless otherwise specified, carried out directly on the eluate by incubation for 30 min in presence of at least 100 mol excess of the inhibitor. The zinc enzymes were reactivated by addition of $ZnCl_2$ in 20 % excess amounts compared to the concentration of penten. In case of the carboxypeptidases 5 ml of the fractions from the gel chromatography column were dialyzed towards 1 000 ml of 5×10^{-4} M EDTA or penten in standard buffer for 24 h at 4 °C.

Determination of enzymatic activity. Enzymatic activity towards casein, LNA and BAME was determined as described in the preceding paper.[13] Activity towards GPNA was measured at 40 °C according to the method of Erlanger *et al.* [14] as described by Bauer and Löfqvist.[15] Collagenolytic activity was determined at 40 °C according to the method of Wünsch and Heidrich.[16] Enzymatic activity towards all other substrates was assayed at 40 °C by the ninhydrin method.[17]

RESULTS

In order to examine if other metal ions than Ca^{2+} were present in Pronase an analysis using atomic absorption spectrometry was performed. The results obtained are collected in Table 1. The only metal ions found in substantial amounts except calcium were zinc and magnesium. Other ions like Co, Cu, and Cd were found only in trace amounts and were assumed insignificant for the enzymatic activity of the Pronase components.

Since no requirement for magnesium ions has been reported for proteolytic enzymes, these ions in Pronase were regarded as a residue from the culture medium of the actinomycete and their functional role was thus not further investigated. Zinc, however, has been observed as prosthetic metal ion in several proteinases of microbial origin [18,19] Assuming an average molecular weight of 30 000 dalton [13] for the Pronase components, the zinc content corresponded to one zinc atom for every fourth protein molecule. It was thus of interest to further investigate the role of zinc in Pronase.

Table 1. The amounts of metal ions (in ppm) found in commercial Pronase-P.

Calcium	99 000
Magnesium	720
Zinc	310
Cobalt	8
Nickel	9
Manganese	12
Cadmium	2
Copper	23
Lead	12

* *Non-standard abbreviations.* Pentaethylenehexamine, penten; diisopropylfluorophosphate, DFP; L-leucine-β-naphthylamide.HCl, LNA; *N*-benzoyl-L-arginine methyl ester, BAME; 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-L- glycyl-L-prolyl-D-arginine, collagenase substrate A; glutaryl-L-phenylalanine-*p*-nitroanilide, GPNA; Acetyl, Ac; carbobenzoxy, CBZ; standard buffer, 0.03 M borate buffer, pH 7.5, 0.03 M in $CaCl_2$ and containing 0.02 % NaN_3.

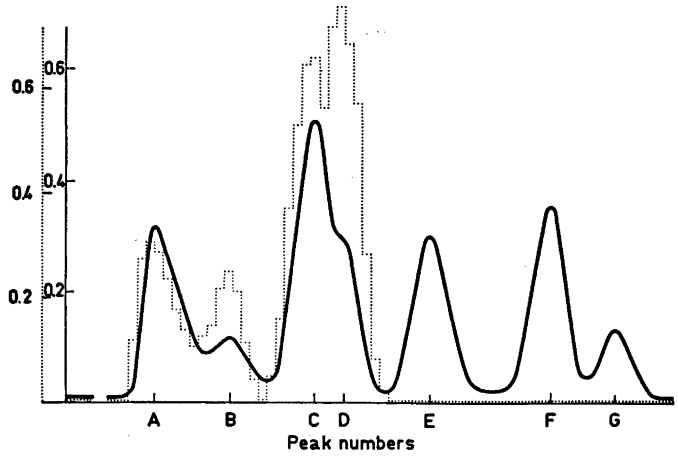

Fig. 1. Elution diagram showing the zinc content found in different peaks of Pronase-P separated by gel chromatography on a 2.5 × 90 cm Sephadex G-75 Superfine bed. —, Absorbance at 280 nm; ···, ppm Zn/ml.

This has to be done in presence of calcium ions, since these were reported to stabilize the Pronase components. Consequently EDTA, which chelates both zinc and calcium ions, could not be used to remove the zinc ions. The addition of 10^2 molar excess of 1,10-phenantrolin, a commonly used chelating agent for zinc, gave only a slight inhibition of the activity of Pronase. Pentaethylenehexamine (penten) on the other hand was found to be more potent as a chelating agent for zinc.

Pronase (1 mg/ml) dissolved in a buffer containing 30 mM Ca^{2+} was treated with 1 mM penten and 1 mM DFP. After incubation for half an hour at room temperature no residual activity towards LNA, BAME or casein at neutral or alkaline pH could be observed. By addition of zinc to a final concentration of 1.2 mM approximately 65 % of the activity towards casein when analyzed at pH 7.5 and the leucineaminopeptidase activity were restored. No activity towards BAME or casein at pH 10 could, however, be detected under these conditions. These inhibition studies indicated that Pronase contained enzymes with two entirely different types of catalytic mechanisms, namely zinc proteinases and serine proteinases.

In order to investigate if zinc ions were bound to various Pronase components, Pronase was separated by gel chromatography on Sephadex G-75 Superfine,[13] and each fraction was analyzed for its zinc content by atomic absorption spectrometry (Fig. 1). It is seen that zinc is bound to Pronase components eluted in the first four peaks (A – D) of the elution diagram. No significant amount of zinc was detected in the remaining peaks E – G. In total, 65 % of the zinc present in the Pronase-P preparation was recovered in peaks A – D. The ratio of zinc content to A_{280} absorbance differed from peak to peak (Fig. 1). In peaks A and C the zinc content was low, around 1 ppm per unit of OD_{280} nm, while in peaks B and D it was around 2.5 times higher. These results thus show that there are zinc proteinases present in Pronase, and that these components are eluted in the first four peaks (A – D) from the gel chromatography column.

Zinc endopeptidases. The various zinc-containing Pronase components present in peaks A – D were further investigated as to substrate specificity. Thus aliquot of the fractions collected from the separation shown in Fig. 1 were treated with DFP in order to inhibit the serine proteinases and analyzed for activity towards casein at pH 7.5 and collagenase substrate A. Enzymatic activity towards both these substrates was found in the two peaks A and C (Fig. 2). A substantial tailing was found for peak A, but no indications for

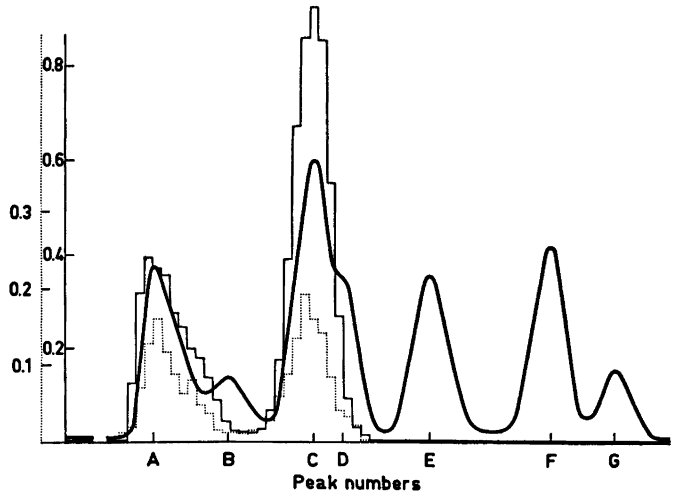

Fig. 2. Zinc endopeptidases found in Pronase-P after separation of 80 mg by gel chromatography on a 2.5 × 90 cm Sephadex G-75 Superfine bed. The enzymatic activities were measured against casein at pH 7.5 and collagenase substrate A on fractions preincubated with 1 mM DFP for 30 min and calculated as optical density per ml eluate and minute, —, Caseinolytic activity (pH 7.5) OD_{280} nm/min ml eluate; ···, collagenase activity OD_{410} nm/min ml eluate.

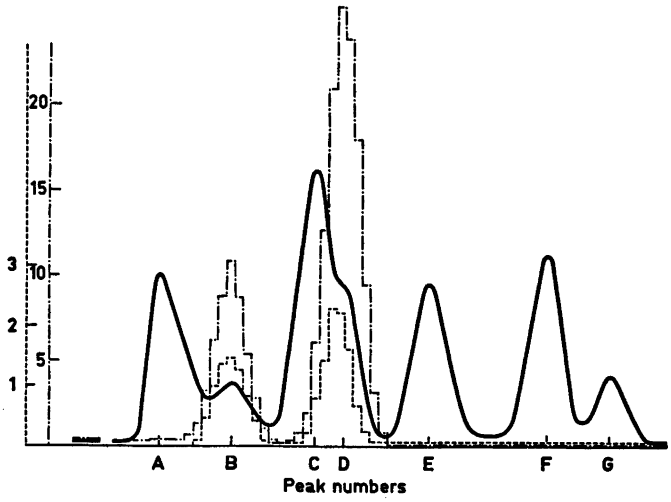

Fig. 3. Zinc aminopeptidases found in Pronase-P after separation of 80 mg by gel chromatography on a 2.5 × 90 cm Sephadex G-75 Superfine bed. The enzymatic activities were measured against LNA and pro-tyr and calculated as optical density per ml eluate and minute. — · —, LNA activity OD_{560} nm/min ml eluate; - - -, pro-tyr activity OD_{570} nm/min ml eluate.

heterogeneity in substrate specificity between different Pronase components within either one of peaks A and C [13] was found. Activity was also observed towards a number of other substrates, CBZ-gly-phe-NH_2, CBZ-gly-tyr-NH_2, CBZ-gly-leu-NH_2, CBZ-trp-leu-NH_2, and Ac-phe-tyr-NH_2, but also these substrates failed to indicate any differences in substrate specificity between the Pronase components in each of peaks A and C.

The DFP-treated fractions of peaks A–D were also made 1 mM with respect to penten

and tested for activity towards casein at pH 7.5 and collagenase substrate A. No residual enzymatic activity was found. The activity could, however, be completely restored by addition of zinc in excess amounts (1.2 mM). It was thus concluded that Pronase components eluted in peak A and C could be classified as zinc endopeptidases with neutral proteinase and collagenase activity.

Peaks A and C were found to contain low amounts of zinc compared to peaks B and D (Fig. 1), and it was questioned if the zinc endopeptidases had been depleted of zinc during the separation of Pronase on the gel chromatography column. This, however, did not seem to be the case to any substantial degree, since the enzymatic activity measured for the endopeptidases after reactivation with excess amounts of zinc was only 5 – 10 % higher than that observed prior to the penten inhibition.

Zinc aminopeptidases. Each fraction from the elution diagram shown in Fig. 1 was also tested for aminopeptidase activity using LNA and pro-tyr as substrates. The observed activities (Fig. 3) show that all the aminopeptidase activity in Pronase was present in peaks B and D. A complete inactivation of the LNA active fractions was observed by the addition of penten to a final concentration of 1 mM. The activity was restored by addition of excess amounts (1.2 mM) of zinc, as was the case for the zinc endopeptidases. Consequently it can be concluded that the aminopeptidases in Pronase are zinc exopeptidases.

Carboxypeptidases. A third category of enzymes found in Pronase are the carboxypeptidases, which have been described to have a substrate specificity similar to the mammalian carboxypeptidase A (E.C. 3.4.2.1) [2] using CBZ-gly-leu as substrate. Carboxypeptidase activity was found in peaks A, C, D and in the region between peaks D and E (Fig. 4). The activity in peaks A and C showed exactly the same elution profile as the zinc endopeptidases. Thus CBZ-gly-leu did not seem to be completely selective as a substrate in searching for carboxypeptidases in Pronase. The activity found in peak D and in the region between peak D and E, however, did not show any endopeptidase activity and thus seemed to correspond to components with true carboxypeptidase activity. Other substrates like CBZ-glu-tyr, CBZ-gly-phe and Ac-phe-tyr were also hydrolyzed by these carboxypeptidases.

In contrast to the zinc endopeptidases and the aminopeptidases the carboxypeptidase activity in peak D and in the region between

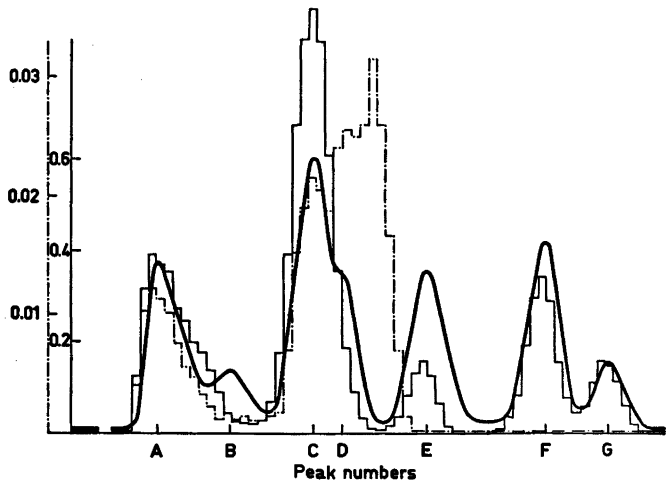

Fig. 4. Carboxypeptidase activity against CBZ-gly-leu and caseinolytic activity at pH 7.5 found in Pronase after separation of 80 mg by gel chromatography on a 2.5 × 90 cm Sephadex G-75 Superfine bed. The enzymatic activities are calculated as optical density per ml eluate and minute. —, Caseinolytic activity (pH 7.5) OD_{280} nm min ml eluate; —·—, CBZ-gly-leu activity OD_{570} nm/min ml eluate.

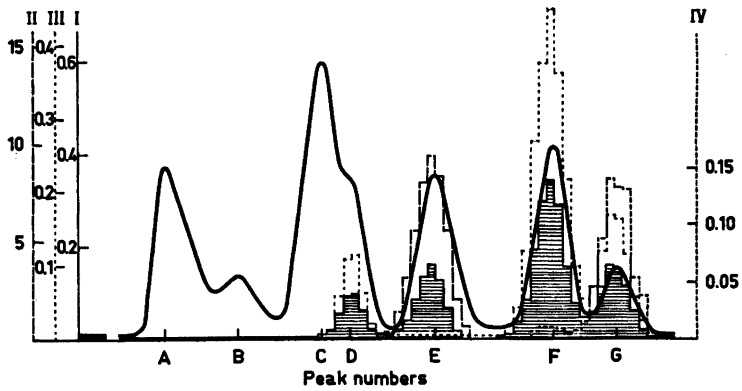

Fig. 5. Serine proteinases found in Pronase-P after separation of 80 mg by gel chromatography on a 2.5 × 90 cm Sephadex G-75 Superfine bed. The enzymatic activities were measured against casein at pH 7.5 and 10, BAME and GPNA on fractions preincubated with 1 mM penten for 30 min and calculated as optical density per ml eluate and minute. I, Caseinolytic activity (pH 7.5) OD_{280} nm/min ml eluate; II, BAME activity OD_{253} nm/min ml eluate; III, caseinolytic activity (pH 10) OD_{280} nm/min ml eluate; IV, GPNA activity OD_{410} nm/min ml eluate. Roman numerals refer to the various enzymatic activities.

peaks D and E was not inhibited by 1 mM penten. Not even 10 mM penten followed by filtration on Sephadex G-25 or dialysis towards 0.5 mM penten or EDTA had any effect on the activity. Nor did 1 mM DFP show any effect on the carboxypeptidase activity. When the fractions were made 1 mM with respect to $HgCl_2$, however, the components were irreversibly inactivated.

Since the total amount of carboxypeptidase activity in the investigated batch of Pronase-P was found to be much lower than could be expected from the results of Narahashi *et al.*,[2] several other batches of Pronase were also analyzed for this activity. Large variations between different batches were found, and the Pronase I [4,18] for example contained 20 times more activity than the batch used in this investigation. The carboxypeptidase in Pronase thus seemed to be rather instable during the isolation procedure used for Pronase-P.

Serine endopeptidases. Another aliquot of each of the fractions from the elution diagram shown in Fig. 1 was made 1 mM with respect to penten to inhibit the zinc enzymes. A residual activity towards casein at neutral pH was found in peaks D, E, F, and G as shown in Fig. 5. The original activity in E, F, and G was unaffected by penten. Although the residual activity in peak D was found in the same fractions as the collagenase activity presented in Fig. 2. no activity was found when tested against collagenase substrate A or LNA in the presence of penten.

Subsequent incubation with DFP of these fractions showing neutral proteinase activity gave a complete inactivation, and no activity towards casein at pH 7.5 or pH 10, BAME or GPNA could be detected. This further established the presence of at least four different serine proteinases in Pronase.

Further examinations concerning the substrate specificity of the serine enzymes in peaks D – G were performed. Activity towards casein at pH 10 was found in peaks D, F, and G, while no such activity was found in peak E. On the other hand peak E was the only peak showing activity towards BAME and thus corresponded to the trypsin-like enzyme found in Pronase.[5,9]

Peaks D – G were also tested towards the chymotrypsin substrate GPNA. Activity was mainly found in peak G, as has already been reported by Bauer and Löfqvist,[15] but a small amount of activity was also present in peak F. Minor activities towards CBZ-glu-tyr, CBZ-gly-phe and Ac-phe-tyr were also found in peak F. These activities were not inhibited by 1 mM DFP or 1 mM penten. Dialysis towards 0.5 mM penten was, however, sufficient to cause an inactivation. The activities could then also be restored by the addition of excess

amounts of Zn^{2+}. By preparative polyacrylamide gel electrophoresis of the eluate in peak F it was possible to distinguish along with the main component in band position 1, also two minor bands in position 2 and 3. No neutral proteinase activity could be detected in these minor bands. They contained, however, all the carboxypeptidase activity found in peak F. It is thus likely that the weak band in position 2 observed in peak F of Pronase IV by Löfqvist and Klevhag [13] is not equivalent to the alkaline proteinase found in band position 2 in Pronase I.

Among the substrates tested CBZ-ala-val-OMe was found to be rather specific for the serine proteinase present in peak D. This enzyme was also active towards casein at pH 10, which further strengthened the suggestion [13] that the subtilisin-like component [7] in Pronase is found in this peak.

DISCUSSION

By use of the gel chromatography separation method of Löfqvist and Klevhag [13] Pronase has been found to be a mixture of mainly zinc- and serine-proteinases. The zinc enzymes are found in the first four peaks (A–D) of the elution diagram. Along with the zinc proteinases peak D also contained carboxypeptidase activity as well as a serine proteinase. The additional serine proteinases were eluted in peaks E, F, and G. The zinc enzymes were according to their substrate specificity divided into endo- and exopeptidases.

Zinc endopeptidases. Zinc has been found to be essential for the enzymatic activity of the endopeptidases eluted in peak A and C and could be exchanged reversibly in presence of 30 mM Ca^{2+}. It was thus concluded that these enzymes are zinc proteinases. The content of zinc corresponds to 0.8 and 0.5 zinc atoms per mol enzyme in peak A and C, respectively, as calculated from the molecular weights (50 000 and 28 000 dalton) [13] and an assumed ε(1 %, 1 cm) at 280 nm of 10. A considerable heterogeneity of the material eluted in peak A and C was found [13] by analysis on polyacrylamide gel electrophoresis and in accordance with the above calculated low zinc values it was questioned to which extent the components present in peak A and C were active enzymes. In the earlier separation of Pronase on polyacrylamide gel electrophoresis at pH 6.8 for 9 h [4] it is, for example, observed that one of the components in peak A (Band 8) was enzymatically inactive. A separation of Pronase on preparative polyacrylamide gel electrophoresis was thus performed in order to examine, whether the various components found within peak A and C were enzymatically active. As

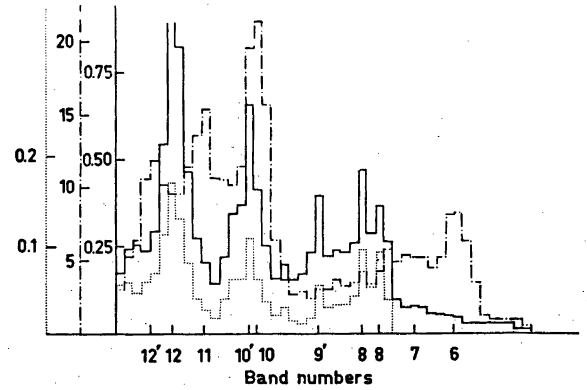

Fig. 6. Histogram showing the enzymatic activities of Pronase-P found in the different gel fractions of a small (1 × 10 cm) preparative polyacrylamide gel after 6 h electrophoresis (cathodic migration) in 0.38 M boric acid – 0.01 M CaO buffer. The gel fractions (0.5 mm thick) were extracted at pH 7.5 with standard buffer, incubated for half an hour with 1 mM DFP and tested for activity towards casein at pH 7.5 and 10 as well as LNA. The activities found are given in optical density per ml extract and hour. Band numbers are given as suggested by Löfqvist and Sjöberg [4] from the staining found in a duplicate run. —, Caseinolytic activity (pH 7.5) OD_{280} nm/h ml eluate; . . ., collagenase activity OD_{410} nm/h ml eluate; — · —, LNA activity OD_{560} nm/h ml eluate.

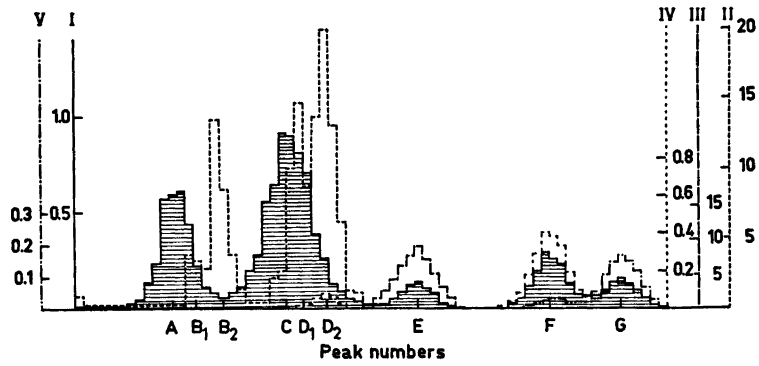

Fig. 7. Analysis of the heterogeneity of Pronase-P due to molecular size by separation of 1 mg on a 2.5 × 90 cm Sephadex G-75 Superfine bed. The separated components were identified by their enzymatic activities towards casein at pH 7.5 and 10, LNA, BAME and GPNA. The amount of activity found is calculated as optical density per ml eluate and hour. I, Caseinolytic activity (pH 7.5) OD_{280} nm/h ml eluate; II, LNA activity OD_{560} nm/h ml eluate; III, BAME activity OD_{253} nm/h ml eluate; IV, caseinolytic activity (pH 10) OD_{280} nm/h ml eluate; V, GPNA activity OD_{410} nm/h ml eluate. Roman numerals refer to the various enzymatic activities.

shown in Fig. 6 each component was active towards both casein at pH 7.5 and collagenase substrate A. Consequently no indications were obtained for the presence of any zinc-free components in peak A or C.

Since a considerable loss in neutral proteinase activity was reported when high concentrations of Pronase (80 mg/ml) were separated on gel chromatography [13] and much more stable conditions were obtained at lower concentrations (1.5 mg/ml), it was also of interest to investigate if a less pronounced heterogeneity might occur at low enzyme concentrations. Thus a separation on a 2.5 × 90 cm Sephadex G-75 Superfine bed was performed purely for analytical purposes (Fig. 7). Considering the improved resolution, which could be expected at this low sample load (and demonstrated by the separation results obtained for the leucine aminopeptidases), peak A was eluted less heterogeneous than in the 80 mg separations. The board elution profile of peak C, however, indicated that this material was heterogeneous even after separation at these more stable conditions. Consequently it was concluded that the poor stability of the zinc endopeptidases in Pronase during the gel chromatography separations [13] could not explain the observed heterogeneity. This seemed to be the result of unstable conditions during the fermentation and isolation of Pronase.

Since peaks A and C were distinctly separated and no intermediate sized components were found, it was, however, considered likely that the components in each of the peaks represented genuine endopeptidases produced by the actinomycete. The molecular weights of these two endopeptidases would thus be 50 000 and 28 000 dalton, respectively. Their content of zinc calculated per mol of enzyme also differed with a factor of about two. The possibility that the larger endopeptidase is a dimer of the smaller one can thus not be excluded. In that case the Kl Strain of *Streptomyces griseus* would only have produced one zinc endopeptidase. Further investigations are in progress to examine the identity of the zinc endopeptidases in Pronase.

Zinc aminopeptidases. The aminopeptidases in Pronase are in this investigation found to contain approximately one zinc atom per mol enzyme assuming an ε (1 %, 1 cm) at 280 nm of 10. Furtheron it is shown that zinc is essential for the enzymatic activity and that the zinc ion can be reversibly removed from the aminopeptidases in a medium containing 30 mM Ca^{2+}. It is thus concluded that zinc is the original prosthetic metal ion of the aminopeptidases. Recently, however, Vosbeck *et al.*[6] found calcium or strontium "as the preferred metal ion" of two aminopeptidases isolated

from Pronase. By dialysis of Pronase against different metal ions calcium was earlier [10] shown to be essential for the aminopeptidase activity. Calcium as well as zinc thus seems to be essential for the activity of the aminopeptidases in Pronase. It is, however, most likely that calcium plays a different role from that of zinc, and in analogy to the findings on thermolysin,[12,20] another zinc- and calcium-dependent proteinase, it is assumed that the calcium ions act as stabilizers, contributing to a stable and enzymatically active tertiary structure of the enzyme molecules by formation of internal salt linkages between carboxyl groups present in different parts of their polypeptide chains. The results on thermolysine also showed that this enzyme is rapidly autolyzed in calcium-free solutions. This gives a possible explanation to the "inhibitory" effect of Zn^{2+} noted by Narahashi and Yanagita [10] for the aminopeptidases in Pronase, and it is most likely that the aminopeptidases in Pronase are calcium-stabilized zinc enzymes.

The aminopeptidases in Pronase were on gel chromatography eluted in two different peaks (B and D). Peak B was not completely separated from peak A and thus pure aminopeptidase activity is only obtained in the later part of peak B. In the case of peak D the alkaline proteinase (Band 9) as well as the carboxypeptidase (Band 5) are eluted together with the aminopeptidase activity, and no part of the peak contained pure aminopeptidase activity.

From the molecular weights estimated for the components in peak B and D, 37 000 and 25 000 dalton respectively,[13] and the absence of LNA-active components with intermediate molecular weights, it is most likely that at least two different zinc aminopeptidases are produced by the actinomycete.

A considerable heterogeneity was, however, observed within both peak B and D and 4–6 components with LNA-activity were found by analytical gel chromatography (Fig. 7), and gel electrophoresis (Fig. 6). No major differences in functional properties were, however, indicated between these components (4, 6, 13), and at this stage of investigation they are assumed to be products of limited proteolysis of the aminopeptidases originally produced by the actinomycete. The favourable stability of the aminopeptidases during the course of separation [13] also indicates that this limited proteolysis has occurred during the fermentation or isolation procedure of Pronase. It is thus uncertain to which extent the zinc aminopeptidases present in Pronase are identical to the ones originally produced by the actinomycete.

Carboxypeptidases. The carboxypeptidases in peak D and in the region between peak D and E could not be inhibited by either penten or EDTA in presence of 30 mM Ca^{2+}. In calcium-free media, however, Narahashi and Yanagita [10] observed inactivation of the CBZ-gly-leu activity by EDTA. It thus seems likely that also the components with carboxypeptidase activity in Pronase need calcium ions as stabilizers of their tertiary structure and the observed heterogeneity is probably due to limited proteolysis. Thus it is likely that only one carboxypeptidase is produced by the Kl Strain of *Streptomyces griseus*.

The nature of the active site of this carboxypeptidase could, however, not be revealed by inhibition experiments. In other carboxypeptidases with similar properties,[22] however, zinc was found to be so firmly bound so it could not be released even by prolonged dialysis against strong chelating agents. It can thus not be excluded that also the carboxypeptidase in Pronase is a metallo enzyme. Indications for this were indirectly obtained by examination of two minor components with true carboxypeptidase activity found in peak F. These components were, due to their substrate specificity and the poor stability indicated for the Pronase carboxypeptidase assumed to be partly digested products of the original carboxypeptidase and they were found to contain a firmly bound zinc atom as prosthetic metal ion. It is thus proposed that the carboxypeptidase in peak D or the region between D and E also is a zinc enzyme.

Serine endopeptidases. Four serine endopeptidases were as expected found in Pronase. They were eluted in chromatography peaks D, E, F, and G, respectively. From recent investigations [15,22] it can also be concluded that they do represent different gene products, out of which one is described as a subtilisin-like enzyme, one as a trypsin-like enzyme, while the remaining two show properties similar to both

Table 2. The heterogeneity of Pronase in terms of components identified on gel chromatography and gel electrophoresis and the corresponding number of original enzymes produced by *Streptomyces griseus* Strain Kl.

Gel chromatography peak	Electrophoretically separated Pronase components [a] present in the various gel chromatography peaks [b]				Suggested number of enzymes produced by *Streptomyces griseus* Strain Kl
	Zinc endopeptidases	Zinc aminopeptidases	Carboxypeptidase	Serine endopeptidases	
A	8, 9′	–	–	–	1
B	–	6, 7	–	–	1
C	10′, 12, 13	–	–	–	1
D	–	10, 11, 12′, 14	5	9	3
E	–	–	–	4	1
F	–	–	–	1, (2)	1
G	–	–	–	3	1

[a] The components are numbered as in Ref. 13. [b] Each component has in case of incomplete separation between different peaks only been assigned to the peak in which its main activity is found.

chymotrypsin and elastase. Together with the main components each peak was also found to contain minor constituents.[13] These minor constituents in peak F were further examined and found to have a substrate specificity completely different from the main component. This further stresses the need for thorough purification control of the Pronase enzymes before detailed characterization of their properties is carried out.[15]

The summarized results of the heterogeneity found for the different categories of proteolytic enzymes in Pronase are given in Table 2. The assumed number of original enzymes produced by the *Streptomyces griseus* Strain Kl is 9, out of which 4 are serine enzymes and at least 4 are zinc enzymes. Further 9 zinc-depending major components are found in the particular batch of Pronase investigated. There is, however, so far no evidence indicating that these components should represent original enzymes. At this stage of investigation it is therefore most relevant to classify them as products of limited proteolysis.

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Dioxolanylium Ions Derived from Carbohydrates. II

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Reaction of methyl 2,3:4,6-di-*O*-benzylidene-α-D-mannopyranoside with triphenylmethyl fluoroborate gave the 2,3-benzoxonium ion (*2*); the 4,6-benzylidene group did not react. A similar treatment of 3-*O*-benzoyl-5,6-*O*-benzylidene-1,2-*O*-isopropylidene-α-D-glucofuranose gave the 5,6-benzoxonium ion (*5*), and 1,2:3,4-di-*O*-benzylidene-β-D-arabinopyranose yielded the 3,4-benzoxonium ion (*11*). In the latter case no hydride abstraction took place from the 1,2-benzylidene group. A study of a number of compounds showed that 4,6-, 3,5-, and 1,2-*O*-benzylidene groups, attached to pyranose or furanose rings, did not undergo hydride abstraction with triphenylmethyl fluoroborate. The reactions of the ions *2*, *5*, and *11* with water and with bromide ions were studied.

In the preceding paper[1] it was shown that benzoxonium ions could be generated by reaction of benzylidene derivatives with triphenylmethyl fluoroborate in acetonitrile solution. The dioxolanylium ring was shown to undergo *cis*-opening when hydrolysed whereas reaction with bromide ions lead to *trans*-opening and formation of bromo-deoxy sugars. In the present paper further examples of these reactions are described, mainly in the hexose series, and the behaviour of various types of benzylidene compounds towards triphenylmethyl fluoroborate has been investigated.

Treatment of methyl 2,3:4,6-di-*O*-benzylidene-α-D-mannopyranoside (*1*) with triphenylmethyl fluoroborate in acetonitrile gave the 2,3-benzoxonium ion (*2*) in quantitative yield after 16 h. The ion was quite stable and its NMR spectrum could be measured in deuterioacetonitrile (Table 1). On prolonged reaction with triphenylmethyl fluoroborate a slow decomposition took place, but no hydride abstraction from the 4,6-*O*-benzylidene group was observed. Lack of reactivity of certain types of benzylidene derivatives was found in a number of other cases. Thus the following compounds could not be induced to react with triphenylmethyl fluoroborate in acetonitrile: methyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside, methyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-α-D-galactopyranoside, 3,5-*O*-benzylidene-6-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-glucofuranose, 1,2:4,6-di-*O*-benzylidene-3-*O*-benzoyl-α-D-glucopyranose, and 1,2:3,5-di-*O*-benzylidene-6-*O*-benzoyl-α-D-glucofuranose. From these results it appears that benzoxonium ions with 6-membered rings cannot be formed under the conditions used; the latter two examples furthermore show that 1,2-benzoxonium ions are not formed. However, while 1,2-*O*-benzylidene groups were isomerized by triphenylmethyl fluoroborate to mixtures of *endo* and *exo* isomers 4,6-*O*-benzylidene groups were completely unaffected. This was further confirmed by treating 1,2:3,4-di-*O*-benzylidene-β-D-arabinopyranose (*10*) with triphenylmethyl fluoroborate which gave the 3,4-benzoxonium ion (*11*) as the sole product. NMR spectra of the two diastereomers of *11* are shown in Table 1. The dibenzoxonium ion which could be formed by hydride abstraction from *11* was not detected. It is known to be stable in anhydrous hydrogen fluoride solution.[2]

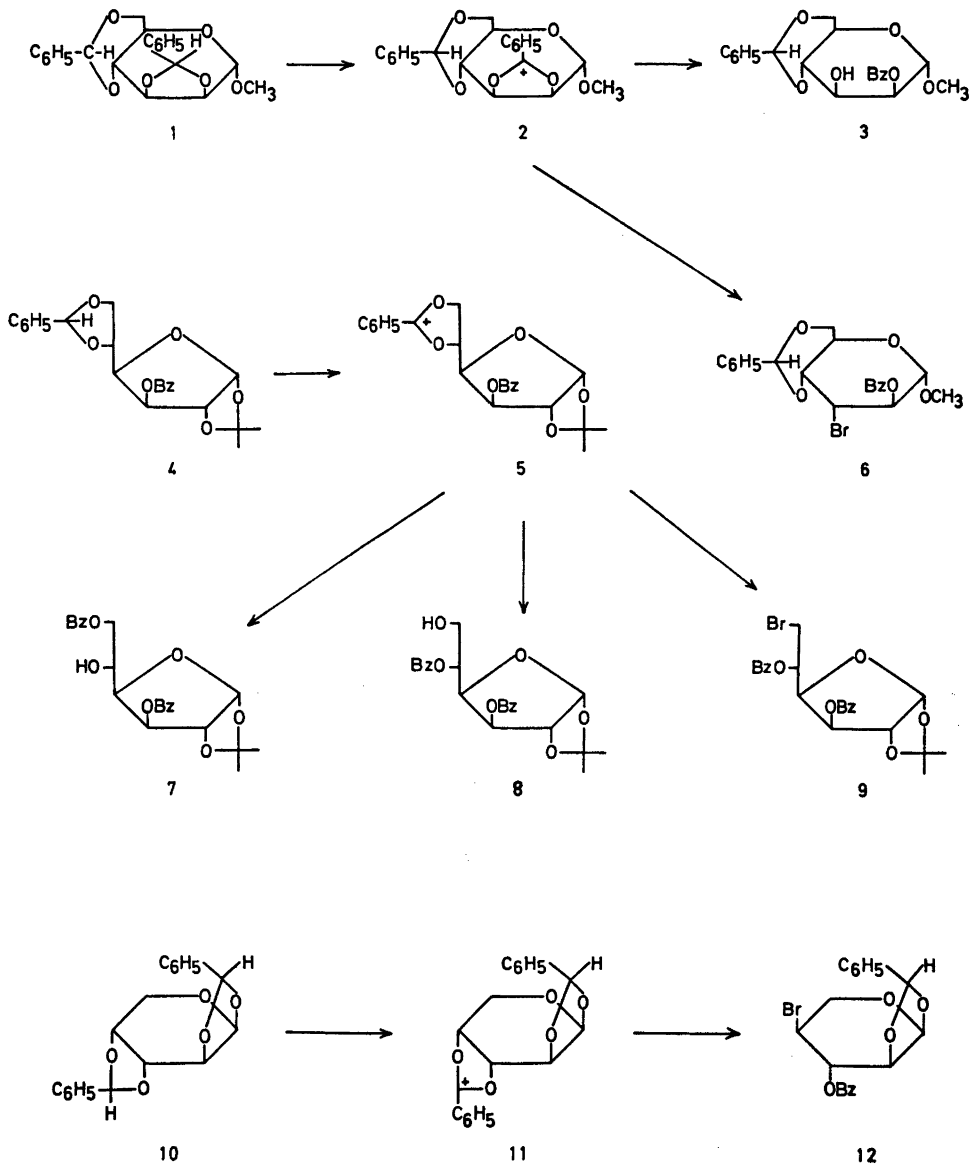
Treatment of 3-*O*-benzoyl-5,6-*O*-benzylidene-1,2-O-isopropylidene-α-D-glucofuranose (*4*) with triphenylmethyl fluoroborate rapidly gave the benzoxonium ion (*5*) as seen from its NMR spectrum in deuterioacetonitrile (Table 1). The corresponding 3-*O*-mesylated ion was obtained by Hanessian and Staub.[3] Barton *et al.*[4] have shown that isopropylidene groups will react with triphenylmethyl fluoroborate. This reac-

Table 1. Proton NMR spectra of benzoxonium ions and products prepared from them. Chemical shifts are in ppm relative to tetramethylsilane, coupling constants in Hz.

Compound	Solvent	H1	H2	H3	H4	H5	H6	H6′	J_{12}	J_{23}	J_{34}	J_{45}	J_{56}	J_{56}'	J_{66}'		
2	CD_3CN	5.59	5.75	6.27	~4.4		3.7–4.1		0	8.5	~8					OCH_3 3.54	benzylidene H 5.74
5	CD_3CN	6.15	4.80	5.66	5.24	6.27	5.76	5.62	3.7	0	3.5	2.6	9.0	8.1	9.3		isoprop. 1.55; 1.32
3	$CDCl_3$	4.76	5.37	4.2	3.7	———		4.4	1.5	3.5						OCH_3 3.33	benzylidene 5.58
7	$CDCl_3$	6.01	4.70	5.61	4.44	4.13	4.74	4.45	3.7	<0.5	2.6	9.2	2.5	5.8	11.9		isoprop. 1.34; 1.39
8	$CDCl_3$	5.98	4.65	5.55	4.82	5.46	4.07	4.03	3.7	<0.5	3.0	9.2	3.2	3.7	12.8		isoprop. 1.32; 1.59

Compound	Solvent	H1	H2	H3	H4	H5	H5′	J_{12}	J_{23}	J_{34}	J_{45}	J_{45}'	J_{55}'	benzylidene H
11 endo-H [a]	CD_3CN	5.53	4.60	5.9	5.9			5.2	1.8					5.68
11 exo-H [a]	CD_3CN	5.58	4.46	5.9	5.9	3.92–4.03		5.2	1.5					5.53
12 endo-H [a]	$CDCl_3$	5.40	3.91	5.83	3.6	———	4.0	4.2	5.3	6.9				6.31
12 exo-H [a]	$CDCl_3$	5.32	3.75	5.87	3.5	———	4.1	4.5	3.5	4.5				

[a] Assignment of structure is based on the chemical shift of the benzylidene proton.[14]



tion is, however, slower than the reaction with benzylidene groups and it was not observed. When the benzoxonium ion (*5*) was kept in acetonitrile solution in the presence of excess triphenylmethyl fluoroborate for several days a slow decomposition took place.

The benzoxonium ions were reacted with water and with bromide ions as in the preceding paper.[1] Treatment of *2* with water gave the 2-*O*-benzoate (*3*), with an axial *O*-benzoyl group, as the sole product, in agreement with the results of King and Allbutt.[5] The 5,6-benzoxonium ion (*5*) gave a mixture of the 6-*O*-benzoate (*7*) and the 5-*O*-benzoate (*8*) on hydrolysis.

Reaction of *2* with bromide ions lead to *trans*-opening of the dioxolanylium ring and gave the 3-bromo-3-deoxy-altroside (*6*) as the only product. The formation of the *trans*-diaxial product is in agreement with the results

that King and Allbutt [6] found for nucleophilic opening of dioxolanylium rings fused to bicyclic systems. The 5,6-benzoxonium ion (*5*) gave only the 6-bromo-derivative (*9*) on reaction with bromide ions.[3] Finally, the benzoxonium ion (*11*) when treated with bromide ions gave the 4-bromo-4-deoxy-L-xylose derivative (*12*) as a mixture of the two diastereomers.

EXPERIMENTAL

For details of chromatography and NMR spectroscopy see the preceding paper.[1]

Methyl 2,3:4,6-di-O-benzylidene-α-D-mannopyranoside (1). Methyl α-D-mannopyranoside (9.7 g) and benzaldehyde (11.5 g) were boiled for 10 h with *p*-toluenesulfonic acid (100 mg) in chloroform (200 ml) with a Soxhlet extractor containing 50 g of 4 Å molecular sieves. The solution was then washed with aqueous sodium hydrogencarbonate and water, dried and evaporated. The residue was crystallized from ethanol–chloroform to give 8.5 g of *1*. The mother liquor was evaporated and the residue was dissolved in chloroform and boiled for 1 h with *p*-toluenesulfonic acid (100 mg). Work up as described above gave 3.4 g of *1*. After an additional equilibration the total yield of *1* was 13.7 g, m.p. 160–175 °C. One recrystallization from ethanol-chloroform gave 11.9 g of a product with m.p. 176–177 °C (reported [7] m.p. 180–181 °C).

Conversion of benzylidene derivatives to hydroxy-benzoates

The benzoxonium ions were prepared by reaction of the benzylidene derivatives with a 10–25 % molar excess of triphenylmethyl fluoroborate in dry acetonitrile at room temp. Hydrolysis and chromatography was performed as described in the preceding paper.[1]

Methyl 2,3:4,6-di-O-benzylidene-α-D-mannopyranoside (1) (520 mg) gave the benzoxonium ion (*2*) after reaction with triphenylmethyl fluoroborate for 16 h. Hydrolysis and chromatography yielded 364 mg (67 %) of methyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-mannopyranoside (*3*) as a syrup, $[\alpha]_D^{21}$ −33.8° (*c* 1.3, $CHCl_3$). (Found: C 65.13; H 5.82. Calc. for $C_{21}H_{22}O_7$: C 65.27; H 5.74). An NMR spectrum (Table 1) proved the structure. Debenzoylation with sodium methoxide in methanol and recrystallization from ethanol-water gave methyl 4,6-*O*-benzylidene-α-D-mannopyranoside, m.p. 145–145.5 °C (reported [7] m.p. 146–147 °C). A mixed m.p. with an authentic sample gave no depression.

3-O-Benzoyl-5,6-O-benzylidene-1,2-O-isopropylidene-α-D-glucofuranose (4) [8] (533 mg) gave the benzoxonium ion (*5*) after reaction with triphenylmethyl fluoroborate for 1 h. Hydrolysis and chromatography yielded two products. The fast moving fraction (183 mg, 33 %) was 3,6-di-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-glucofuranose (*7*), m.p. 108–110 °C, $[\alpha]_D^{20}$ −4.4° (*c* 1.7, $CHCl_3$), (reported [9] m.p. 108–109 °C, $[\alpha]_D$ −4.6°). The slow moving fraction (213 mg, 38 %) was the 3,5-di-*O*-benzoate (*8*) as a syrup, $[\alpha]_D^{21}$ −111.3° (*c* 1.6, $CHCl_3$). (Found: C 64.34; H 5.77. Calc. for $C_{23}H_{24}O_8$: C 64.48; H 5.65). Benzoylation of (*8*) with benzoyl chloride in pyridine gave 3,5,6-tri-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-glucofuranose, m.p. 118–120 °C (reported [10] m.p. 119–120 °C). A mixed m.p. with an authentic sample gave no depression.

Conversion of benzylidene derivatives to bromo-deoxy-benzoates

The benzoxonium ions were prepared as described above and treated with dry tetraethylammonium bromide (3 molar equiv.) in acetonitrile for 2 h. Work up and chromatography as described in the preceding [1] paper gave the products.

Methyl 2,3:4,6-di-O-benzylidene-α-D-mannopyranoside (*1*) (552 mg) gave 388 mg (50 %) of methyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-bromo-3-deoxy-α-D-altropyranoside (*6*). Crystallization from ethyl acetate–pentane gave the pure product, m.p. 134–136 °C. (Found: C 56.25; H 4.62; Br 17.91. Calc. for $C_{21}H_{21}BrO_6$: C 56.13; H 4.71; Br 17.79). The product was identical with a sample prepared by benzoylation of methyl 4,6-*O*-benzylidene-3-bromo-3-deoxy-α-D-altropyranoside,[11] m.p. 136.5–137.5 °C, $[\alpha]_D^{21}$ +1.6° (*c* 1.5, $CHCl_3$). A mixed m.p. gave no depression.

In a separate experiment 3.3 g of *1* was converted to the 2,3-benzoxonium ion (*2*) which was treated with tetraethylammonium bromide to give the bromo-compound (*6*) in acetonitrile solution. Stirring with saturated aqueous sodium hydrogencarbonate (50 ml) for 30 min gave a crystalline precipitate which was filtered off and washed with water. Extraction with pentane (3 × 50 ml) removed most of the triphenylmethane. The product was then dissolved in ethyl acetate (50 ml) and treated with activated carbon. Evaporation of the ethyl acetate to *ca.* 10 ml and addition of pentane (40 ml) precipitated 1.75 g (43 %) of *6*, m.p. 135–137 °C. One recrystallization from ethyl acetate–pentane gave 1.60 g, m.p. 136–138 °C, $[\alpha]_D^{20}$ +1.2° (*c* 1.2, $CHCl_3$).

3-O-Benzoyl-5,6-O-benzylidene-1,2-O-isopropylidene-α-D-glucofuranose (4) (454 mg) gave after chromatography 427 mg (79 %) of 3,5-di-*O*-benzoyl-6-bromo-6-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (*9*) as a syrup, $[\alpha]_D^{21}$ −114.5° (*c* 1.2, $CHCl_3$). (Found. C 56.41; H 4.78; Br 16.13. Calc. for $C_{23}H_{23}O_7Br$: C 56.22;

H 4.72; Br 16.26). A product obtained by benzoylation of 5-*O*-benzoyl-6-bromo-6-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose [12] had $[\alpha]_D^{21}$ −119.8° (*c* 1.4, $CHCl_3$). NMR spectra of the two products were identical.

1,2:3,4-Di-O-*benzylidene-β*-D-*arabinopyranose* [13] (10) (508 mg) gave the 3,4-benzoxonium ion (*11*) after treatment with triphenylmethyl fluoroborate for 4 h in acetonitrile solution. Reaction with tetraethylammonium bromide and work up as described above yielded 324 mg (51 %) of a mixture of the diastereomeric 3-*O*-benzoyl-1,2-*O*-benzylidene-4-bromo-4-deoxy-α-L-xylopyranoses (*12*). Crystallization from ethyl acetate (3 ml)-pentane (10 ml) gave 102 mg, m.p. 124–125 °C. Recrystallization gave the pure *endo*-H isomer, m.p. 125–126 °C, $[\alpha]_D^{21}$ −43.3° (*c* 1.7, $CHCl_3$). (Found: C 56.43; H 4.31; Br 19.72. Calc. for $C_{19}H_{17}BrO_5$: C 56.31; H 4.22; Br 19.72). The *exo*-H isomer could not be obtained pure, but preparative TLC (benzene) gave a product which contained 80 % *exo*-H isomer, allowing it to be identified through its NMR spectrum (Table 1).

Microanalyses were performed by Dr. A. Bernhardt, Mikroanalytisches Laboratorium or by Novo Microanalytical Laboratories.

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On the Occurrence of 1-*O*-Alkylglycerols and 1-*O*-(2-Methoxyalkyl)glycerols* in Human Colostrum, Human Milk, Cow's Milk, Sheep's Milk, Human Red Bone Marrow, Red Cells, Blood Plasma and a Uterine Carcinoma

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1-*O*-Alkylglycerols and 1-*O*-(2-methoxyalkyl)-glycerols were isolated from the neutral lipids and phospholipids of human colostrum, human milk, cow's milk, sheep's milk, human red bone marrow, red cells, blood plasma, and a uterine carcinoma. Human colostrum has a higher content of unsubstituted glycerol ethers in the neutral lipids than human milk. Human milk contains nearly 10 times more unsubstituted glycerol ethers than cow's milk and twice as much as sheep's milk. The highest percentage of unsubstituted glycerol ethers in neutral lipids was found in the human red bone marrow and the uterine carcinoma. The methoxy-substituted glycerol ethers were found both in the neutral lipids and in the phospholipids of all the tissues studied but only in trace quantities. Glycerol ethers with 16 and 18 carbon atoms in the long hydrocarbon chains are the principal components of both the unsubstituted and the 2-methoxy-substitued glycerol ethers. A poly-unsaturated methoxy-substituted glycerol ether, 1-*O*-(2-methoxydocosahexaenyl)glycerol, was found in the neutral lipids and phospholipids of red blood cells.

The physiological effect of the glycerol ethers that is best documented is the stimulation of the bone marrow. Sandler [1] reported that batyl alcohol (1-octadecylglycerol) had a stimulatory effect on the erythrocyte count of both normal rats and those poisoned with benzene. It was confirmed by several investigators that both optically active and racemic batyl alcohol stimulated erythropoesis, thrombopoesis and granulopoesis.[2–10] Chimyl alcohol also has a stimulatory effect on haemopoesis [9] but selachyl alcohol (*cis*-9-octadecenylglycerol) has no haemopoetic activity.[7,10]

The glycerol ethers occur in the tissues in the form of diesters [11,12] and alkyl acyl phosphatides.[13–17] A high level of glycerol ether lipids was found in a variety of transplantable tumours in animals [18–19] and in human tumours.[20]

2-Methoxy-substituted glycerol ethers have been isolated from Greenland shark liver oil.[21] These substituted glycerol ethers have antibiotic activity and also inhibited the dissemination and growth of several experimental tumours in mice.[22,23] The aim of the present investigation was to study methoxy-substituted as well as ordinary, unsubstituted glycerol ethers in human milk, red bone marrow, red blood corpuscles, and blood plasma. A human uterine tumour has also been studied. Milk from two ruminant species, cow and sheep, is included for comparison with human milk. The composition of the two groups of glycerol ethers has also been determined.

MATERIAL AND METHODS

Material. The human milk was pooled milk from a milk bank at Sahlgren's hospital, Gothenburg. Colostrum was collected during the first two days after delivery, "transition milk" during days 3 – 7 and the third sample of milk was from lactating women 8 days to 3 months

* "Alkyl" also includes unsaturated carbon chains except those with a double bond adjacent to the glycerol ether oxygen.

after delivery. The cow's milk was fresh pasteurized milk and the sheep's milk was fresh but unpasteurized.

Red blood cells and plasma were obtained by centrifugation of blood from blood donor bottles. The cells were washed twice with one volume of 0.9 % NaCl. The red bone marrow was collected from the long bones of several autopsy cases free from any blood diseases.

The uterine carcinoma was obtained from the Department of Gynecology, Sahlgren's hospital. The tumour could not be dissected out from the part of the uterus obtained. The large tumour analyzed thus included some normal uterine tissue.

Extraction of lipids. The blood plasma and the milk were freeze-dried before the extraction of the lipids. The red bone marrow, the red cells and the uterine carcinoma were extracted directly. One part by weight of the freeze-dried or the fresh material was extracted with 12 volumes of a chloroform – methanol mixture, 2:1 (v/v) or a mixture of chloroform – ethanol, 2:1 (v/v). The mixture was boiled under reflux for 90 min. After cooling at room temperature, it was filtered. The residue was boiled for another 90 min in chloroform – methanol or chloroform – ethanol. The extract was evaporated to half its volume and then partitioned against one-fifth volume of 0.1 % NaCl. After separation of the chloroform phase, the NaCl solution was extracted 3 times with chloroform. The solvents were evaporated from the lipid extract and the residue was dried under a stream of nitrogen.

Separation of neutral lipids, phospholipids, glycerol ethers, and isopropylidene derivatives of glycerol ethers. 10 g of lipid material was dissolved in chloroform and applied to a column of 1350 g of silicic acid (silicic acid for lipid chromatography, Bio-Rad Lab., Richmond, Calif.). The neutral lipid fraction was eluted with chloroform and chloroform – methanol 9:1 (or chloroform – ethanol 9:1). The main phospholipid fraction was eluted with chloroform – methanol 1:1 (or chloroform – ethanol 1:1) but due to tailing the last parts had to be eluted with methanol (or ethanol).

The neutral lipid fraction was saponified by boiling in 1 M KOH in ethanol for 1 h. The phospholipids were treated according to Thompson and Lee.[24] The material was refluxed with acetic acid – acetic anhydride 3:2 for 8 h. The mixture was made alkaline with 6 M KOH in ethanol and refluxed for a further 2 h. The non-saponifiable material was extracted into ether. Fatty acid salts remaining in the material were removed by treatment with a small amount of lithium aluminium hydride in ether solution. In a few cases the lipid fraction was directly treated with lithium aluminium hydride to cleave the ester bonds. After acidification of the reduction mixture with hydrochloric acid, the organic material was extracted into ether. The material was chromatographed on silicic acid columns. Hydrocarbons, sterols and alcohols were eluted with a mixture of light petroleum (b.p. 60 – 80 °C) and ethyl ether (19:1 v/v). The unsubstituted and methoxy-substituted glycerol ethers were then eluted with ethyl ether. The column chromatography was checked by thin-layer chromatography (Silica gel G, Merck. Developing solvent: trimethylpentane/ethyl acetate/methanol, 50:40:5). To transform the glycerol ethers into their isopropylidene derivatives the material from the fractions containing glycerol ethers was treated with acetone in the presence of perchloric acid.[25] The crude mixture of isopropylidene derivatives was purified by chromatography on silicic acid columns (and checked by TLC). The isopropylidene derivatives of the unsubstituted glycerol ethers were eluted with a mixture of 1 % ether in light petroleum (b.p. 60 – 80 °C) and those of the methoxy-substituted glycerol ethers with a mixture of 5 % ether in light petroleum. The amounts of unsubstituted and methoxy-substituted glycerol ethers were determined by weighing the fractions after evaporation of the solvents under a stream of nitrogen. In some cases with very small amounts of material available, preparative thin-layer chromatography was used (Silica gel G, layer 0.5 – 1 mm). When trimethylpentane/ethyl acetate, 70:20, was used as developing solvent, the isopropylidene derivatives of the unsubstituted and the methoxy-substituted glycerol ethers were obtained from the bands with $R_F \approx 0.6$ and $R_F \approx 0.3$, respectively. The isopropylidene derivatives were used for determination of the compositions by means of gas chromatography and mass spectrometry.

Gas-liquid chromatography. The gas chromatography was performed with a Perkin-Elmer F 11 instrument equipped with a flame ionization detector. A 180 cm × 2 mm i.d. stainless steel column, packed with Gas Chrom Q 80 – 100 mesh containing 1 % Apiezon L was used. The column temperature was in most cases 220 °C. The flow was 30 ml helium/min.

Gas-liquid chromatography-mass spectrometry. The GLC – MS was carried out with the LKB 9000 combination instrument. The operating conditions of the mass spectrometer were: electron energy 70 eV, ion source temperature 270 °C, trap current 60 μA and accelerating voltage 3.5 kV.

The gas chromatography on the combined instrument was carried out at 220 °C using a 300 cm × 2 mm i.d. glass column packed with Gas Chrom Q 80 – 100 mesh, containing 1 % Apiezon L.

RESULTS AND DISCUSSION

The quantitative data on the amounts of glycerol ethers in the lipids are given in Table 1. The content of lipids in human colostrum as

Table 1. The content of unsubstituted and methoxy-substituted glycerol ethers in neutral lipids and in phospholipids isolated from human colostrum, human milk, cow's milk, sheep's milk, human red bone marrow, human red cells, human blood plasma, and a human uterine carcinoma.

Material	Lipids % (w/w)	Neutral lipids (N) and phospholipids (P) in total lipids % (w/w)		Unsubstituted glycerol ethers in N and P [a] % (w/w)		Methoxy-substituted glycerol ethers in N and P % (w/w)	
		N	P	N	P	N	P
Human colostrum, 1–2 days	2.9	96.4	3.6	0.19	0.16	trace	trace
Human "transition milk", 3–7 days	2.8	98.2	1.8	0.14	0.20	trace	trace
Human milk, 8 days–3 months	2.8	99.0	1.0	0.10	0.18	trace	trace
Cow's milk	2.9	99.0	1.0	0.01	0.16	trace	trace
Sheep's milk	7.2	99.6	0.4	0.02	0.14	trace	trace
Human red bone marrow		98.2	1.8	0.33	2.0	trace	trace
Human red blood cells	0.3	32.5	67.5	0.08	0.75	trace	trace
Human blood plasma	0.5	64.6	35.4	0.05	0.63	trace	trace
Uterine carcinoma	1.0	48.3	51.7	0.42	0.50	trace	trace

[a] The figures should be multiplied by a factor of about 2.0–2.5 to get the content of the original glycerol ether lipids in the lipid fractions.

well as in transition milk and milk collected from 8 days to 3 months after delivery is about the same. The percentage of phospholipids in the lipids decreases from 3.6 % in the colostrum to 1.0 % in the milk from the second week of lactation and onwards. The content of glycerol ethers in the phospholipids is about the same, 0.2 %, in the tree types of human milk, whereas the glycerol ether content in the neutral lipids is highest in colostrum, 0.19 %, and decreases to 0.14 % during the first week and then to 0.10 %. The neutral lipids of cow's milk and sheep's milk contain 0.01 and 0.02 % glycerol ethers, respectively. A comparison of human milk with cow's and sheep's milk shows that human milk per volume contains nearly 10 times more glycerol ethers than cow's milk and twice the amount found in sheep's milk. Trace quantitites of (2-methoxyalkyl)glycerols were found in the neutral lipids as well as in the phospholipids in all the samples of milk studied.

The phospholipids of human red bone marrow, red blood cells and blood plasma are fairly rich in alkylglycerols. The highest figure, 2 %, was found for the red bone marrow. The neutral lipids of the bone marrow are also rich in glycerol ethers in contrast to the neutral lipids of the red blood cells and the blood plasma. In both the neutral lipids and the phospholipids of the uterine carcinoma high levels of glycerol ethers were found. Minute amounts of 1-*O*-(2-methoxyalkyl)glycerols were found in both the neutral lipids and the phospholipids of all tissues investigated.

Composition of the glycerol ethers. The composition of the unsubstituted glycerol ethers of the neutral lipids and of the phospholipids is given in Table 2. The compounds with even-numbered, long chains are predominant; the compounds with odd-numbered chains usually constitute less than 5 % of the whole mixture. The unsubstituted glycerol ethers in the neutral lipids of cow's milk and sheep's milk contain somewhat more odd-numbered, long chains than human milk and also branched alkyl chains. The glycerol ethers with 16 and 18 carbon atoms in the long chains (16:0 chimyl, 18:0 batyl, and 18:1 selachyl alcohol) are the principal components. The compounds with 16 carbon atoms in the long chains are mainly

Table 2. Percentage composition of unsubstituted glycerol ethers from neutral lipids (N) and phospholipids (P).

Long chain component	Human colostrum, 1–2 days		Human "transition milk", 3–7 days		Human milk 8 days–3 months		Cow's milk		Sheep's milk		Human red bone marrow		Human red blood cells		Human blood plasma		Uterine carcinoma	
	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
< 14:0			0.2	0.4			0.8*[a]		0.1[e]					0.2	0.5	0.4		
14:0	0.5	0.7	0.5	0.7	0.9	0.8	7.6[b]		4.5[f]	tr.	0.5	0.7	3.2	0.6	3.1	1.4	0.9	0.4
15:0	0.5	0.4	0.5	0.4	0.5	0.6	7.7[c]		2.6[g]	tr.	0.4	0.5	0.7	0.4	0.9	1.0	0.4	0.1
16:0	33.8	32.5	26.0	27.7	24.8	25.3	32.0[d]	46.9	29.2[h]	27.6[k]	33.2	27.9	23.8	30.3	19.5	35.1	29.4	25.6
16:1	1.5	1.7	1.1	1.6	3.2	4.6	1.4	tr.	0.3	1.3	tr.	4.3	16.8	tr.	9.2	tr.	1.0	6.3
17:0	0.7	1.1	1.2	3.8	1.4	1.8	0.9		3.6[i]		1.1	0.7	0.8	1.1	0.7	2.0	0.7	0.9
17:1	0.6	1.0	tr.	1.1	1.6	1.5	1.8		tr.				1.6	1.0	1.7	1.9	1.3	1.2
18:0	21.2	19.1	21.5	17.8	21.8	19.0	29.5	38.0	41.9	52.8	27.0	19.3	11.1	34.8	11.1	19.1	16.3	23.7
18:1	29.7	29.2	38.3	34.1	37.5	34.7	17.7	15.1	17.8	18.3	20.3	28.6	31.8	18.3	41.0	21.7	43.8	37.9
19:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1		tr.		0.2	0.2	0.6	0.1	0.2	0.1	0.1	0.1
19:1	0.3	0.3	0.1	0.2	0.4	0.5	tr				0.2	0.4	0.8	0.2	0.4	0.4	0.3	0.2
20.0	1.6	2.1	1.4	1.8	0.9	1.6	0.2		tr.		3.0	3.1	1.4	2.4	1.4	3.5	0.8	1.1
20:1	1.2	2.4	1.4	2.1	1.7	2.4	0.3				4.2	3.9	2.3	2.4	3.2	4.1	1.8	1.1
21:0 and 1	0.1	0.1	0.1	0.3	0.3	0.5					0.2	0.3	tr.			0.2		
22:0	1.3	1.4	1.0	1.2	0.7	0.9					1.4	1.6	tr.	1.3	1.2	2.2	2.8	0.4
22:1	3.0	3.7	3.1	2.9	2.7	2.8					5.5	4.6	2.1	3.7	5.2	6.9	0.4	1.0
23:0 and 1	0.3	tr.	0.2	0.2	0.1	0.6												
24:0	0.5	0.4	0.3	0.6	tr.	0.4					0.2	0.4	tr.	0.6	0.1			
24:1	3.1	3.8	3.0	3.0	1.4	1.9					2.6	3.5	3.0	2.6	0.6			

* Glycerol ethers with branched hydrocarbon chains: [a] 0.3 % [b] 1.8 % [c] 4.5 % [d] 4.2 % [e] tr. [f] 0.7 % [g] 1.0 % [h] 1.3 % [i] 1.4 % [k] 3.1 %.

Table 3. Percentage of saturated components in the glycerol ethers of neutral lipids and phospholipids from various human material.

	Sat. % Neutral lipids	Sat. % Phospholipids
Colostrum	61	58
"Transition milk", 3–7 days	53	55
Milk, 8 days – 3 months	51	51
Red bone marrow	67	55
Red blood cells	42	72
Blood plasma	39	65
Uterine carcinoma	51	52

saturated. The degree of unsaturation increases with the chain length and the compounds with 24 carbon atoms are mainly unsaturated. The proportion of saturated glycerol ethers in the neutral lipids is higher in the colostrum than in other human milk samples. The red bone marrow has a higher proportion of saturated lipids than other human tissues studied (see Table 3). Only the saturated glycerol ethers, chimyl and batyl alcohol, have been found to stimulate the formation of blood cells.[1–10] The glycerol ethers of the neutral lipids in the bone marrow might be precursors to the glycerol ether phospholipids in the blood cells. It is interesting to note that the phospholipids of the red cells are relatively saturated (Table 3).

The composition of the methoxy-substituted glycerol ethers occurring in neutral lipids and phospholipids is demonstrated in Table 4. As the methoxy-substituted glycerol ethers are found only in minute quantities it was difficult to isolate them in a pure form. The principal components are 2-methoxy-substituted hexadecyl-, hexadecenyl-, and octadecenyl glycerol ethers. A characteristic feature of the methoxy-substituted glycerol ethers is the high content of ethers with 16 carbon atoms in the long alkyl chain. A poly-unsaturated methoxy-substituted glycerol ether with 6 double bonds, 1-*O*-(2-methoxydocosahexaenyl)glycerol was found in both the neutral lipids and the phospholipids of red blood cells. This poly-unsaturated glycerol ether was first found in Greenland shark liver oil.[26]

In the present study we have thus demonstrated that 2-methoxy-substituted glycerol ethers are of common occurrence but in minute

Table 4. Percentage composition of methoxy-substituted glycerol ethers from neutral lipids (N) and phospholipids (P).

Long chain component	Human "transition milk", 3–7 days		Human milk, 8 days–3 months		Cow's milk		Sheep's milk	Human red bone marrow		Human red blood cells		Uterine carcinoma
	N	P	N	P	N	P	P	N	P	N	P	P
14:0	1.5	0.7	tr.	tr.						2.2	tr.	2.1
15:0	0.9	1.1	tr.	tr.						2.0	tr.	1.0
16:0	71.6	84.9	75.8	77.4	92.0	91.3	predominant	26.8	15.2	54.7	28.6	77.1
16:1					5.3	8.7	tr.	21.4	49.1		27.7	
17:0	0.8	0.5	0.6	0.3				2.5	0.6	2.2	2.1	0.2
17:1	3.5	1.0	3.3	2.7				6.4	3.5	2.2	8.2	2.7
18:0	1.8	2.6	1.8	1.2	2.0	tr.	tr.	7.8	2.1	1.7	3.7	0.9
18:1	19.9	8.0	18.5	16.7	0.7	tr.	tr.	20.0	16.4	20.7	19.3	16.0
19:0		0.5		0.7				5.1	2.7	0.4	0.6	
19:1		0.7		0.1				3.2	4.5	1.1	2.3	
20:0				0.2						tr.	tr.	
20:1				0.2				4.5	1.8	3.2	tr.	
21:0				0.1								
21:1				0.2				2.3	2.6			
22:0				tr.							tr.	
22:1				0.2				tr.	1.5			
22:6										9.6	7.5	

quantities. In some experiments the isolation of the glycerol ethers has been performed using ethanol instead of methanol to allay any suspicion that the methoxy-substituted glycerol ethers could be artefacts from methanol treatment.

The physiological functions of the two types of glycerol ethers, the unsubstituted and the 2-methoxy-substituted ones, have not been clarified. The chimyl and batyl alcohols stimulate blood cell formation [1-10] and the 2-methoxy-substituted glycerol ethers have inhibitory effects on tumour growth and dissemination of metastases.[22,23] Further studies to elucidate the metabolism and physiological functions of the methoxy-substituted as well as of the unsubstituted, ordinary glycerol ethers are therefore indicated.

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On the Occurrence of 1-*O*-(2-Methoxyalkyl)glycerols and 1-*O*-Phytanylglycerol in Marine Animals

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1-*O*-(2-Methoxyalkyl)glycerols were isolated from the lipids of herring fillets, Baltic herring fillets, mackerel fillets, marine crayfish, fresh-water crayfish, shrimps, sea mussels, and cod liver oil. They were found both in the neutral lipids and in the phospholipids in considerably higher amounts than in mammalian tissues. The methoxy-substituted glycerol ethers had a high content of ethers with 16 carbon atoms in the long alkyl chains. The compounds with 16 and 18 carbon atoms together usually constituted over 90 %. The composition is roughly similar in the neutral lipids and in the phospholipids. A poly-unsaturated compound, 1-*O*-(2-methoxydocosahexaenyl)glycerol was found in the neutral lipids of mackerel fillets and cod liver oil and in the phospholipids of shrimps. A glycerol ether with a phytanyl chain was isolated from the liver oil of cod caught in the Baltic sea. The content and composition of the unsubstituted glycerol ethers have been determined for comparison.

2-Methoxy-substituted glycerol ethers were first isolated from Greenland shark liver oil.[1] They were also found in human milk, cow's milk, sheep's milk, and different human tissues, *viz.* red bone marrow, red cells, blood plasma, and in a uterine carcinoma.[2] The methoxy-substituted glycerol ethers had antibiotic activity and inhibited the dissemination and growth of several experimental tumours in mice.[3,4] As they occur in a relatively high concentration in shark liver oil compared to the minute amounts found in the mammalian tissues studied, it was of interest to determine the content of 2-methoxy-substituted glycerol ethers in different marine animals. The distribution of the compounds between the neutral lipids and the phospholipids was also studied.

MATERIAL AND METHODS

The material studied were fillets from herring (*Clupea harengus*), Baltic herring (*Clupea harengus*), mackerel (*Scomber scomber*), marine crayfish (*Nephrops norvegicus*), fresh-water crayfish (*Astacus fluviatilis*), shrimps (*Pandalus borealis*), and sea mussels (*Mytilus edulis*). Only the edible parts of the crayfishes, shrimps and sea mussels were included in the samples investigated. Both a commercial sample of cod liver oil and liver oil from cod caught in the Baltic sea were analyzed.

The extraction of the lipids, the separation of neutral lipids and phospholipids, the isolation of glycerol ethers and their isopropylidene derivatives, gas chromatography and mass spectrometry were performed as earlier described.[2] As before,[2] ethanol was used instead of methanol in some experiments in order to exclude the possibility of artefacts from methanol treatment.

RESULTS AND DISCUSSIONS

The methoxy-substituted glycerol ethers like the unsubstituted ones occur both in the neutral lipids and in the phospholipids (Table 1). The percentage of both groups of glycerol ethers is usually much higher in the phospholipids than in the neutral lipids. Due to the large proportion of neutral lipids in herring, Baltic herring, and mackerel, about the same quantities of glycerol ethers are found in the two lipid fractions of these species.

The content of methoxy-substituted glycerol ethers is higher in the neutral lipids and especi-

Table 1. The content of unsubstituted and methoxy-substituted glycerol ethers in the neutral lipids (N) and the phospholipids (P) isolated from marine animals.

Material	Lipids	Neutral lipids (N) and phospholipids (P) in total lipids		Unsubstituted glycerol ethers [a] in N and P		Methoxy-substituted glycerol ethers [a] in N and P	
	% (w/w)	% (w/w)		% (w/w)		% (w/w)	
		N	P	N	P	N	P
Herring fillets	17.3	94.4	5.6	0.05	0.57	0.02	0.14
Baltic herring fillets	10.4	91.4	8.6	0.07	0.54	0.01	0.14
Mackerel fillets	20.7	95.5	4.5	0.06	1.10	0.02	0.22
Marine crayfish	2.3	53.9	46.1	1.20	1.30	0.17	0.35
Fresh-water crayfish	4.3	69.6	30.4	0.65	1.70	0.07	0.06
Shrimps	2.8	47.6	52.4	1.20	1.70	0.08	0.19
Sea mussels	2.1	54.9	45.1	1.10	1.90	0.08	0.47
Cod liver oil, commercial sample	100	100		0.05		0.03	
Liver oil from cod caught in the Baltic sea	100	100		0.05 [b]		0.02	

[a] The figures should be multiplied by a factor of about 2.0–2.5 to get the content of the original glycerol ether lipids in the lipid fractions. [b] In addition this cod liver oil contained 0.03 % phytanyl glycerol ethers.

ally in the phospholipids of the marine animals studied than in the lipids of the mammalian tissues investigated.[2] The principal components of the methoxy-substituted glycerol ethers are the same in the marine animals as in the mammalian tissues. The compounds with 16 and 18 carbon atoms in the long hydrocarbon chains amount to over 90 % of the methoxy-substituted glycerol ethers of both the neutral lipids and the phospholipids (Table 2). The compounds with C_{16} chains, especially, are usually found in high concentrations. Only small amounts of methoxy-substituted glycerol ethers with less than 16 carbon atoms in the long alkyl chains are found. A poly-unsaturated methoxy glycerol ether, 1-*O*-(2-methoxydocosahexaenyl)glycerol, was found in the neutral lipids of mackerel fillets, in the liver oil from the cod caught in the Baltic sea, and in the phospholipids of shrimps. Such a poly-unsaturated methoxy-substituted glycerol ether was first isolated from Greenland shark liver oil [5] and has also been found in the lipids of human red blood cells.[2]

The content of unsubstituted glycerol ethers in the lipids of the herring, Baltic herring and mackerel fillets (Table 1) is comparable with the amounts of these glycerol ethers found in the mammalian tissues.[2] A somewhat higher content of unsubstituted glycerol ethers was found in the crayfish, shrimps, and mussels. Cod liver oil contains small quantities of glycerol ethers compared to the large amounts found in shark liver oil.[1,6] The composition of the unsubstituted glycerol ethers of the neutral lipids and of the phospholipids is roughly similar (Table 3). The compounds with even-numbered long hydrocarbon chains are the principal components and among them those with 16 and 18 carbon atoms dominate. The unsubstituted glycerol ethers of the neutral lipids of herring fillets, mackerel fillets, crayfish, shrimps, and sea mussels contain more saturated C_{16} and C_{18} components than cod liver oil. Fairly large amounts of C_{14} compounds are present in the unsubstituted glycerol ethers of both the neutral lipids and the phospholipids of herring and mackerel fillets.

Table 2. The percentage composition of methoxy-substituted glycerol ethers in neutral lipids (N) and phospholipids (P) from marine animals.

Long chain component	Herring fillets		Baltic herring fillets		Mackerel fillets		Marine crayfish		Fresh-water crayfish		Shrimps		Sea mussels		Cod liver oil, commercial sample	Liver oil from cod caught in the Baltic sea
	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	N
14:0	0.3	0.2					0.9	0.7	1.4	1.4						tr.
14:1							0.1									0.9
15:0		0.3	1.7		tr.		2.5	0.4	2.2	1.3						0.2
15:1							1.1	0.4								0.7
16:0	30.0	41.7	29.5	36.6	46.9	54.6	49.6	27.2	53.1	32.3	45.4	23.5	51.7	66.3	4.4	3.3
16:1	37.1	24.4	21.4	17.7	32.4	28.0	31.0	40.6	2.4	9.8	30.7	42.7	8.7	10.9	55.7	15.1
17:0	1.7	2.0	1.0	1.1	1.0	0.9	2.2	1.6	3.6	3.4	1.9	0.9	2.9	1.7	1.2	1.3
17:1	3.2	3.4	4.0	4.1	3.0	3.7	2.4	4.4	2.8	2.5	3.7	4.6		1.0	2.4	5.7
18:0	4.5	5.1	3.4	1.6	1.4	0.8	4.2	0.9	28.2	16.7	7.3	0.9	20.5	9.0	0.9	0.8
18:1	20.1	21.6	39.0	35.1	14.8	12.0	4.1	23.3	4.4	28.1	8.1	25.7	5.5	7.4	34.5	69.5
19:0	0.4	0.3		0.3		tr.	0.3	0.1	0.9	1.0	1.1	0.4	1.3	0.5	0.1	0.5
19:1	0.5	0.3		0.5	tr.		0.4	0.2	0.3	1.4	0.4	0.6	tr.	tr.	0.2	0.3
20:0	1.6	0.2	tr.	0.3			0.6	0.1	0.6	0.9	1.4	tr.	8.1	3.2	0.1	
20:1	0.2	0.2		0.9	tr.		tr.	0.1	0.1	1.2		0.3	1.3			
20:2																
21:0	0.1	tr.														
21:1	tr.	0.1														
22:0	0.2	tr.		0.4												
22:1	0.1	0.2		1.4			0.6								0.5	
22 poly					0.5							0.4				1.7

Table 3. The percentage composition of unsubstituted glycerol ethers in neutral lipids (N) and phospholipids (P) from marine animals.

Long chain component	Herring fillets		Baltic herring fillets		Mackerel fillets		Marine crayfish		Fresh-water crayfish		Shrimps		Sea mussels		Cod liver oil, commercial sample	Liver oil from cod caught in the Baltic sea
	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	N
12:0 and 1			0.5										0.9		1.4	1.6
14:0	15.2	14.3	13.9	16.8	12.0	6.5	3.8	17.3	4.1	2.6	4.8	5.0	3.0	8.5	5.5	1.2
14:1	5.9	3.9	6.4	3.8	1.6	1.1	0.6	2.8			0.5	0.2			15.5	4.9
15:0	2.2	2.6	1.2	1.7	2.3	1.9	3.2	6.6	tr.	tr.	2.8	3.1	0.9	2.7	0.5	tr.
15:1	tr.	1.3					1.6	5.1							1.9	1.1
16:0	41.0	36.2	57.7	52.2	43.7	40.5	36.4	27.5	41.5	48.0	44.5	50.9	20.8	51.2	13.4	3.6
16:1	13.1	10.8	3.9	3.9	6.6	3.4	12.6	24.3	5.9	8.7	8.4	10.3	5.9	2.8	19.2	14.7
17:0	0.8	3.2	0.1	1.1	2.4	2.3	2.7	1.1	3.6	3.7	2.9	3.6	4.8	5.4	1.8	
17:1	0.5	1.3					2.2	1.2	2.2	2.1				tr.	2.2	1.7
18:0	3.3	4.6	1.6	2.7	4.6	9.5	10.8	4.3	29.3	24.5	10.9	13.0	41.6	17.5	2.7	2.3
18:1	14.1	19.2	8.8	15.9	22.6	31.4	12.4	5.4	10.4	7.3	10.3	10.7	9.3	4.4	23.2	33.3
18:2			tr.				2.4	1.2						2.1		
19:0	0.3	tr.			0.1	0.1	0.5	0.3	1.3	1.7	0.2	0.1	1.1	0.3	0.1	
19:1	0.3	0.3	0.1		0.2	0.9	1.2	0.5	0.4	0.3	0.9	0.7	tr.	0.1	0.7	0.1
20.0	0.2	0.3	0.4	0.3	0.3	0.2	0.6	0.2	0.4	0.4	0.4	0.2	0.1	0.1	0.2	33.4 [a]
20:1	2.8	1.8	4.1	1.2	3.1	1.6	5.9	1.8	0.9	0.7	12.3	2.2	11.0	4.6	8.2	0.7
20:2							0.7	0.1								0.6
21:0	tr.	tr.											tr.		0.1	
21:1	tr.	tr.	0.2		0.2						0.3	tr.			0.1	0.1
22:0	tr.	tr.											0.4	0.1	0.7	
22:1	0.3	0.2	1.1	0.4	0.3	0.6	2.4	0.3	tr.	tr.	0.8	tr.	0.2	0.2	2.6	0.3
22 poly																0.4

[a] Phytanyl 33.3.

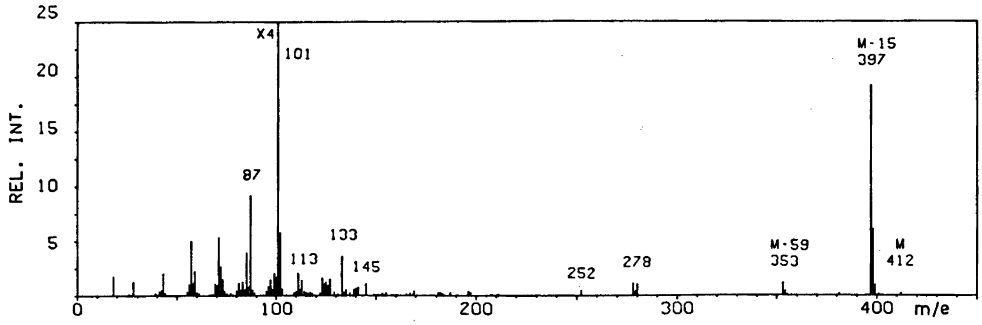

Fig. 1. Mass spectrum of the isopropylidene derivative of phytanylglycerol, isolated from cod liver oil.

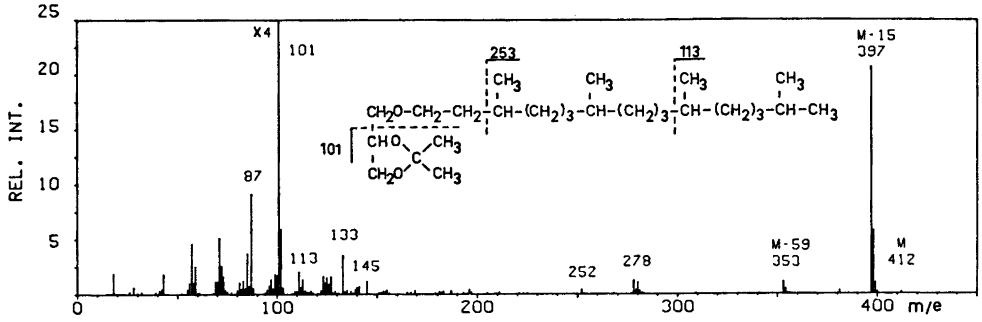

Fig. 2. Mass spectrum of synthetic 1-*O*-phytanyl-2,3-*O*-isopropylideneglycerol.

In the liver oil from cod caught in the Baltic sea a glycerol ether with a phytanyl chain was found. The phytanyl glycerol ether amounted to about a third of the whole glycerol ether mixture. A somewhat higher R_F-value in thin-layer chromatography (TLC) than for batyl alcohol both before and after acetonation indicated that the compound could be a glycerol ether with a branched hydrocarbon chain. This was supported by the IR spectrum of the compound, which showed strong bands at about 3450 (OH), 2960 (CH_3), 1365 – 1375 doublet ($C(CH_3)_2$), and 1100 cm^{-1} (C – O – C).[7] The mass spectrum indicated a molecular weight of 412 for the isopropylidene derivative. For comparison 1-*O*-phytanyl-2,3-*O*-isopropylideneglycerol and 1-*O*-phytanylglycerol were synthesized from dihydrophytol and isopropylideneglycerol in the same manner as earlier described.[8] The IR spectra of the synthesized compounds were identical with those of the compounds from cod liver oil, both as free glycerol ethers and as isopropylidene derivatives. The R_F-values in TLC were also identical. Analyses by GLC – MS of the isopropylidene derivatives gave the same retention times and identical mass spectra (Figs. 1 and 2).

A diphytanyl glycerol ether isolated from *Halobacterium cutirubrum* has been studied in detail by Joo *et al.*[7] They found that the diether had the unusual L-configuration. They also found small amounts of α-monophytanylglycerol ($[\alpha]_D - 0.95°$) which, however, had been formed from the diether during acid hydrolysis.[9] Unfortunately the amount of the phytanyl ether isolated from cod liver oil was not enough for determination of the optical rotation.

The phytanyl chain probably originates from chlorophyll. The composition of the glycerol ethers is thus obviously dependent on the diet of the animals. The glycerol ethers of the freshwater crayfish are somewhat more saturated than those of the marine crayfish. This is also true for Baltic herring fillets compared to the herring fillets. These differences might be related to different dietary intake or other environmental factors, *e.g.* water temperature.

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Reaction of Acetylated Methyl Glycosides with Hydrogen Bromide

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Treatment of acetylated methyl glycopyranosides with hydrogen bromide in acetic acid gave, in addition to the expected pyranosyl bromides, large amounts of furanosyl bromides. The reaction was studied with a number of methyl hexopyranosides and pentopyranosides and in almost all cases furanosyl bromides were found to be the major products. Methyl tri-*O*-acetyl-β-D-ribopyranoside was almost completely converted into a mixture of the anomeric tri-*O*-acetyl-D-ribofuranosyl bromides by treatment with hydrogen bromide in acetic acid for 2 h.

It was reported previously that treatment of methyl tri-*O*-acetyl-β-D-arabinopyranoside with hydrogen bromide lead to formation of tri-*O*-acetyl-D-arabinofuranosyl bromide in addition to the expected pyranosyl bromide. A mechanism for the ring-contraction was proposed, and it was shown that the benzoylated arabinoside did not undergo ring-contraction with hydrogen bromide.[1]

In the present paper the behaviour of a number of acetylated glycosides towards hydrogen bromide in acetic acid is described. In most cases the reactions were studied by NMR spectroscopy only. In these experiments the acetylated glycosides were dissolved in a solution of 30 % hydrogen bromide in glacial acetic acid in an NMR sample tube and kept at room temperature. NMR spectra were then run at intervals at 90 MHz until no further changes were observed. In Table 1 are given the results of a number of such experiments.

The products formed were identified from the chemical shifts of the anomeric protons of the glycosyl bromides, which were the final products. The relative amounts of products were calculated from the integrated spectra. Authentic spectra of the products shown in Table 1 were obtained from fully acetylated pyranoses and furanoses under the same conditions. Fully acetylated sugars form glycosyl bromides rapidly and ring-changes do not take place, even on prolonged treatment with hydrogen bromide.

The results obtained from these experiments show that when the reactions of acetylated methyl pyranosides with hydrogen bromide are completed furanosyl bromides are the main product in almost all cases (Table 1). Methyl tri-*O*-acetyl-β-D-arabinopyranoside, which was investigated previously,[1] undergoes ring-contraction to a smaller extent than any of the other methyl glycopyranosides investigated. Methyl tri-*O*-acetyl-β-D-ribopyranoside is almost completely converted into a mixture of the anomeric tri-*O*-acetyl-D-ribofuranosyl bromides.

In some cases signals, which could not be ascribed to pyranosyl or furanosyl bromides, were observed. These are probably due to the formation of open-chain, *aldehydo*-derivatives. Only in the case of methyl tri-*O*-acetyl-α-D-lyxopyranoside was such a compound formed in larger amounts; it probably has the structure (*11*). The formation of open-chain derivatives by acetolysis of acetylated methyl glycosides is well known.[2,3]

Acetylated methyl α-D-mannofuranoside and ethyl β-D-galactofuranoside underwent ring-expansion to some extent to give pyranosyl bromides when treated with hydrogen bromide (Table 1). This was also the case with methyl tri-*O*-acetyl-α-D-arabinofuranoside.[1] According to the proposed mechanism [1] ring-changes take place with the methyl glycosides and not with glycosyl bromides. Since methyl furanosides give glycosyl bromides more rapidly

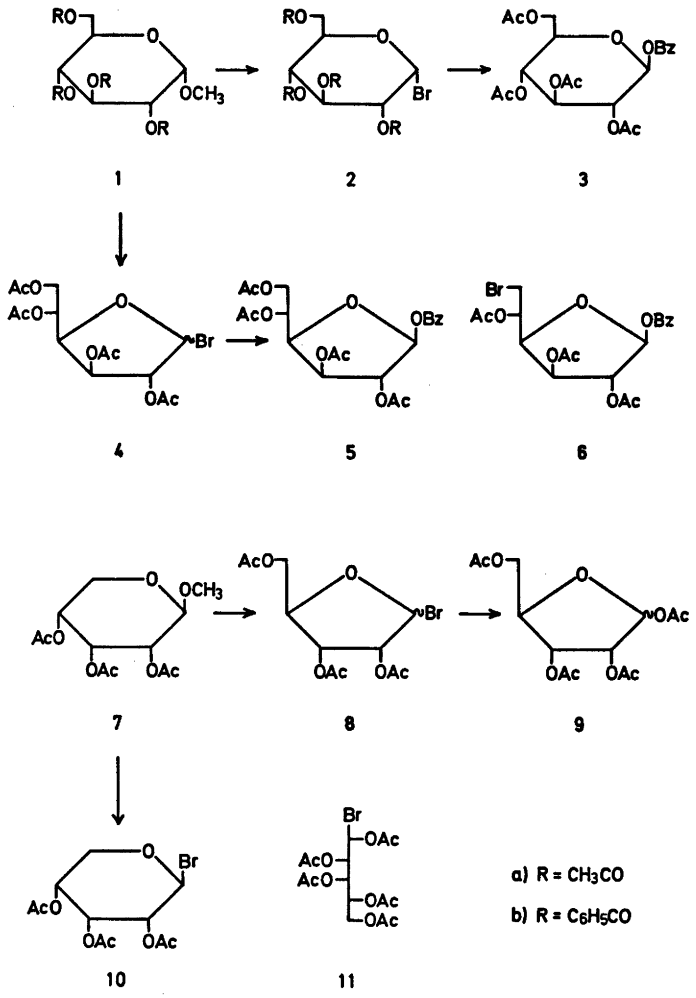
Table 1. Chemical shifts and coupling constants of acetylated glycosyl bromides formed from acetylated glycosides in hydrogen bromide-acetic acid.

Compound, fully acetylated	δ-Value H1, J_{12}(Hz) % Yield				fur./ pyr.	Time for completion of reaction h
	pyranose	α-furanose	β-furanose	*aldehydo*-form		
Methyl α-glucopyranoside	6.79 3.8 41	6.99 4.8 26	6.51 ~0 33	6.82 ~0 trace	1.44	24
Methyl β-glucopyranoside	6.79 3.8 40	6.98 4.8 25	6.51 35		1.50	24
Methyl α-mannopyranoside	6.63 ~0 42	6.58 ~0 46	6.78 3.0 12		1.4	24
Methyl α-mannofuranoside	6.63 ~0 10	6.58 ~0 72	6.78 3.0 18		9	1
Methyl α-galactopyranoside	6.83 3.8 32		6.58 ~0 68		2.1	5
Methyl β-galactopyranoside	6.83 3.8 33		6.58 ~0 67		2.0	5
Ethyl β-galactofuranoside	6.83 3.8 16		6.58 ~0 84		6	2
Phenyl β-galactopyranoside	6.83 3.8 93		6.58 ~0 7		0.08	3
Methyl α-xylopyranoside	6.76 4.0 28	6.97 5.0 41	6.50 ~0 31	6.82 ~0 trace	2.6	2
Methyl β-xylopyranoside	6.76 4.0 45	6.97 5.0 31	6.50 ~0 24	6.82 ~0 trace	1.2	2
Methyl α-lyxopyranoside	6.53 ~0 9	6.62 ~0 50		6.83 ~0 34	5.5	2
Methyl β-arabinopyranoside	6.80 3.5 50	6.57 ~0 50			1	2
Methyl β-ribopyranoside	6.59 ~0 9	6.87 4.0 36	6.54 ~0 55	6.82 ~0 trace	10	2
2,3,4-Tri-*O*-acetyl-xylopyranose	6.76 4.0 65	6.97 17	6.50 18		0.5	0.5

than pyranosides (Table 1) it is understandable that they undergo ring-expansion to a small extent only.

Phenyl tetra-*O*-acetyl-β-D-galactopyranoside gave only a small amount of furanosyl bromide when treated with hydrogen bromide. 2,3,4-Tri-*O*-acetyl-D-xylopyranose gave considerable amounts of furanosyl bromides, but not as much as the corresponding methyl xylopyranosides.

In order to confirm the results shown in Table 1 products were isolated and characterized in two cases. Treatment of methyl tetra-*O*-acetyl-α-D-glucopyranoside (*1a*) with hydrogen bromide in acetic acid for 20 h gave a mixture of glycosyl bromides (*2a*) and (*4*) which were very unstable. They were therefore treated with silver benzoate to give 1-*O*-benzoates. From this product the pyranose (*3*) and the furanose (*5*) were isolated. Besides, 2,3,5,6-tetra-*O*-acetyl-D-glucofuranose was obtained, probably resulting from hydrolysis of the bromide (*4*). A small amount of the 6-bromo-6-deoxy-glucofuranose derivative (*6*) was



also isolated. Thus the reaction of (*1a*) with hydrogen bromide gives a complicated mixture of products, and it is understable that Zemplén [4] could not isolate acetobromoglucose from this reaction.

Treatment of the tetrabenzoate (*1b*) with hydrogen bromide gave a good yield of tetra-*O*-benzoyl-α-D-glucopyranosyl bromide (*2b*).

Methyl tri-*O*-acetyl-β-D-ribopyranoside (*7*) is almost completely converted into a mixture of the anomeric furanosyl bromides (*8*) when treated with hydrogen bromide (Table 1). Since these bromides are unstable the reaction mixture was treated with acetic anhydride and zinc bromide in order to convert them into the tetraacetates (9) which could then be isolated in 58 % yield.

EXPERIMENTAL

Melting points are uncorrected. NMR spectra were recorded on a Bruker HX-90E instrument. The spectra shown in Table 1 were measured on solutions which contained *ca.* 10 % carbohydrate in 30 % HBr/HOAc. The chemical shifts were measured relative to internal chloroform. Thin layer chromatography (TLC) was performed on silica gel PF_{254} (Merck): for preparative work 1 mm layers on 20 × 40 cm plates were used. Spots were visualized with UV light or by charring with a hot wire.

*Methyl tetra-*O*-acetyl-α-*D*-glucopyranoside (1a)* (1.0 g) was dissolved in 4 ml of 30 % HBr/HOAc and the solution was kept at room temp. for 20 h. It was then diluted with dichloromethane, washed with water and aqueous $NaHCO_3$, dried ($MgSO_4$) and evaporated. The crude product (1.15 g) was dissolved in dry acetonitrile (25 ml) and stirred over night with silver benzoate (3.0 g). The mixture was filtered through carbon and the solvent was evaporated. The residue was dissolved in dichloromethane and washed with aqueous $NaHCO_3$, dried and evaporated. The syrupy product was separated into several fractions by preparative TLC using diethyl ether – pentane (2:1) as eluent.

The fastest moving fraction gave 77 mg (6 %) of 2,3,5-tri-*O*-acetyl-1-*O*-benzoyl-6-bromo-6-deoxy-β-D-glucofuranose (*6*) as a syrup, $[\alpha]_D^{20}$ −64.7° (*c* 2.1, $CHCl_3$). (Found: C 48.34; H 4.60; Br 17.12. Calc. for $C_{19}H_{21}BrO_9$: C 48.22; H 4.47; Br 16.89). A 100 MHz NMR spectrum gave the following δ-values and coupling constants (Hz): H1 6.43; H2 5.30; H3 5.55; H4 4.69; H5 5.25; H6 3.67; H6′ 3.76. J_{12} ~0; J_{13} 0.3; J_{23} 0.8; J_{34} 4.7; J_{45} 9.4; J_{56} 3.2; $J_{56'}$ 4.0; $J_{66'}$ −11.8.

The next two fractions could not be identified. The fourth fraction gave 351 mg (28 %) of a mixture of (*3*) and (*5*) in a ratio of 4:1 as seen from an NMR spectrum. Crystallization and recrystallization from ethanol gave tetra-*O*-acetyl-1-*O*-benzoyl-β-D-glucopyranose (*3*), m.p. 140 – 141 °C, $[\alpha]_D^{20}$ −25.5° (*c* 2.9, $CHCl_3$) (reported [5] m.p. 143 – 145°, $[\alpha]_D$ −26.6°). 1-*O*-Benzoyl-tetra-*O*-acetyl-β-D-glucofuranose (*5*) was identified in the mother liquor from (*3*) by comparing its NMR spectrum with that of an authentic sample.[6]

The last fraction gave 140 mg (14.5 %) of a product which consisted mainly of 2,3,5,6-tetra-*O*-acetyl-D-glucofuranose as seen from an NMR spectrum. Acetylation gave a mixture of the anomeric penta-*O*-acetyl-D-glucofuranoses the NMR spectra of which were identical with those previously reported.[7]

*Methyl tetra-*O*-benzoyl-α-*D*-glucopyranoside (1b)* (1.0 g) was dissolved in dichloromethane (1.0 ml) and HBr/HOAc (5.0 ml) was added. The solution was kept for 3 days at room temp. and worked up as described above. The product was crystallized from ether to give 705 mg (65 %) of tetra-*O*-benzoyl-α-D-glucopyranosyl bromide (*2b*), m.p. 122 – 124 °C. One recrystallization gave the pure product, m.p. 125 – 125.5 °C, $[\alpha]_D^{20}$ +123.1° (*c* 1.4, $CHCl_3$) (reported [8] m.p. 129 – 130 °C (corr.), $[\alpha]_D$ +124°). The material in the mother liquor consisted of the same bromide and *ca.* 10 % unreacted (*1b*) as seen from an NMR spectrum. No furanosyl bromide could be detected.

*Methyl tri-*O*-acetyl-β-*D*-ribopyranoside (7)* (2.19 g) was kept in HBr/HOAc (6.0 ml) for 1.5 h at room temp. Acetic anhydride (35 ml) and anhydrous zinc bromide (100 mg) were then added and the mixture was stirred for 15 min. It was then poured on ice and stirred for 3 h. The product was extracted with chloroform; the solution was washed with water and aqueous $NaHCO_3$, dried and evaporated. The product (2.31 g) consisted mainly of a mixture of the anomeric tetra-*O*-acetyl-D-ribofuranoses (9) in an α:β ratio of 1:3 as seen from an NMR spectrum. Crystallization from ethanol gave 510 mg (21 %) of the β-anomer (*β9*), m.p. 55 – 58 °C. Preparative TLC of the material in the mother liquor gave 892 mg (37 %) of α,β-mixture. Crystallization of this from ethanol gave 300 mg of (*β9*), m.p. 56 – 58 °C. Recrystallization from ethanol gave a product with m.p. 57 – 59 °C, $[\alpha]_D^{20}$ −12.7° (*c* 4.5, $CHCl_3$). This is the low melting form of tetra-*O*-acetyl-β-D-ribofuranose;[9] we have not been able to obtain the high melting form.[10] An NMR spectrum was in agreement with the structure and showed no impurities.

Microanalyses were performed by Dr. A. Bernhard Mikroanalytisches Laboratorium.

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Studies on Organophosphorus Compounds. IX.* Hexamethylphosphoric Triamide (HMPA) as Reagent in a New Quinoline Synthesis

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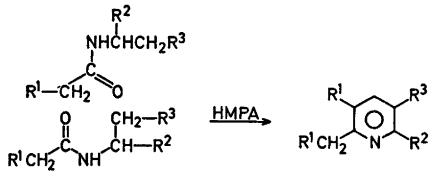
Acetanilides, refluxed in DMF and HMPA, produce 2-dimethylamino-quinolines in 40 – 76 %. For other anilides the yields decreased when the size of the acyl-group increased. A mechanism is suggested in which the anilide first produces the corresponding *N*,*N*-dimethyl-amidine, which by further reaction with DMF gives the quinoline. The kinetics of the formation of the amidines showed that the reactions were autocatalyzed by $(Me_2N)_3P(O)OH$. Also it was found that acetanilide, when refluxed in HMPA and acetic acid derivatives such as phenylacetate and *N*,*N*-dimethyl-acetamide, gave the corresponding 2-dimethylamino-4-methyl-quinoline. Furthermore 23 – 54 % yield of 2-dimethylamino-4-methyl-quinolines were formed when anilines were refluxed in acetic acid and HMPA.

In an earlier investigation [1] it was demonstrated that gentle reflux of secondary carboxamides (RCONHR′, where R or/and R′ is aromatic) in hexamethylphosphoric triamide (HMPA) produced the corresponding *N*,*N*-dimethyl-amidines in fair yields. However, if R or R′ were able to form sufficiently stable carbonium ions fragmentation reactions [2] were found. It was also found that gentle reflux of aliphatic secondary carboxamides produced pyridines,[3] eqn. 1, and it was suggested that the pyridine was formed by the reaction of two different carboxamide molecules. This was confirmed by the observation that a mixture of acetanilide and *N*-isopropylpivaloamide heated in HMPA formed 2-*t*-butyl-6-methylpyridine. In a similar experiment it was attempted to prepare 5,6,7,8-tetrahydroquinoline from acetanilide and *N*-cyclohexyl-formamide by heating in HMPA. Quite unexpectedly the formamide reacted in a different way with acetanilide and instead 2-dimethylamino-quinoline was obtained in 51 % yield. As this new quinoline synthesis was very promising – easy available starting materials, simple reaction conditions, fair yield *etc.* – a thorough investigation was made on this reaction and this paper describes the results of that.



(1)

RESULTS AND DISCUSSION

In the reaction of acetanilide and *N*-cyclohexylformamide with HMPA at reflux temperature cyclohexylamine is split off and 2-dimethylamino-quinoline is produced. It was therefore expected that dimethylformamide (DMF) should undergo a similar reaction and that was indeed found, (Table 1).

Furthermore DMF is a very convenient reagent as side reactions, shown in eqn. 1, are excluded in this quinoline synthesis. The substituents R on the aromatic ring of the acetanilide give no systematic variations of the yields of the quinolines, which are in the order of

* Part VIII, Vesterager, N. O., Pedersen, E. B. and Lawesson, S.-O. *Tetrahedron 30* (1974) 2509.

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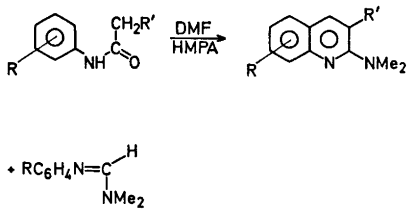
Table 1.

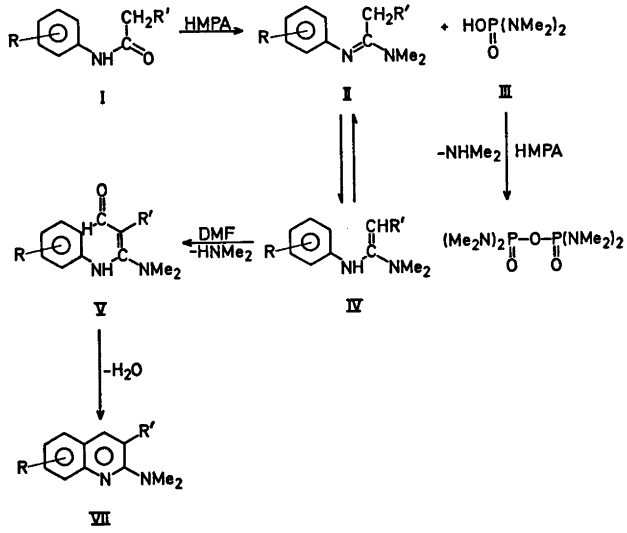


R	R′	Quinoline (%)	Amidine (%)
o-CH_3	H	76	3
p-CH_3	H	61	
o-OCH_3	H	40	
p-OCH_3	H	51	
b-benzo	H	59	
H	H	72	< 1
H	CH_3	53	5
H	Et	26	~ 10
H	i-Pr	0	22

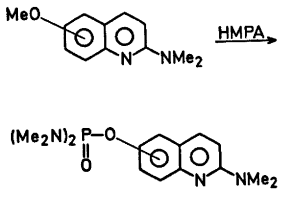
40 – 76 %. However, the substituents R′ had a marked influence on the yields, which decreased from 72 % to 0 %, when hydrogen was replaced by the isopropyl group, (Table 1).

Concerning the mechanism (Scheme 1) it is suggested that the first step is the formation of the corresponding *N*,*N*-dimethyl-amidine II, which is a known reaction when acetanilide is heated in HMPA.[1] In fact, when *N*,*N*-dimethyl-*N*′-phenyl-acetamidine is refluxed (as the corresponding anilide) in DMF and HMPA, 2-dimethylaminoquinoline was obtained in 60 % yield. The amidine II is then assumed to be in a tautomeric equilibrium with the highly reactive enamine IV, which undergoes a reaction with DMF to form V. The intermediate V then tautomerizes to its enol-form VI, which subsequently undergoes a ring closure reaction followed by elimination of water to form the quinoline VII. It might be possible that the hydroxyl group of the enol VI is replaced by a dimethylamino group as ketones are known to produce the corresponding *N*,*N*-dimethyl-enamines by heating in HMPA.[4] If this is the case, a ring closure reaction followed by elimination of dimethylamine also can give the quinoline VII. Octamethylpyrophosphoramide, OMPA, was isolated in the reaction of *p*-methyl- and *p*-methoxy-acetanilide with DMF and HMPA. Its presence in the reaction mixture is best explained by the reaction of primarily formed phosphoric acid derivative III with HMPA, (Scheme 1).

Furthermore, support of the suggested mechanism was also provided by the generation of dimethylamine, which easily could be followed during the reaction by the colour change of silica gel (light blue→dark blue) placed in a drying tube on top of the reflux-condenser. In the synthesis of 6- and 8-methoxy-2-dimethylamino-quinolines the yields were slightly reduced because of further reaction of the methoxy-group with HMPA, (eqn. 2).

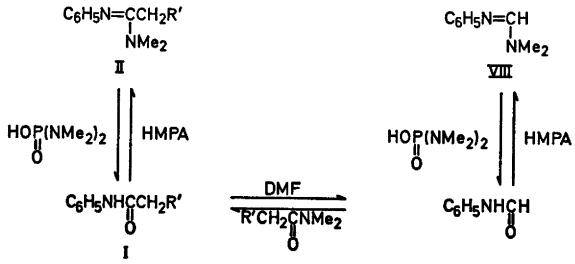


Scheme 1.

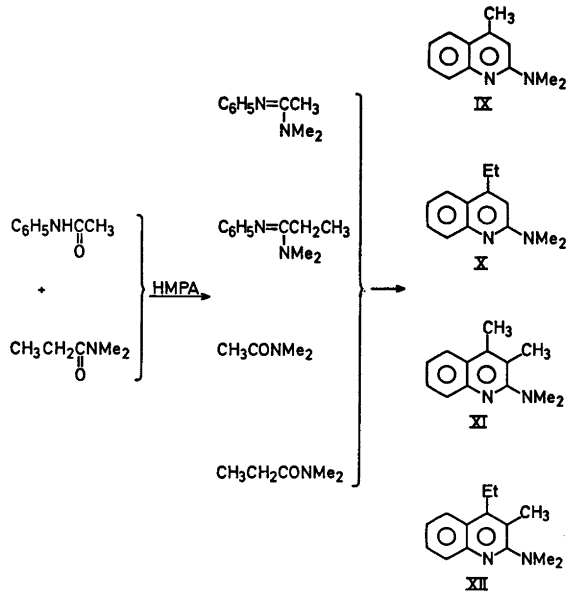


(2)

In the reaction of isovaleranilide no quinoline VII was isolated, but instead II (R′=i-Pr), VIII and *N*,*N*-dimethylvaleramide were found besides some starting material. As III is likely to be present in the reaction mixture (Scheme 2) the suggested equilibrium is quite appropriate. This equilibrium is shifted from VIII towards II as the amidine II is used up in the quinoline syntheses. This was further substantiated in the reaction of acetanilide with HMPA and DMF where only 1 % of VIII was isolated and the yield of the quinoline VII was as high as 72 % whereas the yields of VIII and VII in the reaction of valeramide were 22 % and 0 %, respectively, (Table 1). Equilibria as suggested in Scheme 2 can also produce a very complex reaction mixture in other cases. Thus the quinolines, IX – XII are found in the reaction of acetanilide with *N*,*N*-dimethyl-propionamide and HMPA, (Scheme 3). If the above equilibria were of minor importance, only the quinoline X should be formed by analogy to the formation of the quinoline VII (Scheme 1). It is quite obvious that, if *p*-methyl-acetanilide is refluxed in *N*,*N*-dimethyl-acetamide and HMPA, transacylation reactions cannot interfere with the quinoline synthesis and a high yield is obtained of the corresponding quinoline, (Table 2). Also phenyl acetate can be used in the reaction with acetanilide and HMPA and

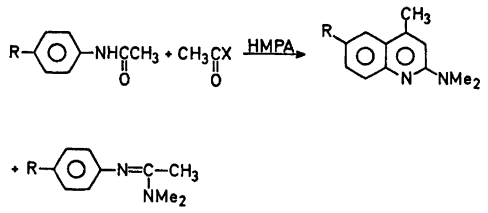


Scheme 2.



Scheme 3.

Table 2.

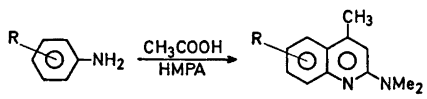


R	X	Quinoline (%)	Amidine (%)
CH_3	NMe_2	46	
H	OC_6H_5	52	
CH_3	p-CH_3-C_6H_4NH	18	44
CH_3O	p-CH_3O-C_6H_4NH	6	76
Cl	p-Cl-C_6H_4NH		64

the corresponding 2-dimethylamino-4-methyl-quinoline was obtained. In this reaction phenol should be produced in a similar way as dimethylamine did in the reaction of DMF with IV, (Scheme 1). In fact phenol was produced, but it reacted with HMPA as it has been shown recently [5] and $C_6H_5OPO(NMe_2)_2$ and $(C_6H_5O)_2$-$PO(NMe_2)$ were found in the reaction mixture. Furthermore in the synthesis of *N*-aryl-*N'*,*N'*-dimethylacetamidines from acetanilides in some cases the corresponding 2-dimethylamino-4-methyl-quinolines were obtained as by-products (Table 2). These by-products are produced by the reaction of the formed amidine with the starting material and clearly demonstrate that 2-dimethylamino-quinolines may be formed whenever an acetic acid derivative is heated together with an acetanilide in HMPA.

2-Dimethylamino-4-methyl-quinolines are formed when anilines are refluxed in acetic acid and HMPA (Table 3). The mechanism of this reaction is easily understood as it is known that acetic acid by heating in HMPA produces *N*,*N*-dimethyl-acetamide.[6] The so formed acetamide then undergoes transacylation reaction with the anilines to form acetanilides, which then produce the quinoline by reaction with the acetamide. By refluxing aniline in propionic acid and HMPA the corresponding quinoline, XII, was obtained in 17 %. The relatively low yield is not surprising as the size of the acyl group also has a profound influence on the yield in the reaction of the carboxamides I with DMF and HMPA (Table 1).

Table 3.



R	%
H	39
o-CH_3	35
p-CH_3	54
p-Cl	40
p-OCH_3	23

KINETICS

The reaction given in eqn. 3, which is observed when no DMF is added, is also the first step in the quinoline synthesis, Scheme 1.

$$\underset{\text{I}}{p\text{-R}-C_6H_4NHCOCH_3} \xrightarrow{\text{HMPA}} p\text{-R}-C_6H_4N=\underset{\text{NMe}_2}{\underset{|}{C}}CH_3 + \underset{\text{O}}{\underset{\|}{HOP}}(NMe_2)_2 \qquad (3)$$

II III

The conversion of the amide I to the amidine II in HMPA can easily be followed by NMR when no DMF is added as the *ortho*-protons to the amido-group in the aromatic ring of the acetanilides fall at a different field in NMR than all other aromatic protons of I and II. It

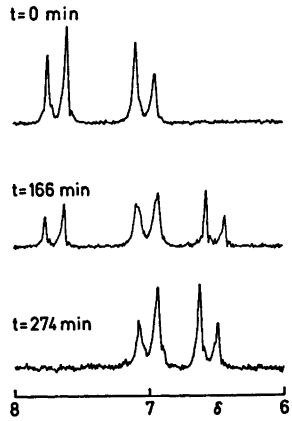


Fig. 1. Reaction of *p*-acetotoluidide with HMPA followed by NMR of aromatic hydrogen atoms.

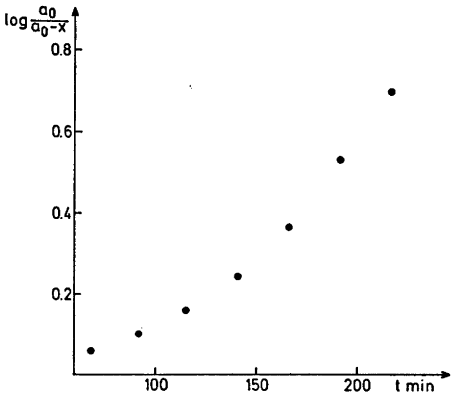


Fig. 2. Log $a_0/(a_0-x)$ *versus* t. 1.000 mol kg^{-1} *p*-acetotoluidide in HMPA.

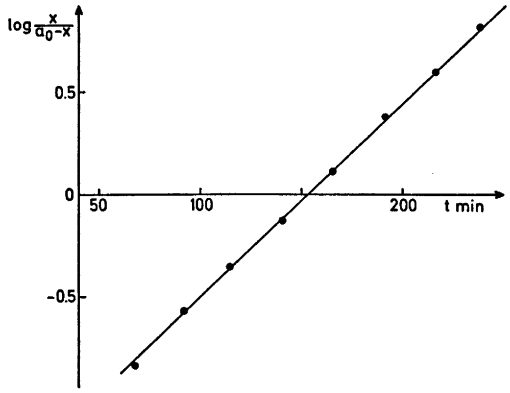


Fig. 3. Log $x/(a_0-x)$ *versus* t. 1.000 mol kg^{-1} *p*-acetotoluidide in HMPA.

should be noted that the number of aromatic hydrogens is constant during the reaction. An example is given in Fig. 1 for the reaction of *p*-acetotoluidide with HMPA at 210 °C. At this temperature only traces of dimethylamine could be detected. This indicates that almost no OMPA is formed by the reaction of III with HMPA at this temperature as dimethylamine is produced in this reaction, (Scheme 1).

The most simple approach to the kinetics of the reaction given in eqn. 3 is to assume that the reaction is pseudo first order with regard to the amide I if excess of HMPA is used. However, if log $a_0/(a_0-x)$, where a_0 is starting concentration of I and x the consumption of I, is plotted against the time, (Fig. 2), this reaction order is easily ruled out as no linear correlation is found. Instead if it is assumed that the reaction is autocatalysed the rate equations will be as follows:

$$d(a_0-x)/dt = -k(a_0-x)(x+c_0) \quad (4)$$

or

$$[1/(a_0+c_0)] \ln (x+c_0)/(a_0-x) = kt+K \quad (5)$$

where a_0 is initial concentration of I, x consumption of I as well as concentration of the catalyst produced during the reaction, c_0 is initial concentration of the catalyst, k rate constant, t time, and K constant. For *p*-acetotoluidide heated in HMPA, $c_0 = 0$, at 210 °C there is then found an excellent linear correlation when log $x/(a_0-x)$ is plotted against t, (Fig. 3).

At the start of the reaction where no catalyst is present this correlation is obviously not found. An induction period of 50 – 60 min was needed to produce the amount of catalyst, about 0.1 equivalent, necessary for the good linear correlation.

The rate constants for the reaction of *para* substituted acetanilides with HMPA at 210 °C were calculated by the least squares method (*para*-substituent, rate constant mol^{-1} kg min^{-1} given): Cl, 2.28×10^{-2}; H, 2.31×10^{-2}; CH_3, 2.22×10^{-2}; OCH_3, 2.11×10^{-2}. These rate constants can be correlated with σ_p^+ values but the found Hammett ϱ-value is so close to zero as 0.05 that no reasonable conclusion about the mechanism can be drawn.

In order to find out if III was the catalyst in the reaction it was prepared *in situ*

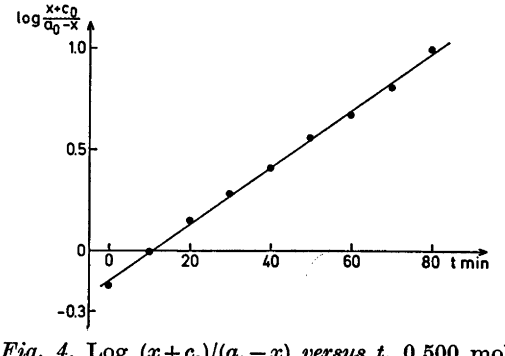
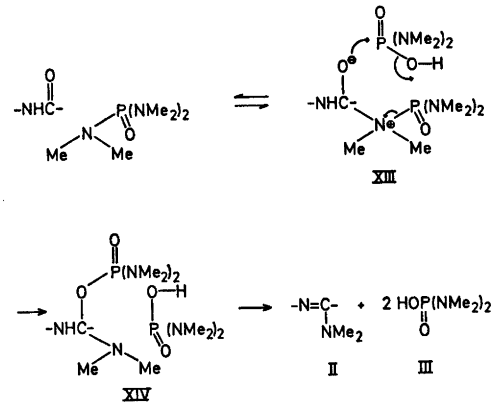
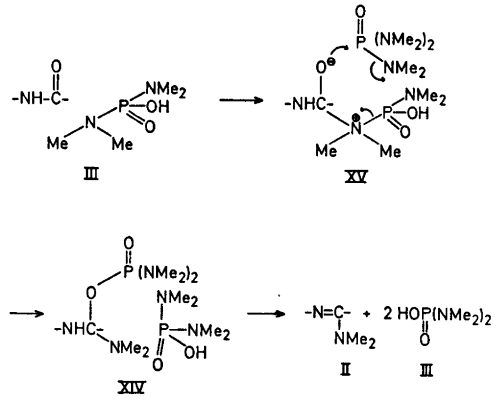


Fig. 4. Log $(x+c_0)/(a_0-x)$ *versus* t. 0.500 mol kg^{-1} NaOH, 0.500 mol kg^{-1} $(Me_2N)_2P(O)Cl$ and 1.000 mol kg^{-1} *p*-acetotoluidide in HMPA.

from $(Me_2N)_2P(O)Cl$ and powdered NaOH (1:1) in HMPA and then combined with *p*-acetotoluidide and heated. In fact if the reaction was followed by NMR and c_0 was assumed to be equal to the initial concentration of $(Me_2N)_2P(O)Cl$ and NaOH in eqn. 5, a linear correlation was obtained between log $(x+c_0)/(a_0-x)$ and t, (Fig. 4). Furthermore the rate constant, 2.19×10^{-2} mol^{-1} kg min^{-1} calculated by the least squares method was very close to the one obtained when no catalyst was added. Interestingly when the catalyst was added no induction periode was needed, instead 10 % of *p*-acetotoluidide was converted to the corresponding amidine even before the reaction temperature of 210 °C was reached. As the kinetics shows that the phosphoric acid derivative III is formed and also acts as a catalyst in the reaction of secondary carboxamides with HMPA, two mechanisms can easily be set up for the formation of the amidines; Schemes 4 and 5. In the first HMPA and the carboxamide are in equilibrium with the additions complex XIII, which in the rate determining step reacts with III to give XIV and III is then recovered. Compound XIV then produces the amidine II by an elimination reaction. In the other mechanism the carboxamide by reaction with III forms the additions complex XV, which by reaction with HMPA also produces XIV and III. The last step is then the same as above. The phosphoric acid derivative III is probably partly deprotonated by the amidine formed during the reaction or by HMPA. The nucleophilicity of the dimethylamino-groups of III is then increased compared to those of HMPA. As the first step in both mechanisms depends on the nucleophilicity of a dimethylamino-group in HMPA and III, respectively, the latter mechanism seems to be the most probable one.



Scheme 4.



Scheme 5.

CONCLUSION

2-Dimethylamino-quinoline has earlier been prepared from a variety of quinoline derivatives [7–12] and thus from not easily available starting materials. Also manystep-ring-closure reactions have been used for the synthesis of, *e.g.* 2-dimethylamino-4-methyl-quinolines.[13] In this paper a method is presented giving the same types of quinolines as mentioned above [7–13] but with easily available, simple and cheap starting materials and with a one-pot reaction. We thus strongly feel, that the dimethylamino-quinoline synthesis presented here seems to be the method of choice and, to the best of our knowledge and judgement, superior to all known procedures.

EXPERIMENTAL

In all experiments commercial HMPA dried over molecular sieves (3A) was used. NMR spectra were recorded on a Varian A-60 spectrometer (s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet). The microanalyses were performed by Løvens Kemiske Fabrik, Copenhagen.

2-Dimethylamino-quinoline. Acetanilide (13.5 g), 10 ml DMF and 50 HMPA were heated on a silicon-oil bath (250 °C) for 16 h. The reflux temperature increased during this time from 205 °C to 240 °C. The reaction mixture was

allowed to cool to 100 °C and was then poured into 400 ml 2 M NaOH and extracted with 3 × 200 ml diethyl ether. The organic phase was washed with 2 × 100 ml H_2O, dried over K_2CO_3 and the ether was stripped off. Distillation 95 – 110 °C/0.1 mmHg followed by recrystallization from light petroleum (60 – 80 °C) gave 11.5 g (67 %) of the title compound, m.p. 70 – 71 °C, lit.[11] m.p. 70 – 71 °C. Preparative TLC of the mother liquid using silica gel as the supporting material and elution with ether – light petroleum (1:4) gave further 0.93 g (5 %) of the title compound, $R_F = 0.18$, and 0.1 g (< 1 %) of *N,N*-dimethyl-*N'*-phenyl-formamidine, $R_F = 0.06$.

2-Dimethylamino-8-methyl-quinoline. *o*-Acetotoluidide (14.9 g), 10 ml DMF and 50 ml HMPA were heated as above and worked up as above. Distillation 100 – 110 °C/0.07 mmHg gave a fraction, which was chromatographed on a silica gel column. Using ether – light petroleum (1:10) for elution 14.1 g (76 %) of the title compound was obtained; b.p. 94 – 96 °C/0.05 mmHg; $n_D^{23} = 1.6367$; NMR δ ($CDCl_3$): 2.65 (s, 3 H), 3.08(s, 6 H), 6.72(d, $J = 9$ Hz, 1 H), 6.9 – 7.5(m, 3 H), 7.73(d, $J = 9$ Hz, 1 H); IR(Film): 1610 cm^{-1} (strong); UV (C_6H_{12}): $\lambda_{max} = 251$ nm (log $\varepsilon = 4.55$) and 354 nm (log $\varepsilon = 3.80$). (Found: C 77.34; H 7.54; N 15.07. $C_{12}H_{14}N_2$ requires: C 77.38; H 7.58; N 15.04). Further elution with ether gave 0.43 g (3 %) of *N,N*-dimethyl-*N'*-*o*-tolyl-formamidine; b.p. 128 – 130 °C/12 mmHg; $n_D^{21} = 1.5772$; NMR δ ($CDCl_3$): 2.26 (s, 3 H), 2.93(s, 6 H), 6.4 – 7.2(m, 4 H), 7.38(s, 1 H); IR(Film): 1640 cm^{-1} (strong). (Found: C 73.98; H 8.65; N 16.81. $C_{10}H_{14}N_2$ requires: C 74.03; H 8.70; N 17.27).

2-Dimethylamino-6-methyl-quinoline. *p*-Acetotoluidide (14.9 g), 10 ml DMF and 50 ml HMPA were heated as above. The reaction mixture was allowed to cool to 100 °C and was then poured into 400 ml 2 M NaOH and extracted with 3 × 200 ml ether. The combined ether phases were washed with 2 × 100 ml water, dried over K_2CO_3 and the ether was stripped off. Distillation 118 – 122 °C/0.3 mmHg and subsequent recrystallization from light petroleum (60 – 80 °C) gave 11.3 g (61 %) of the title compound; m.p. 78 – 79 °C; NMR δ ($CDCl_3$): 2.38(s, 3 H), 3.10(s, 6 H). 6.72(d, $J = 9$ Hz, 1 H), 7.1 – 7.8(m, 4 H); IR(CCl_4): 1610 cm^{-1} (strong); UV (C_6H_{12}): $\lambda_{max} = 250$ nm (log $\varepsilon = 4.54$), 357 nm (log $\varepsilon = 3.77$). (Found: C 77.50; H 7.64; N 15.06. $C_{12}H_{14}N_2$ requires: C 77.38; H 7.58; N 15.04). The combined water phases were then extracted with 3 × 100 ml $CHCl_3$ and distillation 112 – 118 °C/0.1 mmHg gave 5.2 g OMPA.

2-Dimethylamino-6-methoxy-quinoline. *p*-Acetanisidide (16.5 g), 10 ml DMF and 50 ml HMPA were heated as above for 16 h and worked up in a similar way. The ether extract gave by distillation at 134 – 142 °C/0.1 mmHg and subsequent recrystallization from light petroleum (60 – 80 °C) 10.3 g (51 %) of the title compound; m.p. 71 – 72 °C; NMR δ ($CDCl_3$): 3.11(s, 6 H), 3.60(s, 3 H), 6.7 – 7.9(m, 5 H); IR(CCl_4): 1615 cm^{-1} (strong); UV (C_6H_{12}): $\lambda_{max} = 243$ nm (log $\varepsilon = 4.54$) and 366 nm (log $\varepsilon = 3.76$). (Found: C 71.23; H 7.02; N 13.97. $C_{12}H_{14}N_2O$ requires: C 71.26; H 6.98; N 13.85). Preparative TLC of the distillation residue using silica gel as supporting material and ether for elution gave 1.46 g (5 %) of 6-bis(dimethylamino)-phosphinyloxy-2-dimethylaminoquinoline; m.p. 80 – 82 °C; NMR δ ($CDCl_3$): 2.72(d, $J = 10$ Hz, 12 H), 3.15(s, 6 H), 6.85(d, $J = 9$ Hz, 1 H), 7.2 – 7.9(m, 4 H); IR(KBr): 1620 cm^{-1} (strong). (Found: H 7.01; N 17.10. $C_{15}H_{23}N_4O_2P$ requires: H 7.19; N 17.38). From the $CHCl_3$ extract there was obtained 3.5 g OMPA by distillation 118 – 122 °C/0.2 mmHg.

2-Dimethylamino-8-methoxy-quinoline. *o*-Acetanisidide (16.5 g), 10 ml DMF and 50 ml HMPA were heated as above for 16 h. Work up as for 2-dimethylamino-quinoline followed by distillation 130 – 140 °C/0.1 mmHg and subsequent recrystallization from light petroleum (60 – 80 °C) gave 8.0 g (40 %) of the title compound; m.p. 105 – 107 °C; NMR δ ($CDCl_3$): 3.17(s, 6 H), 3.97(s, 3 H), 6.7 – 7.3(m, 4 H), 7.78(d, $J = 9$ Hz, 1 H); IR(CCl_4): 1610 cm^{-1} (strong); UV (C_6H_{12}): $\lambda_{max} = 270$ nm (log $\varepsilon = 4.43$) and 356 nm (log $\varepsilon = 3.59$). (Found: C 71.12; H 6.93; N 13.86. $C_{12}H_{14}N_2O$ requires: C 71.26; H 6.98; N 13.85). Preparative TLC of the distillation residue using silica gel as supporting material and acetone – $CHCl_3$ (1:9) for elution gave 1.77 g (5 %) of 8-bis(dimethylamino)phosphinyloxy-2-dimethylamino-quinoline; NMR δ ($CDCl_3$): 2.78(d, $J = 10$ Hz, 12 H), 3.17(s, 6 H), 6.85(d, $J = 9$ Hz, 1 H), 7.0 – 7.7(m, 3 H), 7.80(d, $J = 9$ Hz, 1 H); IR(CCl_4): 1620 and 1630 cm^{-1} (strong); UV (C_6H_{12}): $\lambda_{max} = 260$ nm (log $\varepsilon = 4.46$) and 353 nm (log $\varepsilon = 3.68$). (Found: C 55.63; H 7.38; N 17.12. $C_{15}H_{23}N_4O_2P$ requires: C 55.89; H 7.19; N 17.38).

2-Dimethylamino-benzo[h]quinoline. *N*-α-Naphthylacetamide (18.5 g), 10 ml DMF and 50 ml HMPA were heated as above for 16 h. Work up as for 2-dimethylamino-quinoline and followed by recrystallization gave 13.0 g (59 %) of the title compound; m.p. 92 – 93 °C; NMR δ ($CDCl_3$): 3.12(s, 6 H), 6.68(d, $J = 9$ Hz, 1 H), 7.4 – 7.9 (m, 6 H), 9.17(m, 1 H); IR(CCl_4): 1610 cm^{-1} (strong); UV (C_6H_{12}): $\lambda_{max} = 241$ nm (log $\varepsilon = 4.72$), 2.91 nm (log $\varepsilon = 4.26$), 321 nm (log $\varepsilon = 3.05$), 363 nm (log $\varepsilon = 3.81$) and 382 nm (log $\varepsilon = 3.81$). (Found: C 81.02; H 6.35; N 12.57. $C_{15}H_{14}N_2$ requires: C 81.05; H 6.35; N 12.60).

2-Dimethylamino-3-methyl-quinoline. *N*-Phenylpropionamide (14.9 g), 10 ml DMF and 50 ml HMPA were heated as above for 25 h. Work up as above gave by distillation 86 – 110 °C/0.15 mmHg a fraction, which was subjected to chromatography on a silica gel column. Elution with ether – light petroleum (40 – 60 °C) (1:1) gave 9.8 g (53 %) of the title compound; b.p. 158 – 160 °C/11 mmHg; $n_D^{26} = 1.6161$; NMR δ

($CDCl_3$): 2.35(d, $J=1.0$ Hz, 3 H), 2.95(s, 6 H), 7.1 – 8.0 (m, 5 H); IR(CCl_4): 1615 cm^{-1} (strong), 1635 cm^{-1} (strong); UV (C_6H_{12}): 253 nm (log $\varepsilon=4.33$) and 333 nm (log $\varepsilon=3.72$). (Found: C 77.21; H 7.72; N 15.09. $C_{12}H_{14}N_2$ requires: C 77.38; H 7.58; N 15.09). Further elution with ether gave 0.94 g (5 %) of *N,N*-dimethyl-*N'*-phenylformamidine.

2-Dimethylamino-3-ethyl-quinoline. *N*-Phenylbutyramide (8.15 g), 5 ml DMF and 25 ml HMPA were heated as above for 12 h. Work up as above gave by distillation a fraction 60 – 100 °C/0.07 mmHg which was subjected to chromatography on a silica gel column. Elution with ether – light petroleum (40 – 60 °C) (1:1) gave 2.6 g (26 %) of the title compound; b.p. 167 – 169 °C/14 mmHg; $n_D^{24}=1.6132$; NMR δ ($CDCl_3$): 1.28 (t, $J=7$ Hz, 3 H), 2.78 (q, $J=7$ Hz, 2 H), 2.93 (s, 6 H), 7.1 – 8.0 (m, 5 H); IR(CCl_4): 1610 cm^{-1} (strong), 1630 cm^{-1} (strong); UV (C_6H_{12}): 253 nm (log $\varepsilon=4.34$), 333 nm (log $\varepsilon=3.74$). (Found: C 77.64; H 8.20; N 13.97. $C_{13}H_{16}N_2$ requires: C 77.96; H 8.05; N 13.99). Further elution with ether gave 3.7 g of a fraction which was estimated by NMR and GLC to contain about 50 % *N,N*-dimethyl-*N'*-phenyl-formamidine, corresponding to ~10 % yield.

Attempted preparation of 2-dimethylamino-3-isopropylquinoline. *N*-Phenyl-isovaleramide (17.7 g), 10 ml DMF and 50 ml HMPA were heated as above for 18 h. The reaction mixture was taken up in 400 ml 2 M NaOH and extracted with 3 × 200 ml ether. The combined ether phases were washed with 2 × 100 ml H_2O, dried over K_2CO_3 and the ether stripped off. Light petroleum was added and 3.6 g (20 %) of the starting material precipitated. Fractionated distillation of the mother liquid gave 1.5 g (11 %) *N,N*-dimethyl-isovaleramide, b.p. 78 – 80 °C/12 mmHg, $n_D^{20}=1.4421$, lit.[14] b.p. 72 – 74 °C/8 mmHg, $n_D^{20}=1.4412$. Further distillation 130 – 160 °C/11 gave a mixture of 3.2 g (22 %) *N,N*-dimethyl-*N'*-phenyl-formamidine and 3.0 g (15 %) *N,N*-dimethyl-*N'*-phenyl-isovaleramidine (estimated by NMR), the latter compound was purified by preparative TLC using silica gel as supporting material and CH_3CN-MeOH-acetone (1:1:1) for elution, NMR δ ($CDCl_3$): 0.78 (d, $J=6$ Hz, 6 H), ~1.7 (m, 1 H), 2.25 (d, $J=7$ Hz, 2 H), 2.98 (s, 6 H), 6.5 – 7.4(m, 5 H); IR(CCl_4): 1630 cm^{-1} (strong); UV (C_6H_{12}): 245 nm (log $\varepsilon=4.08$). Further distillation 100 – 135 °C/0.08 mmHg and subsequent washing with light petroleum gave 2.1 g 12 % of starting material.

2-Dimethylamino-4-methyl-quinoline. Aniline (4.7 g), acetic acid (10 g) and 50 ml HMPA were heated on a silicone-oil bath (250 °C) for 18 h. The reaction mixture was allowed to cool to 100 °C and was then taken up in 400 ml 2 M NaOH and extracted 3 × 200 ml ether. The combined ether-phases were washed with 2 × 100 ml H_2O, dried over K_2CO_3 and the ether stripped off. Distillation at 90 – 120 °C/0.1 mmHg, and subsequent recrystallization from light petroleum gave 3.6 g (39 %) of the title compound, m.p. 47 – 48 °C, lit.[13] m.p. 47 – 50 °C.

2-Dimethylamino-4,6-dimethyl-quinoline. *p*-Toluidine (5.4 g), acetic acid (10 g) and 50 ml HMPA were heated as above. Work up as above gave after distillation 100 – 140 °C/0.1 mmHg and subsequent recrystallization from light petroleum 5.4 g (54 %) of the title compound m.p. 85 – 87 °C; NMR δ ($CDCl_3$): 2.43 (s, 3 H), 2.50 (d, $J=1.0$ Hz, 3 H), 3.11 (s, 6 H), 6.68 (q, $J=1.0$ Hz, 1 H), 7.1 – 7.8(m, 3 H); IR(CCl_4): 1620 cm^{-1} (strong); UV (C_6H_{12}): 254 nm (log $\varepsilon=4.50$), 356 nm (log $\varepsilon=3.78$). (Found: C 78.08; H 8.21; N 14.00. $C_{13}H_{16}N_2$ requires: C 77.96; H 8.05; N 13.99).

2-Dimethylamino-4,8-dimethyl-quinoline. *o*-Toluidine (5.4 g) acetic acid (10 g) and 50 ml HMPA were heated as above for 16 h. Work up as above gave after distillation 80 – 130 °C/0.05 mmHg and subsequent recrystallization from light petroleum 3.5 g (35 %) of the title compound; m.p. 58 – 60 °C, lit.[13] m.p. 60 – 62 °C.

6-Chloro-2-dimethylamino-4-methyl-quinoline. *p*-Chloroaniline (6.4 g) acetic acid (10 g) and 50 ml HMPA were heated as above for 16 h. Work up as above gave after distillation 110 – 150 °C/0.15 mmHg and subsequent recrystallization from light petroleum 4.4 g (40 %) of the title compound; m.p. 81 – 83 °C; NMR δ ($CDCl_3$): 2.43(d, $J=1.0$ Hz, 3 H), 3.12(s, 6 H), 6.65(q, $J=1.0$ Hz, 1 H), 7.2 – 7.8(m, 3 H); IR(CCl_4): 1620 cm^{-1} (strong); UV (C_6H_{12}): 252 nm (log $\varepsilon=4.53$), 280 nm (log $\varepsilon=4.23$), 288 nm (log $\varepsilon=4.10$), 359 nm (log $\varepsilon=3.82$). (Found: C 65.28; H 5.94; N 12.68. $C_{12}H_{13}ClN_2$ requires: C 65.30; H 5.94; N 12.69).

2-Dimethylamino-6-methoxy-4-methyl-quinoline. *p*-Anisidine (6.2 g) acetic acid (10 g) and 50 ml HMPA were heated as above. Work up as above gave after distillation 130 – 180 °C/0.2 mmHg and subsequent recrystallization from light petroleum 2.5 g (23 %) of the title compound m.p. 84 °C lit.[13] m.p. 87 – 89 °C.

2-Dimethylamino-4-ethyl-3-methyl-quinoline. Aniline (4.7 g) propionic acid (12.6 g) and 50 ml HMPA were heated as above. Work up as above gave the title compound, which was purified on a silica gel column using ether – light petroleum (1:4) for elution to give 1.78 g (17 %) of pure title compound; b.p. 154 – 156 °C/2 mmHg; $n_D^{24}=1.6085$; NMR δ ($CDCl_3$): 1.20 (t, $J=8$ Hz, 3 H), 2.37 (s, 3 H), 2.90 (s, 6 H), 3.00 (q, $J=8$ Hz, 2 H), 7.1 – 8.0 (m, 4 H); IR(CCl_4): 1595 cm^{-1} (strong); UV (C_6H_{12}): 252 nm (log $\varepsilon=4.37$), 333 cm^{-1} (log $\varepsilon=3.70$). (Found: C 78.67; H 8.49; N 12.96. $C_{14}H_{18}N_2$ requires: C 78.46; H 8.47; N 13.07).

N,N-Dimethyl-N'-p-tolyl-acetamidine. *p*-Acetotoluidide (29.8 g) and 100 ml HMPA were heated at 230 °C for 6 h. Work up as for 2-dimethylaminoquinoline and subsequent fractionated distillation gave: *1.* 15.6 g (44 %) of

the title compound, b.p. 80 – 82 °C/0.05 mmHg, $n_D = 1.5670$; NMR δ ($CDCl_3$): 1.82 (s, 3 H), 2.27 (s, 3 H), 2.97 (s, 6 H), 6.3 – 6.7 (m, 2 H), 6.8 – 7.2(m, 2 H); IR(CCl_4): 1625 cm^{-1} (strong); UV (C_6H_{12}): 241 nm (log $\varepsilon = 4.14$). (Found: C 74.98; H 9.22; N 16.02. $C_{11}H_{16}N_2$ requires: C 74.95; H 9.15; N 15.90). *2.* 3.6 g (18 %) 2-dimethylamino-4,6-dimethyl-quinoline b.p. 129 – 131 °C/0.1 mmHg.

N,N-*Dimethyl-N'-(p-methoxyphenyl)-acetamidine.* Acetanisidide 33 g and 100 ml HMPA were heated at 225 °C for 4 h. and the reaction mixture was worked up as above. Distillation 105 – 110 °C/0.2 mmHg gave 29 g, 76 % of the title compound, $n_D^{24} = 1.5717$; NMR δ ($CDCl_3$): 1.82 (s, 3 H), 2.87 (s, 6 H), 3.73 (s, 3 H), 6.5 – 6.9 (m, 4 H). IR(CCl_4): 1620 cm^{-1} (strong): UV (C_6H_{12}): 241 nm (log $\varepsilon = 4.14$). (Found: C 68.95; H 8.44; N 14.56. $C_{11}H_{16}N_2O$ requires: C 68.72; H 8.39; N 14.56). Further distillation 160 – 170 °C/0.5 mmHg and subsequent recrystallization from light petroleum gave 1.38 g (6 %) 2-dimethylamino-6-methoxy-4-methylquinoline.

N'-*(p-Chlorophenyl)*-N,N-dimethyl-*acetamidine. p*-Chloroacetanisidide (30 g) and 100 ml HMPA were heated at 225 °C for 5 h and the reaction mixture was worked up as above. Distillation 90 – 100 °C/0.05 mmHg gave 22.3 g (64 %) of the title compound, $n_D^{24} = 1.5859$; NMR δ ($CDCl_3$): 1.83 (s, 3 H), 2.98 (s, 6 H), 6.4 – 6.7 (m, 2 H), 7.0 – 7.3 (m, 2 H); IR(CCl_4): 1620 cm^{-1} (strong); UV (C_6H_{12}): 248 nm (log $\varepsilon = 4.14$). (Found: C 61.27; H 6.75; N 14.13. $C_{10}H_{13}ClN_2$ requires: C 61.06; H 6.66; N 14.24).

p-*Acetotoluidide (14.9 g)* + N,N-*dimethyl-acetamide (12 g)* and 50 ml HMPA were heated on a silicone-oil (250 °C) bath for 16 h and the reaction mixture was worked up as above. Distillation 120 – 160 °C/ 0.3 mmHg and subsequent recrystallization from light petroleum gave 9.2 g (46 %) of 2-dimethylamino-4,6-dimethyl-quinoline.

Acetanilide (13.5 g) + *phenylacetate (16 g)* and 50 ml HMPA were treated as above for 16 h. The cooled reaction mixture is poured into 400 ml 2 M NaOH and extracted with 4 × 200 ml ether. To the combined ether phases (ether phase 1) was added 150 ml 2 M HCl. The precipitate was filtered off and the ether phase 1 was separated. The precipitate was taken up in the acidic water phase, which then was made alkaline and extracted with ether. This ether phase was dried over Na_2SO_4, and distillation at 110 – 120 °C/0.2 mmHg and subsequent recrystallization from light petroleum gave 9.7 g (52 %) 2-dimethylamino-4-methyl-quinoline. The ether phase 1 was dried over Na_2SO_4 and by distillation at 100 – 150 °C/0.05 mmHg a fraction was obtained, which was subjected to column chromatography using silica gel as the supporting material. Elution with ether gave 1.67 g dimethylamidodiphenyl phosphate; b.p. 137 – 140 °C/0.05 mmHg, lit.[5] b.p. 154 °C/0.2 mmHg; $n_D^{26} = 1.5402$, lit./ $n_D^{25} = 1.5407$. Further elution with MeOH gave 3.36 g bis(dimethylamido)phenyl phosphate; b.p. 160 – 161 °C/10 mmHg, lit.[5] b.p. 154 – 155 °C/9 mmHg; $n_D^{25} = 1.5061$, lit.[5] $n_D^{25} = 1.5037$.

N,N-*Dimethylpropionamide* + *acetanilide.* To a solution of *N,N*-dimethylpropionamide in HMPA (prepared by heating propionic acid (5.7 g) in HMPA (50 ml) at reflux temperature for 2 h)[6] acetanilide (6.75 g) was added. The mixture was heated for 17 h on a silicon oil-bath (250 °C), poured into 400 ml 2 M NaOH and extracted with 4 × 200 ml ether. The combined ether phases were washed with 2 × 100 ml H_2O and dried over K_2CO_3. Distillation 86 – 150 °C/0.05 mmHg gave a fraction which was subjected to column chromatography using silica gel as the supporting material.

1. Elution with ether – light petroleum (1:4) gave 1.9 g of a mixture of XI and XII (1:1) which could be separated by preparative GLC (Perkin-Elmer F 21, 5 % Se 30). 3,4-Dimethyl-2-dimethylamino-quinoline (XI), NMR ($CDCl_3$): 2.35 (s, 3 H), 2.50 (s, 3 H), 2.90 (s, 6 H), 7.1 – 8.0(m, 4 H); IR(CCl_4): 1610 cm^{-1} (strong); UV (C_6H_{12}): 250 nm (log $\varepsilon = 4.38$), 334 nm (log $\varepsilon = 3.68$).

2. Further elution with ether – light petroleum (2:3) gave 3.2 g of a mixture of IX and X (1:3) which could be separated by preparative GLC as above. 2-Dimethylamino-4-ethyl-quinoline (X), NMR ($CDCl_3$): 1.31 (t, $J = 7$ Hz, 3 H), 2.93 (q, $J = 7$ Hz, 2 H), 3.15 (s, 6 H), 6.70 (s, 1 H), 7.0 – 7.9 (m, 4 H); IR(CCl_4): 1620 cm^{-1} (strong), UV (C/H_{12}): 250 nm (log $\varepsilon = 4.55$), 350 nm (log $\varepsilon = 3.77$).

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Reactions of Aminophosphines with Isothiocyanates. Dipolar Ionic Products

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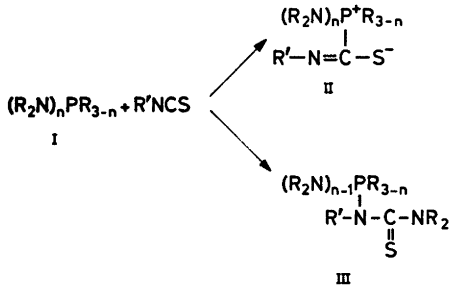
The reactions of three methyl substituted aminophosphines with methyl- and phenylisothiocyanate have been studied. The products are shown by ^1H and ^{31}P NMR spectroscopy to be dipolar ions, and analogous to those formed by trialkylphosphines. The dipolar ionic compounds derived from trimethylphosphine and methiodides of all dipolar ionic compounds have been prepared for comparison.

Aminophosphines (I) have been claimed by Oertel *et al.*[1] to react with isothiocyanates to give insertion products (III). They were formulated as insertion products because they (i) gave thioureas on hydrolysis and (ii) displayed an IR band at 1490 cm^{-1} "characteristic of thioureas". The alternative structure II, which is analogous to that of the known trialkylphosphine isothiocyanate adducts,[2,3] was not considered. We have reinvestigated the reaction and present evidence that product structure corresponds to II.



The investigation has been limited to the reaction of I, R = CH_3, $n = 1,2,3$ with methyl- and phenylisothiocyanate. A crystalline precipitate was generally formed upon mixing the aminophosphine and the isothiocyanate in ether or pentane at −20 °C. Ethyl- and isopropylisothiocyanate react similarly, but no compound could be isolated from *tert*-butylisothiocyanate. Also, we were unable to isolate any reaction product when the aminophosphine was *P*-phenyl substituted. Yields, melting points, and microanalyses of the compounds prepared and their methiodides IV are given in Table 1. Two of the compounds (IIa and IIb) are, judging from their melting points, identical to products obtained by Oertel *et al.* and described as insertion compounds.[1]

Assignment of structure. The adducts are assigned structure II on the basis of their ^1H and ^{31}P NMR spectra (Table 2): (i) Each compound, regardless of the number of amino groups, displays only one $(CH_3)_2N$ doublet with a rather large coupling constant (9.3 − 10.8 Hz). Since this is a coupling to phosphorus,* its magnitude and the fact that all R_2N groups are equivalent indicate that no R_2N group is separated from phosphorus by insertion of RNCS in a P−N bond. (ii) Dissociation to I and isothiocyanate is evident for several of the adducts as observed from their ^1H NMR spectra in $CDCl_3$. This is most unlikely for compounds with structure III. (iii) The ^{31}P chemical shifts are in the range expected for phosphonium compounds (general chemical shift range for phosphonium compounds containing R_2N groups −65 to −25 ppm).[4] The chemical shifts

* All couplings in Table 2 are shown by ^{31}P decoupling to be P···H couplings.

Table 1. Reaction products of aminophosphines with isothiocyanates. Dipolar ionic compounds and their methiodides.

No.	Compound	Yield, %	M.p., °C	Analyses (C, H, N, S)
IIa	$(Me_2N)_3P^+$ $MeNCS^-$	65 [a]	66 – 67 [b]	Found: 40.68, 8.99, 24.00, 13.65 Calc.: 40.70, 8.91, 23.75, 13.54
IVa	$(Me_2N)_3P^+$ I^- $MeNCSMe$	90 [a]	70 – 72	Found: 28.49, 6.04, 14.94, 8.40 Calc.: 28.60, 6.35, 14.85, 8.47
IIb	$(Me_2N)_3P^+$ $PhNCS^-$	90	70 – 70.5 [c]	Found: 52.19, 7.74, 18.76, 10.66 Calc.: 52.20, 7.75, 18.75, 10.72
IVb	$(Me_2N)_3P^+$ I^- $PhNCSMe$	85 [a]	111 – 112	Found: 37.60, 5.97, 12.74, 7.27 Calc.: 38.18, 5.92, 12.73, 7.29
IIc	$(Me_2N)_2P^+Me$ $MeNCS^-$	25	56.5 – 58 [d]	Found: 40.33, 8.95, 20.19, 15.40 Calc.: 40.56, 8.75, 20.27, 15.47
IVc	$(Me_2N)_2P^+Me$ I^- $MeNCSMe$	65 [a]	88.5 – 89	Found: 27.40, 5.95, 12.10, 8.92 Calc.: 27.51, 6.06, 12.03, 9.18
IId	$(Me_2N)_2P^+Me$ $PhNCS^-$	90	58 – 59	Found: 53.39, 7.47, 15.60, 11.81 Calc.: 53.51, 7.48, 15.60, 11.91
IVd	$(Me_2N)_2P^+Me$ I^- $PhNCSMe$	60 [a]	102 – 103.5	Found: 37.95, 5.64, 10.43, 7.53 Calc.: 37.96, 5.64, 10.22, 7.80
IIe	$Me_2NP^+Me_2$ $MeNCS^-$	65	87.5 – 88	Found: 40.08, 8.42, 15.70, 17.79 Calc.: 40.40, 8.44, 15.73, 18.00
IVe	$Me_2NP^+Me_2$ I^- $MeNCSMe$	70 [e]	118 – 118.5	Found: 25.85, 5.66, 8.75, 9.86 Calc.: 26.10, 5.64, 8.71, 9.95
IIf	$Me_2NP^+Me_2$ $PhNCS^-$	70 [e]	98 – 99	Found: 55.38, 7.22, 11.90, 13.16 Calc.: 55.00, 7.13, 11.68, 13.37
IVf	$Me_2NP^+Me_2$ I^- $PhNCSMe$	50 [f]	145 – 146	Found: 37.55, 5.38, 7.26, 8.33 Calc.: 37.70, 5.24, 7.33, 8.39
IIg	P^+Me_3 $MeNCS^-$	90	136 – 138	Found: 39.95, 8.13, 9.47, [g] Calc.: 40.25, 8.11, 9.39, 21.49
IVg	P^+Me_3 I^- $MeNCSMe$	80 [f]	160 – 161	Found. 24.84, 5.14, 4.84, 10.92 Calc.: 24.75, 5.19, 4.81, 11.01

Table 1. Continued.

IIh	P^+Me_3		85	90.5–92	Found: 56.90, 6.78, 6.71, 15.21
	PhNCS$^-$				Calc.: 56.84, 6.67, 6.63, 15.18
IVh	P^+Me_3	I^-	60 [f]	138–138.5	Found: 37.58, 4.86, 3.97, 8.66
	PhNCSMe				Calc.: 37.41, 4.85, 3.97, 9.08

[a] Dissolved in acetone and reprecipitated with ether. Lit. values[1] for the claimed insertion products: [b] 66, [c] 70, [d] oil, b.p. 120–122/0.15 mmHg. [e] Recrystallized from acetone. [f] Recrystallized from 2-propanol. [g] Exploded.

Table 2. ^1H and ^{31}P NMR data [a] for aminophosphines, dipolar ionic products with isothiocyanates, and their methiodides.

Compound	$\delta(^1H)$ CH_3NP	CH_3P	CH_3NC	CH_3S	J_{PNCH}	J_{PCH}	J_{PCNCH}	J_{PCSCH}	$\delta(^{31}P)$
Ia	2.48				9.1				−123.0
IIa	2.86		3.44		9.3		4.8		−31.7
IVa	2.95		3.72	2.73	10.1		4.4	1.7	−34.6
IIb	2.82				9.3				−32.0
IVb	3.05			2.14	10.2			1.8	−34.1
Ic	2.65	1.23			9.2	7.1			−86.3
IIc	2.82	2.02	3.42		9.9	12.6	4.4		−43.3
IVc	2.94	2.55	3.70	2.76	10.6	12.9	4.0	1.2	−50.5
IId	2.80	2.02			9.9	12.6			−44.5
IVd	3.01	2.54		2.23	10.5	13.3		1.3	−51.7
Ie	2.54	1.08			10.3	4.8			−39.6
IIe	2.83	1.99	3.41		10.8	12.5	4.2		−36.4
IVe	2.94	2.53	3.72	2.71	10.8	13.2	3.7	1.3	−53.4
IIf	2.88	2.05			10.7	12.5			−37.6
IVf	2.99	2.63		2.16	10.7	13.6		1.2	−53.8
Ig		1.02				1.7			61.2
IIg		1.96	3.40			13.4	4.4		−6.2
IVg		2.51	3.77	2.68		14.2	3.8	1.5	−29.4
IIh		1.87				13.3			−8.4
IVh		2.59		2.15		14.2		1.4	−30.4

[a] 1–5 % solutions in $CDCl_3$ at *ca.* 35 °C. Chemical shifts in ppm, $\delta(^1H)$ from TMS, $\delta(^{31}P)$ from external 85 % H_3PO_4, coupling constants J in Hz.

of III are expected to be much closer to those of I. (iv) The coupling constants J_{PCH} in the products are larger than those in I in agreement with the formulation of the compounds as phosphonium compounds.[5] (v) The ^1H chemical shifts are close to those found for similar phosphonium compounds [6] and agree with a deshielding of the protons by P^+, relative to the protons of I.

Methylation of the compounds II results in a further NMR deshielding of all protons and phosphorus in accord with a reduced electron density in the methiodides IV (Table 2). Apart from the deshielding and the doublet (due to coupling to phosphorus) from the S-CH_3 group the spectra of II and IV are very much alike, as expected for compounds with similar structures.

For comparison we have included the analogous compounds (II, $n=0$) derived from trimethylphosphine (I, $n=0$) in the investigation. The NMR spectra of these (Table 2) are very similar to those of the compounds described above. Since tertiary phosphines are not able to give insertion compounds analogous to III, but are known to give dipolar ionic compounds

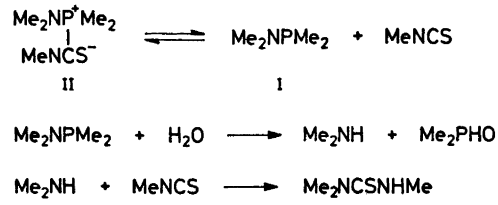
with isothiocyanates,[2,3] this similarity of the NMR spectra support the evidence given above for the dipolar ions structure of the aminophosphine adducts.

Compounds II and IV are expected to exist as mixtures of *E* and *Z* isomers due to restricted rotation about the C=N bond.

IVZ IVE

However, we were in no case able to observe more than one set of ^{1}H NMR signals at temperatures down to −50 °C in $CDCl_3$. These results indicate that II and IV exist mainly as one of the *E*−*Z* isomers, and we suggest that to be the *Z* isomer for the following reasons. (i) The rather large J_{PCNCH} coupling constant in I and IV (R′=CH_3) indicate a *trans* configuration for P^+ and CH_3 about the C=N bond. (ii) The *Z* isomer is probably the most stable isomer for both steric ($-P^+$ is larger than S^- and probably also SCH_3) and electronic reasons (the lonepair of nitrogen being closer to $-P^+$).

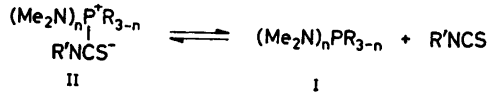
Properties of adducts II. The compounds are colourless to yellow solids which are easily hydrolysed by moist air. The hydrolysis products are thioureas, as found by Oertel *et al.*,[1] and various phosphorus acids. These hydrolysis products are explained by the fact that II is reversibly dissociated to I and isothiocyanate. Hydrolysis of I, a fast process, gives Me_2NH which subsequently combines with the isothiocyanate to give a thiourea, *e.g.*



The dissociation of II to I and isothiocyanate is perceptible from the smell of the solid II, and in most cases is also observable by ^{1}H NMR in $CDCl_3$ solution (Table 3). The values in Table 3 show that dissociation occurs more readily in the order of R′: Me < Ph < Bu^t. This variation is probably due to increasing steric hindrance in II with larger R′. The steric hindrance is expected to be largest for $(Me_2N)_3P$ and smallest for PMe_3, in accordance with the values which show a larger variation with R′ in the former case. An increase in the dissociation of II is also seen with decreasing number of amino groups (decreasing *n*). This variation shows that dimethylamino groups stabilize II relative to I + R′NCS, probably by stabilizing the phosphonium centre by $p\pi$-$d\pi$ overlap.[6] The values in the last column show that substituting a Me group on phosphorus for a Ph group strongly increases the dissociation of II. This is expected from the well-known reduced nucleophilicity of tertiary phosphines when phenyl groups are introduced.[7]

The compounds II are thermally rather un-

Table 3. Degree of dissociation of II to I and R′NCS [100 × mol I/(mol I + mol II) at equilibrium, starting solutions 2.0×10^{-1} M of II in $CDCl_3$, *t ca.* 35 °C, calculated from ^{1}H NMR integral values].



R′NCS	I $(Me_2N)_3P$	$(Me_2N)_2PMe$	Me_2NPMe_2	PMe_3	$(Me_2N)_2PPh$
MeNCS	< 1	< 1	3	27	*ca.* 97 [a]
PhNCS	25	16	17	33	*ca.* 98 [a]
Bu^{t}NCS	> 95 [a]				

[a] 2.0×10^{-1} M in aminophosphine and isothiocyanate.

stable. Although stable for several months at −20 °C, they decompose within a few days at room temperature. From the ^{1}H NMR spectra of the decomposing compounds in $CDCl_3$ it seems that II is first transformed into two new compounds. These subsequently decompose, and the presence of at least three new compounds is established by ^{31}P decoupling experiments. Likewise we obtained mixtures upon attempts to distil the compounds following Oertel *et al.* who isolated some of their products by distillation. One of the compounds in the mixtures may have the structure III, but it seems unlikely that the products obtained by Oertel *et al.* in any case were pure III. The thermal decomposition of II is under further investigation in our laboratories.

The IR spectra of II showed a strong band at 1475−1515 cm^{-1} (KBr). This band is assigned (mainly) to a C=N stretching vibration because a band is found in the same range in the IR spectra of IIg and IIh, and because the band is shifted to higher wavenumbers in the methiodides IV. Although thioamides and thioureas have a band in the same region (the B-band [8]), it is nevertheless not solely characteristic of thioureas, as implied by Oertel *et al.*

EXPERIMENTAL

Microanalyses were carried out at the Microanalysis Department of Department of General and Organic Chemistry, the H. C. Ørsted Institute. ^{1}H and ^{31}P NMR spectra were obtained on a Bruker HX 90E spectrometer. Tris(dimethylamino)-phosphine and dimethylaminodimethylphosphine were prepared according to the literature.[9] Trimethylphosphine was obtained from its silver iodide complex [10] by pyrolysis. All preparations were performed in a nitrogen atmosphere.

Bis(dimethylamino)methylphosphine. To a stirred solution of $(Me_2N)_2PCl$ [11] (12.4 g) in dry ethyl ether (75 ml), kept at −78 °C, was added MeLi (1.6 M in ether, 50 ml). After 2 h at 20 °C the reaction mixture was set aside for LiCl to precipitate, the solution decanted and the solvent evaporated under reduced pressure. Vacuum distillation through a 15 cm Vigreux column gave $(Me_2N)_2MeP$ (5.4 g, 50 %), b.p. 37−39 °C/13 mmHg (lit.[12] 64−67 °C/49−50 mmHg). According to its ^{1}H NMR spectrum the product contained a small amount of $(Me_2N)_3P$ (3−6 %), but was otherwise pure.

Dipolar ionic compounds (II). The phosphine (4 mmol) in dry ether or pentane (5−10 ml) was cooled to −78 °C and methyl- or phenylisothiocyanate (6 mmol) added with stirring. After 24 h at −20 °C (−78 °C in case of IIc) the colourless or yellow crystals were filtered off, washed with cold ether or pentane and dried 5 min *in vacuo* at 20 °C. The crude compounds were analytically pure except those for which solvents of crystallisation are given in Table 1.

Methiodides (IV). To a methylene chloride or acetone solution of II was added the corresponding isothiocyanate (*ca.* mol:mol) in order to suppress dissociation, and then excess methyl iodide at −78 °C. After 1 h at 20 °C the solvent was evaporated and the residue recrystallized from the solvent given in Table 1.

Acknowledgements. Financial support from the Danish Scientific Research Foundation for purchase of the Bruker NMR apparatus is greatly acknowledged. Dr. O. Larsen has kindly delivered samples of IIg and IIh.

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Bromination of Enamines from Methyl Isopropyl Ketone. A Possible Route to Bromomethyl Isopropyl Ketone

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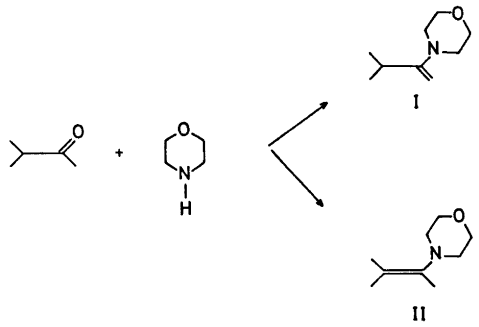
The relative amounts of the various bromo ketones obtained by reacting the morpholine enamines of methyl isopropyl ketone with bromine under different conditions were determined. Mechanistic details of the reaction are briefly discussed. A convenient method of preparing bromomethyl isopropyl ketone *via* the morpholine enamine is described and the optimum conditions for this reaction are presented.

Enamines react with elementary halogens to form α-haloimmonium salts, which on hydrolysis yield the corresponding α-halocarbonyl compounds.[1–7] It has also been reported that certain imines from methyl ketones react with *N*-halosuccinimides to yield halomethyl ketones involving enamine intermediates.[8,9]

When an unsymmetric ketone with α- and α′-hydrogens, *e.g.* methyl isopropyl ketone, is converted to the corresponding tertiary enamine using a secondary amine, *e.g.* morpholine, two isomeric tertiary enamine products are possible, I and II (Scheme 1). The isomers I and II are interconvertible and the equilibrium ratio of I:II is 29:71 at room temperature. However the equilibration is a rather slow process in absence of heat and/or acid, and the isolation of I and II in pure state has been reported.[10]



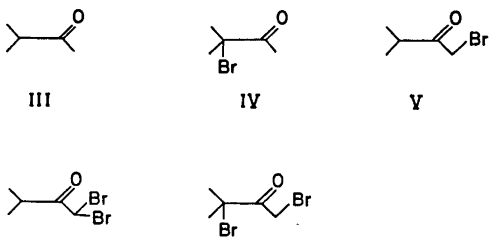
Scheme 1.

α-Halogenation of ketones occurs in the enolized ketone. When unsymmetric ketones are halogenated, mixtures of isomeric haloketones are usually obtained, due to formation of isomeric enols. In some cases a 1:1 ratio of isomeric α-haloketones is reported.[11] It is difficult to prepare halomethyl ketones by direct halogenation of ketones. However, Gaudry and Marquet have reported that bromination of a number of methyl ketones in carbon tetrachloride containing a few percent of methanol affords high yields of the bromomethyl ketone.[12]

No reports of halogenation of tautomerizable tertiary enamines have been found in the literature. The purpose of the present investigation is to ascertain whether or not transformation of a ketone to the corresponding tertiary enamine offers a means of overcoming difficulties encountered by direct halogenation of the ketone. We wish to report some results obtained by bromination of the morpholine enamines from methyl isopropyl ketone. Results from the bromination of the corresponding dimethylamino and pyrrolidino enamines will be reported later, due to the fact that these experiments led to a negative product balance, and the reason for this is not yet fully understood. Control experiments showed that the recovery of ketone was 92 % and of bromine 96 % when the morpholine enamine I was treated with one equivalent of bromine and subsequently hydrolyzed. The recovery of ketone in experiments with dimethylamino and

pyrrolidino enamines was considerably lower.

The enamines I, II as well as I+II in equilibrium mixture were allowed to react with 0.5, 1.0, and 2.0 equivalents of bromine in methylene chloride at −78 °C and room temperature. The reaction between the enamines and bromine is instantaneous and the reaction mixture was quenched with water after one minute followed by removal and analysis (GLC) of the organic layer. The ketones III−VII (Scheme 2) were identified and the results of these experiments are summarized in Table 1.



Scheme 2.

These results may be interpreted in the following way. At −78 °C the enamines are rapidly converted to the corresponding α-bromoimmonium salts, which are relatively stable towards further bromination. Consequently the yields of dibromoketones are low in these experiments. At room temperature the α-bromoimmonium salts which constitute the primary products undergo further reaction prior to hydrolysis. The yield of the dibromo ketone VII is rather high when one equivalent of bromine is used and this has led us to propose the intermediates VIII and IX (Scheme 3).

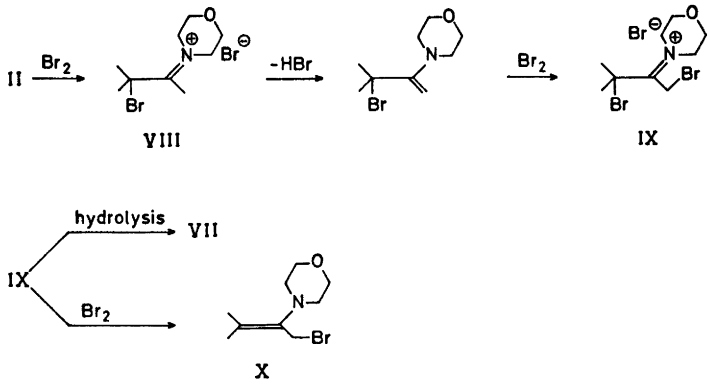
It should be noted that the yield of V far exceeds the amount of the corresponding enamine I present in the equilibrium mixture and as much as *ca.* 14 % of V is found when a pure sample of the other isomer II is brominated. This unexpected result is explained by the reaction sequence in Scheme 3. The dibromoimmonium salt IX is debrominated to the bromo enamine X which is then protonated or brominated on the nitrogen atom. These cationic species are not prone to react with the electrophile bromine.

This explanation is further supported by time interval measurements carried out on the reaction between the enamines and bromine at room temperature (see Table 2). One equivalent of bromine was used throughout. The table

Table 1. Product distribution obtained by bromination of the enamines under conditions given in the text.

Enamine	Equiv. Br_2	Temp. °C	Products after hydrolysis (%) III	IV	V	VI	VII	Others [e]
I[a]	0.55	−78	45	–	55	–	–	–
I[a]	0.55	Rt	47	–	53	–	–	–
I[a]	1.09	−78	2	8	90	< 0.5	–	trace
I[a]	1.09	Rt	6	< 0.5	84	0.5	9	trace
I[b]	2.0	−78	5	7	85	1	2	< 1
I[b]	2.0	Rt	2	6	88	1	2	< 1
II[c]	0.5	−78	42	56	1	–	1	–
II[c]	0.5	Rt	80	17	trace	–	1	1
II[c]	1.0	−78	1	95	1	–	3	–
II[c]	1.0	Rt	29	11	14	–	46	< 0.5
II[c]	2.0	−78	trace	92	1	–	7	trace
II[c]	2.0	Rt	trace	81	1	–	18	1
I+II[d]	0.5	−78	49	26	24	–	–	1
I+II[d]	0.5	Rt	70	5	23	–	–	2
I+II[d]	1.0	−78	2	64	27	trace	8	trace
I+II[d]	1.0	Rt	19	8	47	–	26	< 0.5
I+II[d]	2.0	−78	2	67	27	–	3	–
I+II[d]	2.0	Rt	2	53	27	trace	19	–

[a] I:II = 89.5:10.5. [b] I:II = 91.0:9.0. Determined from NMR spectrum [c] No trace of I was detected in NMR spectrum. [d] Equilibrium mixture I:II = 29.0:71.0. [e] Unidentified peaks in the gas chromatogrammes.



Scheme 3.

Table 2. Time interval measurements of product distribution obtained by bromination of the enamines with one equivalent of bromine at room temperature.

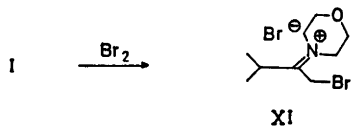
Enamine	Time min	Products after hydrolysis (%) III	IV	V	VI	VII	Others [e]
	1	19	1	54	trace	26	–
I[a]	30	13	3	61	trace	22	–
	1440	17	12	57	–	13	2
	1	31	6	8	–	56	1
II[b]	30	30	2	30	trace	37	1
	1440	29	11	32	–	19	9[d]
	1	21	5	37	trace	36	< 1
I+II[c]	30	20	3	50	trace	27	1
	1440	22	13	47	–	10	5

[a] I:II = 85:15. [b] I 98 %. [c] Equilibrium mixture I:II = 29:71. [d] Distributed over seven minor peaks in the gas chromatogramme. [e] Unidentified peaks in the gas chromatogrammes.

clearly shows that for the dibrominated product the bromination is reversible under these conditions.

When two equivalents of bromine are used the yields of dibrominated ketones are low which might be explained by rapid *N*-bromination of the bromo enamines formed in the reaction mixture when the primarily formed monobromo immonium salts are deprotonated. This indicates that bromination of the nitrogen atom proceeds more rapidly than bromination of the β-carbon in bromo enamines. Excess bromine enables *N*-bromination go almost to completion and consequently the concentration of free bromo enamine susceptible to attack of bromine on the β-carbon is low.

Yields of the 1,1-dibromoketone VI are very low in all experiments. However, spiking experiments showed that this is not due to a rearrangement of VI to VII during hydrolysis and work up. The low yields of dibromoketones from the bromination of I can probably be explained on the basis of a more rapid proton transfer from the methyl group in the immonium salt VIII, than from the bromomethyl group or from the isopropyl group in XI (Scheme 4). Therefore



Scheme 4.

we assign the amounts of VII as coming from the minor amounts of II present in the starting enamine. The proton is transferred to an unbrominated enamine which is consequently withdrawn from further bromination by this protonation. The amounts of III are somewhat too small to account for this proton transfer, but some parent ketone is apparently lost during hydrolysis and work up. The slower proton transfer from XI than from VIII is probably due to steric hindrance in the base attack.

Table 1 shows that it is possible to prepare bromomethyl isopropyl ketone V in a high yield by the reaction of the least substituted enamine I with bromine. The experiments accounted for in Table 1 were performed on a 10 mmolar scale and we have also tried to discover the optimum conditions for synthesis on a larger scale. In this context some experimental findings are of importance. In the experiments listed in Tables 1 and 2 the enamine was added to a stirred solution of bromine. The reverse addition procedure is unsuitable since it leads to considerable amounts, approximately 40 %, of unbrominated ketone, in addition to a rather high yield of the dibromomethyl ketone VI. This can be explained by a proton transfer from the monobromo immonium salt to an unbrominated enamine, followed by a second bromination of the bromo enamine thus formed. This can be avoided by the addition of enamine to bromine. A direct scale-up also yielded rather large amounts of dibromo ketones, and it is possible that this may be caused by heat evolved during the reaction.

The enamine I is the kinetically controlled product when it is prepared according to the method described by White and Weingarten.[13] In this reaction hydrocarbons are preferred as solvents, and using pentane we attempted a direct bromination of the crude enamine. However, it was found to give rise to practical difficulties. The bromo immonium salts formed precipitated as a sticky mass which made stirring impossible. However, methylene chloride is known to be a good solvent for ion pairs [14] and we found it to be a more favourable solvent. Use of low concentrations of reactants (< 1 M) gave a homogeneous reaction mixture. Therefore the most suitable procedure for bromination of the enamine I to the bromomethyl ketone V was to use a type of "flow" reactor described below.

The yield of V determined by NMR analysis of the distilled crude product was 57 % calculated from the amount of starting enamine. A disadvantage is that the yield of the enamine when prepared according to Ref. 13 is only 60 – 70 % and use of more forced conditions in the synthesis of the enamine results in mixtures of tautomeric enamines. Thus the actual yield of the bromoketone calculated from the starting ketone is 30 – 40 %. A considerable advantage is that the product contains only trace amounts of the isomeric monobromo ketone IV. The desired bromomethyl ketone can easily be separated from unbrominated and dibrominated ketones by distillation.

EXPERIMENTAL

The enamines were prepared according to White and Weingarten.[13] Separation of the isomers was conducted according to Pocar *et al.*[10] NMR-spectra were recorded on a Varian A-60A or a JEOL C-60 HL spectrometer.

Procedure for the experiments in Table 1. 10 mmol of the enamine in 20 ml of methylene chloride was added in one portion with vigorous stirring to a solution of the calculated amount of bromine in 20 ml of methylene chloride. After one minute the reaction was quenched with 20 ml of water and the mixture subsequently stirred for 1 h to complete the hydrolysis. The organic layer was separated and washed with 10 ml of 2 % $NaHSO_3$-solution and 2 × 10 ml of water and then dried over anhydrous magnesium sulphate. The filtered solution was analyzed by GLC, using a PYE M 64 Gas Chromatograph with a FID and 270 cm × 6 mm, 12 % QF-1 column at 120 °C. Chromatogrammes were recorded on a Philips recorder with a Disc integrator, and the relative amounts of the various ketones were determined from the integrals. Corrections were made for different FID responses of the various ketones. The peaks in the chromatogrammes were well resolved. The figures in Table 1 are the average values of three GLC determinations and the accuracy is within ± 1 %. The identities of the ketones were determined by means of GLC-MS, using a PYE M 84 Gas Chromatograph with a FID and an LKB 9000 Mass Spectrometer. Mass spectral analysis was aided by comparison with authentic sample spectra.

Procedure for the experiments in Table 2. 10 mmol of the enamine in 10 ml of methylene chloride was added to a stirred solution of 10

mmol of bromine in 90 ml of methylene chloride. 5 ml aliquots were hydrolyzed with 5 ml of water and analyzed by GLC.

Preparation of bromomethyl isopropyl ketone. A typical procedure was: 39.0 g (0.454 mol) of methyl isopropyl ketone and 120 ml of morpholine were dissolved in 1000 ml of isopentane. The mixture was cooled to −5 °C and a solution of 25 ml (0.227 mol) of titanium tetrachloride in 100 ml of isopentane was added with stirring. The reaction mixture was stirred at −5 °C overnight and filtered. Evaporation of the solvent at reduced pressure afforded the crude enamine *I* almost free of the other isomer. The yield of the enamine was 44.9 g (64 %) estimated from the NMR spectrum. The crude enamine was dissolved in 500 ml of methylene chloride and transferred to a graduated dropping funnel. An equivalent amount of bromine was dissolved in 500 ml of methylene chloride and subsequently transferred to a second graduated dropping funnel. The reagents were mixid in the following way. The two dropping funnels were mounted on a 250 ml fournecked flask fitted with a mechanical stirrer, drying tube and an outlet stopcock from the bottom of the flask. The flask was cooled with dry ice-ethanol. (The cooling device was actually a plastic bag tied around the bottom outlet pipe with copper wire, the plastic bag being supported by a polystyrene foam beaker, cut into two halves and reassembled around the bag). The bromine solution was added as 25 ml aliquots to the reaction flask with stirring and cooling. Each addition was followed by a rapid addition of a 25 ml portion of enamine solution. The resulting mixture was stirred for an additional 30−40 s. Thereafter it was tapped off *via* the bottom outlet pipe into a 2 l threenecked flask, fitted with a stirrer, where it was quenched with a 25 ml portion of water. A small amount of approximately 10 % $NaHSO_3$-solution was added portionwise to the hydrolysate to destroy excess of bromine. After the reaction was complete, stirring with water was continued for another 40 min. The organic layer was separated and the aqueous layer extracted with 100 ml of methylene chloride. The combined organic layers were then washed three times with 200 ml of water and dried over anhydrous magnesium sulphate. The solvent was evaporated at reduced pressure, and the residue was distilled "bulb to bulb" at a reduced pressure. A final fractionation with a Vidmer column afforded 22.0 g of pure (> 95 %) bromomethyl isopropyl ketone, 46 % yield calculated on the starting enamine.

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Preparation of Sepharose*-bound α-Ketosides of *N*-Acetylneuraminic Acid and their Interaction with *Vibrio cholerae* Neuraminidase

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The 2-aminoethyl and the 2-aminoethylaminocarbonylmethyl α-ketosides of *N*-acetylneuraminic acid and the 2-aminoethylamide of the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid have been synthesized and coupled to cyanogen bromide activated Sepharose. In the resulting polymers, the *N*-acetylneuraminic acid groups are bound to the matrix by means of α-ketoside (*N*-acetylneuraminic acid-Sepharose) and amide (*N*-acetylneuraminamide-Sepharose) linkages, respectively. Columns of *N*-acetylneuraminic acid-Sepharose equilibrated with buffers of pH 5.5 or 9 adsorbed *Vibrio cholerae* neuraminidase very strongly. The enzyme was effectively desorbed in high yield from the polymer by addition of the benzyl α-ketoside of *N*-acetylneuraminic acid to the eluent. The transformation of the *N*-acetylneuraminic acid-Sepharose to the 7-carbon analogue of *N*-acetylneuraminic acid-Sepharose resulted in a polymer having a slightly weaker affinity for the enzyme. *N*-Acetylneuraminamide-Sepharose did not significantly adsorb the neutraminidase.

Previous studies on the specificity of neuraminidase,[1-3] have revealed that the carboxyl group in the glyconic part of neuraminidase labile α-ketosides of *N*-acetylneuraminic acid, seems essential for the enzymatic cleavage of the α-ketosidic bond. It has also been demonstrated [4-5] that *Vibrio cholerae* neuraminidase is activated by divalent cations such as calcium and manganese, which presumably in conjunction with the carboxyl group in the substrate are essential for the formation of the enzyme-substrate complex.[5] In order to study further the interaction of *N*-acetylneuraminic acid derivatives and neuraminidase, attempts were made to prepare matrix-bound α-ketosides of *N*-acetylneuraminic acid. The enzyme-substrate binding properties have previously been utilized in the purification of *Vibrio cholerae* neuraminidase using erythrocyte stromata.[4]

The present paper reports the coupling of the 2-aminoethylamide of the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid and of the 2-aminoethyl and the 2-aminoethylaminocarbonylmethyl α-ketosides of *N*-acetylneuraminic acid to cyanogen bromide activated Sepharose and some properties of the polymers. In addition, the transformation of matrix-bound *N*-acetylneuraminic acid to its 7-carbon analogue will be described.

The synthesis of the 2-aminoethylaminocarbonylmethyl α-ketoside of *N*-acetylneuraminic acid was conveniently performed by treatment of the previously reported butoxycarbonyl α-ketoside of tetra-*O*-acetyl-*N*-acetylneuraminic acid [2] with anhydrous ethylene diamine. The purification of the nonulosaminic acid derivative, was facilated by the fact that it was not adsorbed by Amberlite IRC 50 (H^+). The 2-aminoethylamide of the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid, having a hydrophilic aglycone, was similarly prepared by treatment of the corresponding methyl ester with ethylene diamine.

The coupling of the 2-aminoethyl [3] and the 2-aminoethylaminocarbonylmethyl α-ketosides of *N*-acetylneuraminic acid and of the 2-aminoethylamide of the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid to Sepharose was per-

* Sepharose is the commercial name for beaded agarose from Pharmacia Fine Chemicals, Sweden.

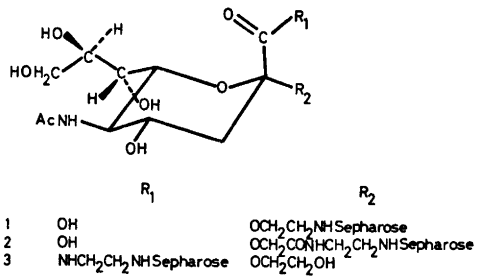

Fig. 1. Sepharose-bound 2-aminoethyl α-ketoside (1) 2-aminoethylaminocarbonylmethyl α-ketoside (2) and 2-aminoethylamide of the 2-hydroxyethyl α-ketoside (3) of *N*-acetylneuraminic acid.

formed as previously described,[6-7] resulting in polymers having the *N*-acetylneuraminic acid groups fixed to the matrix by means of α-ketoside (*N*-acetylneuraminic acid-Sepharose) and amide (*N*-acetylneuraminamide-Sepharose) linkages, respectively (Fig. 1).

Oxidation of the matrix-bound nonulosaminic acid with periodic acid and reduction of the resulting formyl derivative with sodium borohydride afforded the Sepharose-bound 7-carbon analogues of *N*-acetylneuraminic acid. The polymer beads were not destroyed when treated with periodic acid and sodium borohydride. Further, the beaded form remained unaffected when the Sepharose derivatives were freeze dried or treated with boiling water. On assaying *N*-acetylneuraminic acid-Sepharose at 37 °C with neuraminidase, free *N*-acetylneuraminic acid was released. In contrast the *N*-acetylneuraminamide-Sepharose was resistant to the hydrolytic action of the neuraminidase as well as to 0.05 M hydrochloric acid. By treatment with 0.5 M sulfuric acid at 80 °C, however, the amide linkage could be hydrolyzed and the resulting nonulosaminic acid determined. Under these hydrolyzing conditions, the released acid is probably partly destroyed resulting in an underestimate of the bound *N*-acetylneuraminic acid. Table 1 shows the amounts of *N*-acetylneuraminic acid in different Sepharose conjugates.

All of the *N*-acetylneuraminic acid-Sepharose preparations adsorbed *Vibrio cholerae* neuraminidase very strongly, type V showing a high enzyme binding capacity. Sodium acetate buffer pH 5.5 which according to Mohr and Schramm,[4] is effective in eluting neuraminidase adsorbed to erythrocyte stromata, eluted the enzyme from columns of the Sepharose derivatives only very slowly. The 7-carbon analogue of *N*-acetylneuraminic acid-Sepharose, prepared by periodic acid oxidation and sodium borohydride reduction of the matrix-bound *N*-acetylneuraminic acid, showed a slightly weaker affinity for the enzyme. Attempts to elute the neutraminidase

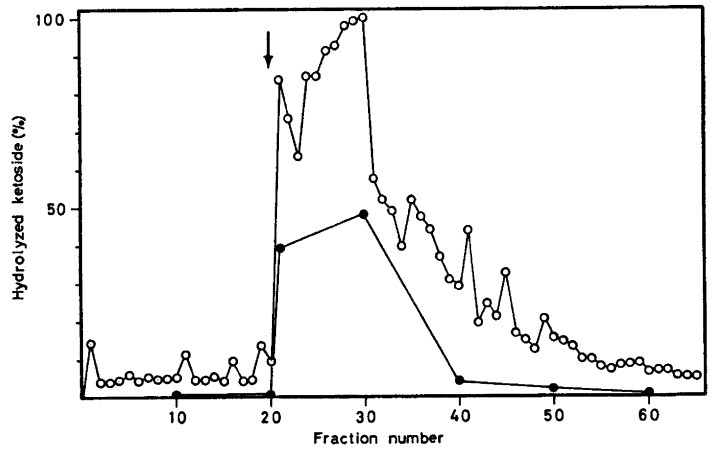

Fig. 2. Activity of *Vibrio cholerae* neuraminidase in the effluent from a column of *N*-acetylneuraminic acid-Sepharose (type V). The enzyme was eluted with 0.1 M sodium acetate buffer pH 5.5, 0.05 M with respect to calcium chloride, containing 200 μg benzyl α-ketoside of *N*-acetylneuraminic acid per ml buffer. The arrow denotes the start of the addition of the benzyl α-ketoside. Incubation was performed for 30 min (●) and 20 h (○). For further details see text.

with sodium borate buffer pH 9 were also unsuccessful. With either of the aforementioned buffers, serum albumin was eluted in a volume corresponding to that of the column. By means of sodium acetate buffer pH 5.5 containing 200 μg benzyl α-ketoside of *N*-acetylneuraminic acid per ml, however, the neuraminidase was desorbed in high yield from columns of *N*-acetylneuraminic acid-Sepharose, demonstrating a specific adsorption of the enzyme to the polymer (Fig. 2).

Vibrio cholerae neuraminidase, when applied to a column of *N*-acetylneuraminamide-Sepharose and eluted with sodium acetate or sodium borate buffer pH 5.5 or 9.0, respectively, was eluted with the total bed volume. In accordance with previous findings,[1-3] the carboxyl group in the glyconic part of the substrate seems to play a dominant role in the formation of the enzyme-substrate complex.

The present results demonstrate that *N*-acetylneuraminic acid-Sepharose and its derivatives, being water insoluble substrates of *Vibrio cholerae* neuraminidase, should be useful for enzyme-substrate binding studies and for the purification of neuraminidases from other sources, as well as for studies on virus and bacteria.

EXPERIMENTAL

Material and methods. The same general methods were used as previously reported.[1-3] Neuraminidase from *Vibrio cholerae* (glycoprotein *N*-acetylneuraminyl-hydrolase, EC 3.2.1.18) was purchased from Behring-Werke, Marburg; 1 ml containing 500 units (producer's specification). The 2-aminoethyl α-ketoside and the benzyl α-ketoside of *N*-acetylneuraminic acid were prepared as previously described.[3,1,8]

Syntheses. 2-Benzyloxyethyl α-ketoside of tetra-O-acetyl-N-acetylneuraminic acid. Peracetylated *N*-acetylneuraminic acid (1 g) was transformed to its 2-chloro derivative as described by Meindl and Tuppy [8] and condensed with 2-benzyloxyethanol (5 g) in the presence of silver carbonate (0.5 g) and pulverized Drierite (2 g). After reacting overnight, the mixture was treated as previously described for the preparation of the butoxycarbonylmethyl α-ketoside of *N*-acetylneuraminic acid.[2] The product obtained from the chloroform layer was crystallized from water. The yield was 280 mg (24 %) m.p. 165–169 °C (decomp.), $[\alpha]_D^{25}$ −12° (*c* 1.0; methanol) Found: C 55.06; H 6.13; N 2.24. $C_{28}H_{37}NO_{14}$ requires C 54.99; H 6.10; N 2.29).

2-Hydroxyethyl α-ketoside of methyl tetra-O-acetyl-N-acetylneuraminate. The peracetylated benzyloxyethyl α-ketoside of *N*-acetylneuraminic acid was transformed to its methyl ester with diazomethane. A solution of the ester (200 mg) in ethanol–water (1:1, 20 ml) was added to palladium oxide (100 mg) in ethanol–water and hydrogenated overnight at room temperature. Removal of catalyst and evaporation of solvent resulted in a chromatographically pure product which was dissolved in water and freeze-dried. The yield was 150 mg (88 %). $[\alpha]_D^{25}$ −20° (*c* 1.0; chloroform). (Found: C 48.50; H 5.95; N 2.55. $C_{21}H_{32}NO_{14}$ requires C 48.27; H 6.17; N 2.68).

2-Aminoethylamide of 2-hydroxyethyl α-ketoside of N-acetylneuraminic acid. The 2-hydroxyethyl α-ketoside of the methyl tetra-*O*-acetyl-*N*-acetylneuraminate (20 mg) was dissolved in anhydrous ethylene diamine (0.2 ml). After being left overnight at room temperature, the reaction mixture was concentrated to dryness and the residue dried for several hours over diphosphorus pentoxide in vacuum at room temperature, dissolved in methanol and precipitated with ethyl ether. The resulting chromatographically homogeneous product (10 mg) was dissolved in 0.1 M sodium carbonate buffer pH 10.2 (2 ml). This solutions was used in the coupling procedure.

2-Aminoethylaminocarbonylmethyl α-ketoside of N-acetylneuraminic acid. A solution of crystalline butoxycarbonyl α-ketoside of tetra-*O*-acetyl-*N*-acetylneuraminic acid [2] (100 mg) in anhydrous ethylene diamine (0.5 ml) was left overnight at room temperature. After evaporation of solvent, the residue was taken up in water and the solution put on a 1 × 5 cm Amberlite IRC 50 (H^+) column. Elution with water resulted in a chromatographically pure α-ketoside. After freeze-drying, the yield was 55 mg (76 %). $[\alpha]_D^{25}$ −19 °(*c* 1.0; water) (Found: C 41.99; H 6.74; N 9.68. $C_{15}H_{27}N_3O_{10}.H_2O$ requires C 42.15; H 6.84; N 9.83).

Periodate oxidation. The matrix-bound *N*-acetylneuraminic acid (0.4 ml) was treated with a solution of periodic acid (20 mg) in water (1 ml) for 30 min at room temperature. The resulting 6-formylhexulosonic acid derivative was filtered off and washed with water, 0.1 M hydrochloric acid, and water.

Sodium borohydride reduction. Sepharose-bound α-ketoside of *N*-acetylneuraminic acid (0.2 ml) after periodate oxidation was treated with sodium borohydride (20 mg) in water (1 ml). After 60 min at room temperature, the resulting 7-carbon analogue of the *N*-acetylneuraminic acid derivative was filtered off and washed as above.

Preparations of columns. Activation of Sepharose (2 ml) with cyanogen bromide was performed as previously described.[6-7] The pH of the reaction mixture was held at 11 by means of 0.1 or 2 M sodium hydroxide. After 30 to 60 min at room temperature, crushed ice was added to the reaction mixture. The acti-

Table 1. Amounts of *N*-acetylneuraminic acid, released from different Sepharose conjugates by treatment with neuraminidase or acid.

α-Ketoside of *N*-acetylneuraminic acid	Type of Sepharose	Cyanogen bromide used for activation (mg/ml wet gel)	Released *N*-acetylneuraminic acid (μmol/ml wet gel) with neuraminidase	with 0.05 M hydrochloric acid	Type
2-aminoethyl	2 B	300	0.3	0.6	I
2-aminoethyl	2 B	50	0.04	0.1	II
2-aminoethylamino-carbonylmethyl	2 B	50	0.04	0.1	III
2-aminoethylamino-carbonylmethyl	4 B	50	0.7	1.6	IV
2-aminoethylamino-carbonylmethyl	4 B	300	1.3	3.2	V
2-aminoethylamide of 2-hydroxyethyl	4 B	300	–	0.2[a]	VI

[a] Hydrolysis with 0.5 M sulfuric acid. See text.

vated Sepharose was rapidly filtered off and washed cold 0.1 M sodium carbonate buffer pH 10.2 (20 ml). The *N*-acetylneuraminic acid derivative having a free primary amino group (10 mg), dissolved in the same buffer as above (2 ml), was added to the polymer and the mixture was left overnight at 5 °C. The mixture was filtered, the filtrate being saved for further couplings, and the polymer was washed with 0.1 M sodium hydroxide, 0.1 M hydrochloric acid and distilled water. Columns of the Sepharose derivatives were equilibrated with the appropriate buffer and the column experiments were performed at *ca.* 0 °C.

The enzyme labile fraction of the bound *N*-acetylneuraminic acid was determined by incubation of the Sepharose derivative (0.2 ml) with neuraminidase (100 μl) for 24 h at 37 °C in 0.05 M Tris-maleate buffer pH 6.40, (0.005 M with respect to calcium chloride) in a total volume of 0.60 ml. Acid hydrolysis of α-ketosidically bound *N*-acetylneuraminic acid was performed by heating the Sepharose derivative (0.2 ml) in 0.1 M hydrochloric acid (0.2 ml) for 60 min at 60 °C. Samples of 200 μl were analyzed according to Warren.[9] The amount of *N*-acetylneuraminic acid, bound as amide was estimated by treatment of the polymer (0.2 ml) with 1 M sulfuric acid (0.2 ml) at 80 °C for 60 min and samples of 200 μl were treated as above. The results from the couplings are shown in Table 1.

Column experiments. The neuraminidase (0.5 ml) was applied to 5 × 5 mm Sepharose columns which, after 15 min, were washed with 0.1 M sodium acetate buffer pH 5.5 and sodium borate buffer pH 9, both being 0.01 M with respect to calcium chloride. Fractions (2 ml) were collected and a flow rate of about 0.2–0.3 ml/min was used. To each fraction was added a solution of benzyl α-ketoside of *N*-acetylneuraminic acid (400 μg) in buffer (50 μl). After incubation for 30 min and 20 h at 37 °C, aliquots of 200 μl were removed from each fraction and released *N*-acetylneuraminic acid analyzed as above. When using sodium borate buffer pH 9, the effluent was adjusted to pH 7 with 0.1 M ice-cold hydrochloric acid prior to the incubation with benzyl α-ketoside of *N*-acetylneuraminic acid.

Desorption of neuraminidase from the columns was performed with 0.1 M sodium acetate buffer pH 5.5, 0.01 M with respect to calcium chloride, containing 200 μg of the benzyl α-ketoside of *N*-acetylneuraminic acid per ml buffer. The effluent was directly incubated for 30 min and 20 h at 37 °C and aliquots (200 μl) from the 2 ml fractions were analyzed as above. In all experiments the temperature of the Sepharose columns was kept at *ca.* 0 °C.

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Some New α-(Cyclopolymethylenepyrazolyl-2)propionic Acids. Resolution of α-(Cycloheptapyrazolyl-2)propionic Acid and Determination of its Absolute Configuration

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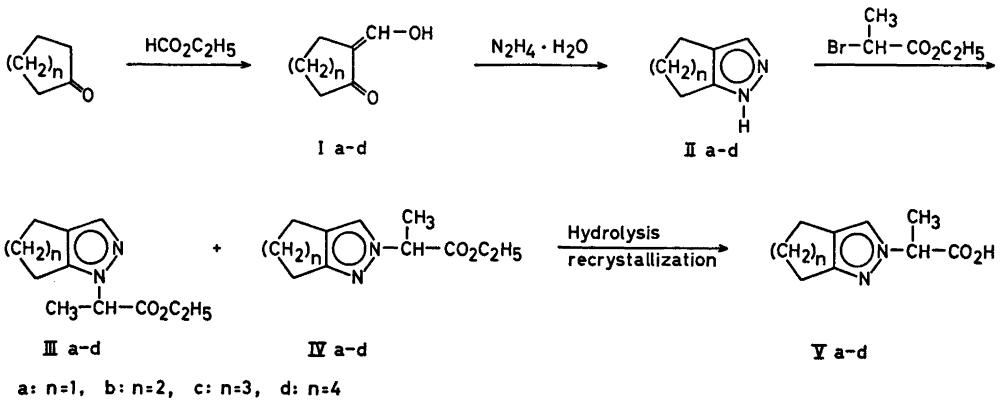
α-(Cyclopentapyrazolyl-2)propionic acid (Va), α-(cycloheptapyrazolyl-2)propionic acid (Vc), and α-(cyclooctapyrazolyl-2)propionic acid (Vd) have been synthesized from the corresponding cyclomethylenepyrazoles and ethyl α-bromopropionate by hydrolysis of the ester product. The reaction of cyclopentapyrazole (IIa) or 4,5,6,7-tetrahydroindazole (IIb) with ethyl α-bromopropionate in alcoholic sodium ethoxide solution gave a ratio of 1-ester to 2-ester of about 40:60. Alkylation of IIa with ethyl α-bromopropionate and without base yielded an ester ratio of 70:30, while IIb yielded the ratio 31:69. The acid Vc was resolved into its optical antipodes by fractional crystallization of its (−)-α-(β-naphthyl)ethylamine and (−)-ephedrine salts giving acids with $[\alpha]_D^{25} = +46.9$ and −46.5°, respectively, in chloroform. The absolute configuration of Vc was determined by means of circular dichroism measurements.

Some α-propionic acid derivatives of benzotriazole,[1,2] indazole,[3] and 4,5,6,7-tetrahydroindazole [4] have been synthesized earlier for stereochemical studies and studies of synthetic plant hormones. Three α-(cyclomethylenepyrazolyl-2)propionic acids have now been synthesized. The compounds are under examination with regard to auxin activity [5] and antimalarial action.[6]

The title compounds were synthesized from the corresponding cycloalkanones according to Scheme 1.

The cycloalkanones were formylated with ethyl formate in ether solution with sodium methoxide as base to give the α-hydroxymethylenecycloalkanones, which were then reacted



Scheme 1.

with hydrazine monohydrate under formation of the cyclopolymethylenepyrazoles IIa–d. The method used was, with slight modification, that of Ainsworth [7] for synthesis of 4,5,6,7-tetrahydroindazole. A higher yield of the cyclomethylenepyrazoles (IIa–c) was obtained by adding 99 % hydrazine hydrate directly to the dried ether solution of Ia–c.

The alkylation with alkylhalides of unsymmetrically substituted pyrazoles with unsubstituted nitrogen generally gives a mixture of isomers due to the ambident character of the pyrazole nucleus.[8] No rules have been established for predicting the course of reaction from the nature of the substituents in the pyrazole nucleus. The entering position is also influenced by the alkylating agent and the experimental conditions.[9]

The reaction between IIa–d and ethyl α-bromopropionate without solvent at 120° C gave both 1- and 2-ester. The ratio between 1-ester and 2-ester was dependent on the pyrazole used. IIa–c formed esters in the ratio 70:30, 31:69, and 19:81, respectively. A considerable amount of II remained unreacted. The analysis of the reaction products was done after neutralization with potassium carbonate solution and extraction with ether. The acid obtained after hydrolysis and recrystallization from ethanol-water was pure Va–d. Both IIa and IIb gave an ester ratio of about 40:60 on reaction with ethyl α-bromopropionate in alcoholic sodium ethoxide solution at room temperature.

The chemical shifts in DMSO-d_6 for the vinyl protons in II and the corresponding III and IV are well separated. The isomeric composition of the reaction mixture and the amount of unreacted IIa–d were determined from the peak areas of the different vinyl protons in the ^{1}H NMR spectra by cutting out and weighing the peaks and by GLC analysis. The position of the α-propionic acid group in Va–d was established by ^{1}H NMR spectroscopy according to Gustafsson.[10]

PRELIMINARY TESTS ON RESOLUTION

Preliminary tests for resolving Vc were done with the common alkaloids and some synthetic bases. Crystalline salts were formed with (–)-ephedrine, (+)-α-(β-naphthyl)ethylamine, dehydroabietylamine and (–)-α-phenethylamine in ethyl acetate giving levorotatory acid. The cinchonidine salt formed in ethyl acetate yielded dextrorotatory acid. Oils were obtained with cinchonine and brucine in ethyl acetate. On the basis of these results the resolution was performed with (–)-ephedrine and (–)-α-(β-naphthyl)ethylamine with ethyl acetate as solvent.

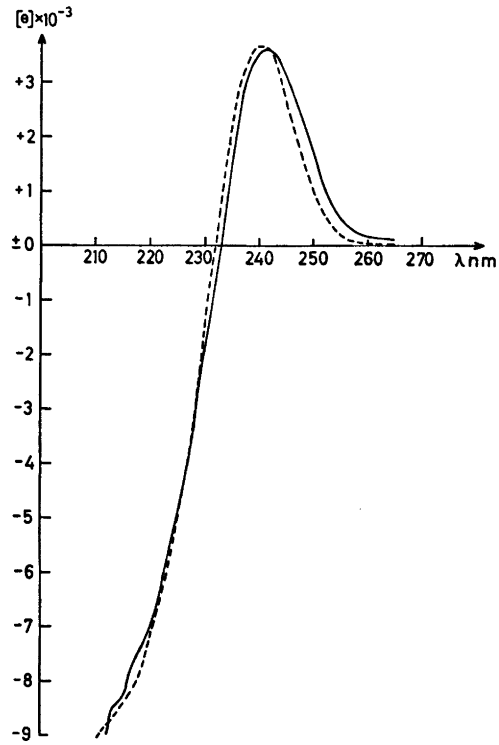


Fig. 1. CD spectra of D(–)Vb (——, $c = 3.96 \times 10^{-3}$ g/100 ml) and (–)Vc (- - -, $c = 3.88 \times 10^{-3}$ g/100 ml) in methanol.

DETERMINATION OF THE ABSOLUTE CONFIGURATION

The circular dichroism (CD) curves of D(–)Vb and (–)Vc were examined for configurational correlations. Fig. 1 clearly shows that (–)Vc has the D(*R*)-configuration. The absolute configuration of L(+)Vb was unequivocally established by direct synthesis *via* L(+)alanine.[11]

EXPERIMENTAL

The optical activity was measured with a Perkin-Elmer 141 spectropolarimeter in 1 ml micro cells of 10 cm length and with chloroform as solvent. The circular dichroism (CD) curves were recorded at 27 °C in 1 mm cells on a Cary 60 spectropolarimeter, equipped with a circular dichroism accessory. The IR spectra were recorded on a Perkin-Elmer 157 spectrophotometer and the UV spectra on a Spectronic 505 spectrophotometer. The ^{1}H NMR spectra were recorded on a Varian A-60 spectrometer in DMSO-d_6 solutions of about 15 % concentration with TMS as internal standard.

Gas-liquid chromatography (GLC) was performed on a Perkin-Elmer 990 gas chromatograph fitted with a flame ionization detector. Column temperature 150 °C, injection port temperature 240 °C and detector temperature 240 °C. Nitrogen carrier gas, flow rate 47 ml/min. Column: Chromosorb W, AW, DMCS-treated, 100 – 200 mesh, coated with 7.5 % UCON 50LB 550X, packed in a 3.0 m glass column AW, DMCS-treated, I.D. 3.5 mm. The column was conditioned at 200 °C before use. The mass spectra were recorded on an LKB 9000 A gas chromatograph-mass spectrometer operating at 70 eV. The melting points were determined with a hot stage microscope and are uncorrected. The microanalyses were carried out at the Analytical Department, Institute of Chemistry, University of Uppsala.

(+)-Ephedrine was isolated from its hydrochloride (Fluka AG $[\alpha]_{546}^{20} = +39 \pm 2°$ (*c* 11.5; water) by addition of 2 M sodium hydroxide and subsequent extraction with ether. The ether solution was dried over magnesium sulphate, the ether evaporated and the residue distilled. B.p.$_{0.2}$ 115 – 116 °C.

α-Hydroxymethylenecyclopentanone (Ia). A mixture of 84.0 g (1.0 mol) cyclopentanone distilled and dried over magnesium sulphate and 110 g (1.5 mol) dried ethyl formate was added with stirring at 15 °C to 54.0 g (1.0 mol) sodium methoxide in 2.0 l dry diethyl ether in a 5 l three-necked flask equipped with condenser, stirrer, dropping funnel, and drying tubes. The mixture was stirred at room temperature for 6 h, and allowed to stand overnight, after which 200 ml water was added and the mixture stirred until a solution was obtained. The ether phase was washed with 20 ml water. The combined water phase was acidified with 165 ml 6 M hydrochloric acid and extracted three times with 80 ml portions of ether. The ether solution was washed with a saturated aqueous solution of sodium chloride and then dried over anhydrous sodium sulphate. The boiling point from an earlier run was 67 – 73 °C at 1 mm torr (lit.[12,13] B.p.$_{11}$ 90 – 95 °C).

α-Hydroxymethylenecycloheptanone (Ic). A dry ether solution of α-hydroxymethylenecycloheptanone (Ic) was prepared in the same way from 44.5 g (0.40 mol) dried and distilled cycloheptanone, 44.4 g (0.60 mol) dried ethyl formate and 21.6 g (0.40 mol) sodium methoxide.

α-Hydroxymethylenecyclooctanone (Id). α-Hydroxymethylenecyclooctanone (Id) was prepared in the same way from 50.0 g (0.40 mol) cyclooctanone, 44.4 g (0.60 mol) ethyl formate and 21.6 g (0.40 mol) sodium methoxide in 1.0 l dried ether. 32.2 g (53 %) Id was obtained as a colourless viscous oil with b.p.$_{12}$ 104 – 106 °C and n_D^{22} 1.5154 (lit.[14] B.p.$_{0.1}$ 70 – 71 °C).

Cyclpolyomethylenepyrazoles (II). A slight excess of 99 % hydrazine hydrate (based on the amount of cycloalkanone used in the formylation step) was added dropwise with stirring to the ether solution of I in an ice bath. After standing at room temperature overnight the ether solution was concentrated in a rotary evaporator and the residue fractionated at reduced pressure. Distillation gave the corresponding cyclopolymethylenepyrazole as a clear viscous oil which crystallized to a white solid on standing at room temperature.

Cyclopentapyrazole (IIa). The ether solution of Ia previously described was reacted with 52.0 g (1.04 mol) 99 % hydrazine hydrate. Yield 50.2 g (46 % based on the amount of cyclopentanone used). B.p.$_{30}$ 165 °C. M.p. 57 °C (lit.[15] M.p. 57 – 59 °C). ^{1}H NMR (DMSO-d_6): δ 2.48 (m, $-CH_2-$), δ 7.15 (s, $-CH=$).

Cycloheptapyrazole (IIc). Cycloheptapyrazole was obtained from the ether solution of Ic and 21.0 g (0.42 mol) 99 % hydrazine hydrate. Yield 40.6 g (75 % based on the amount of cycloheptanone used). B. p.$_{1.2}$ 125 – 128 °C (lit.$_{0.5}$ [16,17] 118 – 120 °C) ^{1}H NMR (DMSO-d_6): δ 1.65 (m, $-CH_2-$), δ 2.50 (m, $-CH_2-C=$), δ 7.11 (s, $-CH=$).

Cyclooctapyrazole (IId). Cyclooctapyrazole was synthesized from 32.2 g (0.21 mol) of Id and 11.0 g (0.22 mol) 99 % hydrazine hydrate in 150 ml dry ether.

Yield 26.6 g (85 %). B.p.$_{1.5}$ 160 – 165 °C. M.p. 45 °C (lit.[18] M.p. 45 °C) ^{1}H NMR (DMSO-d_6): δ 1.42 (broad, $-CH_2-$), δ 2.54 (m, $-CH_2-C=$), δ 7.10 (s. $-CH=$).

D,L-α-*(Cyclopentapyrazolyl-2)propionic acid (Va).* 54.0 g (0.50 mol) of IIa and 108.6 g (0.60 mol) ethyl α-propionate were warmed at 120 °C for 4 h. After cooling, 100 ml water was added and the mixture was extracted three times with 100 ml portions of ether. The ether phase was washed with water and dried over magnesium sulphate. A GLC test showed that the ether solution contained 1-ester and 2-ester in the ratio 70:30. The ether was distilled off in a rotary evaporator and the residue was fractionated at reduced pressure. 34.1 g product with b.p.$_{0.5}$ 134 – 136° C was obtained. 20 % of IIa remained unreacted. ^{1}H NMR (DMSO-d_6): δ 6.95 (s, $-CH=$; IIIa), δ 7.21 (s, $-CH=$; IVa).

10.0 g of the product was hydrolyzed with

50 ml 2 M sodium hydroxide at 100 °C for 2 h. After cooling, the hydrolysate was extracted with ether. The water phase was then acidified with concentrated hydrochloric acid to pH 3.5 and placed in a refrigerator. 6.0 g product was obtained containing both 1-acid and 2-acid. After recrystallization twice from ethanol-water (2:1) and once from ethanol, no 1-isomer was found by ^{1}H NMR analysis. M.p. 170–171 °C. ^{1}H NMR (DMSO-d_6): δ 1.57 (d, 3 H, CH_3-), δ 2.46 (complex m, 6 H, $-CH_2-$), δ 4.89 (q, 1 H, α-H), δ 7.21 (s, 1 H, $-CH=$). IR (KBr), C=O 5.8 μ. UV, λ_{max} 233.4 nm (log ε 3.82). [Found (179.4, titration): C 59.87; H 8.73; N 15.65. Calc. for $C_9H_{12}N_2O_2$ (180.2): C 59.99;H 6.71; N 15.54].

D,L-α-*(Cycloheptapyrazolyl-2)propionic acid (Vc).* The acid Vc was synthesized in the same way from 40.0 g (0.29 mol) cycloheptapyrazole and 63.4 g (0.35 mol) ethyl α-bromopropionate. 33.8 g product was obtained after distillation. B.p. $_{0,1-0.2}$ 126–134 °C. Hydrolysis and recrystallization once from ethanol-water and twice from ethyl acetate yielded 18.5 g of Vc. M.p. 154–159 °C. IR (KBr) C=O 5.8 μ. UV λ_{max} 231.2 nm (log ε 3.83). ^{1}H NMR (DMSO-d_6): δ 1.55 (complex m and d, 9 H, $-CH_2-$ and CH_3-), δ 2.50 (complex m, 4 H, $-CH_2-\underset{|}{C}=$), δ 4.82 (q, 1 H, α-H), δ 7.28 (s, 1 H, $-CH=$). [Found (208.2, MS): C 63.36; H 7.64; N 13.54. Calc. for $C_{11}H_{16}N_2O_2$ (208.2): C 63.45; H 7.73; N 13.45].

D,L-α-*(Cyclooctapyrazolyl-2)propionic acid (Vd).* 58.6 g (0.39 mol) cyclooctapyrazole was reacted with 81.5 g (0.45 mol) of ethyl α-bromopropionate in the same way. 33.5 g product was obtained with b.p.$_{0.8}$ 153–156 °C. Hydrolysis and recrystallization from ethanol-water (2:1) yielded 16.2 g of Vd. M.p. 107 °C. IR (KBr) C=O 5.8 μ. UV λ_{max} 231.5 nm (log ε 3.76). ^{1}H NMR (DMSO-d_6): δ 1.57 (complex m and d, 11 H, $-CH_2-$ and CH_3-), δ 2.54 (complex m, 4 H, $-CH_2-\underset{|}{C}=$), δ 4.93 (q, 1 H, α-H), δ 7.36 (s, 1 H, $-CH=$). [Found (222.2, MS): C 64.61; H 8.19; N 12.43. Calc. for $C_{12}H_{18}N_2O_2$ (222.3): C 64.82; H 8.16; N 12.60].

Alkylation of cyclopentapyrazole and 4,5,6,7-tetrahydroindazole with ethyl α-bromopropionate in alkaline medium. 1.0 g (9 mmol) of IIa was dissolved in a sodium ethoxide solution made from 0.30 g (13 mmol) sodium in 7 ml 99 % ethanol. 5.0 g (22 mmol) ethyl α-bromopropionate was the added with stirring for a few minutes. The temperature increased to 52 °C and a white precipitate of sodium bromide was formed. After 25 min the reaction mixture became neutral. The mixture was concentrated in a rotary evaporator, and 5 ml of water was added to the residue. The mixture was extracted with three 10 ml portions of ether, the organic layer was dried, and the ether was distilled off. A ^{1}H NMR analysis of the mixture showed that 70 % of IIa was transformed into an ester mixture consisting of 42 % IIIa and 58 % IVa.

Alkylation of IIb in the same way gave 41 % IIIb and 59 % IVb of the total amount of ester formed. A good analytical separation of IIb, IIIb, and IVb by GLC was obtained with retention times 54, 93, and 102.5 min., respectively. The retention times for IIa, IIIa, and IVa were 27, 45, and 53 min.

Table 1.

Crystallization	Ethyl acetate (ml)	Salt obtained (g)	$[\alpha]_D^{25}$ of the acid (°)
1	780	12.5	−16
2	250	10.3	−27
3	180	8.8	−31
4	150	7.1	−37
5	130	6.0	−40
6	85	5.1	−45
7	65	4.3	−46
8	50	3.8	−45
9	50	3.2	−45

Preliminary tests on resolution of α-(cycloheptapyrazolyl-2)propionic acid (Vc). Preliminary experiments on resolution were performed with common alkaloids and some synthetic bases. 1 mmol of Vc and 1 mmol base were dissolved in a small amount of solvent and the salt obtained was filtered off and dried. The acid was liberated by adding 2 M sodium hydroxide. The organic base was removed by extraction several times with chloroform. The water solution was then acidified with 2 M hydrochloric acid to pH 3.5. The acid was filtered off and dried, and, the optical activity was determined in chloroform.

D*(R) (−)α-(Cycloheptapyrazolyl-2)propionic acid.* 12.5 g (0.060 mol) of Vc and 10.0 g (0.060 mol) (−)-ephedrine were dissolved in 780 ml of boiling ethyl acetate. After one night in a refrigerator the salt was filtered off and recrystallized several times from ethyl acetate. Acid was liberated from 0.10 g salt after each recrystallization and the optical activity was determined in chloroform. The results are given in Table 1.

The acid was liberated from its (−)-ephedrine salt by adding 2 M sodium hydroxide. After extraction with chloroform the water phase was acidified to pH 3.5 with concentrated hydrochloric acid. The product was recrystallized from ethanol–water (2:1) and dried over phosphorus pentoxide in vacuum. 1.1 g of Vc was obtained with m.p. 177–178.5 °C. The optical activity of (−)Vc in some different solvents is given in Table 2.

L(+)-α-*(Cycloheptapyrazolyl-2) propionic acid.* The mother liquor from the first crystallization

Table 2. The optical activity of (−)Vc in some different solvents. g = gram acid dissolved in the solvent to 10.0 ml

Solvent	$[\alpha]_D^{25}$ (°)	$[\alpha]_{365}^{25}$ (°)	g acid
Dimethyl formamide	+11.8	+33.8	0.0801
Glacial acetic acid	+1.5	+7.4	0.0830
Dimethyl sulphoxide	−0.4	−5.4	0.0790
Methanol	−7.5	−31.9	0.0800
Ethanol (abs.)	−11.8	−45.6	0.0811
Chloroform	−46.5	−156	0.0400

Table 3.

Crystallization	Ethyl acetate (ml)	Salt obtained (g)	$[\alpha]_D^{25}$ of the acid (°)
1	130	7.8	+24
2	120	6.6	+28
3	120	5.8	+30
4	120	5.1	+35
5	160	4.4	+37
6	190	3.7	+41
7	120	2.7	+42
8	160	2.2	+43
9	210	1.3	+42

was evaporated to dryness. 10.5 g salt was isolated from which the acid was liberated as described earlier. 4.6 g acid with $[\alpha]_D^{25} = +17°$ was obtained. This acid (0.022 mol) and 3.77 g (0.022 mol) (−)-α-(β-naphthyl)ethylamine [19] were dissolved in 130 ml of boiling ethyl acetate and the salt allowed to crystallize in a refrigerator. After nine recrystallizations the optical activity remained constant. The course of the resolution is given in Table 3. The acid was then liberated and recrystallized from ethanol–water (2:1). 0.50 g acid in white glistening crystals was obtained with m.p. 178–180 °C. $[\alpha]_D^{25} = +46.9°$ (*c* 0.398).

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1-*O*-(2-Hydroxyalkyl)glycerols Isolated from Greenland Shark Liver Oil

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2-Hydroxy-substituted glycerol ethers with 14, 16, and 18 carbon atoms in the long carbon chains have been found in the unsaponifiable fraction of Greenland shark liver oil. The mass spectrum of the isopropylidene derivative of the predominant 16:1 hydroxy compound from shark liver oil and that of synthetically prepared 1-*O*-(2-hydroxy-4-hexadecenyl)-2,3-*O*-isopropylideneglycerol were identical.

Methoxy-substituted glycerol ethers with 16:0 16:1, and 18:1 * as the predominating carbon chains have been isolated from shark liver oil.[1] They have also been found in trace quantities in the lipids from other marine animals [2] and from mammals including man.[3] In isolating larger quantities of methoxy-substituted glycerol ethers from other lipids in the unsaponifiable fraction of Greenland shark liver oil, the enrichment steps were checked by thin-layer chromatography (TLC). A small spot with an R_F-value lower than that of the methoxy glycerol ethers was observed. By a series of concentration steps the unknown compounds were obtained in milligram quantities. The R_F-values, compared with those of the unsubstituted and the methoxy-substituted glycerol ethers and their isopropylidene derivatives and later also with synthetically prepared 1-*O*-(2-hydroxyhexadecyl)glycerol [4] and 1-*O*-(2-hydroxyhexadecenyl)glycerol, indicated that the unknown compounds could be hydroxy-substituted glycerol ethers. This was also supported by the gas chromatographic – mass spectrometric (GLC–MS) analysis. The retention time and the mass spectrum of the compound, corresponding to the predominant peak of the gas chromatogram, were identical with the retention time and the mass spectrum of synthetically prepared 1-*O*-(2-hydroxy-4-hexadecenyl)-2,3-*O*-isopropylideneglycerol (Figs. 1 and 2). Hydroxy-substituted tetradecyl, tetradecenyl, hexadecyl, and octadecenyl glycerol ethers were also indicated. It is not likely that the position of the double bond differs from that in the methoxy-substituted glycerol ethers.[1] Therefore the compounds found would be 1-*O*-(2-hydroxytetradecyl)glycerol, 1-*O*-(2-hydroxy-4-tetradecenyl)glycerol, 1-*O*-(2-hydroxyhexadecyl)glycerol,[4,5] 1-*O*-(2-hydroxy-4-hexadecenyl)glycerol, and 1-*O*-(2-hydroxy-4-octadecenyl)glycerol.

In investigations on the biosyntheses of alk-1-enyl glycerol ethers from the corresponding alkyl compounds Blank *et al.*[6] and Snyder *et al.*[7] found an unidentified compound that behaved similarly to a β-hydroxy-*O*-alkylglycerol. It has been proposed [7] that a glycerol ether compound with a substituent such as hydroxy in the 2-position of the alkyl chain could be an intermediate in the biosynthesis of the alk-1-enyl lipids from the alkyl ones. However, in experiments by Muramatsu and Schmid,[8] labelled 1-*O*-(2-hydroxyheptadecyl)-glycerol, which had been formed in rat brain after administration of 1,2-heptadecanediol-2-^{14}C, did not form observable amounts of labelled *O*-alk-1-enyl ether.

* The first figure denotes the number of carbon atoms in the long carbon chain (the methoxy group excluded) and the figure after the colon the number of double bonds.

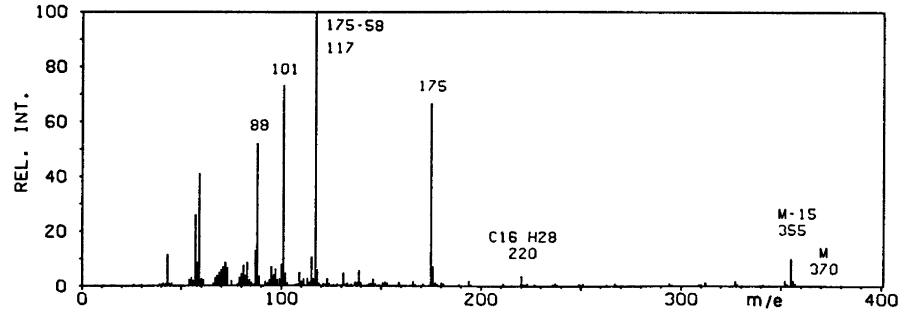


Fig. 1. Mass spectrum of the isopropylidene derivative of 1-*O*-(2-hydroxyhexadecenyl)glycerol, isolated from Greenland shark liver oil.

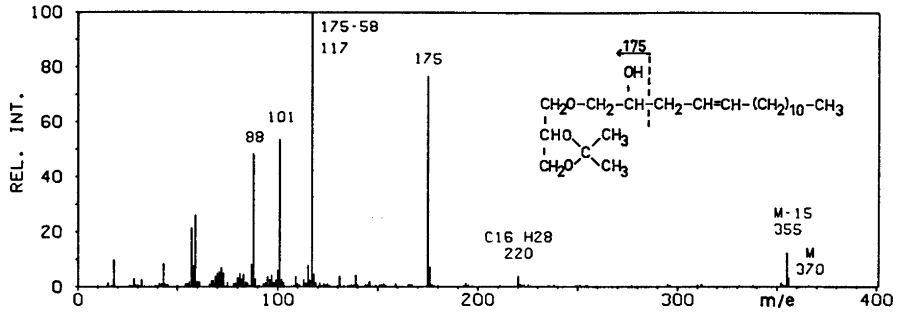


Fig. 2. Mass spectrum of synthetic 1-*O*-(2-hydroxy-4-*cis*-hexadecenyl)-2,3-*O*-isopropylideneglycerol.

EXPERIMENTAL

Enrichment of hydroxy-substituted glycerol ethers from the unsaponifiable fraction of the liver oil from Greenland shark * (*Somniosus microcephalus*) was performed by the following procedure. The unsaponifiable fraction (13 g), obtained after hydrolysis of the liver oil in 1 M ethanolic KOH, was freed from some less polar material by crystallization from acetone (120 ml) at −18 °C. The material (4.0 g) from the filtrate was dissolved in 81 % methanol (125 ml) and extracted continuously with light petroleum, b.p. 40−60 °C, (in an extractor with a sintered disc distributor). The lipids (2.3 g) obtained from the methanol−water phase were chromatographed on a silicic acid (75 g) column (Bio-Sil HA minus 325 mesh, Bio-Rad Laboratories, Richmond Calif). After eluting less polar components with ether, the more polar ones were eluted with methanol. TLC (silica gel G, Merck, developing solvent: trimethylpentane − ethyl acetate − methanol, 50:40:10) showed that the unknown compounds were enriched in the first fractions of the methanol eluate. This material (10 mg) was treated with acetone in the presence of 10^{-2} M $HClO_4$.[9] TLC (developing solvent: trimethylpentane−ethyl acetate, 60:30) indicated that some compounds in the mixture had been transformed to isopropylidene derivatives. Preparative TLC gave 1.4 mg of a material, which was subjected to gas chromatographic-mass spectrometric analysis.

The GLC−MS analysis was performed on a LKB 9000 combination instrument under the following operating conditions: electron energy 70 eV, ion accelerating voltage 3.5 kV, trap current 60 μA, and ion source temperature 270 °C. The gas chromatographic separation was carried out at 210 °C using a 3 m × 3 mm i.d. glass column packed with Gas Chrom Q 80−100 mesh, containing 1 % Apiezon L. The flow was 30 ml helium/min.

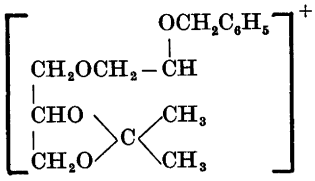
Synthesis of 1-*O*-(2-hydroxy-4-*cis*-hexadecenyl)-2,3-*O*-isopropylideneglycerol

2-Benzyloxy-4-hexadecynyl p-toluenesulfonate and methanesulfonate. Reduction of methyl 2-benzyloxy-4-hexadecynoate [10] (2.95 g) with lithium aluminium hydride gave 2.7 g of crude 2-benzyloxy-4-hexadecyn-1-ol, which by treat-

* The liver oil was supplied by A/S Johan C. Martens and Co., Bergen, Norway.

ment with *p*-toluenesulfonyl chloride (2.0 g) in the presence of dry pyridine [11] gave 3.9 g of almost pure (as shown by TLC) 2-benzyloxy-4-hexadecynyl *p*-toluenesulfonate. The corresponding methanesulfonate was obtained by treatment of the alcohol with methanesulfonyl chloride in the manner described by Baumann and Mangold.[12]

1-O-(2-Benzyloxy-4-hexadecynyl)-2,3-O-isopropylideneglycerol was prepared according to the procedure described by Gupta and Kummerow.[13] Potassium (0.45 g) was granulated by stirring in refluxing dry benzene (25 ml). Isopropylideneglycerol (4.0 g) was slowly added during 15 min. After 1 h, when no more potassium could be observed in the flask, a solution of 2-benzyloxy-4-hexadecynyl *p*-toluenesulfonate (3.9 g) in dry benzene (5 ml) was added. The mixture was refluxed for 5 h. After cooling, ether was added. The benzene–ether solution was washed with water and dried over anhydrous sodium sulfate. The crude product was purified by silicic acid chromatography, yielding 1.57 g (41 %) of almost pure (as shown by TLC) acetonide of 2-benzyloxyhexadecynylglycerol. (On storage the acetonide slowly decomposed into several compounds). The 1-*O*-(2-benzyloxyhexadecynyl)-2,3-*O*-isopropylideneglycerol was also prepared from the methanesulfonate. Tetrahydrofuran was used as solvent instead of benzene. The yield of purified acetonide was 56 % of the calculated amount. The mass spectrum of the acetonide [4] shows a molecular ion peak at $m/e = 458$. The base peak at $m/e = 91$ is probably due to tropylium ions, formed from the benzyl group. Prominent peaks at $m/e = 265$ and $m/e = 313$ correspond to the fragments



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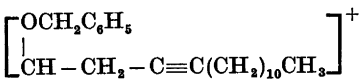

*1-O-(2-Hydroxy-4-*cis*-hexadecenyl)-2,3-O-isopropylideneglycerol.* Selective hydrogenation [14] of 1-*O*-(2-benzyloxy-4-hexadecynyl)-2,3-*O*-isopropylideneglycerol (660 mg) in pyridine solution (5 ml) and with 5 % palladium on barium sulfate (70 mg) as catalyst gave a mixture of benzyloxy- and hydroxy-substituted hexadecenyl compounds (in the proportions 9:1). Chromatography on a silicic acid column (eluent: light petroleum – ether, 9:1) gave 60 mg of 1-*O*-(2-hydroxy-4-hexadecenyl)-2,3-*O*-isopropylideneglycerol (MS in Fig. 2). Hydrolysis of the acetonide in a mixture of ether and concentrated hydrochloric acid [15] and purification by chromatography on a silicic acid column (eluent: ether-methanol, 99:1) gave the free glycerol ether (pure as shown by TLC) as a colourless transparent jelly, which after crystallization at −18 °C melted at about 34 °C (probably polymorphism). The IR spectra of the 1-*O*-(2-hydroxy-4-hexadecenyl)glycerol and of 1-*O*-(2-hydroxyhexadecyl)glycerol were almost identical except in the ranges of the double bond absorptions (≈3010, 1635 and 700 cm^{-1}). The *cis* form of the unsaturated compound was confirmed by the bands at 1635 and 700 cm^{-1}.

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Enethiols. VIII.* 3-Mercapto-5,5-dimethyl-2-cyclohexen-1-one ("Thiodimedone") and Derivatives. Thermal and Photochemical Rearrangements

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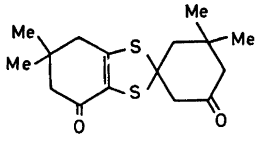
Reaction of 3-chloro-5,5-dimethyl-2-cyclohexen-1-one with Na_2S gave 3-mercapto-5,5-dimethyl-2-cyclohexen-1-one (RSH). Subsequent alkylation of RSH (Na^+ salt) gave the sulfides RSR′ (R′ = CH_3-, $CH_2=CHCH_2-$, $CH_3CH=CHCH_2-$, and CH_3COCH_2-). Similarly RSH (Tl^+ salt) yielded RSR′ (R′ = $HC\equiv CCH_2-$) while RSH (Bu_4N^+ salt) gave (R′ = $H_2C=C=CH-$) by alkylation with $HC\equiv CCH_2Br$. Allylic sulfides (RSR′) underwent thio-Claisen rearrangement and cyclization at about 140 °C in quinoline to 2,6,6-trimethyl-4-oxo-2,3,4,5,6,7-hexahydrobenzothiophene (when R′ = $CH_2=CHCH_2-$) and 2,3,6,6-tetramethyl-4-oxo-2,3,4,5,6,7-hexahydrobenzothiophene (when R′ = $CH_3CH=CHCH_2-$). Rearrangement in acetic anhydride gave 3-acetylthio-2-allyl-5,5-dimethyl-2-cyclohexen-1-one (when R′ = $CH_2=CHCH_2-$). Photolysis of RSR′ (R′ = $CH_2=CHCH_2-$, $CH_3CH=CHCH_2-$, $PhCH_2-$, $CH_3C(O)-$, and $H-$) gave RSSR and a 1,3-dithiole:



This product was obtained in 79 % yield by photolysis of RSSR. Photolysis of RSR′ (R′ = phenyl) gave 3,3-dimethyl-5-oxo-2,3,4,5-tetrahydrodibenzothiophene.

In previous papers dealing with the thermal [1-5] and photochemical [1] behaviour ($Z-E$ isomerization [1,2] and thio-Claisen rearrangements [1-5]) of α,β-unsaturated sulfides, open chain compounds were investigated. As part of this study it seemed desirable to investigate enethiols and sulfides having the corresponding mercapto- and alkylthio group placed in the β-position of an α-β-unsaturated carbonyl compound, but in which intramolecular H-bonding of the enethiol is excluded. Thiodimedone (3-mercapto-5,5-dimethyl-2-cyclohexen-1-one) and the 3-alkylthio derivatives were thought to be suitable representatives of such compounds. 3-Mercapto-5,5-dimethyl-2-cyclohexen-1-one and most of its derivatives were conveniently prepared from the 3-chloro-2-cyclohexen-1-one (for relevant literature, see Refs. 6 – 15). Results on synthesis, thermolysis, and photolysis of these compounds are the subject of the present work.

Synthesis of 3-mercapto-5,5-dimethyl-2-cyclohexen-1-one (thiodimedone) and derivatives (Fig. 1). Dimedone *1*, when treated with PCl_3 gave *2*,[16] which by further reaction with Na_2S gave *3* and small amounts of *4*. Acidification, alkylation or oxidation of the water solution of *3* yielded thiodimedone *5*, the sulfides *6* – *9*, or the disulfide *13*, respectively. Preparation of the unsoluble thallium(I) salt of *5* suspended in HMPA followed by alkylation with 2-propynyl bromide resulted in the formation of the 2-propynyl sulfide *10*. When alkylation was performed in a homogeneous mixture, consisting of the tetrabutylammonium salt of *5* dissolved in CH_2Cl_2 and 2-propynyl bromide, the allenyl sulfide *11* was formed. The acetyl derivative *12* was prepared from acetyl chloride and *5* dissolved in pyridine. Treatment of *1*

* Part VII. See Ref. 1.

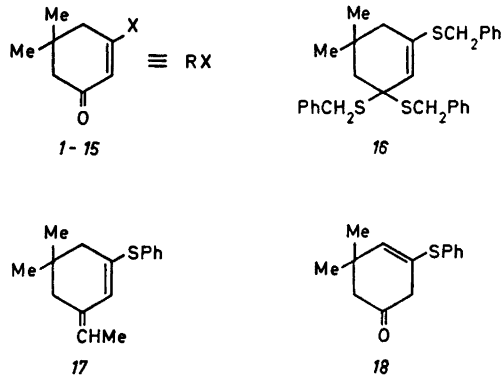


Fig. 1. Compounds *1-18*. 1 (ROH), *2* (RCl), 3 ($RS^{\ominus}$), *4* (RSR), *5* (RSH), *6* ($RSCH_3$), *7* ($RSCH_2CH=CH_2$), *8* ($RSCH_2CH=CHCH_3$), *9* ($RSCH_2COCH_3$), *10* ($RSCH_2C{\equiv}CH$), *11* ($RSCH=C=CH_2$), *12* ($RSCOCH_3$), *13* (RSSR), *14* (RSPh), *15* ($RSCH_2Ph$).

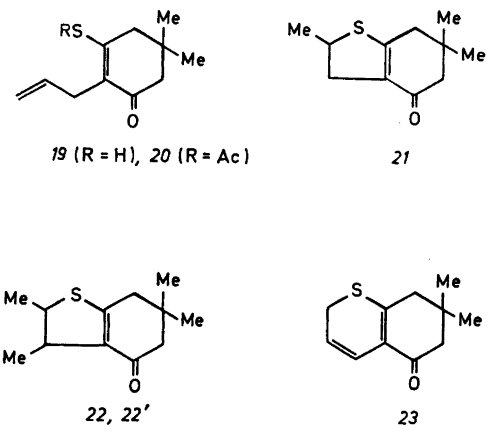
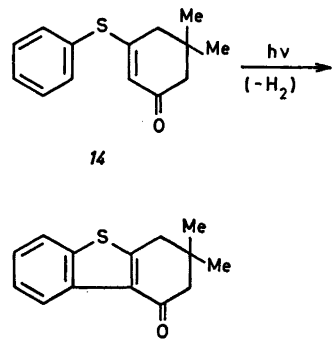


Fig. 2. Compounds *19-23*.

with the appropriate thioles gave the sulfides *14* [7] and *15* [7] together with the thioacetal *16*. When *14* was reacted with ethyllithium a 1,2-addition followed by elimination took place resulting in the formation of *17*, while treatment of *14* with $LiNH_2/NH_3$ gave partly isomerization to *18* (see also Refs. 17, 18).

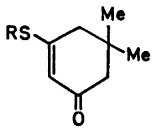
Thermal rearrangements of allylic derivatives. 3-Allyloxy-2-cyclohexen-1-one has been shown [19] to undergo Claisen rearrangement at 190-210 °C. Similarly the 3-allylthio analogues do undergo thio-Claisen rearrangement. Rearrangement in the presence of *p*-toluenesulfonic acid resulted in the formation of 2-allyl-3-mercapto-5,5-dimethyl-2-cyclohexen-1-one *19*, which cyclisized partly during distillation. When the reaction was performed in boiling acetic anhydride, 3-acetylthio-2-allyl-5,5-dimethyl-2-cyclohexen-1-one *20* was isolated. Rearrangement in quinoline at 160 °C yielded the hexahydrobenzothiophene *20*. The 2-butenyl derivative rearranged to *21* somewhat slower under similar conditions. Heating of the 2-propynyl (*10*) and the allenyl (*11*) sulfides in quinoline or pyridine resulted mostly in tarry material However, the 2H-thiapyran *23* could be isolated in a reasonably pure state by rearrangement of *10*.

Photolysis of 3-mercapto-5,5-dimethyl-2-cyclohexen-1-one and sulfide derivatives. Photolysis of the compounds *5*, *7*, *8*, *12*, and *15* (Table 1) did not yield the expected [23-24] 2+2 cycloadducts. Irradiation of the enethiol *5* gave the disulfide *13* which is an ordinary type of product in photolysis of thiols,[25] the 1,3-dithiole *24* and the 1,4-dithiane *25*. The allyl *7*, 2-butenyl *8*, and the benzyl *15* derivatives gave *13* and *24* in variable ratios. In one experiment when *15* was irradiated traces of PhCHO arising from the benzyl group were detected along with *13*. The acetyl derivative *12* yielded *13* and *24* together with EtOAc upon photolysis in EtOH. As might be expected the phenyl derivative *14* did not undergo cleavage reaction at the R-S bond, but instead an intramolecular cyclization-oxidation reaction (eqn. 1) took place, giving the tetrahydrodibenzothiophene *26* in poor yields. Similar reactions have been observed for other types of divinyl sulfides.[26,27] In



(1)

Table 1. Photolysis of 3-mercapto-5,5-dimethyl-2-cyclohexen-1-one and derivatives. Conditions and product distribution.

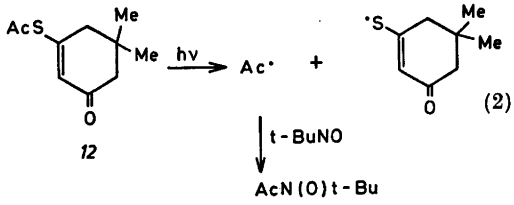


Compound	R	Starting material g (mmol)	Solvent (175–200 ml)	Reaction time (h)	Light [a] source (nm)	Recovered g (%)	*5*, g (%)	*24*, g (%)	Compound, g (%)
5	H	1.52(9.75)	hexane	44	300 py.	0	0.624(41)	0.332(22)	*25*, 0.129(9)
13		5.00(16.0)	EtOH	18	254 qu.	0.26(5)		3.95(79)	
7	$CH_2CH=CH_2$	2.45(12.5)	cyclohexane	16	300 qu.	1.11(45)		trace	
8	$CH_2CH=CHCH_3$	0.75(3.57)	EtOH	19	300 qu.	1.49(61)	0.165(11)		
12	Ac	4.97(25.2)	EtOH	33	254 qu.	0.248(28)	1.08(28)	2.3(59)	EtOAc (GLC)
14	Ph	2.34(10.1)	acetone [b]	72	300 qu.	1.08(46)			*26*, 0.092(4) diacetone-alcohol, 8.5 g
14	Ph	4.56(19.7)	ether [b]	110	300 qu.	3.60(79)			*26*, 0.109(2.4)
14	Ph	3.70(16.0)	dioxan	67	300 py.	1.58(43)			*26*, 0.184(5)
15	$PhCH_2$	2.46(10.00)	ether	11	300 qu.	1.49(61)	0.165(11)		
15	$PhCH_2$	0.80(3.25)	hexane	36	300 py.	0.22(28)	0.10(20)	0	PhCHO, trace

[a] λ_{max} of "Rayonet RS" mercury arcs. Reaction vessel: qu.=quartz, py.=pyrex.
[b] 1 l.

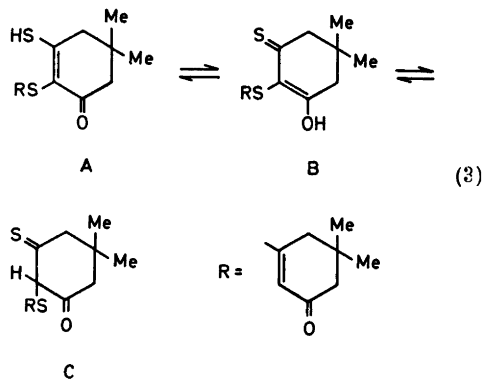
order to find out if *24* could be formed from *13* this compound was irradiated at 254 nm in ethanol. It turned out [28] that *24* was produced in high yields (79 %) and crystallized directly from the solvent. Also sunlight caused this conversion (*13*→*24*) in solution, while in the solid state *13* was quite stable. The possibility that the rearrangement was thermally induced was ruled out, as no conversion was observed when *13* was boiled in ethanol in the dark. More severe conditions (*i.e.* heating in HMPA in the presence of $KHSO_4$)[29,30] which have brought about 3,3-sigmatropic rearrangements of other divinyl-disulfides,[29–32] gave only tar.

Various methods were used in order to determine the mechanisms of the observed photochemical reactions. A hexane solution of the acetyl derivative *12* and 2-methyl-2-nitrosopropane was irradiated at 254 – 300 nm in the ESR spectrograph. The formation of *N*-acetyl-*N*-(*t*-butyl)nitroxide ($a_N = 8$ gauss) [33] indicated that the primary photochemical process was a cleavage of *12* into acetyl and thiyl radicals (eqn. 2). However, no well defined signal from the thiyl radical or trapped thiyl radical could be observed. Also attempts to trap radicals by photolysis of the disulfide *13* were fruitless. The conversion of *13* irradiated with monochromatic light (254 and 300 nm) was measured by the change of the UV spectrum. The change of the UV spectrum obtained in ethanol differed



(2)

clearly from that obtained in $CHCl_3$. In both solvents the absorption at 277 nm disappeared with about equal rates. However, in ethanol an absorption at 350 nm appeared quickly, reached a maximum and then faded out. In $CHCl_3$ only comparatively weak absorption at 350 nm was found. It is believed that different tautomers (eqn. 3) of the same intermediate are formed when the solvent is changed, thus giving rise to different UV-spectra. For comparison, it should be pointed out that *5* showed a behaviour similar to the intermediate in eqn. 3 as *5* revealed absorption at 342 nm in ethanol and DMSO, while in $CHCl_3$ this band was comparatively weak. The suggested intermediate (M) represented by structure A, B, and C in eqn. 3 might be formed by a radical chain process initiated by light (eqn. 4). The propagation step (eqn. 5) consists of a thiyl radical attack on C-2 of *13* (RSSR), giving M and another thiyl radical. This means that the rate of disappearance of *13* is dependent on the concentration of *13*. Measurement of the quantum yield ($\Phi = 0.1$) for disappearance of *13* showed no such dependence on [RSSR], and it seems therefore reasonable to suggest another mechanism. The reaction could proceed through reactions in which two thiyl radicals combine to give M either as free radicals or in a solvent cage (eqn. 7). Finally a concerted [1,3]-sigmatropic process could be suggested (eqn. 8). The conversion of M to *24* might be a thermal cyclisation reaction, but more likely a photochemical process, because it explains the change of the UV spectrum in EtOH. The absorption at 350 nm, ascribed to M, did not change on standing in the dark, while irradiation caused a change as described above.



(3)

Initiation:

$$RSSR \rightarrow RSSR^* \rightarrow 2\ RS\cdot \quad (4)$$

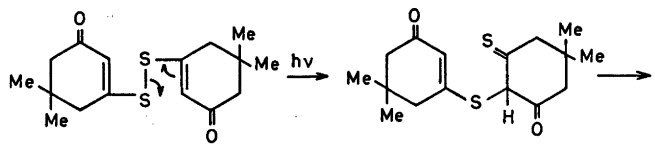
Propagation:

$$RS\cdot + RSSR \xrightarrow{k_1} M + RS\cdot \quad (5)$$

$$\Phi I = -dC/dt = k_1[RS\cdot][RSSR] \quad (6)$$

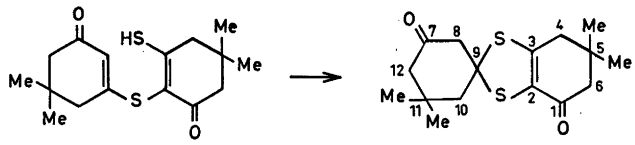
$$2\ RS\cdot \rightarrow M \quad (7)$$

$$RSSR \rightarrow RSSR^* \rightarrow M \quad (8)$$



13

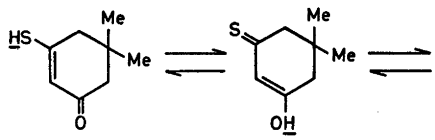
(9)



24

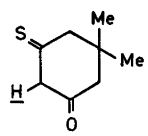
SPECTROSCOPY

Tautomerism of thiodimedone. In principle thiodimedone *5* can exist as the monomeric keto-enethiol (D), thioketo-enol (E), and the ketothioketo (F) structures (eqn. 10). In concentrated solutions or neat also dimer structures



5 (D) 5 (E)

(10)



5 (F)

analogous to the findings for 1,3-cyclohexandione and dimedone should be considered likely.[36,37] The IR spectrum of thiodimedone showed absorption at 2520 cm^{-1} and a broad band at 1680 cm^{-1} assigned, respectively, to the mercapto and the α,β-unsaturated keto group of the keto-enethiol (D). This was the only tautomer detectable by NMR. The signal at δ 6.05 was assigned to the vinyl proton, as an allylic coupling to the protons on C-4 was observed. The broad signal at δ 4.23 could be attributed to a mercapto proton, excluding an enolic proton, which was expected to reveal a signal at lower field (δ 9 – 13). The broadening of this signal was ascribed to proton exchange. Contrary to what is often found for enethiols, no coupling between the proton of the mercapto group on C-3 and the protons on C-4 was present. The structural requirement for a maximum coupling is that the bondings between the hydrogens obtain a planar zig-zag framework, as found for enethiols possessing an intramolecular chelated structure.[38] As only energetical less important intermolecular hydrogen bonding can occur in thiodimedone, the rotation about the C – S bond is less restricted, and the coupling decreases to zero. The UV spectrum of thiodimedone obtained in hexane showed absorption at 268 nm, ascribed to the $\pi - \pi^*$ transition of the α,β-unsaturated keto chromophore, present in the keto-enethiol (A). When the solvent was changed to EtOH or DMSO absorption maxima at 282 and 340 nm were observed. On going from an α,β-unsaturated ketone to the thione the $\pi - \pi^*$ absorption is shifted about 80 nm.[39–42] The strong absorption of 340 nm must therefore arise from structure *5*E. The structure *5*F cannot be excluded because saturated thioketones have only weak absorption above 300 nm and the observed one is strong.

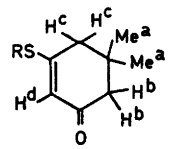
Identification of other compounds. The spectroscopic data of 3-alkylthio derivatives of thiodimedone are shown in Table 3 (NMR) and Table 4 (IR and UV). These compounds gave rise to an IR absorption in the range of 1650 – 1680 cm^{-1} due to an α,β-unsaturated carbonyl group and a band at 1570 – 1595 cm^{-1} corresponding to a carbon-carbon double bond. The UV spectra of compounds *7* – *10*, *15* showed

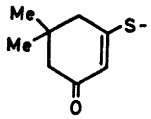
Table 2. Rate of disappearance ($-\Delta C/\Delta t$) of compound *13*, measured by the change in UV absorbance (ΔAbs) for various concentrations (C) of *13*.

$C \times 10^7$ mol/cm³	ΔAbs	$-\Delta C \times 10$/[a] mol/cm³	Δt (s)	$-\frac{\Delta C}{\Delta t} \times 10^{11}$ mol/cm³ s
0.645	0.29	1.45	180	8.06
1.30	0.23	2.30	300	7.67
1.94	0.25	3.45	420	8.23
3.23	0.21	5.25	720	7.23
3.87	0.23	6.90	900	7.67
6.45	0.29	14.50	1800	8.05

[a] ΔC corresponds to 16–23 % conversion of *13*. $\Delta C = \Delta\text{Abs}/\Delta\varepsilon$, where $\Delta\varepsilon$ is the difference between the molar absorbance of compound *13* ($\varepsilon = 21\,400$) and that of compound *24* ($\varepsilon = 1400$) at 277 nm.

Table 3. ^{1}H NMR chemical shift (δ values) and coupling constants (J Hz) of 3-mercapto-5,5-dimethyl-2-cyclohexen-1-one and derivatives. The solvent is CCl_4.



Compound	R	δ_a	δ_b	δ_c	δ_d	δ_e	δ_f	J_{cd}	J_{ef}
5	H[e]	1.05(s)	2.13(s)	2.39(d)	6.05(t)	4.23(s)		1.30	
13		1.06(s)	2.19(s)	2.39(d)	6.03(t)				
7	$\overset{e}{CH_2}=\overset{e}{CH}\text{-}\overset{f}{CH_2}$-	1.05(s)	2.13(s)	2.26(d)	5.73(t)	5.1–6.2(m)	3.46(br.d)		6
8[a]	$\overset{g}{Me}\overset{e}{CH}=\overset{e}{CH}\overset{f}{CH_2}$-	1.05(s)	2.14(s)	2.25(d)	5.73(t)	5.2–9(m)	3.42(br.d)		6
9[b]	$\overset{e}{MeCO}\overset{f}{CH_2}$-	1.05(s)	2.24(s)	2.36(d)	5.78(t)	2.32(s)	3.76(s)		
10	$\overset{e}{HC}\equiv\overset{f}{CCH_2}$-	1.06(s)	2.17(s)	2.30(d)	5.82(t)	2.35(t)	3.60(d)		2.4
11	$\overset{e}{H_2C}=\overset{f}{C}=CH$-	1.08(s)	2.15(s)	2.27(d)	6.18(t)	5.06	5.88	1.30	6.6[c]
12	$\overset{e}{MeCO}$-	1.10(s)	2.20(s)	2.50(d)	6.24(t)	2.37(s)			
14	$\overset{e}{Ph}$-	1.04(s)	2.12(s)	2.33(d)	5.42(t)	7.47(s)			
15	$\overset{e}{Ph}\overset{f}{CH_2}$-	1.02(s)	2.12(s)	2.24(d)	5.82(t)	7.28(s)	3.94(s)		

[a] $\delta_g(J_{eg}\text{Hz}) = 1.74(5)$. [b] Solvent $CDCl_3$. [c] AB_2 spin system.

Table 4. IR and UV spectra of 3-mercapto-5,5-dimethyl-2-cyclohexen-1-one and derivatives.

Compound	IR ν_{max} (cm^{-1}) $\nu_{C=O}$	$\nu_{C=C}$	Other	Conditions	UV,[a] λ_{max} nm (log ε)
5	1680(br.s)	1595	2520(w,br,)	CCl_4	282(4.12), 340(3,74) [b] 268(4.11), 300–310(w) [c] 269, 314(w) [d] 279, 342 [e] 273, 313(w) [f] 280
4	1660(s)	1580(s)		$CHCl_3$	232(3.96), 278(3.97), 313(3.90)
13	1650(s)	1580(s)		KBr	277(4.33)
7	1660(s)	1580(s)		film	288(4.24)
8	1660(s)	1580(s)		CCl_4	293(4.26)
9	1730(s) 1670(s)	1590(s)		CCl_4	287(4.20)
10	1660(s)	1590(s)	3300(m) 3250(m)	film	287(4.22)
11	1660(s)	1590(s)	1930(w)	film	297(4.15)
12	1705(s) 1670(s)	1590(s)		film	226(3.93), 277(3.96)
14	1660(s)	1580(s)		KBr	294(4.20)
15	1660(s)	1570(s)		KBr	282(4.30)
19	1655(s)	1600(s)	2530(m)	film	290, 350(shoulder)
20	1715(s) 1685(s)	1645(w) 1605(w)		film	232, 255, 278
21	1660(s)	1605(s)		film	312(4.13)
22	1660(s)	1600(s)		film	312(4.09)
23	1650(s)	1550(m)		film	

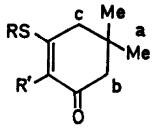
[a] The solvent is EtOH, unless otherwise stated. [b] Solvent: Hexane, [c] ether, [d] DMSO, [e] MeCN, [f] $CHCl_3$.

absorption at 287–292 nm which is ascribed to a $\pi-\pi^*$ transition of the conjugated system. The compounds *4*, *11*, *14* which were further conjugated with an additional carbon-carbon double bond showed a $\pi-\pi^*$ transition at somewhat longer wavelength *i.e.* in the range of 294–313 nm. The disulfide *13* and the acetyl *12* derivatives absorbed at shorter wavelength, namely at 277 nm, than the above mentioned compounds. The spectroscopic data of the products obtained by rearrangement of 3-allylthio and 3-(2-butenyl) derivatives are shown in Table 5 (NMR) and Table 6 (IR and UV). The UV absorptions of the hexahydrobenzothiophenes were found at 312 nm which are about 25 nm higher than the corresponding bands of the starting materials. This is in agreement with the bathochromic effect exhibited by an α-substituent on α,β-unsaturated carbonyl chromophore.

The composition and the molecular weight of the 1,3-dithiole *24* were determined to be $C_{16}H_{22}O_2S_2$ (M = 310) from the elemental analysis and the mass spectrum, respectively. The ^1H NMR spectrum showed signals at δ 1.05–1.15 δ 2.25–2.48, and δ 3.02 with the relative intensity of 1:4:6, while the IR spectrum showed strong bands at 1705, 1660, and 1560 cm^{-1}. Several structures, which were compatible with these data, could be drawn. Among the most plausible suggestions are those, which maintain the carbon skeleton and the positions of substituents of the starting material. However, an unambiguous choice could be made from the ^{13}C-noise decoupled and the off resonance NMR spectra, which are shown in Table 6. The assignment of the signals was based on the data given for the chemical shifts and C–H coupling constants of cyclohexanone [43] and cyclohexenone.[44] Approximate values of the C–H coupling constants were determined from the residual splittings, using the expression given by Ernst,[45] and shown to be of the same size as for methylene groups of cyclohexane.[45]

Compound *25* was shown to have the composition $C_{16}H_{24}O_2S_2$ (molecular weight 312)

Table 5. ^{1}H NMR chemical shift (δ values in ppm relative to TMS) and coupling constants of 2-alkyl-3-mercapto-5,5-dimethyl-2-cyclohexen-1-one and derivatives.



Compound	Solvent	R	R′	δ_a	δ_b	δ_c	δ_d	δ_e	δ_f	δ_g	J_{de}	J_{ef}	J_{eg}	J_{fg}	J_{cf}	J_{cg}
19	(CCl_4)	$H^d\,\overset{e}{CH_2}=\overset{f}{CH}-\overset{g}{CH_2}-$						3.09 (br.d)								
20	($CDCl_3$)	$Ac^d\,CH_2=CH-CH_2-$		1.09 (s)	2.36 (s)	2.69 (br.)	2.50 (s)	3.23 (br.d)	4.8	−6.05 (m)						
21	(CCl_4)	R= $-\overset{e}{CH}(Me^d)-\overset{f,g}{CH_2}-$	=R′			2.33 (t)	1.38 (d)	3.83 (m)	3.08 (m)	2.66 (m)	6.75	8.92	5.74	−15.78[a]	2.36	2.04
22	(CCl_4)	R= $-\overset{e}{CH}(Me^d)-\overset{f}{CH}(Me^g)-$	=R′	0.78 (s)	2.05 (s)	1.97 (br.s)	1.11 (d)	1.19 (d)	2.7	−3.5 (m)	7.0			6.5		
22′		80 %(22)+20 %(22′)		0.78 (s)	2.05 (s)	1.97 (br.s)	1.07 (d)	1.05 (d)	2.5	3.5 (m)	7.0			7.0		
22	(C_6D_6)			1.07 (s)	2.12 (s)	2.33 (br.s)	1.35 (d)	1.11 (d)	2.9	3.5 (m)						
22′				1.07 (s)	2.12 (s)	2.33 (br.s)	1.36 (d)	0.99 (d)	2.9	3.5 (m)						
23[b]	(CCl_4)	R= $-\overset{d}{CH_2}-\overset{e}{CH}=\overset{f}{CH}-$	=R′	1.03 (s)	2.24 (br.s)	2.34 (br.s)	3.42 (q)	5.45 (m)	6.57 (m)		5.1	10.15			0.9	

[a] Rel. sign. [b] J_{df}= 1.6 Mz.

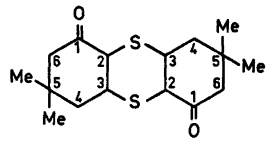
Table 6. ^{13}C NMR data of the noise and off resonance decoupled spectra of compound *24* (*cf.* p. 1081). Chemical shifts (δ values in ppm, relative to TMS) and approximate coupling constants (J_{CH} Hz) evaluated from the expression [44]

$J_{CH}{}^{r} = 2\pi \Delta\nu J_{CH}/\gamma H_2$

Relative intensities of lines (I). The solvent is $CDCl_3$.

	Me	Me	Me	Me	C5(11)	C11(5)	CH_2	CH_2	CH_2	CH_2	CH_2	C9	C2	C3	C1	C7
δ	27.4	28.6	29.4	30.2	35.3	35.9	40.5	49.3	50.5	53.2	57.4	66.9	128.9	152.5	190.0	204.0
I(rel)	206	185	188	203	198	132	171	169	193	206	157	14.7	60	90	90	198
J(Hz)	122±3	122±3	122±3	122±3	–	–	129±5	129±5	129±5	129±5	129±5	–	–	–	–	

Table 7. ^{13}C NMR data of the noise and off resonance decoupled spectrum of compound *25*.



Chemical shifts (δ values in ppm, relative to TMS) and relative intensities of lines (I). The solvent is $CDCl_3$.

	Me	Me	C5	C3	C4	C2	C6	C1
δ	28.3	34.9	39.7	48.4	49.4	54.7	54.8	213
I	162	155	59	112	119	74	211	30
Multiplicity	q	q	s	d	t	d	t	s

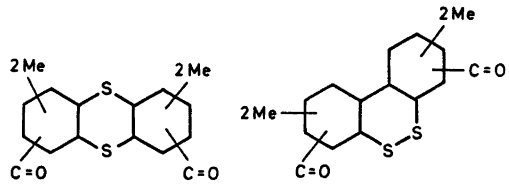
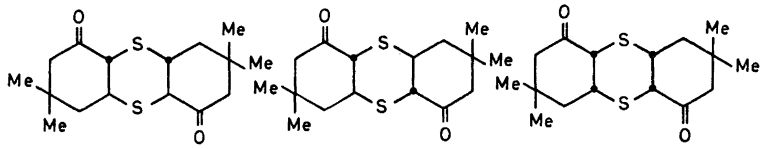


Fig. 3. Possible structures for the photo-dimer *25*.

as determined by the mass spectrum. The noise decoupled ^{13}C NMR spectrum showed 8 signals (Table 7) from the 16 carbon atoms, indicating magnetic equivalent pairs of carbon atoms. The off resonance decoupled spectrum showed the presence of methyl methylene and methine carbon atoms. It was assumed that the carbon skeleton of thiodimedone *5* was maintained in the photo-dimer *25*, which might then have one of the 1,2- or 1,4-dithiane structures shown in Fig. 3. However, the 1,2-dithiane structure was unlikely, as it did not account for the ^1H NMR multiplet signal at δ 3.6 – 4.1 assigned to a pair of equivalent methine protons. The signals due to the methine protons in the α-position to the carbonyl group of the 1,2-dithiane would be found at higher field, *i.e.* at about δ 3. The most probable structures are therefore those represented in Fig. 4, which could not be distinguished based on ground of the available data.

EXPERIMENTAL

^1H NMR spectra were recorded at 60 MHz on a Varian A-60 spectrometer. The temperature of the 15 – 20 % solutions (w/w) were 33 ± 1 °C. TMS was used as internal reference standard and the chemical shifts are expressed in δ-values and are correct within ± 0.02 ppm. ^{13}C spectra were recorded on a Varian XL-100-15 spectrometer operating in the c.w. mode at 25.2 MHz. Internal field-frequency lock was provided by the ^2H resonance of $CDCl_3$ as solvent. Carbon line positions were measured relative to the carbon resonance of internal TMS. Noise-modulated and single-frequency proton decoupling experiments were performed by means of the Varian Gyrocode spin decoupler. The sample solution was contained in a 12 mm tube. 70 eV mass spectra were obtained on a Bell and Howell CEC 21 – 104 single focussing mass spectrometer. The IR spectra were recorded as 5 % solutions, film,



25

Fig. 4. Compound *25*.

or as KBr pellets on a Perkin-Elmer infracord 137 and the UV spectra on a Bausch & Lomb Spectronic 505 spectrophotometer with EtOH as solvent. B.p's are uncorrected. Analyses were made by Novo Industri A/S, Copenhagen.

General procedure for the preparation of the sodium salt of 3-mercapto-5,5-dimethyl-2-cyclohexen-1-one. An amount of 16 g (0.1 mol) 3-chloro-5,5-dimethyl-2-cyclohexen-1-one, *2*, was dropped to a solution of 25 g (0.1 mol) $Na_2S.9H_2O$ in 25 ml water during 2 h. The mixture was kept at about 60 °C until it became homogeneous after further 2 h. This solution was then extracted with diethyl ether (2 × 10 ml), which after drying and evaporation gave small amounts of bis(5,5-dimethyl-2-cyclohexen-3-yl-1-one)sulfide, *4*. The water solution was then used for further reactions.

3-Mercapto-5,5-dimethyl-2-cyclohexen-1-one, 5. Acidification (0.1 mol) of the solution of the sodium salt of *5* with cold 4 M HCl, extraction with ether, drying and evaporation of the ether gave the crude product, which amounted to 14.8 g (95 %). The yield decreased considerably by distillation at 98 °C/0.1 mmHg; $n_D^{25} = 1.5690$. (Found: C 61.49; H 7.79; S 19.47. $C_8H_{12}OS$ requires: C 61.52; H 7.75; S 20.49).

Bis(5,5-dimethyl-2-cyclohexen-1-on-3-yl)-sulfide, 4. Ether extraction of the water solution of the sodium salt of *5* prepared by the general procedure gave after drying and evaporation 0.656 g (5 %) of m.p. 176–177 °C. (Found: C 68.84; H 7.98; S 11.46 $C_{16}H_{22}O_2S$ requires: C 69.04; H 7.97; S 11.50). MS, *m/e* (%): 280(9), 279(38), 278(51), 264(5), 263(23), 207(8), 196(5,) 195(11), 194(57), 156(9), 155(47), 152(14), 151(16), 141(9), 139(9), 138(15), 137(24), 123(9), 112(11), 111(12), 110(77), 95(9), 93(8), 91(8), 79(23), 77(15), 71(17), 68(100), 56(8), 55(22), 53(22).

Bis-(5,5-dimethyl-2-cyclohexen-1-on-3-yl)-disulfide, 13. To a water solution of 0.1 mol of the sodium salt of *5* prepared by the general method were added (excess) 20 g (0.079 mol) of iodine dissolved in 50 ml THF. After about 2 h at room temp. a saturated solution of $Na_2S_2O_3$ was added until the dark colour disappeared, and the mixture was extracted with CH_2Cl_2. This was then stripped off and the residue treated with ether and water. The ether was dried and evaporated giving 14.1 g (91 %) of crystalline product. M.p. 91 °C (ether). (Found: C 61.95; H 7.16; S 20.21. $C_{16}H_{22}O_2S_2$ requires: C 61.92; H 7.15; S 20.6). MS, *m/e* (%): 310(26), 295(9.2), 279(11), 278(37), 277(100), 255(9.2), 254(39), 253(56), 247(7.4), 246(45), 254(19), 220(11), 212(35), 156(26), 155(33), 100(56), 83(56), 71(32), 67(65), 55(37).

5,5-Dimethyl-3-methylthio-2-cyclohexen-1-one, 6. To the solution prepared by the general method were added 16 g (0.11 mol) MeI. The temp. was kept at about 30 °C. The mixture was then allowed to stir overnight. Extraction with ether, drying evaporation of the solvent yielded 15.1 g (89 %) of crystalline compound, m.p. 35 °C (lit. m.p.[6] 34–37 °C).

3-Allylthio-5,5-dimethyl-2-cyclohexen-1-one, 7. An amount of 15 g (0.12 mol) allyl bromide in 25 ml DMSO was added to the water solution of the 0.1 mol sodium salt of *5*. The temp. was allowed to rise to 45 °C. After 2 h the mixture was cooled and extracted with 15 ml ether, which was then evaporated. Extraction with water of the residue dissolved in light petroleum was repeated. Drying and evaporation and distillation gave 18 g (92 %); b.p. 65 °C/10^{-4} mmHg, $n_D^{25} = 1.5520$. (Found: C 67.34; H 8.21; S 16.11. $C_{11}H_{16}OS$ requires: C 67.32; H 8.22; S 16.31).

3-(2-Butenylthio)-5,5-dimethyl-2-cyclohexen-1-one, 8. An amount of 8 g (0.059 mol) of 2-butenyl bromide was added to 0.05 mol of the water solution of the sodium salt of *5*. Stirring overnight, followed by extraction with ether, drying and evaporation of the ether gave 8.7 g (83 %); b.p. 114–116 °C/0.1 mmHg; m.p. 33–34 °C. (Found; C 68.61; H 8.64; S 15.11. $C_{12}H_{18}OS$ requires: C 68.54; H 8.63; S 15.22).

5,5-Dimethyl-3-(propan-2-on-1-ylthio)-2-cyclohexen-1-one, 9. A water solution of 0.1 mol of the sodium salt of *5* was treated with 11 g (0.12 mol) chloroacetone. The exothermal process was finished within ½ h. Extraction with 50 ml of ether, drying, and evaporation of the solvent gave 14.5 g (68 %) of crystalline product; m.p. 65 °C (ether–light petroleum 1/1). (Found: C 62.47: H 7.74; S 15.21 $C_{11}H_{16}O_2S$ requires: C 62.25; H 7.60; S 15.08).

5,5-Dimethyl-3-(2-propynylthio)-2-cyclohexen-1-one, 10. An amount of 1.80 g (11.5 mmol) of *5* was dropped to 3.0 g (12 mmol) of thallium(I) ethoxide dissolved in 25 ml benzene. The solid product was filtered off and washed with benzene and ether, giving an mount of 3.9 g (95 %). Then 3.59 g (10 mmol) of this salt were suspended in 15 ml HMPA and 1.5 g (12.6 mmol) of 2-propynyl bromide were added. The mixture was stirred overnight and extracted with ether and water. The ether was stripped off and extraction was repeated with light petroleum and water. Drying and evaporation of the solvent yielded 1.77 g (89 %) which were distilled quantitatively. B.p. 80 °C/10^{-4} mmHg; $n_D^{25} = 1.5608$; (Found: C 68.23; H 7.04; S 16.23. $C_{11}H_{14}OS$ requires: C 68.02; H 7.27; S 16.5).

3-Allenylthio-5,5-dimethyl-2-cyclohexen-1-one, 11. An amount of 4.62 g (29.5 mmol) of *5* dissolved in 30 ml CH_2Cl_2 was added to 10.5 g (31.0 mmol) of tetrabutylammonium hydrogensulfate and 3 g (75 mmol) of NaOH in 30 ml water. Then 4 g (34 mmol) of 2-propynyl bromide were added and the mixture stirred for 18 h. The CH_2Cl_2 phase was evaporated and the residue was treated with ether and water. Drying and evaporation yielded 4.9 g (86 %) of *11*. However, the yield decreased considerably by distillation which gave 1.58 g of b.p. 70 °C/10^{-4} mmHg; $n_D^{25} = 1.5705$. (Found: C 67.94;

H 7.14; S 15.95. $C_{11}H_{14}OS$ requires: C 68.02; H 7.27; S 16.5).

3-Acetylthio-5,5-dimethyl-cyclohexen-1-one, 12. Acidification and work up (without distillation) of a solution prepared by the general method gave *5* which was then treated with 10 g pyridine and 100 ml ether. An amount of 9 g (0.115 mol) acetyl chloride was dropped to the mixture and after 1 h the pyridinium hydrochloride was filtered off and the residue washed with water. The ether was dried over $NaHCO_3$. Evaporation gave 19 g and distillation yielded 15.82 g (75 %); b.p. 96 °C/0.05 mmHg; $n_D^{25} = 1.5314$. (Found: C 60.79; H 7.13; S 15.80. $C_{10}H_{14}O_2S$ requires; C 60.59; H 7.12; S 16.15).

1,3,3-Tribenzylthio-5,5-dimethyl-1-cyclohexene, 16, and 3-benzylthio-5,5-dimethyl-2-cyclohexen-1-one, 15. Preparation by the method of Campaigne.[7] An amount of 14 g (0.1 mol) of dimedone, *1*, and 24.8 g (0.2 mol) of phenylmethanethiol were mixed in 200 ml ethanol. The solution was saturated with HCl gas. A precipitate of 1.16 g (3.6 %) of *16* was formed (theoretical yield 31.9 g). M.p. 77–82 °C. (Found: C 72.73; H 6.68; S 19.88. $C_{29}H_{32}S_3$ requires: C 73.09; H 6.77; S 20.14). NMR (CCl_4): δ 0.96 (6 H, s), 1.83 (4 H, br.s), 3.53 (2 H, s), 3.70 (4 H, s), 5.18 (1 H, s), 7.22 (15 H, s). IR ν_{max}(KBr) (cm^{-1}) 1590. UV (EtOH) λ_{max} (log ε): 212 (4.46), 252 nm (4.07). The alcohol solution was poured into 300 ml water in which 150 g Na_2CO_3 was dissolved. A precipitate was filtered, and then dissolved, dried and the solvent evaporated, giving 5.5 g (22 %) of *15*. M.p. 84–85 °C. (Found: C 73.11; H 7.29; S 13.11. $C_{15}H_{18}OS$ requires: C 73.14; H 7.37; S 12.99).

Treatment of 3-alkylthio-5,5-dimethyl-2-cyclohexen-1-ones with nucleophiles

3-Ethyliden-5,5-dimethyl-1-phenylthio-cyclohexene, 17. An amount of 11.6 g (0.05 mol) of *14* dissolved in 50 ml THF was treated with 0.05 mol EtLi in 50 ml THF solution at −10 °C. After about 1 h, 15 g (0.106 mol) MeI were added and the mixture allowed to stir for 1 h. The solvent was stripped off and extracted with ether and water. The ether phase was dried and evaporated, giving 12.0 g of a liquid. After a short time it became milky and separated into two layers. The mixture was treated with ether again, dried and the solvent evaporated. Distillation yielded 9.4 g (77 %) of b.p. 123–124 °C/0.2 mmHg; $n_D^{25} = 1.5909$. IR ν_{max}(film) (cm^{-1}) 3050(m), 2900(s), 1570(m). NMR (CCl_4): δ 0.89 and 0.92 (6 H, s), 1.70 (3 H, br. d, 7), 2.02 (4 H, br. s), 5.1–5.5 (1 H, m), 6.24 and 6.56 (1 H, br.s), 7.28 (5 H, br.s.). (Found: C 78.24; H 8.00; S 13.17. $C_{16}H_{20}S$ requires: C 78.65; H 8.25; S 13.1).

5,5-Dimethyl-3-phenylthio-3-cyclohexen-1-one, 18. An amount of 2.32 g (0.01 mol) of *15* dissolved in 10 ml of dry ether was dropped to a suspension of $LiNH_2$ in liq. NH_3 (prepared from 400 mg Li). The NH_3 was evaporated and the residue acidified with H_2O and NH_4Cl. Extraction with ether drying and evaporation of the solvent gave 2.05 g of crude product, which was fractionated on a column, giving 1.85 g (80 %) of starting material and 0.19 g (8 %) of *19*. IR ν_{max}(film) (cm^{-1}) 1710(s). MS, *m/e* (%): 232(56), 219(6), 218(16), 217(100), 189(14), 176(6), 164(10), 156(6), 113(10), 111(12), 110(26), 109(26), 107(56), 95(8), 93(6), 91(8), 81(15), 80(12), 79(68), 78(10), 77(43), 69(10), 67(17), 66(8), 65(15), 55(12), 53(19).

Rearrangement of 7

2-Allyl-3-mercapto-5,5-dimethyl-2-cyclohexen-1-one, 19. Traces of *p*-toluenesulfonic acid were added to 5.3 g (27 mmol) of *7* and kept at 100 C for 4½ h in a Vigreaux distillation apparatus. Distillation gave 3 fractions, which consisted of compounds *19* and *21*, as shown by the NMR and IR spectra.

3-Acetylthio-2-allyl-5,5-dimethyl-2-cyclohexen-1-one, 20. An amount of 2.68 g (13.7 mmol) of *7* in 10 ml acetic anhydride was boiled at 140 °C for 2 h. The acetic anhydride was stripped off and the residue dissolved in ether, which was then extracted with 2 M aqueous Na_2CO_3. Drying and evaporation of the ether yielded 2.87 g crude product. 1.40 of this was purified with PLC yielding 0.5 g (38 %) of *20* and 0.26 g of an unidentified mixture. B.p. of *20* 60° C/10^{-4} mmHg; $n_D^{25} = 1.5290$; (Found: C 65.09; H 7.51; S 13.41 $C_{13}H_{18}O_2S$ requires: C 65.53; H 7.61; S 13.41).

2,6,6-Trimethyl-4-oxo-2,3,4,5,6,7-hexahydrobenzothiophene. 21. An amount of 2.05 g (10.4 mmol) of *7* in 5 ml quinoline was heated to 140 °C. After 3 h the starting material was converted. The mixture was extracted with ether and 4 M HCl. The ether layer was dried and evaporated. Crude product amounted to 1.78 g. Distillation gave 0.73 g (36 %) of b.p. 100 °C/0.01 mmHg; $n_D^{25} = 1.5535$; (Found: C 67.26; H 7.92; S 15.99. $C_{11}H_{16}OS$ requires: C 67.32; H 8.22; S 16.31).

Rearrangement of 8

2,3,6,6-Tetramethyl-4-oxo-2,3,4,5,6,7-hexahydrobenzothiophene, 22. An amount of 1.77 g (8.3 mmol) of *8* in 3 ml quinoline was heated for 5 h. Extraction with 4 M HCl and ether gave after drying and evaporation of the ether 1.40 g, which was distilled. B.p. 100 °C/0.01 mmHg, giving 1.17 g of a mixture of 27 % starting material and 73 % product. The experiment was repeated with 0.585 g of this mixture at a temp. of 160 °C and a reaction time of 3 h. Work up as above gave 0.348 g (yield 39 %) of b.p.

100 °C/0.01 mmHg; $n_D^{25} = 1.5420$; (Found: C 67.79; H 8.42; S 14.99. $C_{12}H_{18}OS$ requires C 68.54; H 8.63; S 15.22).

Rearrangement of *10*

7,7-Dimethyl-5-oxo-2,5,6,7,8-pentahydrobenzothiopyran, 23. An amount of 1.19 g (6.2 mmol) of *10* was boiled in 4 ml pyridine for 3½ h. The solvent was evaporated and the residue distilled. B.p. 100 °C/0.01 mmHg; $n_D^{25} = 1.5796$.

General procedure by photolysis of 3-alkylthio- and 3-arylthio-5,5-dimethyl-2-cyclohexen-1-ones. A solution of the compound was irradiated in a "Rayonet RS" photoreactor (for details see Table 1). A flow of dry and oxygen-free N_2 through the solution was maintained during the photolysis. The reaction was followed by UV spectroscopy and TLC. The solvent was evaporated and the residue was fractionated on a silica gel column or plate (about 100 g silica gel/g of product) with benzene – ethyl acetate = 4:1 (w/w) as eluent. However, the mixture obtained by photolysis of *14* was worked up using ether – light petroleum = 1:1 (w/w) as eluent.

The 1,3-dithiole, 24. M.p. 167 – 169 °C; (Found: C 61.90; H 7.12; S 20.63. $C_{16}H_{22}O_2S_2$ requires: C 61.92; H 7.15; S 20.6). NMR ($CDCl_3$): δ 1.05 – 1.15(12 H, 3 s); 2.25 – 2.48 (8 H, br. singlets and AB splittings); 3.02 (2 H, br.s). IR ν_{max}($CHCl_3$) (cm^{-1}), 1710(m-s); 1660(s); 1560(m.) UV ($CHCl_3$) λ_{max} (log ε), 250 (3.71), 350 nm (3.71). MS, *m/e* (%): 310(33), 308(5), 277(5) 256(8), 255(21), 254(68), 253(100), 239(32), 214(8), 213(16), 212(72), 178(20), 156(11), 155(8), 83(48), 55(54).

The 1,4-dithiane, 25. M.p. 172 – 174 °C; NMR ($CDCl_3$): δ 0.93 – 1.1(12 H, br.s); 1.7 – 2.7 (8 H, m); 3.1 – 3.3(2 H, m); 3.6 – 4.1 (2 H, m). IR $\nu_{max}CHCl_3$(cm^{-1}): 1705. MS, *m/e* (%): 312(2), 311(9), 310(4), 309(6), 308(8), 297(18), 282(12), 281(56), 280(40), 279(30), 278(10), 277(17), 263(16), 261(14), 260(75), 248(20), 247(100), 246(14), 245(10), 206(22), 195(40), 181(20), 157(23), 156(67), 155(8), 141(25), 125(77), 97(38), 91(22), 83(56), 55(46), 43(26), 41(53).

3,3-Dimethyl-5-oxo-2,3,4,5-tetrahydrodibenzothiophene, 26. M.p. 100 – 102 °C (Found: C 73.88; H 6.16; S 13.86. $C_{14}H_{14}OS$ requires: C 73.02; H 6.13; S 13.90). NMR (CCl_4): δ 1.15 (6 H, s), 2.36 (2 H, s), 2.90 (2 H, s), 7.1 (3, H, m), 8.50 – 8.65 (1 H, m). IR ν_{max}(KBr) (cm^{-1}) 1650. UV (EtOH): λ_{max} (log ε): 220 (4.08), 243 (3.70), 303 nm (3.48).

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Derivatives of Hydrazine. XII. Rotational Isomerism of Methyl Dithiocarbamates and Dithiocarbazates

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The ^{1}H NMR spectra have been recorded for a series of methyl dithiocarbamates and dithiocarbazates carrying one or more *N*-alkyl substituents. Signals attributed to the occurrence of *Z* and *E* isomers have been observed. The multiplicity, position, and solvent shifts at −20 °C are tabulated. The assignment of the *Z* and *E* isomers is based upon comparison of the spectral data with those of two cyclic dithiocarbazates which are necessarily *Z* isomers. The assignment is in harmony with results obtained for methyl diselenocarbamates and diselenocarbazates and furthermore support Bauman's interpretation of the ^{1}H NMR spectra of thionocarbamate esters.

A study of the variable temperature ^{1}H NMR spectra in a series of *Se*-methyl N^2-methyldiselenocarbazates and -carbamates [1] has demonstrated the occurrence of hindered rotation around the N^2-C bond (Fig. 1, Y = Se). Based on these results three deductions were possible. (1) The N^2-CH_3 group *cis* to the C=Y group always gives rise to a signal at lower field than the same group *trans* to the C=Y group. (2) In nitrobenzene solution, the low-field N^2-methyl resonance is shifted downfield relative to $CDCl_3$ solution while the high-field *N*-methyl resonance is shifted upfield. (3) In CCl_4 solution, the signal from the N^2-CH_3 group in the *Z* isomer is displaced towards higher field relative to the position in $CDCl_3$ solution, while the signal from the N^2-CH_3 group in the *E* isomer is almost unaffected by this change in solvent.

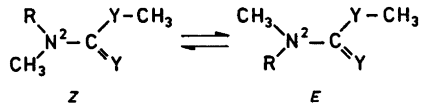


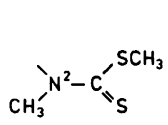
Fig. 1.

Compound	R	Compound	R
1	NH_2	*4*	CH_3
2	$NHCH_3$	*5*	CH_3CH_2
3	$N(CH_3)_2$	*6*	$(CH_3)_2CH$

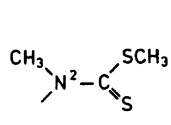
The generalisations (1) and (3) have been found to be valid for the corresponding sulfur compounds. In order to obtain well-resolved signals from the *Z* and *E* isomers it was necessary to work below the m.p. of nitrobenzene (6 °C) and accordingly the validity of the generalisation (2) could not be tested. Holloway and Gitlitz [2] showed that hindered rotation of dithiocarbamate esters leads to the appearance of signals assigned to *E* and *Z* forms by low temperature ^{1}H NMR spectroscopy. This has also been demonstrated in the case of *N,N*-diisopropyldithiocarbazic esters [3] and *O*-phenyl *N,N*-diisopropylthiocarbazate,[4] but in the two last cases the occurrence of additional dipolar forms represented an extra complication. Since these can only occur in carbazates containing the unsubstituted N^2-H group, we chose for the present study the dithiocarbazates *1*—*3* (Fig. 1, Y = S). The well-known isomerism [2,5] of methyl dimethyldithiocarbamate (*4*) was reinvestigated. Methyl *N*-ethyl-*N*-methyldithiocarbamate (*5*) and methyl *N*-isopropyl-*N*-methyldithiocarbamate (*6*) were included for steric comparison with *2* and *3*.

When the ^{1}H NMR spectra of *1*—*6* were recorded at *ca.* 40 °C each type of protons exhibits only one signal (singlet or multiplet). This reflects the state of rapid interconversion on the NMR time scale between the possible

Table 1. ^{1}H NMR chemical shifts (δ) of 3 % solutions of *N*-alkyl substituted methyl dithiocarbazates and dithiocarbamates at −20 °C and 1,3,4-thiadiazolidine-2-thiones at 40 °C in CCl_4, $CDCl_3$, CD_3NO_2, and C_6D_5Br.



(*Z*)



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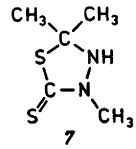

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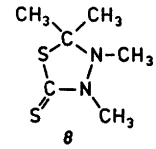

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Signal from	Solvent	Compound 1[d]	2	3	4	5[f]	6[g]	7	8
% *Z*[h]	$CDCl_3$	50	80	100	(50)	45	45	(100)	(100)
	CD_3NO_2	86	94	100	(50)	45	40	(100)	(100)
	CCl_4		75	100	(50)	45	40	(100)	(100)
	C_6D_5Br	50	75	100	(50)	47	41	(100)	(100)
N^2-CH_3 (*Z*)	$CDCl_3$	3.81s	3.68s	3.43s	3.63s	3.54s	3.38s	3.51s	3.51s
	CD_3NO_2	3.71s	3.62s	3.41s	3.54s	3.49s	3.36s	3.45s	3.47s
	CCl_4		3.60s	3.37s	3.56s	3.46s	3.31s	3.43s	3.44s
	C_6D_5Br	3.23s	3.33s	3.13s	3.17s	3.22s	3.13s	3.25s	3.25s
N^3-CH_3 (*Z*)	$CDCl_3$		2.73d[a]	2.62s					2.72s
	CD_3NO_2		2.69[b]	2.64s					2.70s
	CCl_4		2.73d	2.65s					2.66s
	C_6D_5Br		2.27d	2.14s					2.24s
S-CH_3 (*Z*)	$CDCl_3$	2.55s	2.48s[c]	2.48s	2.67s[e]	2.67s	2.68s		
	CD_3NO_2	2.40s	2.39s[c]	2.39s	2.57s[e]	2.59s	2.60s		
	CCl_4		2.39s[c]	2.37s	2.52s[e]	2.55s	2.58s		
	C_6D_5Br	2.43s	2.45s[c]	2.47s	2.58s[e]	2.53s	2.57s		
N^2-CH_3 (*E*)	$CDCl_3$	3.58s	3.48s		3.44s	3.37s	3.19s		
	CD_3NO_2	3.57s	3.48s		3.42s	3.38s	3.21s		
	CCl_4		3.47s		3.44s	3.34s	3.17s		
	C_6D_5Br	3.00s	3.08s		2.80s	2.92s	2.80s		
N^3-CH_3 (*E*)	$CDCl_3$		2.70d						
	CD_3NO_2		2.65[b]						
	CCl_4		2.67d						
	C_6D_5Br		2.40d[a]						
S-CH_3 (*E*)	$CDCl_3$	2.69s	2.68s[c]		2.67s[e]	2.67s	2.68s		
	CD_3NO_2	2.61s	2.65s[c]		2.57s[e]	2.59s	2.60s		
	CCl_4		2.63s[c]		2.52s[e]	2.55s	2.58s		
	C_6D_5Br	2.47s	2.50s[c]		2.58s[e]	2.51s	2.54s		

Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, and sep = septuplet. Center of multiplet is given.

[a] The N^3-CH_3 doublet collapsed on strong irradiation at the resonance frequency of the N^3−H proton. [b] Poorly resolved doublet. [c] The signal was absent in the spectrum of the corresponding S−CD_3 ester. [d] The solubility in CCl_4 was too low for recording the spectrum at −20 °C. [e] Necessarily identical for *Z* and *E* positions. [f] The signals from the ethyl group are ($CDCl_3$): CH_3 (*Z*) 1.30t, (*E*) 1.27t, CH_2 (Z) 3.88q, (*E*) 4.18q. A considerable solvent shift is observed in C_6D_5Br: CH_3 (*Z*) 0.91t, (*E*) 0.97t, CH_2 (*Z*) 3.42q, (*E*) 3.86q. [g] The signals from the isopropyl group are ($CDCl_3$): CH_3 (*Z*) 1.28d, (*E*) 1.22d, CH (*Z*) 4.92sep, (*E*) 6.00sep. A considerable solvent shift is observed in C_6D_5Br: CH_3 (*Z* and *E*) 0.85d, CH (*Z*) 4.67sep, (*E*) 5.98sep. [h] Average values from at least two measurements. The estimated error is 3 %.

rotamers. However, on cooling the signals are broadened. At −20 °C all the compounds *1*, *2*, and *4*−*6* exhibit well-resolved separate signals from the two isomers. As observed in the case of the corresponding selenium compound [1] the dithiocarbazate *3* failed to show the expected splitting of the signals at temperatures down to −40 °C. Since the rotational barriers are expected to be of the same magnitude in compounds *1*−*3* this result is taken to indicate that the population of one of the isomers of *3* is very low. The chemical shift data and the isomer ratios in four solvents are tabulated in Table 1.

In all cases the NMR spectra can be explained by assuming an equilibrium composed of the *Z* and the *E* isomers shown in Fig. 1 (Y=S). In principle, a barrier to rotation exists around both the N^2-C bond and the C−S bond. However, recent CNDO/2 calculations indicate [6] that (i) the energy barrier to rotation around the C−S bond is one order of magnitude lower than that around the C−N bond, and (ii) hindered rotation around the C−S bond leads to only one energetically favourable isomer in the case of compounds such as *1*−*6*. Empirically, a reliable indication of isomerism around the C−S bond has not been observed. In the present case the *Z*/*E* isomer ratio varies with changes in solvent, concentration, and temperature, allowing a clear separation of the signals in two groups, each arising from one isomer. When possible, this was confirmed by spin decoupling experiments and in the case of *2* by recording the spectrum of the *S*-trideuteriomethyl ester.

In order to decide which signals arise from the *Z* and which from the *E* isomer, we chose as model compounds the cyclic dithiocarbazates shown at the top of Table 1. By virtue of their cyclic structures they of necessity have the N^2-CH_3 groups situated in the *Z* position. In accordance with generalisation (3) mentioned above for the corresponding selenium compounds, the signal from this group is displaced towards higher field in CCl_4 solution relative to $CDCl_3$ solution. However, in order to obtain a more firm basis for the assignment the solvent shift was determined in the four solvents $CDCl_3$, CD_3NO_2, CCl_4, and C_6D_5Br. Since the solvent shifts of the N^2-CH_3 group of the *Z* and the *E* forms in these solvents are quite different, it was possible to use this as a criterion for assigning the isomers. Furthermore, generalisation (1) stated above also applies to methyl substituted thioureas,[7] monothiocarbazic esters,[8] and thioamides.[9] The assignment given in Table 1 is in complete accordance with this rule. Finally, if the NMR spectra of the sulfur and selenium compounds corresponding to Fig. 1 are compared they show a striking resemblance in their solvent, concentration and temperature dependence. This is taken to indicate that similar factors are operative within both classes of compounds in determining the *Z*−*E* isomer ratios.

Our results can be compared with those reported by Bauman [10] for thiocarbamate esters. In the case of *O*-methyl *N*-methylthiocarbamate it was demonstrated that *E*−*Z* isomerism gives rise to signal splitting at temperatures below 36 °C. The assignment of *Z* and *E* isomers was based primarily on the generalization (1) mentioned above. This assignment has recently been confirmed by Bauman [11] by using the ability of tris(dipivalomethanato)europium(III) to form complex compounds selectively with the *E* form. It was furthermore shown [10] that the benzene-induced shift (*i.e.* $\delta_{CDCl_3}-\delta_{C_6D_6}$) is greater for the $N-CH_3$ signal from the *E* form than from the *Z* form. This empirical result has also been found to apply in the case of thioamides [12] and thioureas [13] and it is easily seen from Table 1 that it is in agreement with the bromobenzene shifts ($\delta_{CDCl_3}-\delta_{C_6D_5Br}$) observed for the dithiocarbamates *4*−*6*. In our opinion, therefore, the assignment given by Rao *et al.*[14,15] for *O*-methyl *N*-methylthiocarbamate and related compounds should be changed to agree with the assignment given by Bauman. Similarly, the present results suggest that the assignment made by Holloway and Gitlitz [2] for dithiocarbamates should be reversed.

The *Z*−*E* isomer ratios for *1*−*3*, *5* and *6* are qualitatively the same for the corresponding sulfur and selenium compounds.[1] Both dithio- and diselenocarbamates show a small but definite tendency for the larger *N*-alkyl group to occupy a *trans* relationship to $C-SCH_3$ and $C-SeCH_3$, respectively. This trend is more marked in the case of *N*-isopropyl than in the case of *N*-ethyl groups. Since dilution experiments indicate that intermolecular forces can be neglected to a first approximation, this must

be due either to an inductive or to a steric effect. The present results allow no distinction between these possibilities. However, it has been shown [6] that the steric requirements of the $SCH_3/SeCH_3$ groups are much larger than those of the double-bonded sulfur/selenium provided that rotation around the C–S/C–Se bonds is not strongly restricted. In our opinion this is the most likely explanation for the observed trend. In the dithiocarbazates the *Z* forms predominate. This can be explained by invoking intramolecular compensation of the local dipoles in the same way as for diselenocarbazates.[1]

The coalescence temperatures for the *Z*–*E* splitting have in all cases been found to lie between 10 and 40 °C ($CDCl_3$) while those of the selenium analogues [1] are somewhat higher (*e.g. Se*-methyl N^2,N^3-dimethyldiselenocarbazate in C_6H_5Br at *ca.* 70 °C). In agreement with earlier results [2,16] Yoder *et al.*[17] have determined the coalescence temperature for *4* to be 33 °C with a $\Delta G_a^{\ddagger}$ of 15 kcal/mol, while the barrier in the selenoesters has not been determined though it is undoubtedly somewhat higher. By analogy with thioamides and selenoamides [18] (*cf.* Walter [19]) a higher rotational barrier for the central N^2–C bond of the diselenoesters can be explained by a more extensive electron delocalisation of the NCSe group.

Tin(IV) complexes of *N*,*N*-dimethylthio, dithio-, seleno-, diseleno-, and thioselenocarbamates have been described by Tanaka *et al.*[20–23] In the ^1H NMR spectra of the tin(IV) compounds with the mixed ligands Me_2NCOSe- and Me_2NCSSe-, the methyl groups gave rise to a doublet in which one of the signals was observed to be smaller in height and broader than the other. This was explained as due to *trans* coupling probably of 1–2 Hz in those molecules containing the natural abundance of ^{77}Se. In order to test the possibility of broadening of lines by coupling to selenium, the spectra of *4* and the selenium analogue $Me_2NC(Se)SeMe$ were compared in $CDCl_3$ at −40 °C in approximately identical concentrations. Within experimental error identical half-band widths were found for CH_3–N signals in both compounds (1.2 Hz) suggesting the coupling with ^{77}Se to be too small to give rise to the suggested broadening of the lines.

The infrared spectra of the compounds *1*–*6* are remarkably similar to those of the corresponding selenium compounds in the range 1200–4000 cm^{-1}. Below this region the change from CSS to CSeSe is followed by the systematic shift of several strong bands towards lower wave numbers. Especially prominent are peaks in the range 900–1100 cm^{-1}. Normal coordinate analyses for the dimethyldithiocarbamate [24] and the dimethyldiselenocarbamate ions [25] have shown, that bands in this region originate in highly mixed vibrations, so location of the bands with major CS/CSe stretching contributions has not been attempted. The deformational vibration of the CH_3 group bonded to sulfur was established by replacement with the CD_3 group. It occurs around 1430 cm^{-1} and when bonded to selenium around 1415 cm^{-1} in agreement with published evidence.[26] In the deuterated compounds the corresponding bands were observed in the 1000–1030 cm^{-1} range.

EXPERIMENTAL

Conditions and equipment used for the physical measurements were those described in part XI of this series.[1]

Methyl 2,3-dimethyldithiocarbazate (2). An ethanolic solution (4 ml) of methyl iodide (10^{-3} mol) was added to a filtered aqueous solution (5 ml) of potassium 2,3-dimethyldithiocarbazate [27] (10^{-3} mol). The reaction mixture was stirred for 5–10 min at room temperature and the solvent evaporated *in vacuo* with gentle heating until a volume of ½–1 ml was reached. This residue was extracted with benzene, dried, and taken to dryness. The crude product was purified by dissolution in ether and reprecipitation with pentane (−80 °C). The yield was 75 % of colourless crystals, m.p. 16.5–18 °C. (Found: C 31.91; H 6.57; N 18.70. Calc. for $C_4H_{10}N_2S_2$: C 32.00; H 6.71; N 18.66).

Methyl 2-methyl-2-ethyldithiocarbamate (5). Methylethylammonium chloride (40 mmol) was added to powdered potassium hydroxide (40 mmol) in dioxane (15 ml) and the mixture was stirred for 30 min. The resulting amine solution was dried (KOH pellets) and then added to a suspension of powdered KOH (40 mmol) in dioxan (40 ml). Carbon disulfide (40 mmol) in dioxan was added to the vigorously stirred solution over a period of 1 h. At the end potassium methylethyldithiocarbamate was filtered off, washed with dioxane and dry ether and dried *in vacuo*. The reaction with methyl iodide proceeded as described above for *2*. The compound was purified by dissolution in the minimum amount of pentane and cooling to *ca.* −80 °C with scratching. The solid that separated

was quickly collected by centrifugation, washed with a small amount of cold pentane and dried *in vacuo*. The melting point was well below room temperature. Yield 60 %. (Found: C 39.49; H 7.26; N 9.82. Calc. for $C_5H_{11}NS_2$: C 40.24; H 7.43; N 9.38). Though the analysis was not quite satisfactory, the impurities were not discernible in the NMR spectrum.

Methyl 2-methyl-2-isopropyldithiocarbamate (6). The compound was prepared as was the previous compound, except that it was found necessary to add a small amount of water during the generation of the amine. Yield 50 % of a colourless oil, which solidified by immersion in a bath cooled to *ca.* −80 °C. (Found: C 44.30; H 8.20; N 8.56. Calc. for $C_6H_{13}NS_2$: C 44.13; H 8.02; N 8.58).

The preparation of the cyclic dithiocarbazates has been described in a previous communication.[28] The structures have been established by comparison of the spectral properties with those of the corresponding selenium compounds.[29]

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Short Communications

Bacterial Carotenoids. XLV.* Synthesis of Lycopen-20-al and Rhodopin-20(20′)-al

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In the carotenoid field *N*-bromosuccinimide (NBS) has been frequently used for the introduction of carbon-carbon double bonds.[1,2] Although no brominated carotenoid intermediates have been isolated, it is assumed that the reaction proceeds *via* allylic bromides, *cf.* Ref. 3. Thus allylic acetates or ethers are obtained in the presence of acetic acid or alcohol, respectively.[4,5] A remarkable dehydrogenation is reported for canthaxanthin (*1*, Scheme 1), which provided the 15,15′-didehydro derivative with central triple bond.[6,7]

On the basis of this experience NBS-treatment of crocetindial (*2*) was expected to give either the acetylenic analogue or result in allylic substitution.

Under particular conditions NBS-treatment of crocetindial (*2*) resulted in allylic substitution of one of the central methyl groups. After chromatography of the product *3* on alumina, the allylic alcohol *4* was isolated. Characterization of *3* and *4* and a general study of the reaction between NBS and diapocarotenals are published separately.[8]

The dial *4*, containing the desired hydroxy substituent, was used for the synthesis of lycopen-20-al (*6*) and an expected mixture of rhodopin-20-al (*7a*) and rhodopin-20′-al (*7b*), Scheme 1.

The hydroxy-dial *4* (9 mg) was reacted with the ylids of a mixed phosphonium salt (24 % *8* and 76 % *9*, *cf.* Ref. 9) by a general procedure [10] providing in good yield lycopen-20-ol (*10*, 75 % of recovered carotenoid) and a presumed mix-

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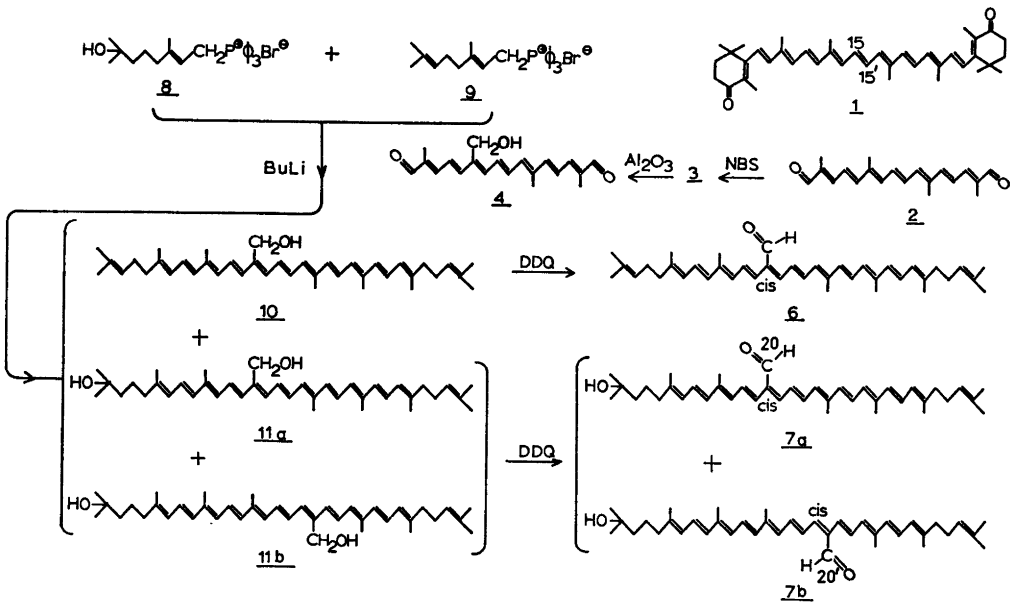

Scheme 1.

ture of rhodopin-20-ol and rhodopin-20′-ol (*11a* and *11b*; 20 % of recovered carotenoid).

Lycopen-20-ol (*10*, 5.4 mg), purified by TLC (kieselgel; 15 % acetone in petroleum ether) had λ_{max} (acetone) for the *trans* isomer (51 % of the stereoisomeric mixture) 345, 363, 450, 471 and 500 nm, for neo U (27 %) 345, 362, 442, 466 and 496 nm and for neo V (23 %) 345, 363, (446), 467 and 495 nm; ν_{max} (KBr) 3300 and 1000 (OH) and 950 cm^{-1} (*trans* CH=CH); *m/e* 552 (M), M−16, M−18, M−69, M−92, M−106, M−108, M−122, M−158, M−174.

Rhodopin-20(20′)-ol (*11a* and *11b*) which could not be separated, had λ_{max} (acetone) 345, 363, 440, 469 and 495 nm (*cis* and *trans*); *m/e* 570 (M), M−2, M−16, M−18, M−92, M−106, M−108, M−158, M−174 and was inseparable from natural rhodopinol (*ex. Thiocystis* sp.[11]) on alumina paper. On electron impact both *10* and *11* showed the elimination of 108, 122 and 174 mass units characteristic of aliphatic carotenoids with in-chain hydroxymethyl groups.[12]

Allylic oxidation of lycopen-20-ol (*10*), best achieved with DDQ [13,14] in dry ether at 0 °C, gave lycopen-20-al (*6*), but only in 7 % yield. Previous attempts to oxidize such allylic alcohols have met with the same difficulties.[14] The synthetic lycopen-20-al (*6*) was obtained as two stereoisomers with λ_{max} (acetone) 363, 502 (very broad) nm and 368, 492 (very broad) nm and *m/e* 550 (M), M−2, M−16, M−18, M−69, M−92, M−106, M−120, M−158, M−172, which could not be separated from those of authentic rhodopinal *ex. Thiocystis* sp.[11]

Allylic oxidation of rhodopin-20(20′)-ol (*11a* and *11b*), effected with DDQ, gave a presumed mixture of *7a* and *7b* in 2 % yield. The cross-conjugated aldehyde (*7a* and *7b*) had λ_{max} (acetone) *ca.* 490 (very broad), *m/e* 568 (M), M−2, M−18, M−73, M−69−18, M−106, M−120, M−120−18, M−158, M−172 and could not be separated from rhodopinal *ex. Thiocystis* sp.[12]

Rhodopin-20-al (*7a*) and lycopen-20-al (*6*) have been isolated from several *Thiorhodaceae* spp.[11,14,15] Also the corresponding allylic alcohols rhodopin-20-ol (*11a*) and lycopen-20-ol (*10*) are naturally occurring.[12,14,15] Structural studies led to derivatives of rhodopin and lycopene with one of the in-chain methyl groups oxidized.[14] Subsequent mass-spectrometric analysis was consistent with structures *7a*, *6*, *11a* and *10*.[21]

The present small scale synthesis of lycopen-20-al (*6*) and lycopen-20-ol (*10*) confirms the structures previously assigned to these carotenoids.

The properties of the mixed rhodopinals (*7a* and *7b*) confirm the previous chromophore assignment, but add no proof for 20- rather than 20′-substitution.

The same exceptional instability, failure to crystallize,[14] characteristic broad electronic spectra with strong absorption in the *cis*-peak region,[14] and the typical fragmentation pattern on electron impact,[12] as well as identical chromatographic properties,[12,14,15] were recorded for both the natural and synthetic pigments.

The synthetic use of the hydroxy-dial *4* for the preparation of other, more stable cross-conjugated carotenals is being pursued.[16]

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The Use of Ethylene Maleic Anhydride for the Preparation of a Water-soluble Polyanionic Derivative of Pepsin. Preparation and Properties

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The pH-activity profile for several enzymes covalently bound to polyanionic or polycationic carriers, may be shifted towards alkaline or acidic pH values, respectively, when compared with the corresponding profile of the native enzyme.[1] It has been shown that the pH-activity profile of pepsin (EC 3.4.23.1) may also be shifted, when coupled to polyelectrolytes as glass and probably agarose and cellulose.[2-4] However, all these modifications give rise to insoluble derivatives. Furthermore, most of the coupling reactions proceed at optimum rate at alkaline pH, where pepsin is rapidly and irreversibly denatured. Ethylene maleic anhydride copolymer (EMA) which is an example of a polyanion may be coupled to proteins [5,6] even at pH values lower than 6.[7] The aim of this work was to determine whether porcine pepsin covalently bound to a soluble polyanionic carrier could be used to catalyse the clotting of milk in the pH range 6 – 6.5 where native porcine pepsin is rapidly inactivated.

In the present paper the preparation of a catalytically active and watersoluble polyanionic enzyme (EMA-pepsin) is described. The coupling was undertaken at pH 5.8. The activity against macromolecular substrates (Haemoglobin and reconstituted skim milk) of EMA-pepsin was measured. The change in activity relative to native pepsin reflected the influence of the electrostatic field, induced by the attached polyelectrolyte chain. A marked enchancement of stability in the pH range above pH 6 was demonstrated in the EMA-modified pepsin.

Pepsin (2 × crystallized porcine pepsin) was purchased from Worthington Biochem. Corp., Freehold, N.Y., U.S.A. Bovine haemoglobin (cryodialyzed) was a gift from Børge Thing, Finseninstitutet, Copenhagen, Denmark. Nonfat low-heat spray-dried milk powder was from West Comp., Fon du Lac, Wisc., U.S.A. Chymosin (Rennin) was a gift from Chr. Hansen's Laboratorium Ltd., Copenhagen, Denmark. Linear ethylene maleic anhydride copolymer (EMA grade 11) was generously provided by Monsanta Ltd., La Salle, Quebec, Canada. The molecular weight was about 20 000 Dalton as determined by sedimentation equilibrium in the ultracentrifuge.[7] Formaldehyde (35 % W/W solution) and sodium borohydride was from Merck, Darmstadt, Germany. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was from Sigma, St. Louis, U.S.A.

200 mg pepsin was dissolved and 400 mg EMA 11 was suspended in 20 ml 0.05 M sodium phosphate buffer pH 5.8. The reaction was carried out at 30 °C for about 20 h in a pH-stat, equipped with a thermostated reaction vessel (25 ml) (Radiometer, Copenhagen, Denmark). During the reaction the pH was kept constantly at 5.8 by addition of 2 M sodium hydroxide. EMA-pepsin and EMA were precipitated with dilute hydrochloric acid (final pH 2.2) and ultracentrifuged for 20 min at 17 000 *g*. The centrifugation was repeated 4 times with intermittent washing of the precipitate with potassium chloride/hydrochloric acid buffer pH 2.2, ionic strength 0.1. Finally the precipitate was redissolved in 0.5 M sodium phosphate, and the pH was adjusted to 5.8. The modified enzyme was stored at −20 °C. The final volume was 10 ml.

Determination of pepsin contents in the precipitate and the supernatants was performed by amino acid analysis [9] for aspartic and glumatic acids, threonine, serine, glycine, and valine and comparison with the contents of these amino acids found in a pepsin solution with known concentration.

The average number of EMA-molecules bound per pepsin molecule was calculated from the number of residual free amino groups. These were determined by the TNBS method according to Fields [8] and by amino acid analysis [9] after reductive methylation with formaldehyde and sodium borohydride according to Means and Feeney.[10]

The rate of proteolysis of reconstituted milk was determined as described by Foltmann [11] with the following modification: Ten ml of reconstituted skim milk and one ml of 0.05 M sodium phosphate buffer pH 6.0, 6.3, or 6.5 were placed in one branch and the enzyme solution (not more than 100 µl) in the other branch of a bifurcated glass tube. Reconstituted milk with pH-values of 6.0, 6.3, and 6.5 at 20 °C was obtained by dissolving the dry skim milk powder in 0.04, 0.01, and 0.001 M calcium chloride, respectively.

For stability measurements, the enzyme was stored in 0.05 M phosphate buffer of pH 6.3 and 6.5 at 30 °C, for varying length of time before determination of the proteolytic activity. The time of coagulation was in all cases between 4 and 5 min.

Measurements of the proteolysis of haemoglobin was carried out according to Kassell and Meitner [12] using 1.25 % haemoglobin solutions, ionic strength 1.0 and 0.010 and pH between 4 and 6, as substrates. Agarose gel electrophoresis was performed according to Ref. 13.

Coupling of EMA to pepsin by the method described here gave yields of about 30 %. Estimates of the yield based on the different amino acids were in good agreement and the

method was reproducible. (Coupling experiments performed at low temperature in acetone-water mixtures according to Centeno,[6] but at pH 5.8 in stead of 7 showed only negligible conjugation). In dilute HCl the EMA-pepsin and free EMA were selectively precipitated, leaving unmodified pepsin in the supernatant. The recovery of EMA-pepsin was 95 % of the yield after four washings and the amount of pepsin in the last wash less than 2 % of the amount in the initial reaction mixture. Isolated EMA-pepsin was stable during storage at 5 °C for at least two months at pH 5.8, and ionic strength about 1.0. Agarose gel electrophoresis of the reaction mixture showed two distinct protein bands, whereas pepsin only gave one.

The pepsin molecule contains only one ε-amino group and one α-amino group. Amino acid analysis after reductive methylation of EMA-pepsin was performed. It was demonstrated that nearly 100 % of the lysine was modified. Modification of the residual free N-terminal α-amino group in pepsin cannot be detected by this method. However, a determination of the residual free primary amino groups of the isolated EMA-pepsin using the TNBS method showed that 50 % of these groups were modified. This makes it reasonable to suggest that mainly lysine was modified with EMA under the selected conditions. Binding of two or more pepsin molecules to the same EMA molecule might be excluded as the agarose gel electrophoresis only showed two bands.

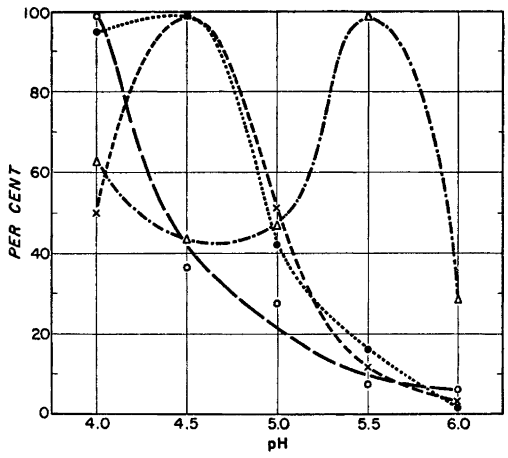

Fig. 1. pH-Activity profiles for pepsin and EMA-pepsin measured at different ionic strength using haemoglobin as substrate. The individual activities were calculated as the mean of four measurements (range ± 5 to 15 %) and expressed as the percentage of the activity at pH-optimum. ●, pepsin ($\Gamma/2 = 1.0$); ○, pepsin ($\Gamma/2 = 0.010$); ×, EMA-pepsin ($\Gamma/2 = 1.0$), △, EMA-pepsin ($\Gamma/2 = 0.010$).

pH-activity profiles of pepsin and EMA-pepsin measured in the pH-range 4 – 6 using haemoglobin as substrates are shown in Fig. 1. The profiles of the polyanionic derivatives of pepsin are displaced towards more alkaline pH-values by 1.5 pH units at low ionic strength ($\Gamma/2 = 0.010$) as compared with the native enzyme. The displacements are less pronounced at higher ionic strength ($\Gamma/2 = 1.0$). The enzymatic activity at 30 °C is expressed as percentage of the maximum activity obtained. The pH-activity profile shift could be attributed to a lowered intrinsic pH arising from a polyanion near the domain of the active site of the enzyme. The theoretical aspects of the electrostatical field prevailing in the microenvironment of polyanionic enzymes are discussed in several papers.[1,14] Another explanation of the pH-shift might be decreasing solubility of EMA-pepsin at pH below 4. However, turbidometric measurement shows that EMA-pepsin is soluble even at pH 3, ionic strength 0.01 and 1, respectively.

Comparison between the activity of pepsin and EMA-pepsin against reconstituted milk is shown in Table 1. The low activity of EMA-pepsin compared with that of pepsin may reflect steric hindrance due to the bulky EMA-group which will reduce the contact between the active centre of the enzyme and a macromolecular substrate. Also interaction between

Table 1. Proteolytic activity of EMA-pepsin relative to unmodified pepsin at different pH-values measured on reconstituted milk.[11]

pH	% Activity
6.0	8.2 (±.7)
6.3	11.6 (±.1)
6.5	14.8 (±.05)

Table 2. Half-lives for proteolytic activity of pepsin and EMA-pepsin at pH 6.3 and 6.5. Half-lives were calculated from the time dependent decrease in proteolytic activity against reconstituted milk. The activity was defined to be 100 % at time zero, and the kinetics of inactivation to be of first order. The coefficient of the correlation found by first order kinetic plots are given in brackets.

pH	Pepsin $t_{1/2}$(min)	EMA-pepsin $t_{1/2}$(min)
6.3	70 (−0.98)	180 (−0.95)
6.5	30 (−1.00)	150 (−1.00)

positively charged substrate groups and EMA might prevent catalysis.

The stability of pepsin and EMA-pepsin in the pH-range above 6.0 was determined using calf chymosin as internal standard. The activity was measured relatively to pepsin at 30 °C. The results are given in Table 2 as half-lives. It is seen that the stability against the irreversible denaturation is three–five times better for EMA-pepsin than for unmodified pepsin.

For pepsin an electrostatic expansion of the negatively charged polypeptide chain may lead to denaturation at pH-values higher than 6. The enchanced stability of EMA-pepsin relative to pepsin may be attributed to different micro-environmental states of the two enzymes.

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Tritium—Protium Exchange in Dextran at 25 °C

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In the dextran (Sephadex®) gels the cross-links are connected by ether linkages to the anhydroglucose residues of the dextran chains. Thus, at each point of attachment of a cross-link an hydroxyl hydrogen is lost and the concentration of hydroxyl groups remaining after cross-linking provides valuable information about the cross-linking structure. Since hydroxyl hydrogens will exchange with deuterium (D) or tritium (T) [1] provided they are accessible, tritium exchange can, thus, be used, giving information about the fraction of accessible groups, to determine the hydroxyl concentration.

The degree of accessibility of the Sephadex hydroxyls is, as yet, unknown. As regards other polysaccharides all the hydroxyls of amylose and starch, notwithstanding some degree of crystallinity in the latter, were reported to exchange with deuterium of deuterated water.[2] The 1,6-linked polysaccharides, *e.g.* dextran, are conformationally very flexible with an extended structure [3] and inaccessibility is unlikely but should be tested for. The lack of crystallinity in a dry sample of the most highly cross-linked Sephadex gel,[4] G-10, while not proof of accessibility, is strongly suggestive of it.

This communication reports an attempt to measure the tritium exchange constant of the parent polymer dextran as a model for the dextran gels. The exchange reaction between tritiated water (HTO) and the gel hydroxyls is

$$\mathrm{HTO} + \mathrm{Gel-OH} \rightleftharpoons \mathrm{Gel-OT} + \mathrm{H_2O}$$

Because of the relatively large difference in their vibrational zero point energies,[5,6] the bond dissociation energies of the different hydrogen isotopes differ sufficiently for the exchange constants to deviate considerably from unity in many systems; the T/H value usually diverging more than the D/H.

Experimental. Dextran® ($\overline{M}_w = 250\,000$; $\overline{M}_n = 113\,000$) was dissolved, without predrying, in double quartz-distilled water to a concentration of approximately 40 %. The concentration (weight fraction of dextran = 0.3604) was then calculated from the polarimetry [7] (Na D line 589 nm) of triplicate gravimetric dilutions (~2 % solutions).

About 15 ml of the 36 % dextran solution were placed in one compartment of a double glass flange (FG 25, Quickfit, Great Britain) chamber and were separated from 15 ml water containing HTO (1 mCi/ml) in the other compartment by a hydrophobic polyvinylidene

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About 15 ml of the 36 % dextran solution were placed in one compartment of a double glass flange (FG 25, Quickfit, Great Britain) chamber and were separated from 15 ml water containing HTO (1 mCi/ml) in the other compartment by a hydrophobic polyvinylidene

fluoride 0.45 μm pore membrane (VF 6, GELMAN, Ann Arbor, Mich., U.S.A.).

The compartments were not stirred because of the high viscosity of the dextran solution. Kel-F grease (3 M, St. Paul, Minn., U.S.A.) was used to seal the membrane to the two flanges which were then held together by clamping the double chamber in a special holder which also served to support the chamber submerged in a water bath kept at 25 ± 0.05 °C.

This membrane has the useful property that although it allowed a rapid exchange of water molecules (the half-time for HTO or $H_2{}^{18}O$ exchange was about 24 h) there was no net water transport for up to 15 days with concentration differences (sucrose) across the membrane of up to 0.2 M.[8] As the dextran concentration used had an osmotic pressure corresponding to about 0.2 M,[9] there should thus be no, or very little, net water transport. There was, in fact, a little, the dextran fraction decreasing from $f_d = 0.3604$ to $f_d = 0.3069$ after 13 weeks. This was due to influx of water from the "water" side since dextran was not detected chemically on the latter side at the end of the experiment.

Duplicate samples were taken from both compartments, suitably diluted (about 500×) and the tritium activities in ten 0.1 ml samples of the dilutions were measured. All samples and dilutions were weighed. The high dilutions ensured that the dextran concentration introduced into the scintillation fluid (Aquasol, NEN. Boston, U.S.A.) was not more than 0.006 %, a concentration which did not affect the tritium counting rate significantly.

Samples taken after 3 weeks had not quite reached equilibrium (estimated > 95 %). Second samples were therefore taken 10 weeks later when equilibrium was assumed; the differences at 3 and 13 weeks were less than 5 %.

The exchange constant $K^{T/H}$ was calculated from the ratio (R) between the tritium activities in the two compartments, *i.e.*,

$$R = \frac{(3/162)K^{T/H}f_d + (2/18)(1-f_d)}{(2/18)}$$

where f_d is the final dextran concentration and $K^{T/H}$ the T/H exchange constant; the figures refer to the numbers of exchange sites on the anhydroglucose residues (3) and water (2) and to the rounded molecular weights of the residues (162) and water (18).

The value of the exchange constant ± the estimated error of the estimate was,

$$K^{T/H} = 1.20 \pm 0.06.$$

The above constant is calculated assuming complete accessibility, which with regard to the structure of dextran and known properties of other related oligosaccharides seems reasonable.

Whether dextran is a satisfactory model for a cross-linked Sephadex is another question. In the 1,4-β-linked cellulose there is a marked tendency for inter-chain coupling to occur when hydroxyls of different chains approach sufficiently closely to form hydrogen bonds [10] which make the groups inaccessible to exchange. In Sephadex, particularly with the more highly cross-linked types where there may be considerable restriction in chain movement, this may also occur and requires further study.

Data on the T/H equilibrium exchange constant are apparently conflicting. In cellulose limited accessibility introduces considerable uncertainty into the calculation of the constant as indeed is the case with most biological macromolecules. Lang and Mason, [1] however, concluded that for cellulose the equilibrium T/H probably exceeded unity. In the open-chain peptides poly D,L-alanine and poly D,L-lysine, T/H and T/D were reported to be 1.21 and 1.09, respectively, [11] compared with the corresponding ratios in the solvent. The T/H ratios were also of a similar order in myoglobin [12] and oxidised ribonuclease [13] although some uncertainty exists in these closed-chain peptides due to the question of accessibility. Lobunez and Karush [14] in a study of the distribution of tritium between gaseous ammonia and dry protein found that the latter was preferred.

The absence of a significant equilibrium isotope effect between tritium and deuterium has been reported in bovine plasma albumin [15] and several other workers have found small or absent isotope effects.[16]

The problem in interpreting the data is much simpler in a polysaccharide such as dextran, where there is only one type of exchange group, and whose random conformation should promote accessibility. It is thus more comparable to the simpler homogeneous open-chain peptide than to a natural peptide constrained in a native protein and the close agreement of the $K^{T/H}$ value reported here with that of the open-chain peptides probably reflects this.

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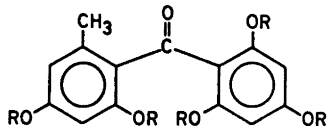
Synthesis of 2,2′,4,4′,6′-Pentahydroxy-6-methylbenzophenone*

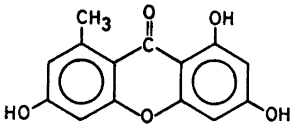
GÖRAN SUNDHOLM

Institute of Chemistry, Organic Department, University of Uppsala, Box 531, S-751 21 Uppsala, Sweden

2,2′,4,4′,6′-Pentahydroxy-6-methylbenzophenone (I) has often been postulated as a precursor of certain polyketides in lower plants, *viz.* xanthones [1] and griseofulvin.[1,2]



I R=H
II R=CH_3
III R=$CH_2C_6H_5$



IV

For biosynthetic studies on lichen xanthones (all of which are related to norlichexanthone, IV) [3] I was needed. There appears, however, to be no record of its synthesis in the literature.

In an attempt to synthesize I, *O,O*-dimethylorsellinoyl chloride was reacted with 2,4,6-trimethoxyphenyl lithium in diethyl ether [4] to give the methylated benzophenone II in ~50 % yield. During demethylation of II with boron tribromide in dichloromethane, I was formed (15 %) together with partially demethylated benzophenones and xanthones.

In another synthesis, *O,O,O*-tribenzylphloroglucinol carboxylic acid was condensed with *O,O*-dibenzylorcinol using trifluoroacetic anhydride in dichloromethane [5] to give the benzylated benzophenone III in high yield. 2,2′,4,4′-Tetrabenzyloxy-6,6′-dimethylbenzophenone was formed as a by-product. Hydrogenolysis of III afforded I quantitatively.

The spectral data (NMR, UV, IR, MS) are in agreement with the pentahydroxy benzophenone I structure. (Cooling of the KBr disc prevented thermally induced formation of IV during the registrating of the IR spectrum of I. When recording the mass spectrum of I, the main part of the sample dehydrated to IV in the direct inlet system before evaporation; however peaks at *m/e* 276, 151, and 153 were present).

The compound is sensitive to moisture even at room temperature. Other 2,2′,6′-hydroxylated benzophenones are known to dehydrate fairly easily under alkaline conditions, even as the methyl ethers.[2]

Because of its great instability, it would be difficult to demonstrate the presence of I in plant material even if appreciable amounts should occur.

Experimental. All melting points are uncorrected. Elemental analyses were performed by the Analytical Department, Institute of Chemistry, Uppsala. All solvents used were of analytical grade. Petroleum ether refers to the fraction boiling at 43 – 55 °C. Column chromatography was done using Merck Silica Gel 60 (70 – 230 mesh); TLC was carried out using Merck's precoated silica gel plates. IR spectra were recorded with a Perkin-Elmer 157. NMR-spectra were recorded on a Varian A-60D, using TMS as the internal standard. UV-spectra were recorded on a Unicam SP 1800.

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2,2′,4,4′,6′-Pentamethoxy-6-methylbenzophenone (II). 1,3,5-Trimethoxybenzene (3.60 g) [8] in ether (25 ml) was treated with butyl lithium (14.3 ml, 16.7 mmol/ml in hexane) under nitrogen at room temperature (1 h). The slurry was slowly added to an ethereal solution (20

* Part 33 of the series Chemical Studies on Lichens. Part 32 in press.

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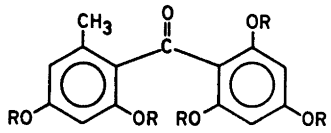

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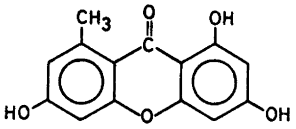

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ml) of *O,O*-dimethylorsellinoyl chloride during 40 min at 0 °C under nitrogen. Water (20 ml) was added, the precipitate dissolved in benzene, washed with water, dried ($MgSO_4$), and evaporated to give a pale yellow oil. Recrystallization from benzene/petroleum ether afforded 2.8 g (47 %) of colourless needles, m.p. 126.5–127 °C. R_F 0.7 [silica gel, dichloromethane-acetone (4:1)]. NMR ($CDCl_3$): δ 2.32 (t 3 H, $J = 0.5$ Hz), 3.54 (s 3 H), 3.64 (s 6 H), 3.78 (s 3 H), 3.80 (s 3 H), 6.07 (s 2 H), 6.31 (m 2 H, $J = 2.2$ Hz, $J = 0.5$ Hz). IR, ν_{max}(KBr) 1667 cm^{-1} (C=O). (Found C 65.6; H 6.4; MW (MS) 346. $C_{19}H_{22}O_6$ requires C 65.8; H 6.4).

Demethylation of benzophenone II. Boron tribromide (750 mg, 6 equiv.) in dichloromethane (7 ml) was added dropwise during 15 min to a stirred solution of II (173 mg, 0.5 mmol) in dichloromethane (6 ml) at room temperature. After 1.5 h an orange product precipitated and the reaction was continued for 6.5 h. Additional boron tribromide (750 mg) was added and stirring continued overnight. Water (50 ml) was added at 0 °C with stirring. After work-up the crude material was separated by TLC [silica gel 0.5 mm, dichloromethane–acetone (4:1) to yield I (R_F 0.10, 20 mg, 15 %; identical with I prepared by hydrogenation of III, see below). IV (R_F 0.28, 35 mg, 27 %), and griseoxanthone C[2] (R_F 0.45, 15 mg, 11 %)]. The identities of the xanthones were confirmed by comparison with authentic samples. The other bands contained mono-, di-, and trimethoxybenzophenones.

Benzyl-O,O,O-tribenzylphloroglucinol carboxylate. To a solution of anhydrous phloroglucinolcarboxylic acid (2 g)[9] and benzyl bromide (8.5 g) in acetone (50 ml) was added freshly roasted K_2CO_3 (13.8 g). The mixture was stirred at room temperature (48 h). After pouring into ether-water, the ether layer was washed with water, dried and evaporated. The product crystallized from hexane/tetrahydrofuran to give colourless needles (3.1 g, 50 %) m.p. 102–103 °C. NMR (C_3D_6O): δ 5.08 (s 2 H), 5.10 (s 4 H), 5.26 (s 2 H), 6.44 (s 2 H), 7.10–7.55 (m 20 H); IR ν_{max}(KBr) 1725 cm^{-1} (C=O). (Found C 79.3; H 5.7; MW (MS) 530. $C_{35}H_{30}O_5$ requires C 79.3; H 5.6).

O,O,O-Tribenzylphloroglucinol carboxylic acid. Benzyl-*O,O,O*-tribenzylphloroglucinol carboxylate (0.53 g) was added to a solution of KOH (1.2 g in 2 ml water) and ethylene glycol (12 ml) and the mixture refluxed (48 h). Water (25 ml) was then added, the solution extracted with ether and the ether layer washed with water. The aqueous extracts were combined, acidified (pH ~2) with 2 M hydrochloric acid, extracted with ether, washed, dried and evaporated. Recrystallization from benzene/petroleum ether gave colourless needles (0.39 g, 89 %); m.p. 134–35 °C. NMR (C_3D_6O): δ 5.12 (s 2 H), 5.16 (s 4 H), 6.47 (s 2 H), 7.12–7.62 (m 15 H); IR ν_{max}(KBr) 3560–2210 (OH), 1700 (sh), and 1690 cm^{-1} (C=O). (Found C 76.3; H 5.5; MW (MS) 440. $C_{28}H_{24}O_5$ requires C 76.4; H 5.5).

2,2',4,4',6'-Pentabenzyloxy-6-methylbenzophenone (III). *O,O,O*-Tribenzylphloroglucinol carboxylic acid (1.32 g) and *O,O*-dibenzylorcinol (4.56 g)[5] were dissolved in dry dichloromethane (25 ml) and freshly distilled trifluoroacetic anhydride (1.5 ml) was dropwise added. After 10 min, the mixture was evaporated and chromatographed on silica gel (750 g, petroleum ether-ether (2:1)) to give unreacted *O,O*-dibenzylorcinol (3.67 g), 2,2',4,4'-tetrabenzyloxy-6,6'-dimethylbenzophenone [170 mg, recrystallized from benzene/petroleum ether to give colourless needles (50 mg); m.p. 124–125 °C (lit. 116–120 °C)[5]], and pentabenzyloxybenzophenone (III) (1.81 g, 83 %, recrystallized from methanol, filtered at −20 °C, dried over P_2O_5 to give 1.44 g of a glassy product that starts melting at 33 °C). NMR (C_3D_6O): δ 2.14 (s 3 H), 4.78 (s 2 H), 4.80 (s 4 H), 5.11 (s 4 H), 6.30 (s 2 H), 6.45 (q 2 H, $J = 1.8$ Hz), 6.85–7.50 (m 25 H); IR ν_{max}(CCl_4) 1665 cm^{-1} (C=O). (Found C 80.8; H 5.8; MW (MS) 726. $C_{49}H_{42}O_6$ requires C 81.0; H 5.8).

2,2',4,4',6'-Pentahydroxy-6-methylbenzophenone (I). The benzophenone III (73 mg) was hydrogenated at room temperature in ethyl acetate over 10 % palladium-carbon (20 mg) at atmospheric pressure until hydrogen uptake ceased. Filtration, evaporation and trituration with petroleum ether yielded I (28 mg, ~100 %) as a pale yellow powder. NMR (C_3D_6O): δ 2.12 (t 3 H, $J = 0.6$ Hz), 5.91 (s 2 H), 6.28 (t 2 H, $J = 0.6$ Hz), 3.5 (broad 2 H), and 10.8 (broad 3 H); IR ν_{max}(KBr) 3900–2900 (strong, OH), 1625 (sh), and 1605 cm^{-1} (C=O); UV (MeOH) ν_{max} 300 (ε 15 000), 310 nm (ε 6 300).

Acknowledgements. I wish to thank my teacher Dr. Johan Santesson for many stimulating and helpful discussions. The costs of this investigation were defrayed by a grant from the Swedish Natural Science Research Council to Dr. Gerd Bendz at this Institute.

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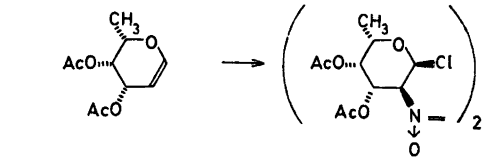
A Synthesis of a 2-Acetamido-2,6-dideoxy-L-galactose Derivative

PER J. GAREGG, BENGT LINDBERG and THOMAS NORBERG

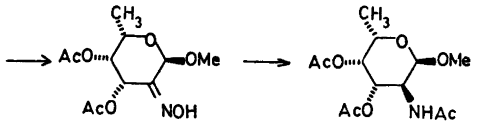
Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-104 05 Stockholm, Sweden

In connection with studies of bacterial polysaccharides, 2-acetamido-2,6-dideoxy-L-galactose (*N*-acetyl-L-fucosamine) was needed. Different syntheses of this sugar, and of its enantiomer, have been described,[1-3] and the synthesis of this sugar *via* the corresponding acetylated glycal, a procedure devised by Serfontein,[4] Lemieux [5-7] and their coworkers, is now reported. The advantage of this procedure is that the desired epimer predominates in the final product and that it is simple.

3,4-Di-*O*-acetyl-1,2,6-trideoxy-L-*lyxo*-hex-1-enopyranose [8] (3,4-di-*O*-acetyl-L-fucal, I) and nitrosyl chloride gave the dimeric nitroso compound (II) in 86 % yield. Treatment of II



I II



III IV

with methanol and pyridine in tetrahydrofuran yielded the glycoside oxime (III) which, without purification, was treated with lithium aluminium hydride and acetylated. GLC of the product revealed the presence of two components (7:3), presumably the methyl 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-α-hexopyranosides with the L-*galacto*- and L-*talo*-configurations. The major component crystallized (20 % yield from II), m.p. 150 °C, $[\alpha]_D$ −144° (chloroform) and NMR spectroscopy in the presence of tris-(dipivaloylmethanoato)europium demonstrated that this was the *galacto*-isomer (IV). The spectral assignments of ring protons shown in Table 1 were confirmed by spin decoupling experiments.

Table 1. ^{1}H NMR data for methyl 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-α-L-*galacto*-hexoside (IV). δ values in ppm from tetramethylsilane (internal) in deuteriochloroform, coupling constants in Hz.

Proton	Compound IV	IV + Eu(DPM)$_3$[a]
H−1	[b]	5.62 ($J_{1,2}$3)
H−2	[b]	7.00 ($J_{2,3}$10)
H−3	[b]	6.15 ($J_{3,4}$ 3.5)
H−4	[b]	5.81 ($J_{4,5}$1)
H−5	4.08 ($J_{4,5}$1)	4.50 ($J_{5,6}$6)
H−6	1.17 ($J_{5,6}$6)	1.35
OCH_3	3.40	3.60 or 3.61
N*H*Ac	5.80	7.15
NHAc		3.61 or 3.60
OAc		2.32, 2.45
NH*Ac* or OAc	2.12 1.97 1.98	

[a] 0.2 mol Eu(DPM)$_3$/mol IV. [b] Unresolved at δ 4.4−5.4.

Experimental. Melting points are corrected. Concentrations were performed at reduced pressure. Optical rotations were recorded at room temperature (20−22 °C) using a Perkin-Elmer 141 instrument. NMR spectra were recorded for all new compounds using a Varian A60-A instrument and were invariably in agreement with the postulated structures. TLC was performed on silica gel F_{254} (Merck). Gas liquid chromatography was performed on a Perkin-Elmer Model 990 instrument at a nitrogen flow of 20 ml/min on an ECNSS column (3 % on Gas-Chrom Q).

Dimeric-3,4-di-O-acetyl-2,6-dideoxy-2-nitroso-α-L-galacto-hexopyranosyl chloride (II). 3,4-Di-*O*-acetyl-1,2,6-trideoxy-L-*lyxo*-hex-1-enopyranose (I) ("di-*O*-acetyl-L-fucal") was obtained in a 52 % yield from L-fucose essentially as described by Iselin and Reichstein [8] who report a 30 % yield for this transformation. The higher yield in the present synthesis may be due to the lower temperature (−10 to −20 °C) used in the reductive 1,2-elimination, the shorter reaction time (1 h against 3 h) or the use of extremely finely powdered zinc. The glycal I (1.42 g, m.p. 48−50 °C, in agreement with the literature value) in ethyl acetate (60 ml) was cooled to −40 °C with stirring while air in the reaction vessel was displaced with nitrogen. Nitrosyl chloride was then passed through the solution at −40 °C for about 20 min. Excess nitrosyl chloride was displaced by nitrogen and the solution was allowed to attain room temperature. Concentration and recrystallization from

chloroform – hexane gave 1.60 g of II, m.p. 147 °C (decomp.), $[\alpha]_D$ −183° (*c* 0.5, in chloroform). (Found: C 42.8; H 4.94; N 4.88; O 34.4; Cl 12.9. $C_{10}H_{14}NO_6Cl$ requires C 42.9; H 5.05; N 5.01; O 34.3; Cl 12.7.)

*Methyl 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-α-*L*-galacto-hexoside (IV).* The above nitrosyl chloride adduct II (1.40 g) was refluxed with methanol (0.32 g) and pyridine (0.79 g) in dry tetrahydrofuran (40 ml) for 1 h and concentrated. The residue was taken up in chloroform and the chloroform solution shaken with water, dried over magnesium sulfate, filtered and concentrated to yield III as a colourless syrup (1.38 g) which was used directly in the next step. The oxime III (1.38 g) in tetrahydrofuran (35 ml) was refluxed under nitrogen with lithium aluminium hydride (0.66 g) for 3 h. Excess hydride was decomposed by adding, in turn, ethyl acetate and 50 % aqueous methanol (135 ml). The mixture was filtered, the filtrate was neutralized (HCl) to pH 4.5, low-boiling solvents were removed by evaporation and the residual water solution was freeze-dried. The product was acetylated overnight at room temperature with acetic anhydride (8 ml) and pyridine (20 ml). The solution was poured into ice-water and the mixture extracted with chloroform. The combined chloroform phases were washed with ice-cold 1 M sulfuric acid, saturated aqueous sodium hydrogen carbonate and finally water, dried over magnesium sulfate, filtered and concentrated to yield 1.44 g of crude product. GLC at 200 °C showed the presence of two components in an approximate ratio of 7:3. The major component crystallized from diethyl ether – hexane to yield 0.31 g of IV, m.p. 150 °C, $[\alpha]_D$ −144° (*c* 0.25, chloroform). (Found: C 51.7; H 6.91; N 4.82. $C_{13}H_{21}NO_7$ requires C 51.5; H 6.98; N 4.62.)

Acknowledgements. This work was supported by grants from the Swedish Natural Science Research Council.

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Synthesis of 3-Deuterio-2,3-diphospho-D-glyceric Acid

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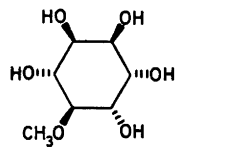
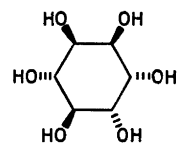
2,3-Diphospho-D-glyceric acid, labelled with deuterium at C-3, was required for NMR studies of its association with hemoglobin [1] and the present paper describes the synthesis of this substance. The key intermediate, 2,3:4,5-di-*O*-cyclohexylidene-D-*manno*-hexodialdose (III) was obtained in a manner similar to that described by Angyal and Hoskinson for the synthesis of 2,3:4,5-di-*O*-isopropylidene-L-*manno*-hexodialdose, an intermediate in the synthesis of L-mannitol from quebrachitol.[2]

D-*chiro*-Inositol (I), obtained by demethylation of pinitol, was converted into the 1,2:5,6-dicyclohexylidene derivative (II) as described by Angyal and co-workers for the enantiomer.[3]



Pinitol



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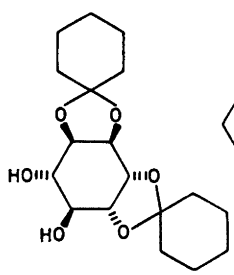

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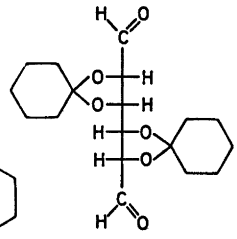

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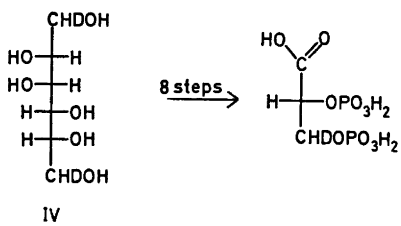

IV

chloroform – hexane gave 1.60 g of II, m.p. 147 °C (decomp.), $[\alpha]_D$ −183° (*c* 0.5, in chloroform). (Found: C 42.8; H 4.94; N 4.88; O 34.4; Cl 12.9. $C_{10}H_{14}NO_6Cl$ requires C 42.9; H 5.05; N 5.01; O 34.3; Cl 12.7.)

*Methyl 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-α-*L*-galacto-hexoside (IV).* The above nitrosyl chloride adduct II (1.40 g) was refluxed with methanol (0.32 g) and pyridine (0.79 g) in dry tetrahydrofuran (40 ml) for 1 h and concentrated. The residue was taken up in chloroform and the chloroform solution shaken with water, dried over magnesium sulfate, filtered and concentrated to yield III as a colourless syrup (1.38 g) which was used directly in the next step. The oxime III (1.38 g) in tetrahydrofuran (35 ml) was refluxed under nitrogen with lithium aluminium hydride (0.66 g) for 3 h. Excess hydride was decomposed by adding, in turn, ethyl acetate and 50 % aqueous methanol (135 ml). The mixture was filtered, the filtrate was neutralized (HCl) to pH 4.5, low-boiling solvents were removed by evaporation and the residual water solution was freeze-dried. The product was acetylated overnight at room temperature with acetic anhydride (8 ml) and pyridine (20 ml). The solution was poured into ice-water and the mixture extracted with chloroform. The combined chloroform phases were washed with ice-cold 1 M sulfuric acid, saturated aqueous sodium hydrogen carbonate and finally water, dried over magnesium sulfate, filtered and concentrated to yield 1.44 g of crude product. GLC at 200 °C showed the presence of two components in an approximate ratio of 7:3. The major component crystallized from diethyl ether – hexane to yield 0.31 g of IV, m.p. 150 °C, $[\alpha]_D$ −144° (*c* 0.25, chloroform). (Found: C 51.7; H 6.91; N 4.82. $C_{13}H_{21}NO_7$ requires C 51.5; H 6.98; N 4.62.)

Acknowledgements. This work was supported by grants from the Swedish Natural Science Research Council.

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Synthesis of 3-Deuterio-2,3-diphospho-D-glyceric Acid

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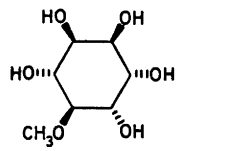
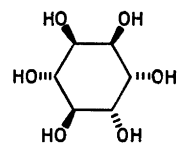
2,3-Diphospho-D-glyceric acid, labelled with deuterium at C-3, was required for NMR studies of its association with hemoglobin [1] and the present paper describes the synthesis of this substance. The key intermediate, 2,3:4,5-di-*O*-cyclohexylidene-D-*manno*-hexodialdose (III) was obtained in a manner similar to that described by Angyal and Hoskinson for the synthesis of 2,3:4,5-di-*O*-isopropylidene-L-*manno*-hexodialdose, an intermediate in the synthesis of L-mannitol from quebrachitol.[2]

D-*chiro*-Inositol (I), obtained by demethylation of pinitol, was converted into the 1,2:5,6-dicyclohexylidene derivative (II) as described by Angyal and co-workers for the enantiomer.[3]



Pinitol



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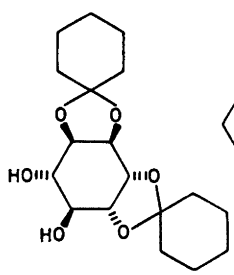

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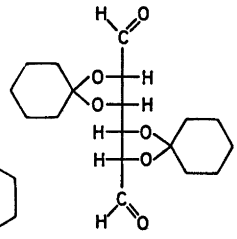

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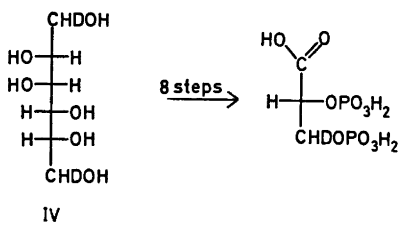

IV

Periodate oxidation afforded the hexodialdo derivative III which, without purification, was converted into 1,6-dideuterio-D-mannitol (IV). The deuterium labelling was confirmed by NMR and by GLC-MS of its hexaacetate. 1,6-Dideuterio-D-mannitol was converted into the title compound by established procedures previously described for unlabelled 2,3-diphospho-D-glyceric acid.[4-6] The various intermediates were characterized by NMR and, when appropriate, by GLC-MS.

Some stereoselectivity during the reduction of the hexodialdo derivative III with sodium borodeuteride, due to asymmetric induction, is not excluded and has been observed in related reactions.[7,8]

Experimental. Concentrations were performed under reduced pressure. Precoated plates with Silica Gel F_{254} (Merck) were used for TLC and Silica Gel 60 (> 230 mesh, Merck) for column chromatography. Light petroleum refers to a fraction with b.p. 60 – 71 °C. NMR spectra were recorded with a Varian A 60-A instrument and were invariably in agreement with the postulated structures. Mass spectra were recorded with a Perkin-Elmer 270 instrument and optical rotations with a Perkin-Elmer 141 polarimeter. GLC was performed with a Perkin Elmer model 900 instrument using a column packed with ECNSS-M (3 %) on Gas-Chrom Q.

1,2:5,6-Di-O-cyclohexylidene-D-chiro-inositol (II). D-*chiro*-Inositol (11 g), cyclohexanone (275 ml), benzene (165 ml), and *p*-toluenesulfonic acid (0.55 g) were heated under reflux using a Dean and Stark separator. When, after 5 h, codistillation of water ceased, aqueous 10 % potassium carbonate (200 ml) was added and the excess reagent was removed by steam distillation. The crystals which formed on cooling the residue were filtered off. TLC (ethyl acetate – light petroleum 3:1) indicated the presence of both di- and tri-cyclohexylidene-D-*chiro*-inositol. The product was taken up in benzene – ethanol 10:1 (60 ml) and treated with acetic acid saturated with hydrogen bromide (0.6 ml) for 45 min at room temperature. The crystals which had separated were filtered off and washed with light petroleum. Recrystallization from ethanol afforded II (10 g) m.p. 208 – 209 °C $[\alpha]_{578}$ +18° (*c* 0.4, chloroform) in agreement with the values reported for its enantiomer. (Found: C 63.7; H 8.3. $C_{18}H_{28}O_6$ requires: C 63.5; H 8.3.)

1,6-Dideuterio-D-mannitol. The dicyclohexylidene-D-*chiro*-inositol (4.1 g), in methanol (800 ml) was treated with sodium metaperiodate (4.1 g) in water (80 ml) for 4 h with stirring at room temperature in the dark. Ethylene glycol (0.3 g) was added and the stirring continued for another 15 min. Precipitated sodium iodate was removed by filtration and the filtrate diluted with water (400 ml) and then extracted with chloroform. The combined chloroform extracts were washed with water, dried over sodium sulfate and concentrated to a syrup (crude III). This was dissolved in dioxane – methanol 3:1 (100 ml) and treated with sodium borodeuteride (0.4 g). The solution was kept at room temperature for 18 h. Excess deuteride was decomposed by adding acetic acid and the solution was concentrated. Boric acid was removed by repeated codistillations with methanol and the product purified by chromatography on a silica gel column using ethyl acetate and light petroleum (3:2) as eluent. The chromatographically pure product was hydrolyzed with aqueous acetic acid (60 %) at 100 °C for 5 h, cooled and concentrated. The resulting syrup was taken up in water (150 ml) and washed with chloroform. The water solution was concentrated to yield crystalline IV (2.0 g) m.p. 165 – 166 °C.

3-Deuterio-2,3-diphospho-D-glyceric acid was obtained from IV as described by Baer and Fischer[4-6] for unlabelled D-mannitol and purified as its barium salt. (Found: C 5.95; H 1.44 (recalculated for D_2H_{10}); Ba 54.5; P 9.59. $(C_3H_3DO_{10}P_2)_2Ba_5.3H_2O$ requires C 5.70; D + H 1.12; Ba 54.3; P 9.80.)

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Intermolecular Hydride Transfer Reactions. I. Electrophilic Aromatic Substitution with 2,6-Dimethoxycarbonylpyrylium Cation

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The reaction of the 2,6-dimethoxycarbonylpyrylium cation with monosubstituted benzene derivatives to 4-substituted-4*H*-pyrans has been studied. Intermolecular hydride transfer between the pyrylium cation and the 4*H*-pyrans may occur, whereby the more stabilized pyrylium ion is formed.

It has previously been shown that 2,6-didimethoxycarbonylpyrylium perchlorate (*1*) reacted with carbonylactivated methyl groups to yield 4-substituted 4*H*-pyrans, and moreover that intermolecular hydride transfer between *1* and 4-alkyl substituted 4*H*-pyrans took place forming the more substituted pyrylium cations.[1]

The present work deals with the reaction of *1* with monosubstituted benzene derivatives (*2*).

Equimolecular amounts of *1* and anisole in acetonitrile solution were allowed to react for 24 h. Treatment with ether deposited a crystalline product for which elemental analysis and ^{1}H NMR data were consistent with the pyrylium salt *3* (R = OCH_3). Formation of the 4*H*-pyran *4*, which has been synthesized earlier,[2] was verified from ^{1}H NMR analysis of the concentrated etheral solution after removing of *3* (R = OCH_3). Reactions leading to *3* and *4* are depicted in Scheme 1.

It is likely that the first step of the process is an electrophilic substitution reaction to form the 4*H*-pyran *5* (R = OCH_3), which is subsequently oxidized by another molecule of *1* to yield *3* (R = OCH_3). The reduction of *1* by intermolecular hydride transfer explains the formation of *4*.

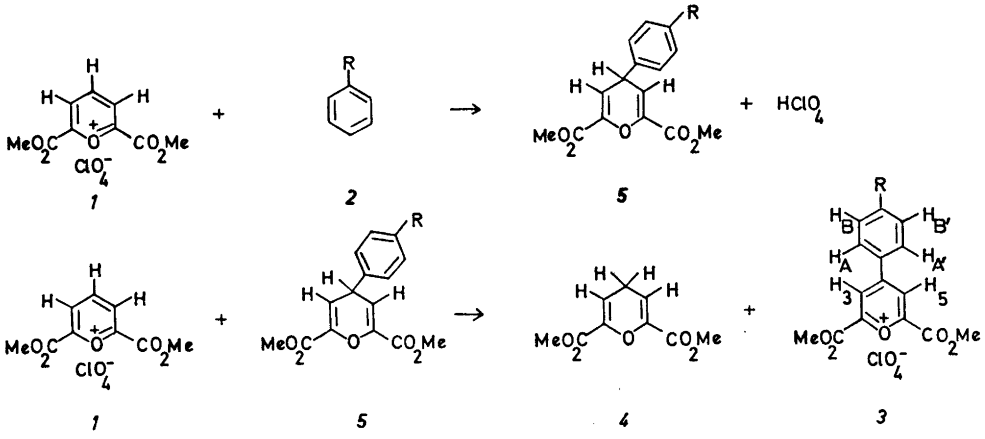
The reaction was repeated in trideuterioacetonitrile solution. The 4*H*-pyran *5* (R = OCH_3) was not formed in detectable amounts as shown by the ^{1}H NMR spectrum of the reaction mixture. The presence of nearly equimolecular amounts of unreacted anisole, the pyrylium cation *3* (R = OCH_3) and the 4*H*-pyran *4* showed that the stoichiometry of the reaction was according to the equation:

$$2(1) + (2) = (3) + (4) + HClO_4$$

The reaction between equimolecular amounts of *1* and dimethylaniline in acetonitrile solution also resulted in isolation of a salt. The product, however, was not the expected pyrylium salt *3* [R = $N(CH_3)_2$], but the hydroperchlorate of the amine *5* [R = $N(CH_3)_2$] in 85 % yield. The reaction was repeated with two molar equivalents of *1* and this yielded the pyrylium salt *3* [R = $N(CH_3)_2$] in 75 % yield and the 4*H*-pyran *4*.

The reaction of *1* with toluene (molar ratio; 2:1) in trifluoroacetic acid solution was carried out in the ^{1}H NMR sample tube. The result as shown by ^{1}H NMR spectra was analogous to that obtained in the reaction of *1* with anisole. Only formation of *3* (R = CH_3) and *4* in equal amounts and the simultaneous disappearance of *1* and toluene were observed. The pyrylium salt *3* (R = CH_3) was isolated when the reaction was quantitative.

These experiments demonstrate that the 4-unsubstituted pyrylium nucleus *1* is a very reactive cation, which readily takes part in both electrophilic aromatic substitution and hydride transfer reactions. Kinetic studies have not been carried out. The results, however, indicate that intermolecular hydride transfer is much faster than substitution in the reaction of *1* with



Scheme 1.

Table 1. The ^1H NMR spectra (δ values) of pyrylium salts *3* recorded in trifluoroacetic acid solution

R	H_3,H_5	$O-CH_3$	$H_A,H_{A'}$	$H_B,H_{B'}$	R	$J_{AB}=J_{A'B'}$ Hz
CH_3	9.23	4.30	8.38	7.68	2.65	10
OCH_3	9.00	4.27	8.50	7.38	4.17	10
$N(CH_3)_2$	8.52	4.22	8.22	7.38	3.57	10

equimolecular amounts of anisole. On the other hand, the inverse is presumably the case in the reaction of *1* with dimethyaniline. It seems likely that the explanation is an increased rate of substitution due to the dimethylamino group and furthermore a decreased rate of hydride transfer due to the formation of the hydroperchlorate of the amine *5* ([R = $N(CH_3)_2$]).

The ^1H NMR shift values of the pyrylium salts *3* are listed in Table 1.

The increased stabilizing effect of substituent R on the pyrylium cation through the series Me, OMe, $N(CH_3)_2$ is reflected in chemical shift positions of the heterocyclic ring protons. The upfield shifts of these protons are in agreement with a decreased positive charge of the pyrylium nucleus.

Experimental. The ^1H NMR spectra were recorded on a Varian A-60A instrument with TMS as internal standard.

*2,6-Dimethoxycarbonyl-4-*p*-methoxyphenylpyrylium perchlorate* (*3*, *R* = *OCH*$_3$). To a solution of 2,6-dimethoxycarbonylpyrylium perchlorate [2] (1.48 g, 0.005 mol) in dry acetonitrile (5 ml) was added anisole (0.540 g, 0.005 mol) at 20 °C with stirring. After 24 h anh. ether (75 ml) was added. The precipitated material was filtered off and recrystallized from acetic acid. The yield was 93 % (0.930 g), m.p. 212–215 °C (decomp.). (Found: C 47.75; H 3.84; Cl 8.76. Calc. for $C_{16}H_{15}O_{10}Cl$: C 47.71; H 3.73; Cl 8.82). ^1H NMR spectrum see Table 1. The filtrate after removal of *3* (R = OCH_3) was concentrated under reduced pressure and the residual material was redissolved in $CDCl_3$. ^1H NMR spectrum of this solution showed signals due to 2,6-dimethoxycarbonyl-4*H*-pyran (*4*) and anisole. ^1H NMR spectrum of 2,6-dimethoxycarbonyl-4*H*-pyran in $CDCl_3$ (δ): 3.05 (triplet; $2H_4$, $J_{3,4}=4$ Hz), 3.81 (singlet; OCH_3) 6.05 (triplet; H_3H_5).

*2,6-Dimethoxycarbonyl-4-*p*-dimethylaminophenyl-4H-pyran*, [*5*, *R* = *N(CH*$_3$)$_2$] *hydroperchlorate.* To a solution of 2,6-dimethoxycarbonylpyrylium perchlorate (0.60 g, 0.002 mol) dissolved in dry acetonitrile (5 ml) was added dimethylaniline (0.24 g, 0.002 mol) at 20 °C with stirring. After 24 h the title compound was filtered off (0.4 g). The filtrate was diluted with ether and additional 0.32 g was collected.

The yield was 85 % (0.720 g), m.p. 224–227 °C (decomp.) (CH_3CN). (Found: C 48.85; H 4.79; N 3.53; Cl 8.53. Calc. for $C_{17}H_{20}O_9ClN$: C 48.87; H 4.79; N 3.35; Cl 8.50). ^1H NMR spectrum in TFA (δ); 3.50 (doublet; $^+NH(CH_3)_2$, $J=5$ Hz), 4.0 (singlet; OCH_3), 4.58 (triplet; H_4, $J_{3,4}=4.0$ Hz), 6.32 (doublet; H_3H_5). The aromatic protons form an AA'BB' system centered at δ 7.67 with a small chemical shift difference between H_A and H_B.

*2,6-Dimethoxycarbonyl-4-*p*-dimethylaminophenylpyrylium perchlorate* [*3*, *R* = *N(CH*$_3$)$_2$] was prepared as *5* [R = $N(CH_3)_2$] from 0.004 mol of pyryliumsalt and 0.002 mol of dimethylaniline. The reaction mixture was left standing for one week. The yield was 75 %, m.p. 280–285 °C (decomp.). (Found: C 49.16; H 4.47; N 3.27; Cl 8.46. Calc. for $C_{17}H_{18}O_9ClN$: C 49.10; H 4.33; N 3.37; Cl 8.54). ^1H NMR spectrum see Table 1. The filtrate, after removal of the title compound, was concentrated under reduced pressure and the residual material was redissolved in $CDCl_3$. ^1H NMR spectrum of this solution showed signals due to the 2,6-dimethoxycarbonyl-4*H*-pyran (*4*).

*2,6-Dimethoxycarbonyl-4-*p*-methylphenylpyrylium perchlorate* (*3*, *R* = *CH*$_3$). 2,6-Dimethoxycarbonylpyrylium perchlorate (0.06 g, 2×10^{-4} mol) and toluene (0.01 g, 10^{-4} mol) was dissolved in trifluoroacetic acid (0.3 ml). The solution was transferred into a ^1H NMR sample tube and left standing at 20 °C. Spectra were recorded regularly and after 12 days the signals due to the added pyrylium salt were undetectable. The solution was diluted with ether and the title compound was collected by filtration: m.p. 195–197 °C (decomp.). (Found: C 49.65; H 4.01. Calc. for $C_{16}H_{15}O_9Cl$; C 49.67, H 3.88). ^1H NMR spectrum see Table 1.

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The Crystal Structure of Two Polarized Ethylenes

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The structures of 1,1-bis-methylthio-2-*p*-bromobenzoyl-2-cyanoethylene and 1,3-dimethyl-2-[*p*-bromobenzoyl-cyanomethylene]-imidazolidine have been determined by X-ray crystallography.

The length of the carbon-carbon double bond in the first compound, 1.369 Å, indicates only a moderate ground state polarization. The second compound shows a twist of 41° between the imidazolidine and O(1)=C(7)−C(8)−C(12)−N(1) planes, and a C=C bond length of 1.448 Å, indicating a strong steric effect.

The observed bond lengths are discussed in their relation to PPP calculations and experimental rotation barriers.

In recent years several investigators have found by NMR lineshape methods that ethylenes with push-pull substituents (polarized ethylenes) have free energies of activation ($\Delta G^{\ddagger}$) to rotation around the double bond lower than 25 kcal/mol, in some cases even lower than 6−7 kcal/mol, which is the practical lower limit for most NMR spectrometers (see Ref. 1 for a recent review).

These barriers should be compared to $\Delta H^{\ddagger}$ = 62.2 kcal/mol for 1,2-dideuterioethylene [2] and 60.0 kcal/mol for *cis*-2-butene.[3] It is a popular concept that low torsional barriers are due to low π-bond orders of the bonds concerned. However, this is not necessarily true, since the barriers measure the energy differences between the ground and transition states, whereas the bond order is a function of the electron interaction in the ground state alone. In polarized ethylenes the transition state energy is determined by the capacity of the two parts of the molecule to stabilize respectively a positive and a negative charge. It is therefore of interest to study the relation between bond lengths and torsional barriers in polarized ethylenes. Some bond lengths resulting from X-ray crystallographic studies of similar compounds have recently been published. The double bond joining the cyclopropene and cyclopentadiene rings in 1,2,3,4-tetrachloro-5,6-dipropylcalicene is 1.37 Å,[4] and the corresponding bond in 1,2,3,4-tetrachloro-5,6-diphenylcalicene is 1.36 Å.[5] No torsional barriers are known for these calicenes, but Kende *et al.*[6] have found barriers in the range 18.0−19.4 kcal/mol for 1-formyl-5,6-dipropylcalicene in different solvents, and Prinzbach [7] has observed temperature dependent NMR spectra of 1,3-di-*tert*-butyl-5,6-dimethylcalicene, from which free energy barriers of 17.8 kcal/mol (in C_6D_6) and 14.4 kcal/mol (in CCl_4) can be calculated. The exocyclic double bond in 6,6-ethylenedithiofulvene is 1.37 Å,[8] as compared to 1.346 Å in 6,6-dimethylfulvene, by an electron diffraction study.[9]

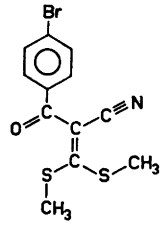
In 2-formyl-6-dimethylaminofulvene the exocyclic double bond is 1.389 Å.[10] The barrier to rotation around this bond could not be measured, probably due to unfavourable conformer distribution, but it is probably lower than the 22.1 kcal/mol barrier found for the simple 6-dimethylaminofulvene.[11]

More recently, Shmueli *et al.*[12] have studied the crystal structure of 1-dimethylamino-2,2-biscarbomethoxyethylene, for which the length of the double bond was found to be 1.380 Å and the free energy barrier to rotation around this bond 15.6 kcal/mol.

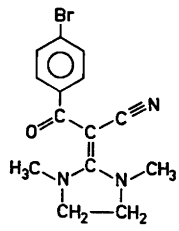
The purpose of the present investigation has been to study the molecular structure of an ethylene with push-pull substituents, for which

the barrier to rotation around the carbon-carbon double bond is known, in order to increase the knowledge of relations between barriers and bond lengths. For this purpose, 1,1-bismethylthio-2-*p*-bromobenzoyl-2-cyanoethylene (TCE) was chosen. The barrier of the bromine-free analogue has been studied by the complete lineshape method, and $\Delta H^{\ddagger} = 13.8$ kcal/mol, $\Delta S^{\ddagger} = -17.3$ e.u. and $\Delta G^{\ddagger}_{300} = 19.0$ kcal/mol were found.[13] From the rate of rotation at the coalescence temperature and assuming the same entropy of activation, TCE has $\Delta H^{\ddagger} = 13.9$ kcal/mol and $\Delta G^{\ddagger}_{300} = 19.1$ kcal/mol.

Furthermore, this study includes the structure of a polarized ethylene, 1,3-dimethyl-2-[*p*-bromobenzoyl-cyanomethylene]-imidazolidine (ICE) (II), for which the NMR spectrum indicates a twisted double bond in solution.[14] The barrier in ICE cannot be studied, but the bromine-free 1,3-dibenzyl analogue has $\Delta G^{\ddagger} = 9.5$ kcal/mol with the planar state as transition state.



I



II

EXPERIMENTAL

The preparation of TCE [15] and the bromine-free analogue of ICE [16] has been described previously. ICE was prepared similarly and was obtained in 82 % yield as pale yellow prisms, m.p. 183.5 – 185 °C after recrystallization from toluene. (Found: C 52.5; H 4.36; Br 25.1; N 13.1; O 4.94. $C_{14}H_{14}BrN_3O$ (320.19) requires C 52.5; H 4.41; Br 25.0; N 13.1; O 5.00).

X-Ray single crystal data for the two compounds were collected on a Picker FACS I automatic diffractometer using graphite monochromated Cu$K\alpha$ radiation. Reflexions up to $2\theta = 124°$ were measured with a 2.0 degree $\theta - 2\theta$ scan. The scanning speed was 1 degree min^{-1}. The background level was determined from 10 second counts on each side of the reflexion.

Lorentz and polarization factors were applied, but no correction was made for extinction or absorption. Atomic scattering factors were taken from the International Tables for X-Ray Crystallography [17] except for hydrogen in which case the values of Stewart, Davidson and Simpson [18] were used. All calculations were performed on a Datasaab D21 – PDP15 dual computer system with programs developed at the Crystallography Group.

CRYSTAL DATA

TCE	$C_{12}H_{10}BrNOS_2$
M =	328.25
Unit cell:	$a = 7.97$, $b = 13.00$, $c = 6.53$ Å
	$\beta = 102.19°$
V =	661.28 Å^3
Z =	2
D_c =	1.648 g cm^{-3}
D_m =	1.658 g cm^{-3}
Absent reflexions:	$0k0$ for k odd
Spacegroup	$P2_1$
ICE	$C_{14}H_{14}BrN_3O$
M =	320.22
Unit cell:	$a = 9.07$, $b = 11.81$,
	$c = 13.45$ Å
	$\beta = 105.08°$
V =	1.391.16 Å^3
Z =	4
D_c =	1.529 g cm^{-3}
D_m =	1.525 g cm^{-3}
Absent reflexions:	$h0l$ for l odd, $0k0$ for k odd
Spacegroup	$P2_1/c$

STRUCTURE DETERMINATION AND REFINEMENT

TCE. In total 1177 independent reflexions were measured of which 1164 were more than two standard deviations above background and used in the structure analysis. The non-hydrogen atom positions were found from successive electron density series starting with the phases of the bromine atom. The hydrogen atoms were included at their expected positions which were compatible with the peaks in a difference synthesis. In the final least-squares treatment using the full matrix all positional parameters were refined. Anisotropic temperature factors were used except for the hydrogen atoms which were given isotropic temperature factors corresponding to those of their parent atoms. The B-values were not refined. At an R-value of 0.037 no further improvement occurred and the refinement was terminated.

Table 1. Fractional positional coordinates in TCE and temperature factors for the hydrogen atoms. The standard deviations (within brackets) are multiplied by 10^4 and 10^3 for the hydrogen atoms.

	x	$\sigma(x)$	y	$\sigma(y)$	z	$\sigma(z)$	B(Å^2)
Br(1)	0.9529	(0.6)	0.4996	(0)	−0.2351	(0.8)	
S(1)	0.1803	(1)	0.7363	(1)	0.5303	(2)	
S(2)	0.4384	(2)	0.8835	(1)	0.7481	(2)	
O(1)	0.3001	(5)	0.6385	(4)	0.2293	(7)	
N(1)	0.7309	(6)	0.8869	(4)	0.3724	(8)	
C(1)	0.7984	(6)	0.5583	(4)	−0.0852	(8)	
C(2)	0.8606	(6)	0.5972	(4)	0.1153	(8)	
C(3)	0.7480	(7)	0.6414	(4)	0.2232	(8)	
C(4)	0.5707	(6)	0.6421	(4)	0.1384	(8)	
C(5)	0.5078	(6)	0.5997	(4)	−0.0594	(8)	
C(6)	0.6204	(6)	0.5593	(3)	−0.1721	(8)	
C(7)	0.4405	(6)	0.6796	(4)	0.2582	(7)	
C(8)	0.4838	(6)	0.7656	(3)	0.4122	(7)	
C(9)	0.3810	(6)	0.7912	(3)	0.5487	(7)	
C(10)	0.1026	(8)	0.7863	(7)	0.7492	(11)	
C(11)	0.6666	(8)	0.8762	(5)	0.8339	(9)	
C(12)	0.6239	(6)	0.8316	(4)	0.3942	(7)	
H(21)	0.969	(9)	0.592	(5)	0.159	(9)	2.91
H(31)	0.788	(8)	0.666	(5)	0.346	(10)	2.61
H(51)	0.387	(7)	0.598	(5)	−0.112	(9)	2.44
H(61)	0.579	(7)	0.541	(5)	−0.347	(9)	2.58
H(101)	0.062	(9)	0.860	(8)	0.700	(11)	4.02
H(102)	0.171	(10)	0.784	(6)	0.885	(13)	4.02
H(103)	−0.032	(8)	0.764	(7)	0.734	(11)	4.02
H(111)	0.725	(9)	0.908	(5)	0.760	(11)	3.06
H(112)	0.702	(8)	0.813	(6)	0.829	(11)	3.06
H(113)	0.747	(8)	0.919	(5)	0.924	(11)	3.06

Table 2. Anisotropic thermal parameters for the non-hydrogen atoms of TCE in the form $\exp[-2\pi^2(h^2a^{*2}U_{11}+k^2b^{*2}U_{22}+l^2c^{*2}U_{33}+2hka^*b^*U_{12}+2klb^*c^*U_{23}+2hla^*c^*U_{13})]$. The standard deviations (within brackets) have been multiplied by 10^4.

	U_{11}	U_{22}	U_{33}	U_{12}	U_{23}	U_{13}
Br(1)	0.0394(4)	0.0581(4)	0.0490(4)	−0.0156(3)	0.0190(3)	0.0073(2)
S(1)	0.0197(6)	0.0490(7)	0.0444(7)	−0.0049(6)	0.0118(5)	−0.0030(5)
S(2)	0.0286(7)	0.0515(8)	0.0491(8)	−0.0228(6)	0.0129(6)	−0.0012(5)
O(1)	0.0245(19)	0.0630(24)	0.0575(26)	−0.0290(22)	0.0150(18)	−0.0133(18)
N(1)	0.0436(26)	0.0535(26)	0.0425(23)	−0.0092(22)	0.0196(22)	−0.0214(23)
C(1)	0.0302(23)	0.0356(25)	0.0388(25)	−0.0094(22)	0.0102(20)	0.0038(18)
C(2)	0.0241(24)	0.0460(25)	0.0410(27)	−0.0117(23)	0.0062(20)	0.0043(21)
C(3)	0.0264(22)	0.0420(24)	0.0302(23)	−0.0120(21)	0.0022(19)	0.0023(20)
C(4)	0.0240(22)	0.0327(23)	0.0308(22)	0.0041(19)	0.0014(19)	0.0018(18)
C(5)	0.0210(22)	0.0393(22)	0.0351(23)	0.0002(19)	0.0022(20)	0.0011(18)
C(6)	0.0332(26)	0.0346(23)	0.0310(24)	−0.0023(18)	0.0059(20)	−0.0024(18)
C(7)	0.0313(27)	0.0341(25)	0.0269(25)	−0.0002(16)	0.0042(22)	0.0015(17)
C(8)	0.0208(20)	0.0342(22)	0.0302(19)	−0.0009(18)	0.0119(17)	0.0013(16)
C(9)	0.0211(19)	0.0316(19)	0.0307(22)	0.0019(19)	−0.0016(17)	0.0016(17)
C(10)	0.0350(29)	0.0801(43)	0.0470(35)	−0.0126(32)	0.0218(25)	0.0059(31)
C(11)	0.0342(28)	0.0544(33)	0.0325(24)	−0.0111(25)	0.0116(21)	−0.0031(22)
C(12)	0.0278(25)	0.0378(22)	0.0288(23)	−0.0058(19)	0.0123(19)	−0.0038(22)

Table 3. Fractional positional parameters for the atoms of ICE and isotropic temperature factors for the hydrogen atoms. The standard deviations (within brackets) are multiplied by 10^4.

	x	$\sigma(x)$	y	$\sigma(y)$	z	$\sigma(z)$	B(Å^2)
Br(1)	0.0143	(0.6)	0.3661	(0.5)	−0.4018	(0.3)	
O(1)	0.4599	(3)	0.2248	(2)	0.0692	(2)	
N(1)	0.2380	(4)	0.5849	(3)	0.0780	(2)	
N(2)	0.6454	(4)	0.3644	(2)	0.2444	(2)	
N(3)	0.4680	(4)	0.4366	(3)	0.3086	(2)	
C(1)	0.1286	(4)	0.3533	(3)	−0.2620	(2)	
C(2)	0.2852	(4)	0.3384	(3)	−0.2404	(2)	
C(3)	0.3665	(4)	0.3277	(3)	−0.1391	(2)	
C(4)	0.2942	(4)	0.3343	(3)	−0.0596	(2)	
C(5)	0.1382	(4)	0.3515	(3)	−0.0833	(3)	
C(6)	0.0541	(4)	0.3601	(3)	−0.1850	(3)	
C(7)	0.3916	(4)	0.3158	(3)	0.0491	(2)	
C(8)	0.3992	(4)	0.4049	(3)	0.1215	(2)	
C(9)	0.5023	(4)	0.4003	(3)	0.2236	(2)	
C(10)	0.7355	(5)	0.3461	(4)	0.1717	(3)	
C(11)	0.3188	(5)	0.4647	(4)	0.3211	(3)	
C(12)	0.3087	(4)	0.5031	(3)	0.0966	(2)	
C(13)	0.7187	(5)	0.3712	(3)	0.3556	(3)	
C(14)	0.6013	(5)	0.4310	(3)	0.3985	(3)	
H(21)	0.343		0.336		−0.302		3.25
H(31)	0.492		0.314		−0.120		3.21
H(51)	0.083		0.358		−0.021		3.52
H(61)	−0.067		0.374		−0.204		3.88
H(101)	0.798		0.265		0.188		4.24
H(102)	0.820		0.414		0.178		4.24
H(103)	0.663		0.344		0.094		4.24
H(111)	0.325		0.548		0.357		4.73
H(112)	0.288		0.402		0.371		4.73
H(113)	0.238		0.465		0.246		4.73
H(131)	0.746		0.287		0.390		4.05
H(132)	0.827		0.420		0.372		4.05
H(141)	0.639		0.515		0.427		3.74
H(142)	0.574		0.382		0.460		3.74

The weight assigned to each observation in the refinement [19] was

$$w = 1 + [(|F_o| - 1.67|F_{min}|)/1.67|F_{min}|]^2$$

Corrections for anomalous dispersion were applied for bromine and sulfur.

ICE. The intensities of 2442 reflexions were recorded of which 2127 were more than 2σ above background and used in the analysis. The structure determination procedure was analogous to that for TCE except that it was not possible to refine the hydrogen positions. The final R-value was 0.043. The weighting scheme used was

$$w = 1 + [(|F_o| - 10.4|F_{min}|)/15.5|F_{min}|]^2$$

DISCUSSION

The final atomic parameters are given in Tables 1–4. Structure factor lists can be obtained on request from the Crystallography Group. Distances and angles are given in Figs. 1 and 2 which also show the atomic numbering and in Tables 5 and 6 where the standard deviations are included. Least-squares planes through various parts of the molecules are given in Tables 7 and 8. Stereoscopic pairs of the molecules are shown in Figs. 3 and 4.

The dimensions of the two bromobenzene groups are normal. The mean value of the C–C bond is 1.388 Å in TCE and 1.384 Å in ICE. The average angle is 120.0° in both structures. In both cases C(7) is about 0.1 Å out of

Table 4. Anisotropic thermal parameters for the non-hydrogen atoms of ICE in the form $\exp[-2\pi^2(h^2a^{*2}U_{11}+k^2b^{*2}U_{22}+l^2c^{*2}U_{33}+2hka^*b^*U_{12}+2klb^*c^*U_{23}+2hla^*c^*U_{13})]$. The standard deviations (within brackets) have been multiplied by 10^4.

	U_{11}	U_{22}	U_{33}	U_{12}	U_{23}	U_{13}
Br(1)	0.0702(3)	0.1003(4)	0.0303(2)	0.0021(2)	−0.0025(2)	−0.0129(3)
O(1)	0.0634(16)	0.0411(13)	0.0346(12)	−0.0012(10)	0.0114(12)	0.0122(12)
N(1)	0.0617(20)	0.0503(18)	0.0489(18)	−0.0008(14)	0.0142(16)	0.0126(16)
N(2)	0.0518(18)	0.0513(17)	0.0306(14)	0.0023(12)	0.0039(13)	0.0055(15)
N(3)	0.0669(20)	0.0518(18)	0.0253(14)	−0.0061(12)	0.0109(14)	0.0000(16)
C(1)	0.0530(21)	0.0489(20)	0.0284(16)	−0.0004(14)	0.0039(15)	−0.0076(17)
C(2)	0.0549(21)	0.0472(20)	0.0303(17)	−0.0020(14)	0.0157(16)	−0.0019(17)
C(3)	0.0501(20)	0.0422(18)	0.0320(16)	−0.0015(13)	0.0140(15)	0.0002(16)
C(4)	0.0472(19)	0.0380(17)	0.0266(15)	−0.0014(12)	0.0093(14)	0.0003(15)
C(5)	0.0449(19)	0.0600(22)	0.0332(17)	−0.0046(15)	0.0140(15)	−0.0023(17)
C(6)	0.0456(20)	0.0612(23)	0.0412(19)	−0.0046(16)	0.0099(16)	−0.0009(18)
C(7)	0.0466(19)	0.0430(18)	0.0264(15)	−0.0009(13)	0.0127(14)	−0.0002(16)
C(8)	0.0473(19)	0.0396(17)	0.0260(15)	−0.0007(13)	0.0090(14)	0.0060(15)
C(9)	0.0543(20)	0.0347(17)	0.0271(15)	0.0019(12)	0.0095(15)	0.0010(15)
C(10)	0.0498(22)	0.0705(26)	0.0501(22)	0.0017(19)	0.0125(18)	0.0049(20)
C(11)	0.0799(29)	0.0670(25)	0.0420(20)	−0.0032(18)	0.0283(20)	0.0142(23)
C(12)	0.0489(19)	0.0469(19)	0.0284(16)	−0.0033(14)	0.0108(14)	0.0005(16)
C(13)	0.0709(26)	0.0540(22)	0.0341(18)	0.0040(16)	−0.0058(18)	−0.0055(20)
C(14)	0.0803(28)	0.0541(22)	0.0275(16)	0.0002(15)	0.0027(18)	−0.0108(21)

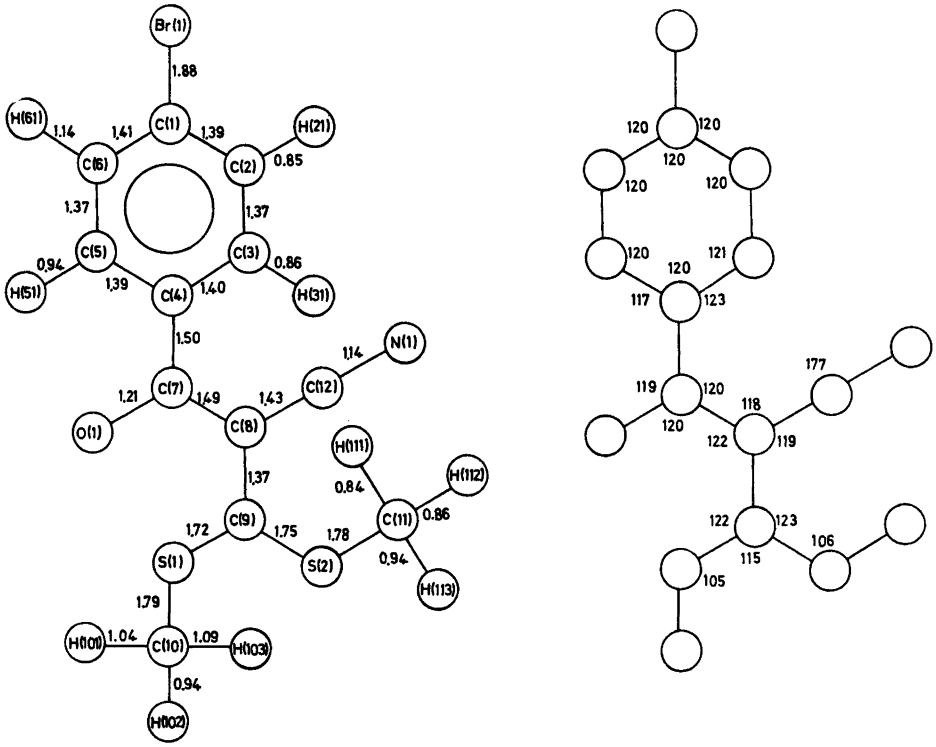


Fig. 1. Bond distances and angles in TCE.

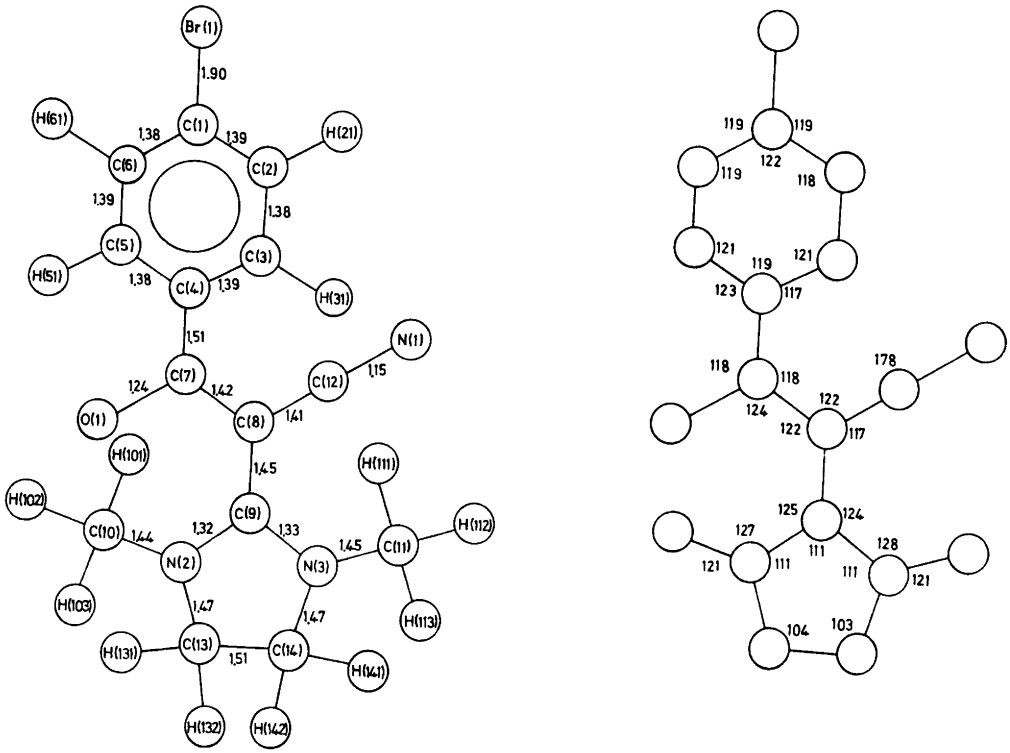


Fig. 2. Bond distances and angles in ICE.

the plane of the ring at the same time as the carbonyl group is twisted 32.1° (TCE) and 60.7° (ICE) from the plane. Whereas in ICE O(1) is near the plane through C(7), C(8) and C(12), it is 0.35 Å off the corresponding plane in TCE. The calculated twist [angle between planes through C(7), C(8), C(12) and C(9), N(2), N(3) and C(9), S(1), S(2) respectively] around the double bond is 15.2° in TCE and 41.1° in ICE. In TCE most of the effect appears to be due to the change in direction of the C(9) – S(1) bond.

The double bond in ethylene is given lengths between 1.332 and 1.339 Å.[20] Using 1.337 Å for this bond, and 1.517 Å for a $C(sp^2)-C(sp^2)$ bond with zero π-bond order and zero rotational barrier,[21] direct proportionality between barriers and bond orders (and a linear relation (4) between bond orders and bond lengths) should have given a C(8) – C(9) bond length of 1.477 Å. The experimental value for this bond, 1.369 Å, shows that no barrier-bond order proportionality exists. To obtain a general idea of electron distribution and bond lengths in TCE, calculations have been performed by the PPP method modified according to Roos and Skancke.[22] The modified matrix elements are given by (1) and (2), where ν refers to atoms directly bonded to μ, and λ to all atoms except μ. Alkyl groups were not taken into account.

$$F_{\mu\mu} = H_{\mu\mu}^{\text{core}} - 1/2 q_\mu \gamma_{\mu\mu} + \sum_{\lambda \neq \mu} q_\lambda \gamma_{\mu\lambda} \quad (1a)$$

$$H_{\mu\mu}^{\text{core}} = W_{\mu\mu} - (n_\mu - 1)\gamma_{\mu\mu} - \sum_{\lambda \neq \mu} n_\lambda \gamma_{\mu\lambda} \quad (1b)$$

$$W_{\mu\mu} = W_{\mu\mu}^\circ + \sum_\nu \Delta W_{\mu\nu} \quad (1c)$$

$$\Delta W_{\mu\nu} = \Delta W_{\mu\nu}^\circ + \delta_{\mu\nu}{}^W (R_{\mu\nu} - R_0) \quad (1d)$$

$$F_{\mu\nu} = \beta_{\mu\nu} - \tfrac{1}{2} p_{\mu\nu} \gamma_{\mu\nu} \quad (2a)$$

$$\beta_{\mu\nu} = \beta_{\mu\nu}^\circ + \delta_{\mu\nu}{}^\beta (R_{\mu\nu} - R_0) \quad (2b)$$

Repulsion integrals between nearest neighbours are given by (3). Other two-centre repulsion integrals are calculated by the

$$\gamma_{\mu\lambda} = \gamma_{\mu\lambda}^\circ + \delta_{\mu\lambda}{}^\gamma (R_{\mu\nu} - R_0) \quad (3)$$

Table 5. Interatomic distances (Å) and angles (°) (TCE), with e.s.d.'s.

Br(1) – C(1)	1.882	(0.006)
S(1) – C(9)	1.724	(0.005)
– C(10)	1.790	(0.008)
S(2) – C(9)	1.752	(0.005)
– C(11)	1.779	(0.006)
O(1) – C(7)	1.212	(0.006)
N(1) – C(12)	1.142	(0.007)
C(1) – C(2)	1.388	(0.007)
– C(6)	1.405	(0.007)
C(2) – C(3)	1.374	(0.008)
C(3) – C(4)	1.396	(0.007)
C(4) – C(5)	1.391	(0.007)
– C(7)	1.502	(0.007)
C(5) – C(6)	1.374	(0.008)
C(7) – C(8)	1.490	(0.007)
C(8) – C(9)	1.369	(0.007)
– C(12)	1.427	(0.007)
C(2) – H(21)	0.85	(0.07)
C(3) – H(31)	0.86	(0.06)
C(5) – H(51)	0.94	(0.06)
C(6) – H(61)	1.14	(0.06)
C(10) – H(101)	1.04	(0.10)
– H(102)	0.94	(0.08)
– H(103)	1.09	(0.07)
C(11) – H(111)	0.84	(0.08)
– H(112)	0.86	(0.08)
– H(113)	0.94	(0.06)
C(9) – S(1) – C(10)	105.4	(0.3)
C(9) – S(2) – C(11)	106.4	(0.3)
Br(1) – C(1) – C(2)	119.6	(0.4)
– C(6)	120.3	(0.4)
C(2) – C(1) – C(6)	120.0	(0.5)
C(1) – C(2) – C(3)	119.5	(0.4)
C(2) – C(3) – C(4)	120.7	(0.5)
C(3) – C(4) – C(7)	122.8	(0.4)
– C(5)	119.7	(0.5)
C(5) – C(4) – C(7)	117.3	(0.4)
C(4) – C(5) – C(6)	119.9	(0.4)
C(1) – C(6) – C(5)	120.0	(0.4)
C(4) – C(7) – O(1)	119.2	(0.5)
O(1) – C(7) – C(8)	120.4	(0.5)
C(4) – C(7) – C(8)	120.4	(0.4)
C(7) – C(8) – C(9)	122.3	(0.4)
– C(12)	117.8	(0.4)
C(9) – C(8) – C(12)	119.3	(0.4)
C(8) – C(9) – S(1)	121.6	(0.3)
– S(2)	123.4	(0.4)
S(1) – C(9) – S(2)	115.0	(0.3)
C(8) – C(12) – N(1)	176.7	(0.5)

Table 6. Interatomic distances (Å) and angles (°) (ICE) with e.s.d.'s.

Br(1) – C(1)	1.902	(0.003)
O(1) – C(7)	1.235	(0.004)
N(1) – C(12)	1.151	(0.005)
N(2) – C(9)	1.324	(0.005)
– C(10)	1.444	(0.006)
– C(13)	1.473	(0.004)
N(3) – C(9)	1.332	(0.005)
– C(11)	1.445	(0.006)
– C(14)	1.472	(0.004)
C(1) – C(2)	1.385	(0.005)
– C(6)	1.378	(0.006)
C(2) – C(3)	1.377	(0.004)
C(3) – C(4)	1.394	(0.005)
C(4) – C(5)	1.382	(0.005)
– C(7)	1.514	(0.004)
C(5) – C(6)	1.385	(0.005)
C(7) – C(8)	1.423	(0.004)
C(8) – C(9)	1.448	(0.004)
– C(12)	1.410	(0.005)
C(13) – C(14)	1.512	(0.006)
C(9) – N(2) – C(10)	126.9	(0.3)
– C(13)	110.8	(0.3)
C(10) – N(2) – C(13)	120.7	(0.3)
C(9) – N(3) – C(11)	127.5	(0.3)
– C(14)	111.4	(0.3)
C(11) – N(3) – C(14)	120.6	(0.3)
Br(1) – C(1) – C(2)	118.9	(0.3)
– C(6)	119.4	(0.3)
C(2) – C(1) – C(6)	121.8	(0.3)
C(1) – C(2) – C(3)	118.4	(0.4)
C(2) – C(3) – C(4)	121.1	(0.3)
C(3) – C(4) – C(5)	119.2	(0.3)
– C(7)	117.4	(0.3)
C(5) – C(4) – C(7)	123.3	(0.3)
C(4) – C(5) – C(6)	120.5	(0.4)
C(1) – C(6) – C(5)	119.1	(0.3)
O(1) – C(7) – C(4)	118.0	(0.3)
– C(8)	124.2	(0.3)
C(4) – C(7) – C(8)	117.8	(0.3)
C(7) – C(8) – C(9)	121.7	(0.3)
– C(12)	121.7	(0.3)
C(9) – C(8) – C(12)	116.6	(0.3)
N(2) – C(9) – N(3)	110.9	(0.3)
– C(8)	124.6	(0.3)
N(3) – C(9) – C(8)	124.4	(0.3)
N(1) – C(12) – C(8)	178.2	(0.3)
N(2) – C(13) – C(14)	103.7	(0.3)
N(3) – C(14) – C(13)	102.5	(0.3)

hard sphere approximation of Parr.[23] The parameters W_0, ΔW_0, β_0, and the different δ values are taken from the work of the Stockholm School and are collected together with the pertinent references in Table 9. The cyano group has not been parametrized, and instead the parameters for pyridine nitrogen have been employed here. The bond lengths were calculated by an iterative procedure according to Sundbom.[24] An idealized geometry was used in the first calculations, assuming a planar molecule with single and double bond lengths taken from similar systems. The imidazolidine ring in ICE was regarded as a regular pentagon, and all other bond angles were assumed to be 120°. Using the π-bond orders from the first calcula-

Table 7. Some least-squares planes in TCE. Equations referred to the crystal axes.

Planes	Equations
(a) C(1) C(2) C(3) C(4) C(5) C(6)	$0.12315X + 0.96337Y - 0.23824Z - 0.65558 = 0.$
(b) O(1) C(7) C(8)	$0.16576X - 0.88006Y + 0.44500Z + 0.41017 = 0.$
(c) C(7) C(8) C(12)	$0.43592X - 0.83236Y + 0.34227Z + 0.28528 = 0.$
(d) S(1) S(2) C(9)	$0.21557X - 0.90845Y + 0.35812Z + 0.44015 = 0.$

Deviations from the planes (Å)

	(a)	(b)	(c)	(d)
Br(1)	−0.011			
S(1)			−0.630	0.000[a]
S(2)			−0.027	0.000[a]
O(1)	−0.698	0.000[a]	−0.345	0.071
N(1)		−0.811	−0.064	−0.765
C(1)	0.010[a]			
C(2)	−0.022[a]			
C(3)	0.015[a]	0.671		
C(4)	0.003[a]	0.013	0.439	
C(5)	−0.015[a]	−0.581		
C(6)	0.008[a]			
C(7)	−0.098	0.000[a]	0.000[a]	0.105
C(8)	0.521	0.000[a]	0.000[a]	−0.035
C(9)		0.206	−0.181	0.000[a]
C(10)			−0.635	0.167
C(11)			1.232	0.887
C(12)		−0.416	0.000[a]	−0.406

[a] Atoms defining plane.

Table 8. Some least-squares planes in ICE. Equations referred to the crystal axes.

Planes	Equations
(a) C(1) C(2) C(3) C(4) C(5) C(6)	$0.10132X + 0.99479Y + 0.01116Z - 0.36201 = 0.$
(b) O(1) C(7) C(8)	$0.71008X + 0.40629Y - 0.57507Z - 0.37812 = 0.$
(c) C(7) C(8) C(12)	$0.65095X + 0.44419Y - 0.61559Z - 0.36494 = 0.$
(d) N(2) N(3) C(9) C(13) C(14)	$0.28933X + 0.90832Y - 0.30206Z - 0.44137 = 0.$

Deviations from the planes (Å)

	(a)	(b)	(c)	(d)
Br(1)	−0.010			
O(1)	−1.071	0.000[a]	−0.097	
N(1)		−0.185	0.021	
N(2)		0.990	0.777	0.031[a]
N(3)		−0.518	−0.657	−0.030[a]
C(1)	−0.005[a]			
C(2)	0.010[a]			
C(3)	−0.005[a]	1.075		
C(4)	−0.004[a]	0.010	0.137	
C(5)	0.009[a]	−1.007		
C(6)	−0.004[a]			
C(7)	−0.090	0.000[a]	0.000[a]	−0.664
C(8)	0.971	0.000[a]	0.000[a]	0.062
C(9)		0.142	0.026	0.000[a]
C(10)				0.402
C(11)				−0.284
C(12)		−0.114	0.000[a]	0.896
C(13)				−0.044[a]
C(14)				0.044[a]

[a] Atoms defining plane.

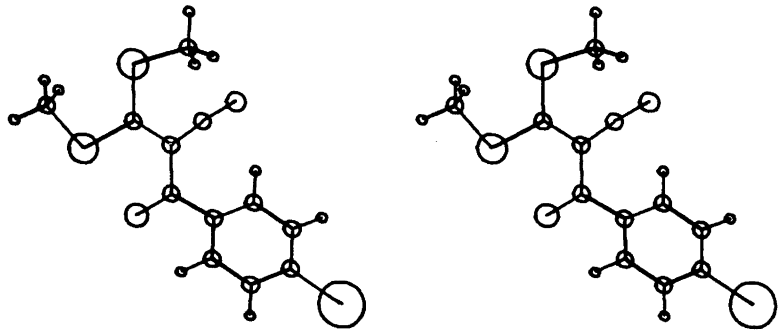


Fig. 3. Stereoscopic pair of TCE.

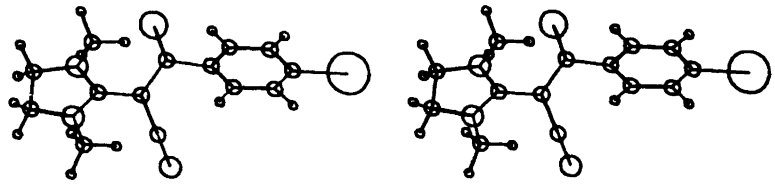


Fig. 4. Stereoscopic pair of ICE.

tion, new bond lengths were calculated by (4), where $R_{\mu\nu}°$ is a standard bond length,

$$R_{\mu\nu} = R_{\mu\nu}° - 0.18\, p_{\mu\nu} \quad (4)$$

1.517 Å for a C–C bond, 1.458 Å for a C–N bond, 1.365 Å for a C–O bond, and 1.773 Å for a C–S bond. The C–Br and C≡N bonds were not iterated. The calculations were repeated until self-consistent bond lengths were obtained, which only required a few cycles. The results are given in Table 10 together with the starting bond lengths and the experimental bond lengths. The agreement between the calculated and the experimental C=C bond length in TCE is quite satisfactory, and the same can also be said about the C–S bonds, for which even the difference in bond length is correctly reproduced. The too large calculated C(8)–C(12) bond length can be ascribed to the use of $R_{\mu\nu}° = 1.517$ Å for this bond, though C(12) is *sp*-hybridized. The too low calculated value for the C(4)–C(7) bond can at least partly be explained by the observed twisting of the benzene ring out of the plane through C(8), C(7), and O(1) of 32.1°. The twisting of the S(1)–C(9)–S(2) group (15.2°) is probably too small to affect the bond lengths significantly. With the adopted conformation the S(1)–O(1) contact distance is 2.673 Å (van der Waals distance 3.25 Å) and C(11)–N(1) is 3.44 Å. H(111) is 2.54 Å from C(12) and 2.55 Å from N(1). Very short non-bonded interactions between sulfur and oxygen have been found earlier, *e.g.* 2.64 Å in two 2-acyl-methylene-1,3-dithietans,[25,26]

Table 9. Parameters (eV).

Atom	$W_{\mu\mu}°$	$\gamma_{\mu\mu}$	Bond (μ–ν)	$\beta_{\mu\nu}°$	$\gamma_{\mu\nu}°$	$\delta_{\mu\nu}{}^{W}$	$\delta_{\mu\nu}{}^{\beta}$	$\delta_{\mu}(\gamma)$	$\Delta W_{\mu}(\gamma)$	$\Delta W_{\gamma}(\mu)$	R_0 (Å)	Ref.
C	−9.89	11.97	C–C	−2.42	6.91	9.22	3.05	−3.99	0.07	0.07	1.397	33
N	−12.57	15.44	C–N	−2.72	7.16	5.6	2.6	−3.99	0.03	0.14	1.338	34
N	−8.52	15.44	C–N	−2.25	6.34	5.6	2.6	−3.99	0.03	0.14	1.338	34
O	−19.60	18.89	C–O	−2.46	9.33	0	0	0	−0.71	0	1.22	35
S	−10.62	9.58	C–S	−1.37	7.28	9.22	3.05	−3.99	−0.70	0	1.714	36
Br	−11.25	9.35	C–Br	−1.84	6.42	0	0	0	−2.01	0	1.880	37

2.41 Å in a 3-acetylmethylene-1,2-dithiole [27] and 2.78 Å in *o*-carboxyphenyl methyl sulfoxide.[28] The nature of this attractive force has been discussed by Kapecki and Baldwin [29] in terms of EHT calculations, and by Pfister-Guillouzo *et al.*[30] in terms of CNDO/2 calculations. The EHT calculations give no conclusive result, and the latter authors conclude that introduction of *d* orbitals in the basis set is necessary to get a proper description of the O···S interaction.

In the benzene ring H(31) is 2.56 Å from C(12) but 2.91 Å from N(1). Whereas thus C(12) has the same hydrogen packing contacts from two sides, the surrounding of N(1) is less symmetrical. This might explain the observed deviation of the C(8)−C(12)−N(1) angle from 180°. H(51) is 2.52 Å (van der Waals distance 2.72 Å) from O(1).

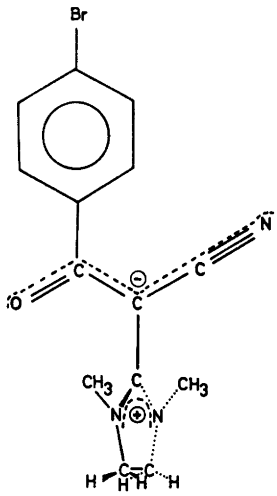


Fig. 5. ICE in the 90° twisted state.

For ICE, the C=C bond length calculated for a planar molecule is 1.388 Å. The difference between this and the experimental value of 1.448 Å must largely be ascribed to a steric interference between the N-methyl groups on one side and the carbonyl and cyano groups on the other causing a considerable twist (41.1°) of the molecule around the double bond. The C(10)−O(1) distance is 2.90 Å compared with the van der Waals distance 3.22 Å, and the C(11)−N(1) distance is 3.46 Å. The shortest O(1)···H distance is 2.27 Å and the shortest N(1)···H distance 2.67 Å. Evidently the interaction with the carbonyl group is the most important one. A case of a twisted double bond, which is only slightly stretched (to 1.345 Å), has recently been reported [31] but this bond is a member of a polycyclic ring system, which may well restrict its expansion.

The experimental C(9)−N(2) and C(9)−N(3) bonds are considerably shorter than those calculated for the planar molecule. This can be ascribed to the twist and reflects the greater contribution in the actual molecule of the amidinium ion structure, which is postulated to stabilize the 90° twisted molecule [14] (Fig. 5). This is compatible with the almost planar arrangement of O(1), C(7), C(8), C(12), and N(1). The imidazolidine group is also nearly planar whereas it for instance in 1-(4-chlorobenzyl)-1-nitroso-2-(4,5-dihydro-2-imidazolyl)hydrazine [32] is considerably puckered. A larger polarization in ICE than in TCE is shown both by experimental and calculated bond lengths in the electron attracting part of the molecule (Table 10). Similarly, ICE shows a more extensive charge separation in the calculated π-electron distribution (see Fig. 6 for the distribution of

Table 10. Bond lengths (Å).

Molecule I				Molecule II			
Bond	PPP Initial	Final	Experimental	Bond	PPP Initial	Final	Experimental
C_8-C_9	1.340	1.362	1.369	C_8-C_9	1.340	1.388	1.448
C_9-S_1	1.730	1.729	1.724	C_9-N_2	1.400	1.378	1.324
C_9-S_2	1.730	1.736	1.752	C_9-N_3	1.400	1.386	1.332
$C_{12}-C_8$	1.450	1.467	1.427	$C_{12}-C_8$	1.450	1.456	1.410
C_7-C_8	1.450	1.465	1.490	C_7-C_8	1.450	1.440	1.423
C_7-O_1	1.220	1.232	1.212	C_7-O_1	1.220	1.242	1.235
C_4-C_7	1.460	1.469	1.502	C_4-C_7	1.460	1.471	1.514

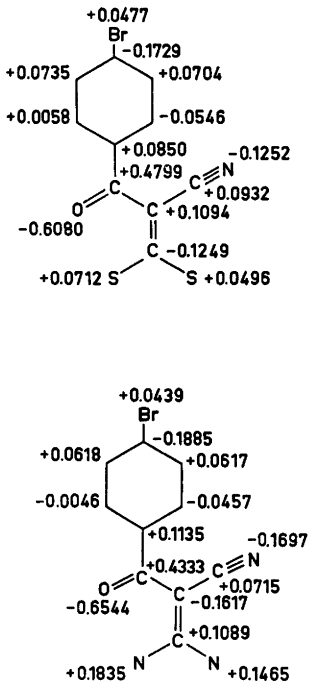


Fig. 6. Formal π-electron charges in TCE (above) and ICE (below).

formal charges). In TCE, the sulfur atoms donate 0.05 and 0.07 electronic units, respectively, whereas in ICE the figures for the nitrogen atoms are 0.15 and 0.18 units. In the latter compound, C(9) is also positive, indicating a partial formation of the amidinium ion structure already in the ground state, whereas in TCE this atom is negative.

Table 11. Intermolecular Br···H, O···H, N···H and H···H contacts less than 3 Å (TCE). i refers to the equivalent position $-x, \frac{1}{2}+y, -z$.

A	B	Unit translation of atom B	Å
Br(1)	H(113^{i})	(2, −1, 1)	2.97
O(1)	H(21)	(−1, 0, 0)	2.64
O(1)	H(102)	(0, 0, −1)	2.94
N(1)	H(113)	(0, 0, −1)	2.98
H(31)	H(103)	(1, 0, 0)	2.92
H(51)	H(102)	(0, 0, −1)	2.95
H(51)	H(113^{i})	(1, −1, 1)	2.94
H(101)	H(111)	(−1, 0, 0)	2.85
H(103)	H(111)	(−1, 0, 0)	2.72
H(103)	H(112)	(−1, 0, 0)	2.41

Table 12. Intermolecular Br···H, O···H, N···H, C···H and H···H contacts less than 3 Å (ICE). i, ii, and iii refer to the equivalent positions $-x, -y, -z, -x, \frac{1}{2}+y, \frac{1}{2}-z$ and $x, \frac{1}{2}-y, \frac{1}{2}+z$.

A	B	Unit translation of atom B	Å
Br(1)	H(101^{iii})	(−1, 0, −1)	3.00
Br(1)	H(132^{i})	(1, 1, 0)	2.88
O(1)	H(21^{iii})	(0, 0, 0)	2.36
O(1)	H(111^{ii})	(1, −1, 0)	2.85
O(1)	H(141^{ii})	(1, −1, 0)	2.63
O(1)	H(142^{iii})	(0, 0, −1)	2.36
N(1)	H(31^{i})	(1, 1, 0)	2.65
N(1)	H(51^{i})	(0, 1, 0)	2.89
N(1)	H(61^{i})	(0, 1, 0)	2.63
N(1)	H(103^{i})	(1, 1, 0)	2.82
N(1)	H(131^{ii})	(1, 0, 0)	2.42
C(1)	H(102^{i})	(1, 1, 0)	2.96
C(3)	H(112^{iii})	(0, 0, −1)	2.82
C(4)	H(112^{iii})	(0, 0, −1)	2.94
C(6)	H(101^{iii})	(−1, 0, −1)	2.91
C(6)	H(102^{i})	(1, 1, 0)	2.89
C(7)	H(21^{iii})	(0, 0, 0)	2.80
C(12)	H(31^{i})	(1, 1, 0)	2.78
H(21)	H(141^{i})	(1, 1, 0)	2.46
H(31)	H(131^{iii})	(0, 0, −1)	2.57
H(31)	H(142^{iii})	(0, 0, −1)	2.59
H(61)	H(101^{iii})	(−1, 0, −1)	2.32
H(61)	H(111^{i})	(0, 1, 0)	2.83
H(61)	H(113^{i})	(0, 1, 0)	2.43
H(111)	H(101^{ii})	(1, 0, 0)	2.80
H(141)	H(111^{i})	(1, 1, 1)	2.94
H(141)	H(112^{i})	(1, 1, 1)	2.81
H(141)	H(142^{i})	(1, 1, 1)	3.00
H(142)	H(111^{i})	(1, 1, 1)	2.54

From Table 11 it is evident that the most important intermolecular contacts of TCE involve the methyl hydrogens. There are thus no hydrogen packing distances less than 3 Å between benzene rings.

Table 12 gives the shortest intermolecular distances in ICE. Here the hydrogen atoms of one benzene ring are in packing contact with those of other benzene rings as well as with those of CH_2- and CH_3-groups. O(1) has apart from the intramolecular interaction with H(103) of 2.27 Å also other very short distances to H(21) (2.36 Å) and H(142) (2.36 Å).

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Composition of the Essential Oil of Sweet Marjoram Obtained by Distillation with Steam and by Extraction and Distillation with Alcohol—Water Mixture

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The steam distilled oil of sweet marjoram (*Majorana hortensis* Moench) and an alcoholic distillate of this herb used for flavouring liqueurs were analysed by gas chromatography, infra-red spectrophotometry, nuclear magnetic resonance and mass spectrometry. 11 monoterpene hydrocarbons, 8 sesquiterpene hydrocarbons, 10 monoterpene alcohols, 5 esters of monoterpene alcohols and 4 other compounds were identified. Additionally, 6 ethyl ethers of monoterpene alcohols and 9 ethyl esters of long-chain fatty acids were found in the alcoholic distillate. Of the 53 components identified in the sweet marjoram aroma concentrates in the present study, 35 are reported for the first time.

Sweet marjoram is obtained from *Majorana hortensis* Moench (*Origanum majorana* L.), which has for centuries been cultivated in the Mediterranean countries and nowadays in the Northern Temperate Zone as well. The commercially available herb is prepared from the dried leaves and flowers. The content of volatile oil varies between 0.5 and 3.5 % depending on the origin of the herb and many other factors.[1]

The composition of sweet marjoram oil was first investigated at the turn of the century, when Biltz [2] found it to consist of 40 % monoterpene hydrocarbons, mostly terpinene. Somewhat later, Wallach [3,4] showed that sweet marjoram contains terpinenol-4 and α-terpineol. After that no further investigations into the aroma compounds of sweet marjoram were published until the sixties. Nicoletti and Baiocchi [5] found that the oil of sweet marjoram contains monoterpene hydrocarbons, terpinenol-4, α-terpineol, linalool, linalyl acetate and *cis*-sabinene hydrate, which had not previously been identified in natural oils. Lossner [6] found *trans*-sabinene hydrate as well, and identified 6 monoterpene hydrocarbons. In the same study, gas chromatographic analysis showed that the oils of sweet marjoram originating from seven different countries were all much the same. Lossner's analysis showed that the German oil contained 1 % α-pinene, 9 % myrcene, 8 % α-terpinene + limonene, 20 % γ-terpinene, 7 % terpinolene, 9 % linalool + *trans*-sabinene hydrate, 3 % linalyl acetate, 13 % *cis*-sabinene hydrate, 23 % terpinenol-4, and 5 % α-terpineol. Ikeda *et al.*[7] analysed the monoterpene hydrocarbons in the sweet marjoram oil by gas chromatography and, in addition to those above, identified α-thujene, camphene, β-pinene, sabinene, α-phellandrene, β-phellandrene, and ocimene by their retention times.

The volatile oil from sweet marjoram grown in India differs substantially in both physical characteristics and chemical composition from that of European and American origin.[1] The following compounds have been identified in the Indian oil: carvacrol,[8] eugenol,[8,9] chavicol,[8] methylchavicol,[8] linalool,[8,9] α-terpineol,[8,9] terpinenol-4,[9] geraniol,[9] and caryophyllene.[8]

The volatile oil is commonly separated by steam distillation; the oil of sweet marjoram produced by this method is used in the flavour industry, pharmacy *etc.* In the production of alcoholic beverages, however, it is considered that a finer quality aroma is obtained if the volatile components are separated by percolation and distillation with an alcohol-water mix-

ture. This work investigates both oil separated by steam distillation and the aroma concentrate used in the preparation of herbal liqueurs. The aims of the study were to identify the aroma compounds from sweet marjoram and to investigate the effect of the different methods of separation on the aroma concentrates.

EXPERIMENTAL

Apparatus. A Hewlett-Packard 7620 A gas chromatograph fitted with a TC detector was used for analysis. The temperature of the injection block and the detector was 240 °C. Four pairs of 6 mm × 3 m stainless steel columns were packed with: 10 % FFAP on 60/80 mesh Chromosorb W AW, 5 % SE-30 on 60/80 mesh Chromosorb G, 15 % Apiezon L on 60/80 mesh Chromosorb W AW, and 5 % LAC-2R-446 on 70/80 mesh Chromosorb G AW DMCS. Peak areas were measured by an Infotronics CRS-11 HSB/42 Integrator. For the preparative work the fractions eluting at the TCD exhaust were collected in 1 mm × 20 cm glass tubes cooled with dry ice.

The IR spectra were measured with samples between plates of KBr on a Perkin-Elmer 521 spectrophotometer equipped with a beam condenser. The mass spectra were measured on a Varian CH 7 combined GLC-MS connected with a Spectro System 100 MS computer. The ionisation energy used was 70 eV, and a 2 mm I.D. × 2 m glass column packed with FFAP or SE-30 was used in the GLC.

The NMR spectra were measured on a Varian A 60 model using carbon tetrachloride as solvent and tetramethylsilane as internal standard.

Materials and procedure. The alcoholic distillate of sweet marjoram used in the investigation was produced in Alko's flavour distillery from East-European plants. The herb is percolated for three weeks with a 55 % ethanol water mixture as the first stage in the preparation of the alcoholic distillate. The percolate is distilled under reduced pressure and a fraction with the desired aroma is collected. For the analysis, the aroma compounds were separated from the alcoholic distillate by extraction into pentane. 500 ml lots of the distillate were diluted with water to 50 % alcohol, saturated with NaCl and shaken in a separating funnel with pentane. The extract was dried over Na_2SO_4 and the pentane distilled off.

The volatile oil was separated from 200 g of sweet marjoram (from the same batch of herb as that used in the preparation of the alcoholic distillate) by 2 h steam distillation. The oil was extracted from the distillate with pentane, the exract dried over Na_2SO_4, and the pentane distilled off to leave 2.2 g oil.

The sweet marjoram oil obtained by steam distillation and the pentane extract from the alcoholic distillate were first fractionated by preparative GLC on a FFAP column, and the fractions collected were in most cases further separated on a second column so that pure compounds could be collected for determining the spectra. The combined GLC-MS analysis using an SE-30 column was made directly from fractions obtained with the FFAP column.

Before analysing the sesquiterpene hydrocarbons by GLC, the steam distilled oil and the pentane extract from the alcoholic distillate were each fractionated by column chromatography. 200 μl samples were added to a 1 cm × 15 cm silica gel column (Kieselgel, 0.2 – 0.5 mm, from Merck) and the hydrocarbons were eluted with 200 ml pentane.

The components were identified by comparing their GLC retention times and spectra with those of authentic samples or with published spectra or, when reference materials were unavailable, by interpreting the spectral data.

Ledene was synthesized by dehydrating ledol with 5 % H_2SO_4, as described by Kiryalov.[10]

RESULTS AND DISCUSSION

The gas chromatogram of the steam distilled sweet marjoram oil on FFAP column is presented in Fig. 1, and that of the pentane extract from the alcoholic distillate in Fig. 2. The compounds identified are shown in Table 1 with their percentage contribution as calculated from the peak areas and identification methods.

The monoterpene hydrocarbons constituted about 30 % of the oil separated by steam distillation and about 44 % of the aroma from the alcoholic distillate. The main components, γ-terpinene and α-terpinene, were separated from the steam distilled oil by preparative gas chromatography. A direct analysis of sweet marjoram oil by combined GLC-MS using a FFAP column identified α-pinene, β-pinene, sabinene, myrcene, α-phellandrene, β-phellandrene, limonene, and terpinolene by comparing retention times and mass spectra with those of authentic samples and α-thujene using a published mass spectrum.[11] Gas chromatographic analysis of the pentane extract of the alcoholic distillate showed that the same compounds were present and in approximately the same relative proportions.

The sesquiterpene hydrocarbons formed less than 3 % of the steam distilled oil and 7.5 % of the aroma fraction of the alcoholic distillate. The same two main components dominated in

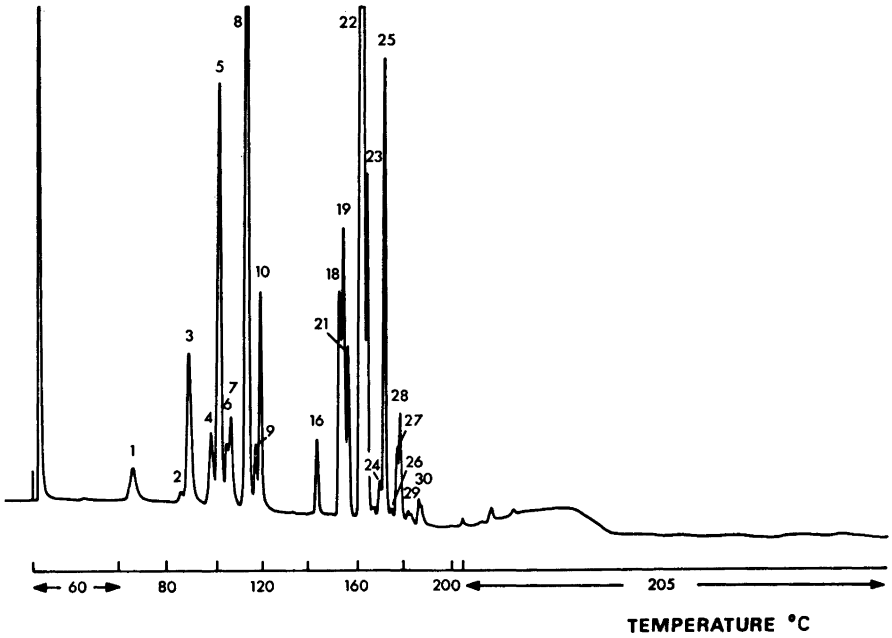


Fig. 1. Gas chromatogram of the oil of sweet marjoram separated by steam distillation. Column: 6 mm × 3 m FFAP.

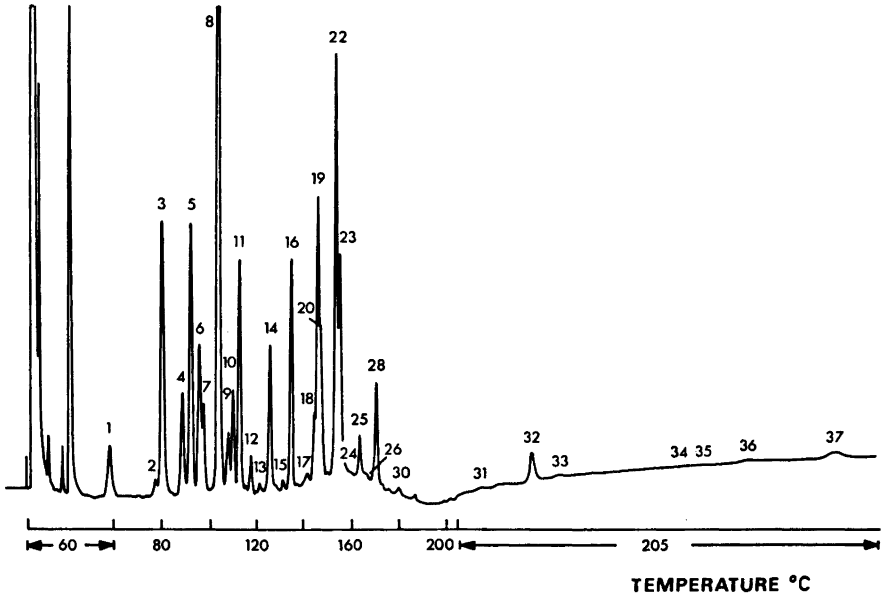


Fig. 2. Gas chromatogram of the pentane extract from the alcoholic distillate of sweet marjoram. Column: 6 mm × 3 m FFAP.

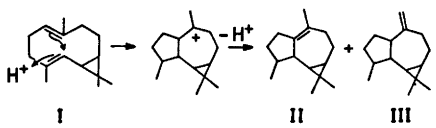
Table 1. Compounds identified in the steam distilled oil and the alcoholic distillate of sweet marjoram.

Peak No.	Compound	Steam distilled oil		Pentane extract of alcoholic distillate	
		Percentage	Evidence for identification	%	Evidence for identification
1	α-Thujene	1.0	MS[11]	1.9	GLC
	α-Pinene		GLC,MS[a]		GLC
2	β-Pinene	0.2	GLC, MS[a]	0.4	GLC
3	Sabinene	2.5	GLC, MS[a]	8.2	GLC
4	Myrcene	1.2	GLC, MS[a]	2.6	GLC
	α-Phellandrene		GLC, MS[a]		GLC
5	α-Terpinene	6.1	IR,[16] MS[11]	6.8	GLC
6	Limonene	0.6	GLC, MS[a]	3.8	GLC
7	β-Phellandrene	0.9	GLC, MS[a]	1.4	GLC
8	γ-Terpinene	14.0	IR,[16] MS[11]	21.3	GLC
	trans-4-Ethoxy-thujane				IR, MS, NMR
9	*p*-Cymene	0.7	GLC, MS[a]	1.1	GLC
10	Terpinolene	2.5	GLC, MS[a]	2.0	GLC
11	*cis*-4-Ethoxy-thujane			5.2	IR, MS, NMR
12	1-Ethoxy-2-*p*-menthene			0.7	IR, MS
13	*trans*-3-Ethoxy-1-*p*-menthene			0.2	IR, MS
14	4-Ethoxy-1-*p*-menthene			3.7	IR, MS, NMR
15	*cis*-3-Ethoxy-1-*p*-menthene			0.2	IR, MS
16	*trans*-Sabinene hydrate	1.0	IR[17] MS	5.1	IR
17	α-Copaene	+	IR[18]	+	IR
18	Linalool	3.3	GLC, MS,[a] IR[a]	0.7	GLC
19	*cis*-Sabinene hydrate	4.0	IR,[17] MS	7.5	IR
20	Linalyl acetate	0.1	GLC	3.4	GLC, IR[a]
21	*cis*-2-*p*-Menthen-1-ol	2.0	IR,[19] MS	0.2	GLC
22	Terpinenol-4	45.5	GLC, IR[a], MS[a]	10.3	GLC, IR
23	Caryophyllene	3.5	GLC, IR[a], MS[a]	6.1	GLC, IR
	trans-2-*p*-Menthen-1-ol		IR,[19] MS		
	4-Terpinenyl acetate				GLC, IR[a]
	Ethyl caprate				GLC, IR[a]
24	*trans*-Piperitol	0.3	IR,[19] MS		
	allo-Aromadendrene	+	MS[20]	+	MS
	β-Farnesene	+	MS	+	MS
	γ-Elemene	+	MS[20]	+	MS
25	α-Terpineol	6.7	GLC, IR[a], MS[a]	0.7	GLC, IR
	α-Terpinyl acetate			+	GLC, IR[a]
	α-Humulene	+	IR[18]	+	IR
26	Neryl acetate	+	GLC, MS	+	GLC, IR[a]
	Ledene	+	IR[a]	+	IR
27	*cis*-Piperitol	0.5	IR,[19] MS		
28	Bicyclogermacrene	1.2	IR, MS	2.7	IR, MS, NMR[12]
	Carvone		GLC, IR,[a] MS[a]		GLC
	Geranyl acetate		GLC		GLC, IR[a]
29	Geraniol	+	GLC, MS[a]		
30	*p*-Cymen-8-ol	0.5	IR[19]		
	Anethol		GLC, IR[a]	+	GLC, IR
	Ethyl laurate			+	GLC, MS
31	Ethyl myristate			+	GLC, IR[a]
32	Ethyl palmitate			1.2	GLC, IR[a], MS
33	Ethyl *trans*-hexadecenoate			+	IR, MS
34	Ethyl stearate			+	GLC, MS[a]
35	Ethyl oleate			+	GLC, IR[a], MS
36	Ethyl linoleate			+	GLC, IR[a], MS
37	Ethyl linolenate			+	GLC, IR[a], MS

[a] Spectrum of an authentic sample.

both samples. The larger of these was identified as caryophyllene. The other was tentatively identified as bicyclogermacrene. Its mass spectrum gave a molecular weight of 204. The IR spectrum had absorption bands at 3010, 1650, and 829 cm^{-1}, corresponding to a trisubstituted double bond. The NMR spectrum is identical with that reported by Nishimura *et al.*[12] for bicyclogermacrene in other respects, although it was not possible to resolve with certainty the cyclopropane ring multiplet from the background noise because of the small sample size available. The same minor components, ledene, α-humulene, α-copaene, β-farnesene, γ-elemene, and alloaromadendrene, were found in the hydrocarbon fractions from both samples. Ledene (II) was identified by comparing its IR spectrum with that of a sample synthesized by the dehydration of ledol.

Ledene (II) has not previously been reported to occur in natural oils. Its occurence, and that of alloaromadendrene (III), together with bicyclogermacrene (I) in the oil of sweet marjoram is in agreement with the suggestion by Nishimura [12] that bicyclogermacrene could be the biogenetic precursor of aromadendrane-type compounds. It is also possible, however, that ledene and alloaromadendrene have been formed from bicyclogermacrene during the isolation or analysis of the volatile oil.



Scheme 1.

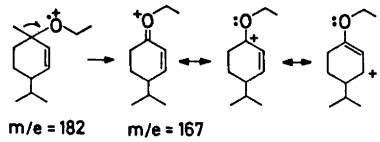
Monoterpene alcohols constituted about 65 % of the steam distilled oil and about 25 % of the aroma fraction from the alcoholic distillate. Ten alcohols were identified in the steam distilled oil: terpinenol-4, α-terpineol, linalool, geraniol, *cis*- and *trans*-sabinene hydrate, *cis*- and *trans*-piperitol, and *cis*- and *trans*-2-*p*-menthen-1-ol. Six of these alcohols, terpinenol-4, α-terpineol, linalool, *cis*- and *trans*-sabinene hydrate, and *cis*-2-*p*-menthen-1-ol were also found in the alcoholic distillate.

In the chromatogram of the pentane extract from the alcoholic distillate there was a group of peaks between the monoterpene hydrocarbons (peaks 1 – 10) and the monoterpene alcohols (starting at peak 16), all of which were absent from the chromatogram of the steam distilled oil. IR, NMR, and MS analysis showed that the peaks represented ethyl ethers of monoterpene alcohols, obviously formed during the preparation of the flavour distillate. These ethers made up about 15 % of the aroma fraction, comparable to the 25 % of the monoterpene alcohols.

The first of this group of components eluted from the FFAP column in peak 8 together with γ-terpinene, from which it could be separated using the SE-30 column. The mass spectrum revealed a molecular weight of 182 and fragments typical of monoterpenes at *m/e* 136, 121, 93. The IR spectrum exhibited the characteristic absorption of the C – H stretching of the cyclopropane ring at 3050 cm^{-1}, the typical double absorption of the *gem*-dimethyl group at 1382 and 1368 cm^{-1}, and the strong absorption band at 1070 cm^{-1} of the ether bond. The NMR spectrum contained two unsymmetrical doublet signals centered at δ 0.88 and 0.96 (each 3 H, $J = 6$ Hz) corresponding to the methyls of an isopropyl group, a 3 H singlet at δ 1.20 ($CH_3 - C - O -$), and a triplet centered at δ 1.10 (3 H, $J = 7$ Hz) and a quartet centered at δ 3.40 (2 H, $J = 7$ Hz) from an ethoxy group. The multiplet appearing at δ 0.1 – 0.5 is clearly caused by the methylene protons of the cyclopropane ring. An ether of ethanol and sabinene hydrate (4-ethoxy-thujane) would fit these data. The spectra from component 11 closely resemble those of the ether in peak 8, the main difference in NMR spectrum being that the signal from one of the methylene protons of the cyclopropane ring appears at lower field strength (δ 0.5 – 0.7) in the spectrum of component 11. It is reasonable that this proton would be less shielded if the ethoxy group were *cis* to the cyclopropane ring. Accordingly, the ether in peak 8 is tentatively identified as *trans*-4-ethoxy-thujane and peak 11 as the corresponding *cis* compound.

The absorption bands at 3020, 1635, and 730 cm^{-1} in the IR spectrum of compound 12 indicate the presence of a double bond, which is most probably *cis* disubstituted. The absorptions at 1380 and 1360 cm^{-1} reveal the typical double peak of the *gem*-dimethyl group. The molecular peak appears in the mass spectrum at *m/e* 182. A structure derived from 2-*p*-

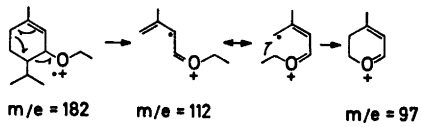
menthene is compatible with these data. The base peak in the mass spectrum is *m/e* 167 (M − 15). With the methyl and ethoxy groups on the same carbon, cleavage at the bond *β* to oxygen would result in a stable (M − 15) ion.



Scheme 2.

The (M − 45) peak too is larger (29 %) here than in the spectra of the other ethers found is sweet marjoram oil. The suggested structure favours this fragmentation as well, since cleavage of the ethoxy group would lead to the formation of a tertiary allylic carbonium ion.

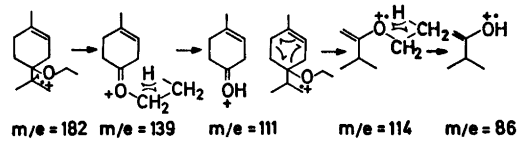
The IR spectrum of compound 13 displayed absorptions at 3010, 830, 1375, 1360 and 1080 cm^{-1}, corresponding to a trisubstituted double bond, a *gem*-dimethyl group, and a C − O bond. The mass spectrum showed the molecular ion peak at *m/e* 182 and the base peak at *m/e* 112. Considering only those ethyl ethers derived from the alcohols already identified in the aroma of sweet marjoram, the ethyl ether of piperitol has the most likely structure to accomodate these data. Cleavage of the ring at the bonds *β* to oxygen and the double bond would lead to the formation of the resonance stabilized *m/e* 112 ion. The base peak in the mass spectrum of piperitol is at *m/e* 84, which have been shown to occur by a corresponding mechanism.[13] Loss of a methyl group from the *m/e* 112 ion would produce a fragment of *m/e* 97, which is in fact the second most abundant ion (45 %) in the spectrum.



Scheme 3.

The IR and mass spectra of compound 15 closely resemble those of compound 13. According to the gas chromatographic behaviour of corresponding alcohols, peak 13 was tentatively identified as the ethyl ether of *trans*-piperitol (*trans*-3-ethoxy-1-*p*-menthene) and peak 15 as the *cis*-compound.

The mass spectrum of component 14 revealed that its molecular weight is also 182. Absorptions in the IR spectrum at 3010, 835, 790, 1385, 1365 and 1070 cm^{-1} were characteristic for a trisubstituted double bond, a *gem*-dimethyl group, and a C − O bond. The NMR spectrum of component 14 had a doublet centered at *δ* 0.87 (6 H, $J = 6.5$ Hz) corresponding to an isopropyl group, a signal at *δ* 1.64 caused by methyl adjacent to a double bond, a multiplet centered at *δ* 5.18 from a proton of the double bond, a triplet centered at *δ* 1.08 (3 H, $J = 7$ Hz) and a quartet centered at *δ* 3.28 (2 H, $J = 7$ Hz) corresponding to an ethoxy group. These data suggest a structure derived from 1-*p*-menthene. The (M − 15) peak in the mass spectrum is only 3 %, while the (M − 43) peak is 52 %. Attachment of the ethoxy group to C-4 would facilitate elimination of the isopropyl group. The *α*-cleavage and rearrangement of hydrogen after *β*-cleavage, which is usual for ethers,[14] would also rationalize the formation of the fragment *m/e* 111 (30 %) as well as the fragments *m/e* 114 (29 %) and 86 (64 %), when the ring cleaves at the bonds *β* to the double bond. The peaks *m/e* = 111 and 86 are also prominent in the mass spectrum of terpinenol-4. Accordingly, component 14 was identified as 4-ethoxy-1-*p*-menthene.



Scheme 4.

The following five monoterpene alcohol acetates were identified in the pentane extract from the alcoholic distillate of sweet marjoram by comparing their retention times and IR spectra with those of authentic samples: linalyl acetate, terpinenyl-4 acetate, *α*-terpinyl acetate, neryl acetate and geranyl acetate. Together these esters made up less than 5 % of the aroma fraction, with linalyl acetate making the largest contribution. The amount of esters found in the steam distilled oil was very low, although linalyl acetate, neryl acetate and geranyl acetate could be identified by GLC.

The only carbonyl compound found in sweet marjoram oil was carvone, which constituted

about a quarter of peak 28. The carvone from the alcoholic distillate formed only a small part of peak 28, but still lent its characteristic odour to this fraction.

The aromatic compounds *p*-cymen-8-ol, *p*-cymene and anethol were found as minor components in the steam distilled oil.

Nine long-chain fatty acid ethyl esters were found in the alcoholic distillate of sweet marjoram. Together they constituted less than 2 % of the aroma fraction of this distillate, and were completely absent from the steam distilled oil. Ethyl caprate, laurate, myristate, palmitate, oleate, linoleate, and linolenate were identified by comparing their retention times, IR and mass spectra with those of authentic samples. The IR spectrum of compound 33 is very similar to those of other unsaturated ethyl esters except that it exhibits the additional feature of the absorption band at 965 cm^{-1}, which is characteristic for a *trans*-disubstituted double bond. The molecular peak in the mass spectrum appears at $m/e = 282$ and the base peak at $m/e = 88$, the latter being characteristically formed in the fragmentation of ethyl esters as a consequence of the McLafferty rearrangement. This implies that components 33 is an ethyl *trans*-hexadecenoate isomer. The position of the double bond could not be settled by the available data.

Clearly the ethyl esters were formed by the action of the ethanol on the herb during the preparation of the alcoholic distillate, and their occurrence suggests the presence of the corresponding acids in sweet marjoram.

The results show that there are marked differences in the composition of the aroma between the sweet marjoram oil obtained by steam distillation and that produced by alcoholic distillation. With the exception of *cis*- and *trans*-sabinene hydrate, the proportion of monoterpene alcohols is clearly lower in the alcoholic distillate than in the steam distilled oil. Lossner [6] claims that *cis*-sabinene hydrate is especially important in the aroma of sweet marjoram. The proportion of mono and sesquiterpene hydrocarbons and esters of monoterpene alcohols is higher in the alcoholic distillate than in the steam distilled oil.

More interesting than these quantitative differences is the formation of new aroma compounds, such as ethyl ethers of monoterpene alcohols and ethyl esters of long-chain fatty acids, during the preparation of the alcoholic distillate. The newly-formed esters are present at quite a low concentration and have a relatively high odour threshold,[15] so that their contribution to the aroma of the alcoholic distillate is slight. The ethyl ethers of the monoterpene alcohols, however, made up about 15 % of the aroma fraction, and by smelling the aroma components as they eluted from the gas chromatograph it was found that many of these ethers had a relatively strong odour, which differed from that of the corresponding alcohols. It is probable, therefore, that the ethers contribute to the aroma of the alcoholic distillate from sweet marjoram.

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Lignin Chromophores. Part II.* The Behaviour of 2,4′- and 4,4′-Dihydroxystilbene Structures towards Oxygen—Alkali**

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The oxidation of 2,4′- and 4,4′-dihydroxystilbene structures, formed in lignins during alkaline delignification processes, has been studied by reacting appropriate model compounds with oxygen in alkaline media. The formation of the different reaction products is outlined in Scheme 1, which shows quinonemethide radicals as common intermediates, arising by a one-electron oxidation step. Quinonemethide radicals from 2,4′-dihydroxystilbenes (VIIIb) undergo ring closure to give 2-arylcoumaran-3-yl radicals (IX, XI) which dimerise (X) or are oxidised to 2-arylcoumarones (XII). Quinonemethide radicals from 4,4′-dihydroxystilbenes (XVb), however, undergo a further one-electron oxidation yielding 4,4′-stilbenequinones (XVI). The latter are subsequently oxidised to benzils (XIV). The significance of hydroxystilbene structures as potential chromophoric systems in alkali and sulfate lignins is briefly discussed.

In Part I of this series [1] the formation of 2,4′- and 4,4′-dihydroxystilbene structures in lignins during pulping has been summarised. Depending on the conditions employed, these stilbene groupings may constitute intermediate or final structures.[2]

Some years ago, it was reported that 2,4′-dihydroxystilbenes during alkaline pulping in the presence of oxygen are extensively converted into 2-arylcoumarones.[2] Recently,[3] this conversion has been interpreted in terms of a two-stage oxidation, proceeding *via* radicals of the quinonemethide type (*cf.* also Ref. 4).

The aim of the present work was to elucidate the action of oxygen in alkali on 2,4′-dihydroxystilbene structures and on the 4,4′-dihydroxy isomers.

RESULTS AND DISCUSSION

The suggested course of oxidation of the two types of dihydroxystilbenes is outlined in Scheme 1.

Oxidation of 2,4′-dihydroxy-3,3′-dimethoxystilbene. After treatment of the diacetate of stilbene I (II) with 2 M sodium hydroxide at 100 °C in the presence of oxygen for 3 h, acetylation of the resulting reaction mixture and separation of the components by column chromatography (see Experimental), the main reaction product was obtained in a crystalline state, m.p. 185–187 °C. The NMR-spectrum, the molecular ion (*m/e* 626) and the mass spectral fragmentation were in accordance with the data for the diacetate of the dimer X. It is suggested that compound X arises by the following route (see Scheme 1): Oxidation of I, formed from II by alkaline hydrolysis, gives the 4′-phenoxy radical (VIIIa). This radical, in its mesomeric quinonemethide form VIIIb, undergoes ring closure to give the 2-arylcoumaran-3-yl radical IX (*cf.* Ref. 3). The latter finally dimerises yielding compound X. Alternatively, the quinonemethide radical

* Part I, see Ref. 1.

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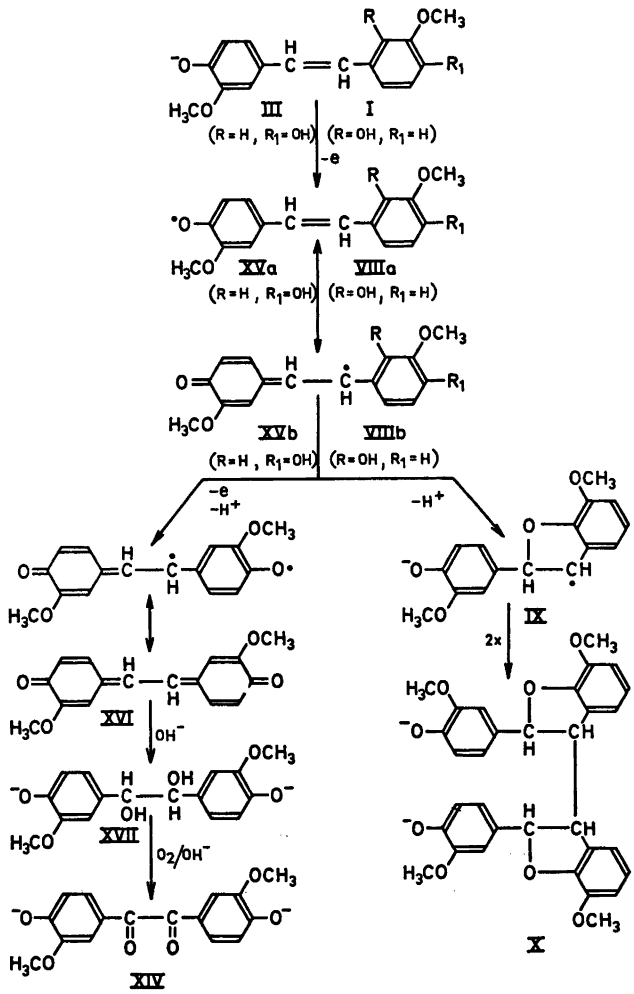


Scheme 1. Oxidation of 2,4′- and 4,4′-dihydroxystilbenes with oxygen in alkali.

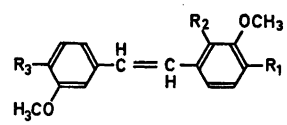
VIIIb dimerises first to give a *bis*-quinonemethide which then by twofold ring closure yields compound X. The formation of *bis*-quinonemethides by oxidation of appropriate hydroxystilbenes (*e.g.* VII) with one-electron oxidants (*e.g.* dichloro-dicyanobenzoquinone, DDQ) has been reported earlier.[5]

It is worthy of note that under similar conditions radical XI behaves differently from radical IX, the former being oxidised to an arylcoumarone derivative (XII).[3] Dimerisation of XI appears to be excluded presumably owing to the steric hindrance caused by the *trans*-arrangement[6] of the methyl and aryl substituents on the coumaran ring in XI.

The reaction sequence proposed for the formation of compound X is based on the following experimental evidence:

The intermediacy of 4′-phenoxy radicals was supported by treating compound VI, in which formation of this type of radical is blocked by etherification of the 4′-hydroxyl group, under similar oxidative conditions. No dimeric product was formed and the starting material was recovered in almost quantitative yield in two stereoisomeric forms (TLC, NMR and mass spectra).

The intermediate formation of radical IX



I $R_1 = H$, $R_2 = R_3 = OH$

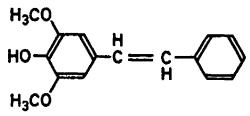
II $R_1 = H$, $R_2 = R_3 = OOCCH_3$

III $R_1 = R_3 = OH$, $R_2 = H$

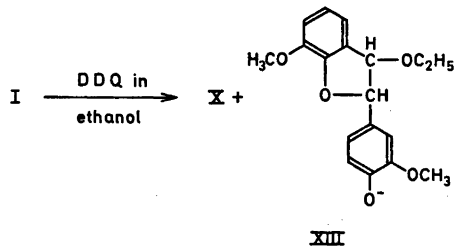
IV $R_1 = R_3 = OOCCH_3$, $R_2 = H$

V $R_1 = H$, $R_2 = OH$, $R_3 = OCH_3$

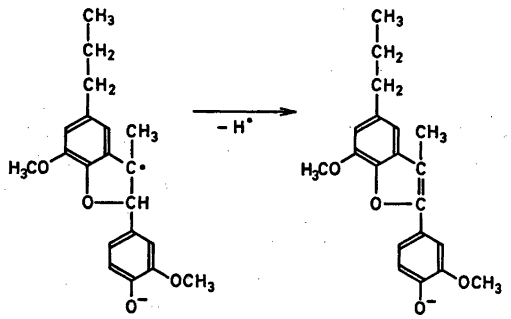
VI $R_1 = H$, $R_2 = OOCCH_3$, $R_3 = OCH_3$



VII



XIII



XI XII

and/or VIIIb during oxidation was demonstrated as follows: The 2,4′-dihydroxystilbene derivative I, when oxidised with an equimolar amount of DDQ in ethanol for 30 min, gave, in addition to compound X, isolated as the diacetate (62 %), another major product (about 12 %) which was identified as the ethoxy-substituted arylcoumaran derivative XIII. It is, thus, concluded that formation of the latter, either by combination of radical IX or its quinonemethide precursor VIIIb with a solvent radical, or by addition of ethanol to the appropriate carbonium ion of the quinonemethide type, competes with dimerisation.

In addition to the dimer (X), small amounts of vanillin, guaiacol, and of three further (unidentified) compounds were detected in the form of their acetates by GLC-MS (*cf.* also Ref. 3).

Oxidation of 4,4′-dihydroxy-3,3′-dimethoxystilbene. Similar oxidative treatment of compound IV and working-up of the resulting reaction mixture gave the acetates of the following compounds: vanillil (XIV, main product), vanillin, guaiacol and acetoguaiacone, as well as a polymeric fraction. The acetates were identified by their melting points, NMR- and mass spectra.

It is suggested that the main product, vanillil (XIV), is formed as shown in Scheme 1.

In the first one-electron oxidation step, a stilbene semiquinone (phenoxy radical XVa and its mesomeric form, the quinonemethide radical XVb) is generated. This step is common to the oxidation of both types of dihydroxystilbenes investigated here. However, whereas the quinonemethide radical VIIIb derived from the 2,4′-dihydroxystilbene I cyclises (formation of IX) and dimerises (formation of X), the radical (XVb) derived from the 4,4′-dihydroxyisomer (III) undergoes a second one-electron oxidation, giving rise to the 4,4′-stilbenequinone XVI. Addition of hydroxyl ions to this *bis*-quinonemethide results in the formation of the anion of hydrovanilloin (XVII) which is then oxidised to vanillil (XIV).

The proposed intermediacy of stilbenequinone XVI and hydrovanilloin (XVII) in the formation of vanillil (XIV) was supported by the fact that XVI and XVII, when treated under similar conditions as used for the reaction of III, afforded XIV in high yields. Treatment of hydrovanilloin (XVII) with alkali in an atmosphere of nitrogen did not yield any XIV.

Vanillil (XIV) was found many years ago [7] in the reaction mixture obtained after oxidation of lignosulfonates by cupric oxide. The

compound was considered to arise from $C_\alpha - C_\alpha$ linked lignin units by oxidative cleavage of the rest of the side chains.[7-9] However, the 1,2-diarylpropane-1,3-dihydroxy structures, shown to be present in lignins.[10-12] constitute a more likely source of vanillil.[13] Their conversion into 4,4'-dihydroxy-3,3'-dimethoxystilbene (III) by the action of alkali has been previously [11,12] illustrated in model experiments and the oxidation of this stilbene with oxygen in alkali *via* the corresponding stilbenequinone to vanillil (*cf.* Ref. 14) has now been demonstrated. In contrast to its behaviour towards cupric oxide and alkali,[7-9] vanillil when treated with oxygen-alkali under the aforementioned conditions does not undergo benzilic rearrangement and oxidative decarboxylation.

Vanillin, the other product formed in appreciable amounts (29 %) from compound IV on the oxidative treatment, presumably arises mainly by oxidative cleavage of the stilbene double bond.[15] However, the formation of guaiacol and acetoguaiacone in minor amounts (3 and 4 %, respectively) indicates that alkaline (non-oxidative) fragmentation may also operate (*cf.* the behaviour of compound II and Ref. 3)

CONCLUSIONS

From the results of the model experiments reported in this work, it may be concluded that hydroxystilbene structures generated in lignins during various pulping processes [2,3] are extensively oxidised by oxygen in alkaline media. Thus, 2,4'-dihydroxystilbene structures yield either arylcoumaran structures by coupling of the intermediate β-radicals with each other or with other radical species, or arylcoumarone structures by oxidation of the β-radical intermediates. 4,4'-Dihydroxystilbene structures are converted to benzil structures by oxidation of the β-radical and stilbenequinone intermediates.

These conversions of dihydroxystilbenes in alkaline media require the presence of oxygen. If air (oxygen) is replaced by nitrogen or if the treatment is carried out in aqueous alkali containing sulfide ions ("white liquor"),* the unchanged dihydroxystilbene can be recovered in a high yield. The isolation of the arylcoumarone XII after treatment of dihydrodehydro-diisoeugenol with white liquor reported previously [3] could only be reproduced if a sufficient amount of oxygen was present in the solution during the treatment.

The results imply that the oxidation of the hydroxystilbene structures formed in lignins during various pulping processes will be extensive during alkali pulping and, particularly, during oxygen-alkali pulping. During sulfate pulping, however, the cooking liquor usually does not contain sufficient oxygen (*cf.* also Refs. 16 and 17) to bring about extensive oxidation of hydroxystilbene structures. The latter may therefore be expected to survive this pulping treatment. Assuming phenoxy- and β-radicals are formed in the first oxidation step by the action of oxidants other than oxygen (*e.g.* quinonoid structures [18]), they would presumably be reduced to the initial hydroxystilbene structures by the reducing sulfur compounds present in the white liquor. In fact, spectrophotometric data indicating the presence of hydroxystilbene structures in lignins from sulfate spent liquors have been reported [18,19] and stilbene III has recently [20] been isolated from a sulfate spent liquor.

The products of oxidation in alkaline media identified in the present work do not include stilbenequinones regarded as possible contributors to the color of sulfite pulps [21] and bisulfite pulps.[22] During the oxidation of 4,4'-dihydroxystilbenes in alkaline solution, stilbenequinone chromophores only appear as intermediates, whereas similar oxidation of 2,4'-dihydroxystilbenes does not even proceed *via* stilbenequinones (*cf.* also Ref. 4). For all these reasons it seems doubtful whether hydroxystilbene structures are converted to chromophoric systems of the stilbenequinone type during the sulfate cook. If such chromophoric systems actually are present in the residual lignins of sulfate pulps or in lignins obtained from sulfate spent liquors, they probably have been formed by autoxidation of the isolated products rather than by oxidation during the pulping process.

Sulfate pulps and sulfate lignins are light-yellow immediately after isolation from the pulping liquor. On exposure to air a rapid

* The term "white liquor" refers to a solution of NaOH (3.5 g) and $Na_2S.9H_2O$ (3.1 g) in water (100 ml).

discolouration takes place, the products assuming the well-known brown colour. Pulps and lignins produced by treatment of wood with oxygen-alkali are significantly lighter in colour than technical kraft pulps and lignins compared at the same degree of delignification. This difference in colour could be explained, at least partly, by the presence of dihydroxystilbene intermediates. Such structures would remain unaffected during sulfate pulping and afford stilbenequinone chromophores on subsequent exposure to air, whereas treatment with oxygen-alkali would remove them by oxidation to arylcoumaran, arylcoumarone and benzil structures.

These oxidation products, like the parent dihydroxystilbenes, do not constitute chromophoric systems *per se*. However, hydroxy-substituted stilbene, arylcoumarone and benzil structures could possibly explain part of the absorption of alkali and sulfate lignins in the near ultraviolet region (*cf.* Refs. 18 and 19). The conjugated double bond in the first two structures and the carbonyl groups in the last structures may function as efficient sensitising groups during the light-induced discoloration and degradation of pulps.[23,24]

EXPERIMENTAL

Melting points are corrected. Evaporations were carried out under reduced pressure.

Thin-layer (TLC) and column chromatography. The reaction mixtures and the various fractions from the column chromatographic separations were investigated by thin-layer chromatography using silica gel HF_{254} (E. Merck, Darmstadt) as adsorbant and light petroleum – ethyl acetate (2:1) as solvent system. Vanillin dissolved in conc. sulfuric acid served as developer. The preparative separations were carried out by column chromatography on silicic acid (Merck 60, 120 – 230 mesh) using the same solvent system.

NMR spectra were recorded on a Perkin-Elmer R-12 spectrometer using deuteriochloroform as solvent and tetramethylsilane (TMS) as internal standard. Chemical shifts (δ values) are given in ppm downfield from TMS.

Mass spectra (MS) were run on a Perkin-Elmer 270 instrument at 20 eV using the direct inlet system or the combination with a gas-liquid chromatograph (GLC); column: silicon OV-1 (3 %) on Gas Chrom Q washed with conc. hydrochloric acid and treated with dimethyldichlorosilane. The temperature of the probe heater was between 110 and 230 °C; detector: 280 °C; injector: 180 °C.

UV spectra were recorded on a DK-2 Beckman spectrophotometer.

Model compounds

The hydroxystilbenes I, III, V and acetoxystilbenes II, IV, and VI used in this study were prepared as described in Part I [1] of this series. Stilbenequinone XVI was obtained by oxidation of stilbene III with DDQ [5] (see below). Hydrovanilloin (XVII) was prepared by electrolytic reduction of vanillin according to Ref. 25.

Treatment of the model compounds with oxygen-alkali. The model compound in the form of the acetate (100 – 300 mg) was heated in 2 M sodium hydroxide (3 – 10 ml) in the presence of oxygen for 3 h at the temperatures given below. After neutralisation with carbon dioxide, the solution was repeatedly extracted with ethyl acetate. The combined extracts were dried (Na_2SO_4) and evaporated. The residue was acetylated with acetic anhydride-pyridine and the resulting mixture of acetylated compounds was separated into its components by preparative column chromatography and/or gas-liquid chromatography. The acetates were identified by their melting points, elemental analyses, NMR and mass spectra.

Compound II (300 mg, *trans*-form, m.p. 138 – 139 °C), in 2 M sodium hydroxide (10 ml), was heated at 100 °C (steam bath) for 3 h, a slow stream of oxygen passing through the solution. The reaction mixture was worked up and separated as described above. The following fractions were obtained from the column:

(1) Monomeric acetylated phenols (15 mg), consisting mainly of vanillin acetate and guaiacol acetate (GLC-MS).

(2) A crystalline fraction (183 mg, 61 %) which after recrystallization from methanol was identified as *bis-[2-(4'-acetoxy-3'-methoxyphenyl)-7-methoxycoumaran-3]-yl* (diacetate of X), m.p. 185 – 187 °C. (Found: C 68.87; H 5.54; O 25.47. $C_{36}H_{34}O_{10}$ (626.36) requires: C 69.03; H 5.43; O 25.54). NMR: δ 7.00 – 6.10 (m, 12 H, arom. H), 5.45 (d, 2 H, 2 H_α), 4.30 – 3.65 (m, 2 H, 2 H_β), 3.89 (s, 6 H, 2 arom. OCH_3), 3.58 (s, 6 H, 2 arom. OCH_3), 2.20 (s. 6 H, 2 arom. $OCCH_3$). MS: 626 (10, M^+), 584 (5), 313 (20), 271 (100), 243 (15).

(3) Mixture containing the diacetate of X and unknown reaction products (26 mg).

(4) Polymeric fraction (about 70 mg).

Compound VI (300 mg, *trans*-form, m.p. 103 – 104 °C) similarly treated gave a mixture of the two stereoisomers of the starting material, indicating isomerisation *via* the *o*-quinonemethide radical or the *o*-quinonemethide carbanion. One of the isomers was obtained in crystalline form. (Found: C 69.48; H 6.06;

O 24.42. $C_{19}H_{20}O_5$ (328.19) requires: C 69.53, H 6.09, O 24.38). The two forms exhibited almost identical mass spectra MS: 328 (24, M+), 286 (100), 271 (20) but migrated at sufficiently different rates to allow separation of the mixture by TLC and preparative column chromatography. The crystalline isomer turns blue and the amorphous greyish-brown on spraying with vanillin – conc. sulfuric acid, followed by heating.

Investigation of the crude acetylated reaction mixture by GLC-MS revealed the presence of traces of the acetates of vanillin, acetoguaiacone and guaiacol.

Compound IV (300 mg, *trans*-form, m.p. 228 – 229 °C) in oxygen-saturated 2 M sodium hydroxide (10 ml) was heated in an autoclave at 180 °C. Column chromatography of the acetylated reaction mixture afforded in the order given: (1) guaiacol acetate (6.9 mg, 3 %); (2) vanillin acetate (67.3 mg, 29 %); (3) acetoguaiacone acetate (9.3 mg, 4 %); (4) *4,4'-diacetoxy-3,3'-dimethoxybenzil* (vanillil diacetate, diacetate of XIV); 116 mg, 50 %; m.p. 138 – 139 °C. NMR: δ 7.76 – 6.96 (m, 6 H, arom. H), 3.87 (s, 6 H, 2 arom. OCH_3), 2.28 (s, 6 H, 2 arom. $OCCH_3$); λ_{max} (methanol) 262 nm (ε, 25.700) and 318 nm (shoulder) (ε, 15 700); (5) Polymeric material (32.5 mg, 14 %).

After treatment of compound IV (150 mg) with white liquor (10 ml) at 180 °C and usual working-up, starting material (100 mg, m.p. 224 – 227 °C) was recovered. GLC-MS analysis of the acetylation mixture after removal of the starting material showed the presence of small amounts of catechol diacetate, guaiacol acetate and of an unknown compound (M+ 306).

Compound II and the 2,4'-dihydroxystilbene derivative obtained from dihydro-dehydro-diisoeugenol [26] were likewise resistent to treatment with white liquor.

3,3'-Dimethoxy-4,4'-stilbenequinone (XVI) (50 mg) and *hydrovanilloin* (XVII) (50 mg) were similarly treated with oxygen-saturated 2 M sodium hydroxide (6 ml) at 180 °C. In both cases, usual working-up provided high yields (about 90 %) of the diacetate of vanillil (XVI), identical by m.p. (137 – 138 °C) as well as NMR and mass spectra with the product obtained from compound IV.

Treatment of XVII with nitrogen-saturated 2 M sodium hydroxide gave a mixture of phenolic substances which did not contain any detectable amount of compound XIV (TLC).

Oxidation of hydroxystilbenes with DDQ

Compound I [1,4] (m.p. 133.5 – 134.5 °C) (100 mg) was dissolved in anhydrous ethanol (5 ml). An equimolar amount of DDQ (85.2 mg, 0.37 mmol) was added to the solution at room temperature with stirring. After 30 min, the solution was evaporated and the resulting oily residue (210 mg) was chromatographed on a column of silica gel. The first fraction gave an amorphous product which on the basis of its NMR- and mass spectra was identified as *2-(4'-hydroxy-3'-methoxyphenyl)-3-ethoxy-7-methoxycoumaran* (XIII) NMR: δ 6.92 – 6.70 (m, 6 H), 5.47 (d, 1 H), 4.98 (d, 1 H), 3.97 – 3.52 (q, 2 H) 3.83 (s, 3 H), 3.75 (s, 3 H), 1.22 (t, 3 H). MS: 316 (32, M+), 287 (14), 285 (15), 270 (100, M – C_2H_5OH).

The second fraction contained compound X, identical with the main product of oxidation from compound II using oxygen in alkali as oxidant. It was characterised as the diacetate by m.p., NMR and mass spectra (see above).

Compound III (400 mg), dissolved in anhydrous ethanol (20 ml), was similarly oxidised with DDQ [5] (341 mg). The red crystalline precipitate formed was centrifuged off and washed with small portions of ethanol. The *3,3'-dimethoxy-4,4'-stilbenequinone* (*XVI*) thus obtained (300 mg, 75 %) did not have a sharp melting point (182 – 185 °C) (lit. 195 – 205 °C and 202 – 206 °C) [5] but was chromatographically pure.

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1,3,5-Trineopentylbenzene. VII.* Friedel-Crafts Reactions of 1,3,5-Trineopentylbenzene with Acyl Chlorides and Preparations of Some Chloro Derivatives of 1,3,5-Trineopentylbenzene

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2-Acetyl-1,3,5-trineopentylbenzene, 2-propanoyl-1,3,5-trineopentylbenzene, 2-benzoyl-1,3,5-trineopentylbenzene, 2-(2-methylpropanoyl)-1,3,5-trineopentylbenzene, and 2,4,6-trineopentylbenzenecarboxylic acid have been prepared by Friedel-Crafts acylation of 1,3,5-trineopentylbenzene with acyl chlorides and aluminum chloride in carbon disulfide. NMR, IR, and mass spectra are reported for these novel compounds.

Ring-chlorinated products were formed when 1,3,5-trineopentylbenzene was treated with antimony(V) chloride in carbon disulfide, and thus 1,3,5-trichloro-2,4,6-trineopentylbenzene could be prepared in good yield.

In the present investigation, larger and larger acyl groups were introduced into the ring of 1,3,5-trineopentylbenzene. In some cases, introduction of such groups into polyalkylbenzenes brings about migration [2–5] or removal [6] of alkyl groups, possibly due to steric strain,[7] and it was considered of interest to study acylation reactions of the sterically hindered 1,3,5-trineopentylbenzene.

RESULTS

Dermer *et al.*[8] have examined a series of metal chlorides to determine their relative efficiencies as Friedel-Crafts catalysts. For the acetylation of toluene, the efficiency was found to decrease in the series: aluminum chloride > antimony(V) chloride > iron(III) chloride > tellurium(II) chloride > tin(IV) chloride. For the preparation of 2-acetyl, 2-propanoyl, 2-benzoyl, and 2-(2-methylpropanoyl)-1,3,5-trineopentylbenzene, and 2,4,6-trineopentylbenzenecarboxylic acid (I)–(V) in the present work, aluminum chloride, iron(III) chloride, and tin(IV) chloride were employed as catalysts, and carbon disulfide, dichloromethane, and nitrobenzene as solvents.

Freshly sublimed aluminum chloride caused dealkylation of 1,3,5-trineopentylbenzene to form 1,3-dineopentylbenzene, neopentylbenzene, and 2-methylbutane. The latter product was probably formed by rearrangement of neopentyl groups which had been removed from the 1,3,5-trineopentylbenzene. 1,3-Dineopentylbenzene was formed as the major product by treating 1,3,5-trineopentylbenzene with freshly sublimed aluminum chloride in carbon disulfide for 24 h at room temperature.

It has been reported that the use of acyl halides as acylating agents causes some complications. Acyl halides often decarbonylate in the presence of Friedel-Crafts catalysts, and alkyl cations are formed, which, in some cases, act as alkylating agents.[9,10] In the present case, freshly sublimed aluminum chloride caused

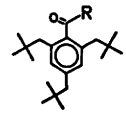

Fig. 1. R = methyl (I), ethyl (II), phenyl (III), isopropyl (IV), or hydroxyl (V).

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decomposition of the acyl chlorides. This reaction was most significant when 2-methylpropanoyl chloride was used.

The dealkylation of 1,3,5-trineopentylbenzene and the decomposition of the acyl chlorides were minimized when commercial aluminum chloride was used without further sublimation. As has been reported for similar reactions, the improvement of the acylation reaction when commercial aluminum chloride was used may be due to trace amounts of iron(III) chloride [11,12] or products of hydrolysis.[13]

It has been demonstrated [14] that the yield of product often decreases rapidly when the amount of catalyst exceeds the molar amount of the aromatic substrate. In the present case, however, an excess of catalyst improved the acylation reaction in accordance with what was reported by Dermer *et al.*[8]

When 1,3,5-trineopentylbenzene was treated with acetyl chloride and iron(III) chloride in carbon disulfide, 2-chloro-1,3,5-trineopentylbenzene was formed as the major product. Treatment of 1,3,5-trineopentylbenzene with iron(III) chloride in carbon disulfide for 20 h at room temperature gave 40 % of 2-chloro-1,3,5-trineopentylbenzene as the only product.

This can be compared with the report that mesitylene was found to react with iron(III) chloride at room temperature in an aromatic solvent to yield 12 – 19 % of 2,2′,4,4′,6,6′-hexamethylbiphenyl in addition to 24 – 36 % of 2-chloromesitylene.[15] Moreover, an increase in yield of biaryl-type product was found in the order toluene < *m*-xylene < mesitylene, which was correlated with an increase in steric hindrance, basicity, and opportunity for hyperconjugative stabilization of the σ-complex.[16] However, recent studies by Nyberg indicate that the biaryl formation is a cation radical reaction.[17] The absence of biaryl products in the case of 1,3,5-trineopentylbenzene may be explained by the much larger steric demands of *tert*-butyl groups. It can be noted that in the treatment of 1,3,5-trineopentylbenzene with freshly sublimed aluminum chloride (see above) no biaryl or chloro-substituted compounds were formed.

When tin(IV) chloride was used, no acetylation or chlorination took place.

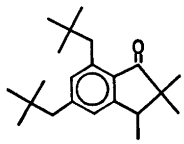
Carbon disulfide was preferred to dichloromethane and nitrobenzene as solvent in the acylation reaction. When 1,3,5-trineopentylbenzene was treated with acetyl chloride and aluminum chloride for 20 h at room temperature in carbon disulfide, dichloromethane, or nitrobenzene, the yields of 2-acetyl-1,3,5-trineopentylbenzene were 80, 30, and 5 %, respectively.

With commercial aluminum chloride as catalyst and carbon disulfide as solvent, compounds (I), (II), and (III) could be prepared in good yields without any side reactions. In the preparation of (IV), under the same conditions, some minor side reactions occurred. If the 2,2-dimethylpropanoylation reaction was interrupted before completion, the only side reaction was the formation of a small amount of 2-chloro-1,3,5-trineopentylbenzene. Attempts to force the reaction toward completion failed because of decomposition of the product (IV).

Compound (V) could be prepared by treating 1,3,5-trineopentylbenzene with oxalyl chloride and aluminum chloride in carbon disulfide.

Attempts to prepare 2-(2,2-dimethylpropanoyl)-1,3,5-trineopentylbenzene *via* acylation failed. The above-mentioned solvents and catalysts, including antimony(V) chloride, were employed. The combination commercial aluminum chloride-dichloromethane was preferred since the main product [*i.e.* (*VI*)] was formed in the highest yield with this combination. The decomposition of 2,2-dimethylpropanoyl chloride was considerable. The main product was formed in about 40 % yield in addition to about 30 different by-products. When the reaction was carried out in carbon disulfide instead of dichloromethane, the yield of the main product decreased and the number of by-products increased. The main product was not 2-(2,2-dimethylpropanoyl)-1,3,5-trineopentylbenzene, however, and none of the other products observed on the gas chromatograms had the same retention time as this compound, which has recently been prepared [18] by a lithium organic reaction. The main product has been identified as 2,2,3-trimethyl-5,7-dineopentyl-1-indanone (*VI*).[19] In this connection, it is interesting to note that successful introduction of the 2,2-dimethylpropanoyl group in mesitylene and a number of polymethylbenzenes has been reported.[7]

It has been reported that aromatic compounds can be chlorinated by antimony(V) chloride,[16] and that chlorinated compounds are formed as by-products when antimony(V) chloride is used



VI

as a Friedel-Crafts catalyst.[8] In the present case, only chlorinated compounds were formed when antimony(V) chloride was used in the presence or absence of an acyl component. 1,3,5-Trineopentylbenzene thus yielded mono-, di- and trichloro-1,3,5-trineopentylbenzene with antimony(V) chloride, parallelling the behavior of mesitylene.[20]

2-Chloro-1,3,5-trineopentylbenzene, 2,4-dichloro-1,3,5-trineopentylbenzene, and 1,3,5-trichloro-2,4,6-trineopentylbenzene were orginally prepared by Márton and Martinson [21] using chlorine gas and iron as a catalyst. In their preparation of 1,3,5-trichloro-2,4,6-trineopentylbenzene, chlorodealkylation occurred and a complex mixture of compounds was formed. In the present case, no side reactions took place, and 1,3,5-trichloro-2,4,6-trineopentylbenzene could be prepared in good yield with antimony(V) chloride as the chlorinating agent.

EXPERIMENTAL

Measurements. Gas chromatographic (GLC) analyses were carried out on a Perkin-Elmer 900 gas chromatograph fitted with a flame ionization detector. The inner diameter of the columns used was 2 mm and the length 2 m. The stationary phase was 3 % of SE-30 silicon gum rubber on Gaschrom Q 100 – 200 mesh. The areas of the peaks on the gas chromatograms were measured by triangulation.

The IR spectra were recorded on a Beckman IR 9 spectrophotometer using potassium bromide pellets. The absorption maxima are reported in cm^{-1} and the intensities are characterized as weak (w), medium (m), strong (s), or very strong (vs).

The NMR spectra were recorded on a Varian A 60 spectrometer. About 10 % by weight solutions in carbon tetrachloride were used. The probe temperature was 35 °C. The chemical shifts are reported in ppm downfield from tetramethylsilane as internal standard. The multiplicities of the peaks are reported as singlet (s), doublet (d), triplet (t), quartet (q), heptet (h) and multiplet (m).

The mass spectra (MS) were determined (at the Department of Medical Biochemistry, University of Göteborg) on an AEI MS 902 mass spectrometer under the following conditions: electron energy 70 eV, accelerating voltage 8 kV and emission 100 μA, or (at the Department of Medical Chemistry, University of Göteborg) on an LKB 9000 mass spectrometer with an electron energy of 70 eV. The latter mass spectrometer was connected to a gas chromatograph with a column containing 3 % of OV-1. The intensities of the peaks are reported in parenthesis as percentages of the base peak. Only the most abundant peaks are reported, together with the parent peaks and the isotope peaks corresponding to the latter.

The melting points were determined on a Kofler micro hot stage.

Materials. Acyl chlorides: Fluka (*purum*). Aluminum chloride: Kistner (*puriss.*). Antimony(V) chloride: Riedel-De Haën (anhydrous, fuming). Silica gel; Merck (neutral, less than 0.08 mm, for column chromatography). Carbon disulfide: Kistner (*purum*). Dichloromethane: Fisher (*p.a.*). The above chemicals were used without further purification, with the exception of aluminum chloride which was sublimed before it was used in some reactions. 1,3,5-Trineopentylbenzene was prepared as described by Martinson and Márton.[22]

Acylations

2-Acetyl-1,3,5-trineopentylbenzene (I). a. With aluminum chloride as catalyst and carbon disulfide as solvent. In a two-necked flask equipped with a magnetic stirrer, a drying tube, and a dropping funnel, 13.9 g (104.1 mmol) of finely powdered aluminum chloride was covered with 50 ml of carbon disulfide, and the flask was then cooled with ice water. The mixture was magnetically stirred and 7.4 ml (104.1 mmol) of acetyl chloride was added. After 15 min 10.0 g (34.7 mmol) of 1,3,5-trineopentylbenzene, dissolved in 20 ml of carbon disulfide, was added dropwise over a period of 30 min. After this addition, the mixture was stirred at room temperature. At regular time intervals small aliquots were withdrawn and analyzed by GLC. The reaction was found to be complete after 15 h, and the flask was then cooled with ice water and the excess of aluminum chloride and acetyl chloride was destroyed with crushed ice. The organic and aqueous layers did not separate well, and therefore the carbon disulfide was evaporated and the aqueous layer was extracted five times with 20 ml portions of hexane. The combined hexane extracts were dried over magnesium sulfate. After evaporation of the solvent, the product was decolorized by adsorption chromatography (alumina, hexane). Recrystallization several times from ethanol (water was cautiously added to the hot solution until turbidity appeared) gave 10.2 g (89 %) of a white crystalline product. *M.p.*: 63 – 65 °C. *IR*: 2962vs, 2910m, 2870m, 1694vs, 1604m, 1565w, 1481s, 1427w, 1396w, 1366s, 1352m, 1280w,

1245s, 1204w, 1148m, 1054m, 980m, 882m, 874m, 780w, 756w, 700w, 607w, 584w, 546w. MS: *m/e* (%) *MS*: 29(13), 41(15), 43(12), 57(41), 147(15), 162(100), 163(13), 219(9), 247(8), 315(20), 330(6.9), 331(1.8), 332(0.2). *NMR*: δ 0.88 (s, 18 H, *tert*-butyl), 0.93 (s, 9 H, *tert*-butyl) 2.28 (s, 3 H, methyl), 2.38 (s, 4 H, methylene), 2.43 (s, 2 H, methylene), 6.75 (s, 2 H, aromatic).

When the acetylation reaction was carried out in 10 ml of carbon disulfide with 1 % of the above-mentioned molar amounts, 2-acetyl-1,3,5-trineopentylbenzene was formed in about 80 % yield after 20 h.

b. With iron(III) chloride as catalyst. The reaction was carried out in 10 ml of carbon disulfide at room temperature with the same molar amounts as in the last experiment described under *a*, but the aluminum chloride was exchanged for iron(III) chloride. A GLC analysis of the mixture after 20 h showed that two products had been formed. 2-Acetyl-1,3,5-trineopentylbenzene had been formed in about 10 % yield, and the other product, which was formed in about 22 % yield, was identified as 2-chloro-1,3,5-trineopentylbenzene.

c. With tin(V) chloride as catalyst. The reaction was carried out at room temperature with the same molar amounts as in *b* but the iron(III) chloride was exchanged for tin(IV) chloride. No products could be detected in the mixture after 20 h (GLC).

d. With dichloromethane as solvent. The reaction was carried out in 10 ml of dichloromethane at room temperature with the same molar amounts as in the last experiment described under *a*. After 20 h, 2-acetyl-1,3,5-trineopentylbenzene had been formed in about 30 % yield (GLC).

e. With nitrobenzene as solvent. The reaction was carried out in 10 ml of nitrobenzene at room temperature with the same amounts of reactants as in *d*. After 20 h, 2-acetyl-1,3,5-trineopentylbenzene had been formed in about 5 % yield (GLC).

2-Propanoyl-1,3,5-trineopentylbenzene (II). The same molar amounts as in the first experiment described under *a* were used, but the acetyl chloride was exchanged for propanoyl chloride. The reaction, which was carried out at room temperature, was found to be complete after 34 h (GLC), and the final yield was 10.1 g (85 %) of white crystals. *M.p.*: 42 – 44 °C. *IR*: 2960vs, 2910m, 2868m, 1705vs, 1609m, 1569w, 1478s, 1465w, 1428w, 1413w, 1395m, 1367s, 1340m, 1228w, 1238s, 1223m, 1202m, 1166w, 1142m, 1076m, 1008w, 948s, 932w, 924w, 882s, 872w, 799w, 790m, 753w, 731w, 656w, 523w. *MS*: 29(19), 41(21), 43(15), 57(49), 71(8), 147(9), 176(13), 204(100), 205(16), 233(18), 260(47), 261(9), 290(6), 315(84), 316(22), 330(27), 331(6), 344(14.6), 345(3.8), 346(0.5). *NMR*: 0.87 (s, 18 H, *tert*-butyl), 0.93 (s, 9 H, *tert*-butyl), 1.11 (t, 3 H, methyl, $J = 7$ Hz), 2.30 (s, 4 H, methylene), 2.43 (s, 2 H, methylene), 2.45 (q, 2 H, methylene, $J = 7$ Hz), 6.76 (s, 2 H, aromatic).

2-Benzoyl-1,3,5-trineopentylbenzene (III). The same molar amounts as in the first experiment described under *a* were used, but the acetyl chloride was exchanged for benzoyl chloride. The reaction, which was carried out at room temperature, was found to be complete after 20 h (GLC). A hexane solution of the crude product was washed several times with 10 % aqueous sodium hydroxide solution to remove the benzoic acid from the organic layer. The organic layer was then washed with water, with dilute hydrochloric acid, and finally again with water. After the solution was dried over magnesium sulfate, the hexane was evaporated, and the product was decolorized by adsorption chromatography (alumina, hexane). Several recrystallizations (ethanol – water) gave 9.2 g (69 %) of crystalline product. *M.p.*: 90 – 92 °C. *IR*: 3062w, 2970vs, 2910m, 2859s, 1675vs, 1603m, 1582w, 1567w, 1477s, 1448m, 1424w, 1393m, 1362s, 1312m, 1288m, 1264s, 1236m, 1199w, 1170m, 1156w, 1116w, 1070w, 1024w, 926s, 897w, 879m, 870w, 797w, 790w, 750w, 706vs, 683m, 648w, 550w. *MS*: 29(39), 39(12), 41(59) 43(26), 57(100), 71(14), 77(14), 91(11), 105(39), 149(18), 209(13), 224(16), 262(16), 265(13), 279(26), 280(33), 335(61), 336(72), 337(18), 377(16), 392(0.9), 393(0.3). *NMR*: 0.82 (s, 18 H, *tert*-butyl), 0.97 (s, 9 H, *tert*-butyl), 2.27 (s, 4 H, methylene), 2.50 (s, 2 H, methylene), 6.87 (s, 2 H, aromatic), 7.1 – 7.7 (m, 5 H, aromatic).

2-(2-Methylpropanoyl)-1,3,5-trineopentylbenzene (IV). The same molar amounts as in the first experiment described under *a* were used, but the acetyl chloride was exchanged for 2-methylpropanoyl chloride. To minimize the decomposition of this acyl chloride, the flask was cooled to – 10 °C before the acyl chloride was added. After this addition and the addition of 1,3,5-trineopentylbenzene, the temperature of the flask was slowly raised to room temperature and the acylation reaction begun. A small amount of gas evolved during the reaction. After 15 h a main product, which was later identified as 2-(2-methylpropanoyl)-1,3,5-trineopentylbenzene, had been formed in about 91 % yield (GLC), and a by-product, which was later identified as 2-chloro-1,3,5-trineopentylbenzene, had been formed in about 1 % yield (GLC). The work-up procedure described for 2-benzoyl-1,3,5-trineopentylbenzene was then applied. The two products and the unreacted 1,3,5-trineopentylbenzene were separated on a column of silica gel. With hexane as eluent, 1,3,5-trineopentylbenzene was eluted, followed by 2-chloro-1,3,5-trineopentylbenzene. 2-(2-Methylpropanoyl)-1,3,5-trineopentylbenzene was eluted with diethyl ether. Several recrystallizations (ethanol – water) of 2-(2-methylpropanoyl)-1,3,5-trineopentylbenzene gave 9.4 g (76 %) of white crystals. *M.p.*: 56 – 58 °C. *IR*: 2968vs, 2910m,

2868m, 1700vs, 1605m, 1567w, 1480s, 1470w, 1426w, 1396m, 1384w, 1366s, 1332w, 1289w, 1239s, 1221m, 1202w, 1135m, 1085w, 990w, 972s, 961w, 917w, 885m, 862w, 796m, 756w, 713w, 647w, 528w. *MS*: 29(6), 41(10), 43(14), 57(19), 71(7), 259(11), 315(100), 316(25), 343(10), 358(0.7), 359(0.2). *NMR*: 0.86 (s, 18 H, *tert*-butyl), 0.93 (s, 9 H, *tert*-butyl), 1.05 (d, 6 H, methyl, $J = 7$ Hz), 2.30 (s, 4 H, methylene), 2.44 (s, 2 H, methylene), 2.64 (h, 1 H, methine, $J = 7$ Hz), 6.77 (s, 2 H, aromatic).

2,4,6-Trineopentylbenzenecarboxylic acid (V). Aluminum chloride (2.53 g, 19.0 mmol) was covered with 20 ml of carbon disulfide and the flask cooled in an ice bath. Oxalyl chloride (1.62 ml, 19.0 mmol) was slowly added, and the mixture was stirred for 15 min before a solution of 4.9 g (17.0 mmol) of 1,3,5-trineopentylbenzene in 10 ml of carbon disulfide was added dropwise over a period of 30 min with continued stirring. The mixture was then reflux for 7 h before being poured into a mixture of 35 g of ice and 5 ml of concentrated hydrochloric acid. The organic layer was separated off, and the aqueous layer was extracted with three 10 ml portions of carbon disulfide. The combined organic layers were washed with two 10 ml portions of water and were then extracted with 20 ml of ice-cold 10 % sodium hydroxide solution. Acidification of the extract with 6 M hydrochloric acid liberated the 2,4,6-trineopentylbenzenecarboxylic acid which soon precipitated. The precipitate was collected and washed with water on a filter and was then dried in a desiccator. The tan-colored crude product (2.3 g) was recrystallized first from acetone and then from carbon tetrachloride to yield 1.9 g (34 %) of white crystals. *M.p.*: 164.5–165.5 °C. *IR*: 2955vs, 2907s, 2870s, 2655m, 2550m, 1688vs, 1606m, 1561w, 1480s, 1451m, 1427m, 1393m, 1364s, 1298s, 1279m, 1261m, 1237m, 1202w, 1182w, 1153w, 1094m, 930w(broad), 879w, 779w, 749w. *MS*: 29(20), 41(25), 43(9), 57(84), 71(5), 164(100), 165(11), 220(38), 221(6), 276(6), 317(10), 332(2.4), 333(0.7). *NMR*: 0.94 (s, 27 H, *tert*-butyl), 2.48 (s, 2 H, methylene), 2.81 (s, 4 H, methylene), 6.87 (s, 2 H, aromatic), 12.27 s, 1 H, carboxylic).

Attempts to prepare 2-(2,2-dimethylpropanoyl)-1,3,5-trineopentylbenzene. 1. With aluminum chloride as catalyst. The reaction was carried out in 30 ml of dichloromethane using 50 % of the molar amounts used in the first experiment described in *a* under the heading 2-acetyl-1,3,5-trineopentylbenzene, but the acetyl chloride was exchanged for 2,2-dimethylpropanoyl chloride. To minimize the decomposition of this acyl chloride, the flask was cooled to −35 °C before the acyl chloride was added. After this addition and the addition of 1,3,5-trineopentylbenzene the temperature of the flask was slowly raised to room temperature, and products started to form. Gases evolved during the reaction. After 10 h the mixture was worked up as described for 2-benzoyl-1,3,5-trineopentylbenzene. The resulting oil was shown by GLC to consist of about 40 % of a main product and about 60 % of a complex mixture of about 30 compounds. The main product has been identified as 2,2,3-trimethyl-5,7-dineopentyl-1-indanone.[19]

2. With antimony(V) chlorine as catalyst. A solution of antimony(V) chloride (1.33 ml, 10.41 mmol) in 20 ml of carbon disulfide was cooled with ice water, and 2,2-dimethylpropanoyl chloride (1.28 ml, 10.41 mmol) was added After 15 min 1,3,5-trineopentylbenzene (1.0 g, 3.47 mmol), dissolved in 10 ml of carbon disulfide, was added over a period of 30 min. The reaction mixture was then stirred at room temperature. After 12 h the reaction mixture was worked up as described for 2-benzoyl-1,3,5-trineopentylbenzene. The crystalline product was shown by GLC to consist of two product. The minor product (3 %) was identified as 2-chloro-1,3,5-trineopentylbenzene and the other one as 2,4-dichloro-1,3,5-trineopentylbenzene. The products were separated on a column of silica gel. With hexane as eluent, 2,4-dichloro-1,3,5-trineopentylbenzene was eluted, followed by 2-chloro-1,3,5-trineopentylbenzene. Yields: 2-chloro-1,3,5-trineopentylbenzene, 12 mg (1 %), 2,4-dichloro-1,3,5-trineopentylbenzene, 1.0 g (80 %). *MS*: The masses of the molecules were determined by high-resolution mass spectrometry. For 2-chloro-1,3,5-trineopentylbenzene the value 322.243 ± 0.005 u was found. The value [23] for $C_{21}H_{35}{}^{35}Cl$ is 322.243 u. 29(15), 41(23), 43(15), 57(100), 71(8), 154(42), 155(6), 156(17), 210(31), 211(6), 212(10), 265(13), 266(10), 307(8), 322(8.5), 323(2.1), 324(3.1), 325(0.8). For 2,4-dichloro-1,3,5-trineopentylbenzene the value 356.202 ± 0.005 u was found for the mass of the molecule. The value [23] for $C_{21}H_{34}{}^{35}Cl_2$ is 356.204 u. 29(10), 41(24), 43(12), 57(100), 71(7), 188(34), 190(22), 244(16), 246(10), 299(10), 300(7), 301(7), 341(4), 356(2.9), 357(0.7), 358(1.9), 359(0.5), 360(0.4). The m.p. and NMR and IR spectra were identical with those reported [21] for the two compounds.

Treatments of 1,3,5-trineopentylbenzene with Friedel-Crafts catalysts. a. With freshly sublimed aluminum chloride. Freshly sublimed aluminum chloride (0.69 g, 5.19 mmol) was covered with 15 ml of carbon disulfide, and a solution of 0.50 g (1.73 mmol) of 1,3,5-trineopentylbenzene in 5 ml of carbon disulfide was added. The mixture was then stirred at room temperature, and after 24 h the composition of the mixture was determined by GLC. Three products were detected, in addition to unreacted 1,3,5-trineopentylbenzene. The mixture was investigated with an LKB 9000 mass spectrometer which was connected to a gas chromatograph, before it was worked up as described for 2-acetyl-1,3,5-trineopentylbenzene. The total yield, including the remaining reactant was 0.40 g, and the products were separated on a column of silica gel with hexane as eluent. The products were identified as 1,3-dineopentylbenzene, neo-

pentylbenzene, and 2-methylbutane. The ratios of the amounts of 1,3,5-trineopentylbenzene, 1,3-dineopentylbenzene, and neopentylbenzene were 0.5:1:0.6, as estimated from the gas chromatogram. *MS*: 1,3-Dineopentylbenzene. 29(16), 41(23), 57(99), 71(5), 77(5), 91(14), 103(5), 104(7), 105(17), 106(100), 107(86), 115(5), 131(5), 161(13), 162(13), 203(14), 218(13.6), 219(2.6), 220(0.1). *NMR*: 1,3-Dineopentylbenzene. 0.92 (s, 18 H, *tert*-butyl), 2.44 (s, 4 H, methylene), 6.6 – 7.2 (m, 4 H, aromatic). The aromatic part of the spectrum clearly indicated that the compound was the *meta* isomer. *MS*: Neopentylbenzene. 29(12), 39(11), 41(24), 51(6), 57(85), 65(15), 91(64), 92(100), 93(7), 105(8), 115(5), 133(12), 148(18.3), 149(2.3), 150(0.1). These values are in accordance with those reported for neopentylbenzene.[24a] *MS*: 2-Methylbutane. 27(25), 29(30), 39(19), 41(73), 42(95), 43(100), 55(7), 56(25), 57(73), 72(11), 73(0.5). These values are in accordance with those reported for 2-methylbutane.[24b]

b. With commercial aluminum chloride. Commercial aluminum chloride (0.28 g, 2.07 mmol) was covered with 10 ml of carbon disulfide. A solution of 0.20 g (0.69 mmol) of 1,3,5-trineopentylbenzene in 5 ml of carbon disulfide was then added dropwise and the mixture was stirred at room temperature. A GLC analysis of the mixture after 20 h showed that only small amounts (altogether < 1 % of the above-mentioned aromatic substrate) of dealkylated products had been formed. No other products could be detected.

c. With iron(III) chloride. The reaction was carried out at room temperature with the same molar amounts as in *b*, but the aluminum chloride was exchanged for iron(III) chloride. A GLC analysis of the mixture after 20 h showed that one product had been formed in about 40 % yield. This product was identified as 2-chloro-1,3,5-trineopentylbenzene. No other products could be detected.

d. With tin(IV) chloride. The same molar amounts as in *b* were used. A GLC analysis of the mixture after 20 h at room temperature showed only starting material.

e. With antimony(V) chloride. The reaction was carried out at room temperature in 30 ml of carbon disulfide with twice the molar amounts used in *a*, but the aluminum chloride was exchanged for antimony(V) chloride. Two products which were later identified as 2,4-dichloro-1,3,5-trineopentylbenzene and 1,3,5-trichloro-2,4,6-trineopentylbenzene were formed. The composition of the mixture changed as follows (GLC): 1 h, 94 % of 2,-4-dichloro-1,3,5-trineopentylbenzene and 6 % of 1,3,5-trichloro-2,4,6-trineopentylbenzene; 2 h, 40 and 60 %, respectively; 4 h, 20 and 80 %, respectively. After 4 h the reaction mixture was worked up as described for 2-acetyl-1,3,5-trineopentylbenzene. The two products were separated on a column of silica gel. With hexane as eluent, 1,3,5-trichloro-2,4,6-trineopentylbenzene was eluted, followed by 2,4-dichloro-1,3,5-trineopentylbenzene. Yields: 2,4-dichloro-1,3,5-trineopentylbenzene 0.15 g (13 %), 1,3,5-trichloro-2,4,6-trineopentylbenzene 0.95 g (70 %). The physical properties of the products were in accordance with those reported.[20]

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Synthesis and Proton Magnetic Resonance Studies of 2,2,3-Trimethyl-5,7-dineopentyl-1-indanone and the Corresponding Indanol and Indan

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2,2,3-Trimethyl-5,7-dineopentyl-1-indanone was formed by a ring-closure reaction when 1,3,5-trineopentylbenzene was treated with 2,2-dimethylpropanoyl chloride or carbon monoxide under Friedel-Crafts conditions. Its structure was determined by means of several spectroscopic methods including the europium-shift NMR technique. 60 MHz NMR studies showed that the 7-methylene group formed an AB spectrum due to the inherent assymmetry in the five-membered ring. Stepwise reduction of 2,2,3-trimethyl-5,7-dineopentyl-1-indanone to the corresponding indanol and indan gave further evidence for the structure. NMR studies of the indanol showed that the configuration of the hydroxyl group at carbon 1 and the methyl group at carbon 3 was *trans*.

In an earlier publication[1] an unsuccessful attempt to prepare 2-(2,2-dimethylpropanoyl)-1,3,5-trineopentylbenzene is described. The main product was isolated from the complex reaction mixture and has now been identified as 2,2,3-trimethyl-5,7-dineopentyl-1-indanone (I). The present paper deals with the preparation and identification of this particular compound and some of its derivatives.

The compound (I) has been prepared in two ways. The first one is by treatment of 1,3,5-trineopentylbenzene with 2,2-dimethylpropanoyl chloride and aluminum chloride in dichloromethane. The second one consists of a reaction between 1,3,5-trineopentylbenzene and carbon monoxide in dichloromethane with aluminum chloride as catalyst.

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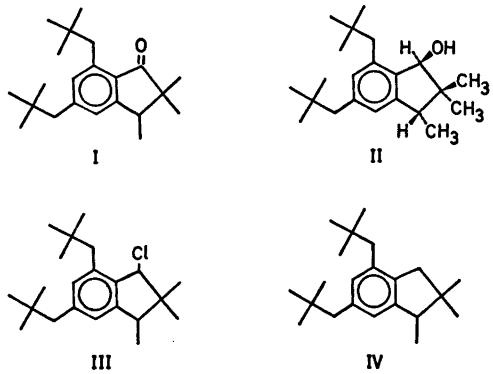
The assigned structure of I is based on the following observations. High-resolution mass spectrometry gave the mass 314.261 ± 0.002 u, which gives the molecular formula $C_{23}H_{34}O$. The presence of two *tert*-butyl groups was indicated by the mass spectrum. The IR spectrum had an absorption at the same wavenumber (1710 cm^{-1}) as the carbonyl stretching of indanone.[2] From the NMR spectrum, it could be concluded that the molecule contains two *meta*-coupled ($J = 1$ Hz) aromatic protons (δ 6.83 and 7.02), two *tert*-butyl groups with different chemical shift values (δ 0.91 and 0.95), two isolated methyl groups with different chemical shift values (δ 1.01 and 1.17), a methyl-methine grouping (δ 1.26 and 3.03, $J = 8$ Hz), a methylene group with magnetically equivalent protons (δ 2.58), and a methylene group in which the protons are nonequivalent (δ 3.09, $J = 12$ Hz).



I II III IV

The 2,2,3-trimethyl-1-indanone structure could be assigned to I, in preference to the 2,3,3-trimethyl-1-indanone structure, on the basis of the chemical shifts. The methine and methyl resonances of 2,2,3-trimethyl-1-indanone have been reported to be δ 3.05 (quartet), 1.03 (singlet), 1.20 (singlet), and 1.28 (doublet), respectively, in deuteriochloroform.[3] A comparision can also be made with the methyl (δ 1.05) and methine (δ 2.64) resonances of the isopropyl group in 2-(2-methylpropanoyl)-1,3,5-trineopentylbenzene,[1] whereas in 1,3,5-triisopropylbenzene the methine protons absorb at δ 2.84.

To get further information on the pattern of substitution in the alicyclic ring, I was reduced by lithium tetrahydridoaluminate to the indanol (II). This resulted in changes in the NMR resonance frequencies, but induced no additional splitting of the methine resonance. This indicates that the methyl groups are in the 2,2,3-positions.

The indanol (II) was converted to the corresponding indan (IV) by treatment with sulfinyl chloride to form the chloro-substituted compound III, followed by treatment with lithium in liquid ammonia. The NMR spectrum of (IV) showed *tert*-butyl, 3-methylene and aromatic resonance at δ 0.91, 2.60, and 6.60, respectively. These values are in close agreement with those in 2,2-dimethyl-4,6-dineopentylindan (δ 0.90 and 0.92, 2.65, 6.60 and 6.69).[4]

The chemical shifts of the aromatic hydrogens and the neopentyl groups in the NMR spectrum of I lead us to suggest that the neopentyl groups are in positions 5 and 7 rather than 4 and 6. The hydrogen in the peri-position in 9-fluorenone absorbs at δ 7.58[5] and the *ortho* hydrogens in acetophenone absorb at δ 7.95.[6] The aromatic hydrogens of I absorb at δ 6.83 and 7.02, which is taken as evidence that they are in the 4 and 6 positions. In addition, the large down-field shift of the magnetically nonequivalent methylene hydrogens in one of the neopentyl groups of I indicates that this group is in the 7 position, deshielded by the carbonyl group.

In an attempt to further confirm the structural assignements above, the effect of $Eu(fod)_3$-d_{27}[7] on the NMR spectrum of I was studied. The paramagnetic pseudocontact shift, Δ_{Eu},[8] for the different hydrogens was estimated from the NMR data and is tabulated in Table 1. From these data it can be found that all the signals shifted in the manner that could be expected for the suggested structure. A plot of the Δ_{Eu}-values *vs.* $(3\cos^2\theta-1)/r^3$ according to the McConnel-Robertson equation[9] gave a good linear correlation (see Fig. 1) if the Eu-ion was placed at a distance of 3.0 Å from the carboxyl oxygen on a straight line connecting the carbon and oxygen atoms of the carbonyl group. The distances r and the angles θ were estimated from a Dreiding molecular model in which the aromatic and five-membered ring and the carbonyl group were coplanar. The suggested non-coplanarity of I (see below) is probably of no importance for these estimations. Much poorer correlation was found with the alternative structure discussed above (see Fig. 2).

Table 1. Δ_{Eu}-values for compound (I). For the designation of the hydrogens see Fig. 1.

Protons	Δ_{Eu} (ppm)
a	0.9
b	1.1
c	1.7
d	1.7
e	1.9
f	2.1
g	4.0
h	5.1
i	5.3
j	5.3

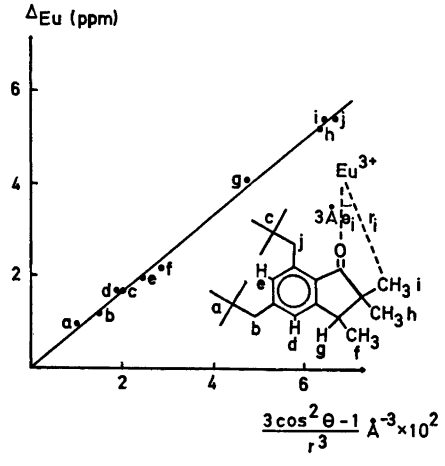


Fig. 1. Plot of Δ_{Eu}-values *vs.* $(3\cos^2\theta-1)/r^3$ for compound (I).

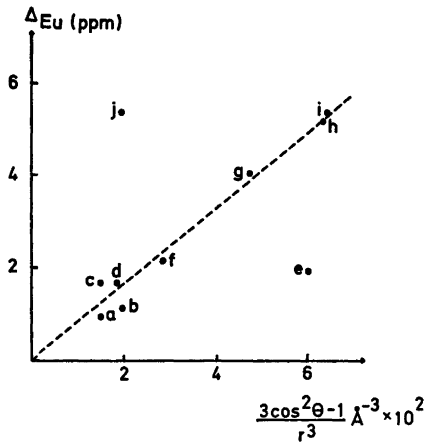


Fig. 2. Plot of Δ_{Eu}-values *vs.* $(3\cos^2\theta-1)/r^3$ for the hypotetical alternative structure 2,2,3-trimethyl-4,6-dineopentyl-1-indanone. The 5- and 7-aromatic hydrogens are designated d and e, respectively. The hydrogens of the methylene groups of the 4- and 6-neopentyl groups are designated b and j, respectively, and the hydrogens of the *tert*-butyl groups of the 4- and 6-neopentyl groups are designated a and c, respectively. For the designation of the other hydrogens see Fig. 1.

The methylene protons of the neopentyl group at carbon 7 of the indanone (I) are magnetically nonequivalent, but those of the neopentyl group at carbon 5 are magnetically equivalent. Heating I to 200 °C in deuteriobromoform caused only a slight decrease of the relative chemical shifts between the two methylene protons of the 7-neopentyl group. If the nonequivalence were due to restricted rotation in the molecule, the barrier to internal rotation, $\Delta G^{\ddagger}$, should have to be at least as high as 23 kcal/mol ($\Delta G^{\ddagger}$(200 °C) = 23.7 kcal/mol), which is considered less probable.

In order to rationalize this nonequivalence, we suggest that the carbonyl group is noncoplanar with the benzene ring in the predominant conformation of I, due to the unsymmetrical pattern of substitution in the five-membered ring. The nonequivalence of the methylene protons of the 7-neopentyl group would thus mainly be induced by the large magnetic field from the carbonyl group.

The distance between the carbonyl group and the methylene protons of the 5-neopentyl group is too large for the asymmetric field from the carbonyl group to be of importance, and thus these protons appear as a singlet.

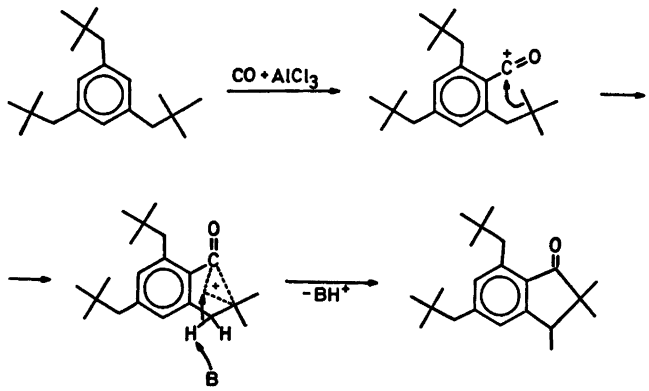
It is interesting to note that 1,2,2-trimethyl-4,6-dineopentylindan (IV) does not show magnetic nonequivalence in the methylene groups. This is explained by the absence of the field from the carbonyl group.

2,2,3-Trimethyl-5,7-dineopentyl-1-indanol (II) has two centers of chirality. Of the two possible (diastereomeric) pairs of enantiomers, only one seems to have been formed in the reduction of I with lithium tetrahydridoaluminate. The close similarity of the shifts of the geminal 2-methyl groups (δ 1.00 and 1.03) indicates that the 1-hydroxyl group and the 3-methyl group are situated on different sides of the plane of the five-membered ring. In contrast it has been reported [3] that the product of sodium tetrahydridoborate-reduction of 2,2,3-trimethyl-1-indanone showed NMR resonances at 0.68 and 1.18 ppm for the geminal 2-methyl groups and a structure with the 1-hydroxyl and the 3-methyl group on the same side of the ring plane was there assigned.

The stereoisomeric difference between II and 2,2,3-trimethyl-1-indanol [3] may be explained by the difference in the reducing agents [10] and/or in steric hindrance.

The suggested mechanism of formation of I with carbon monoxide is outlined in Scheme 1. It is suggested that the same mechanism is valid for the alternative synthesis, including 2,2-dimethylpropanoyl chloride, since this acyl chloride is known to decarbonylate easily under Friedel-Crafts conditions.[11] 1,3,5-Trineopentylbenzene is carbonylated to form an acylium cation, which undergoes an intramolecular reaction between the electron-deficient carbon and a carbon-carbon bond of one of the neighboring *tert*-butyl groups. The methyl group at the two-electron, three-center bond then effects an electrophilic substitution of one of the hydrogens of the methylene group to form the final product. An interesting feature of this mechanism would be that the formation of the three-center bond occurs between the electron-deficient carbon and a carbon-carbon bond and not a carbon-hydrogen bond of a methyl group. The latter mechanism has been suggested for a formation of 1,1-dimethyl-4,6-di-*tert*-butylindan from 2,4,6-tri-*tert*-butylbenzyl alcohol.[12]

We have rejected, as less possible, a mecha-



Scheme 1. Tentative mechanism of formation of 2,2,3-trimethyl-5,7-dineopentyl-1-indanone (I).

nism involving benzylic hydride ion abstraction from 1,3,5-trineopentylbenzene, followed by a 1,2-methyl shift and a carbonylation of the cation thus formed with concomitant ring closure, *cf.* Ref. 13. In a separate study [1] it was shown that commercial aluminum chloride had no effect on 1,3,5-trineopentylbenzene. This is in accordance with the known resistance to oxidation of an aromatic neopentyl group.[14]

EXPERIMENTAL

Measurements. Gas chromatographic (GLC) analyses were carried out on a Perkin-Elmer 900 gas chromatograph fitted with a flame ionization detector. The inner diameter of the columns used was 2 mm and the length 2 m. The stationary phase was 3 % of SE-30 silicon gum rubber on Gaschrom Q 100 – 200 mesh. The areas of the peaks on the gas chromatograms were measured by triangulation.

The IR spectra were recorded on a Beckman IR 9 spectrophotometer using potassium bromide pellets or a potassium bromide cell. The absorption maxima are reported in cm^{-1} and the intensities are characterized as weak (w), medium (m), strong (s), or very strong (vs).

The NMR spectra were recorded on a Varian A 60 spectrometer. About 10 % by weight solutions in carbon tetrachloride were used. The probe temperature was 35 °C. The chemical shifts are reported in ppm downfield from tetramethylsilane as internal standard. The multiplicities of the peaks are reported as singlet (s), doublet (d), and quartet (q). The spectrum of 2,2,3-trimethyl-5,7-dineopentyl-1-indanone (I) was also determined in deuteriochloroform with successive additions of tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-d_6-4,6-octanedione-d_3)europium(III), [Eu(fod)$_3$-d_{27}]. The chemical shift values found for each protons or group of protons for each addition of europium(III) were plotted as functions of the molar ratios X = [Eu(fod)$_3$]/[Compound (I)] and linear correlations were made. The Δ_{Eu}-values were then defined for each proton or group of protons as the difference in the extrapolated chemical shifts for $X = 1$ and the actual chemical shifts for $X = 0$.

The mass spectra (MS) were determined (at the Department of Medical Biochemistry, University of Göteborg) on an AEI 902 mass spectrometer under the following conditions: electron energy 70 eV, accelerating voltage 8 kV and emission 100 μA, or (at the Department of Medical Chemistry, University of Göteborg) on an LKB 9000 mass spectrometer with an electron energy of 70 eV. The latter mass spectrometer was connected to a gas chromatograph with a column containing 3 % of OV-1. The intensities of the peaks are reported in parenthesis as percentages of the base peak. Only the most abundant peaks are reported, together with the parent peaks and the isotope peaks corresponding to the latter.

Materials. Aluminum chloride: Kistner (*puriss*). Dichloromethane: Fisher (*p.a.*). Lithium tetrahydridoaluminate: Metallgesellshaft AG, Frankfurt a. M. Hexane: Fisher (*p.a.*). Sulfinyl chloride: Kistner (*puriss*). 1,3,5-Trineopentylbenzene was prepared as described by Martinson and Márton.[15]

2,2,3-Trimethyl-5,7-dineopentyl-1-indanone (I). a. A stirred suspension of aluminum chloride (7.0 g, 52.2 mmol) in 20 ml of dichloromethane was cooled to −35 °C and 2,2-dimethylpropanoyl chloride (6.7 ml, 52.2 mmol) was added. After 15 min, 1,3,5-trineopentylbenzene (5.0 g, 17.4 mmol) dissolved in 10 ml of dichloromethane, was added dropwise. The temperature of the flask was then slowly raised to room temperature and products started to form. Gases evolved during the reaction, and after 10 h the excess of aluminum chloride and 2,2-dimethylpropanoyl chloride was destroyed with

water. The aqueous layer was then extracted several times with hexane, and the combined dichloromethane and hexane layers were washed with 10 % aqueous sodium hydroxide solution, then with water, then with dilute hydrochloric acid and finally again with water. The organic layer was dried over magnesium sulfate, and the solvent was evaporated. The resulting oil was shown, by GLC, to consist of about 40 % of a main product and about 60 % of a complex mixture of about 30 compounds. Partial separation was performed on a column of silica gel. Unreacted 1,3,5-trineopentylbenzene and about 10 products were eluted with hexane. The main product was eluted with benzene and collected together with some other products. The column chromatography (silica gel, benzene) was repeated several times to give 2,2,3-trimethyl-5,7-dineopentyl-1-indanone of 97 % purity (GLC). The yield was 1.5 g (27 %) of an oil. *IR:* 2960vs, 2910m, 2865s, 1710vs, 1607vs, 1587s, 1480vs, 1427w, 1394m, 1367vs, 1304s, 1237s, 1202s, 1192s, 1160w, 1133m, 1095w, 992m, 974s, 918m, 890s, 873s, 746m. *MS:* 29(18), 41(27), 43(8), 57(7), 173(8), 187(18), 188(8), 202(100), 203(16), 243(13), 258(32), 299(14), 314(15.6), 315(3.9), 316(0.5). The mass of the molecule was determined by high-resolution mass spectrometry, and the value 314.261 ± 0.002 u was found. The value [16] for $C_{22}H_{34}O$ is 314.261 u. *NMR:* δ 0.91 (s, 9 H, *tert*-butyl), 0.95 (s, 9 H, *tert*-butyl), 1.01 (s, 3 H, methyl), 1.17 (s, 3 H, methyl), 1.26 (d, 3 H, methyl, $J = 8$ Hz), 2.58 (s, 2 H, methylene), 3.03 (q, 1 H, methine, $J = 8$ Hz), 3.09 (q, 2 H, methylene, $J = 12$ Hz, $\Delta\nu_{AB} = 11$ Hz), 6.83 (d, 1 H, aromatic, $J = 1$ Hz), 7.02 (d, 1 H, aromatic, $J = 1$ Hz).

b. To a suspension of 210 mg (1.58 mmol) of aluminum chloride in 5 ml of dichloromethane, a solution of 150 mg (0.52 mmol) of 1,3,5-trineopentylbenzene in 5 ml of dichloromethane was added, and a slow stream of carbon monoxide was bubbled into the mixture. A GLC analysis of the mixture after 24 h showed that a main product had been formed in about 60 % yield together with about 15 minor products. The work-up procedure described in *a* was then applied, and the total yield was 163 mg of an oil. The oil was investigated with an LKB 9000 mass spectrometer connected to a gas chromatograph. The MS of the main product was identical with that reported in *a* for 2,2,3-trimethyl-5,7-dineopentyl-1-indanone.

2,2,3-Trimethyl-5,7-dineopentyl-1-indanol (II). 2,2,3-Trimethyl-5,7-dineopentyl-1-indanone (0.2 g, 0.64 mmol) in 5 ml of diethyl ether was added to a suspension of 0.24 g (6.4 mmol) of lithium tetrahydridoaluminate in 20 ml of diethyl ether. The mixture was refluxed overnigt, and the reaction was then found to be complete (GLC). The flask was cooled and the excess of lithium tetrahydridoaluminate was destroyed with water. The mixture was filtered and the precipitate was washed several times with diethyl ether. The organic and aqueous layers were separated, and the organic layer was dried over magnesium sulfate. After evaporation of the solvent, the product was recrystallized from ethanol-water. The yield was 0.16 g (80 %) of white crystals which, according to GLC, were more than 98 % pure. *IR:* 3390vs, 2950vs, 2902s, 2862s, 1610m, 1591m, 1478s, 1414m, 1392m, 1384w, 1362s, 1235s, 1200m, 1039s, 1025s, 989m, 902w, 875s, 836m, 771m, 749w, 685m. *MS*: 29(26), 41(36), 43(34), 55(9), 57(100), 69(8), 71(23), 132(8), 171(16), 185(30), 186(77), 187(17), 188(17), 189(28), 203(11), 204(36), 227(10), 241(23), 242(94), 243(28), 254(23), 259(13), 260(47), 261(10), 283(11), 298(15), 301(15), 316(55.5), 317(13.6), 318(1.8). The mass of the molecule was determined by high-resolution mass spectrometry, and the value 316.277 ± 0.002 u was found. The value [16] for $C_{22}H_{36}O$ is 316.277 u. *NMR*: δ 0.91 (s, 9 H, *tert*-butyl), 0.94 (s, 9 H, *tert*-butyl), 1.00 (s, 3 H, methyl), 1.03 (s, 3 H, methyl), 1.21 (d, 3 H, methyl, $J = 7$ Hz), 1.35 (d, 1 H, hydroxylic, $J = 7$ Hz), 2.46 (s, 2 H, methylene), 2.65 (q, 1 H, methine, $J = 7$ Hz), 2.72 (q, 2 H, methylene, $J = 13$ Hz, $\Delta\nu_{AB} = 37$ Hz), 4.70 (d, 1 H, methine, $J = 7$ Hz), 6.76 (d, 1 H, aromatic, $J = 1$ Hz), 6.79 (d, 1 H, aromatic, $J = 1$ Hz). After addition of a small amount of water the coupling between the hydroxylic proton (δ 1.35) and the methine proton (δ 4.70) disappeared.

3-Chloro-1,2,2-trimethyl-4,6-dineopentylindan (III). 2,2,3-Trimethyl-5,7-dineopentyl-1-indanol (120 mg, 0.38 mmol) was dissolved in 5 ml of hexane, and 0.1 ml (1.3 mmol) of freshly distilled sulfinyl chloride was added. The mixture was stirred overnight at room temperature, and the reaction was then found to be complete according to GLC. The solvent and the excess of sulfinyl chloride were evaporated. The yield was 127 mg (100 %) of a crystalline product which was used directly in the next step.

1,2,2-Trimethyl-4,6-dineopentylindan (IV). 3-Chloro-1,2,2-trimethyl-4,6-dineopentylidane (127 mg, non-purified product from the above reaction) was dissolved in 3 ml of hexane and added to a solution of 26.6 mg (3.8 mmol) of lithium in 15 ml of liquid ammonia. The mixture was stirred for 2 h. The excess of lithium was then destroyed with water, and the mixture was neutralized with 6 M hydrochloric acid. The aqueous layer was extracted several times with hexane, and the combined hexane extracts were dried over magnesium sulfate. After evaporation of the solvent, the product was decolorized by adsorption chromatography (silica gel, hexane). The yield was 110 mg (96 %, computed on the indanol) of an oil which, according to GLC, was more than 98 % pure. *IR*: 2950vs, 2900w, 2860m, 2840w, 2710w, 1614w, 1588m, 1478s, 1465m, 1391m, 1285w, 1236s, 1200m, 1169w, 1115w, 1044w, 875s, 750m, 645w, 420m. *MS*: 29(10), 41(21), 43(16), 57(31), 71(9), 143(7), 157(9), 173(21), 187(29), 188(73), 189(11), 143(100), 244(53), 285(10), 300(20.1), 301(4.9), 302(0.6). *NMR*: δ 0.82 (s, 3 H, methyl), 0.91

(s, 18 H, *tert*-butyl), 1.12 (d, 3 H, methyl, $J=7$ Hz), 1.16 (s, 3 H, methyl), 2.41 (s, 4 H, methylene), 2.60 (s, 2 H, methylene), 2.74 (q, 1 H, methine, $J=7$ Hz), 6.60 (s, 2 H, aromatic).

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Three-dimensional Structure of Usnic Acid

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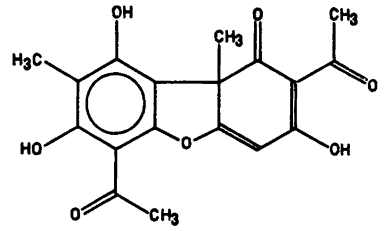
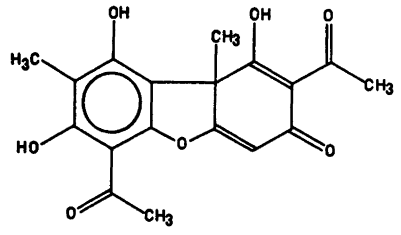
The three-dimensional structure of the very common lichen substance usnic acid, $C_{18}H_{16}O_7$, has been determined. By the use of single crystal *X*-ray diffraction data collected at −110 °C it was possible to determine the positional parameters of both the 50 nonhydrogen atoms and the 32 hydrogens of the asymmetric unit. The two molecules in the asymmetric unit have almost identical conformations. The six oxygens of each molecule are all involved in intramolecular hydrogen bonds of different strengths giving O···O distances ranging from 2.40 to 2.69 Å. Some information on possible racemization mechanisms can be gained from the present study.

Usnic acid, $C_{18}H_{16}O_7$, is one of the most common lichen substances. Up to 20 % of the dry weight of some lichens consist of this yellow acidic enol. Usnic acid, which is toxic to higher animals and exerts hypermetabolic activity, has to some extent been used as an uncoupler of oxidative phosphorylation in studies of the mechanism of this process. The now accepted structural formula, shown in Fig. 1, has been confirmed through total synthesis.[1] The structural formula implies several possibilities for formation of intramolecular hydrogen bonds. The existence of such hydrogen bonds is indicated by the fact that usnic acid, in spite of its high content of proper hydrogen bond donors and acceptors has pronounced lipophilic properties. Spectroscopic studies on usnic acid, using infrared and proton magnetic resonance techniques, have indicated the existence of three hydrogen bonds of markedly different strengths.[2,3] It has been shown [4,5] from determinations of unit cell parameters, space group symmetry and density, that usnic acid crystallizes with two formula units per asymmetric unit. Thus, the existence of strong intermolecular interactions like, *e.g.*, hydrogen bonds leading to a dimerization and a further complication of the hydrogen bond scheme cannot be ruled out. On the other hand the spectroscopical investigations [2] suggested that all of the three hydrogen bonds indicated are intramolecular.

The X-ray structural investigation of usnic acid, reported in the present paper, has been carried out to obtain the three-dimensional structure, and thereby also clarify the hydrogen bond scheme and the nature of the intermolecular interactions. The crystalline specimen used



(a)



(b)

Fig. 1. Proposed bond schemes for the two most plausible tautomers of usnic acid.

Table 1. Unit cell dimensions (Å) for usnic acid. (Space group: $P2_12_12_1$. Composition of asymmetric unit: $C_{36}H_{32}O_{14}$).

	Jones and Palmer[4]	This study 22 °C	−110 °C
a	8.09	8.069	8.042
b	19.10	19.058	18.890
c	20.39	20.326	20.264

was obtained by evaporation of a chloroform solution of (−)-usnic acid.

The space group $P2_12_12_1$ was confirmed by X-ray investigations with precession and De Jong-Bouman techniques using Mo$K\alpha$ radiation. In order to be able to locate not only the 50 independent nonhydrogens atoms, but also the 32 hydrogens, it was decided to collect single crystal X-ray diffraction data at low temperature. Thus, the diffraction data were collected at about −110 °C on a single crystal diffractometer using monochromatized Cu$K\alpha$ radiation. In all, 2405 reflections significantly above the background were observed. The cell dimensions determined are given in Table 1.

The structure was solved by the application of direct methods using the variance-weighted phase-sum formulae for phase determination and refinement.[6] Of the 31 highest peaks in the obtained electron density map, 28 were chemically meaningful and were used in the subsequent difference electron density maps and least-squares refinements to locate the remaining 22 nonhydrogen and 32 hydrogen atoms. After least-squares refinement of the structural model, allowing all the nonhydrogens to vibrate anisotropically and using fixed isotropic temperature factors for all hydrogens, the *R* value became 0.04. No attempts to determine the absolute

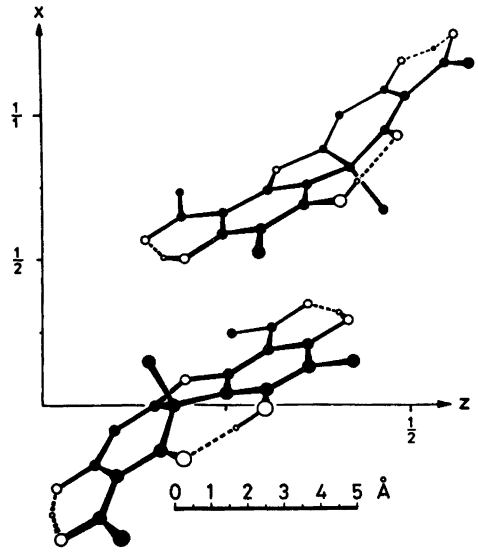


Fig. 2. The molecular geometries of the two molecules of the asymmetric unit. The figures refer to fractional coordinates.

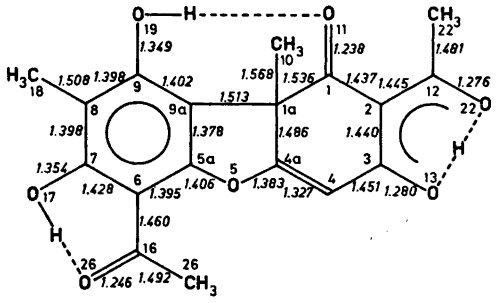


Fig. 3. The obtained intramolecular bond distances averaged over the two molecules of the asymmetric unit.

Table 2. Hydrogen bond distances (Å) and angles (°) in the two independent molecules.

Hydrogen bond	O···O	O−H	H···O	∠O−H···O
O(19)−H···O(11)	2.66	0.99	1.69	166
	2.69	0.84	1.86	170
O(17)−H···O(26)	2.54	0.88	1.69	163
	2.53	1.00	1.58	156
O(13)···H···O(22)	2.40	1.27	1.16	164
	2.41	1.07	1.38	158

configuration from the diffraction data have been made.

The observed molecular geometries and intramolecular distances are shown in Figs. 2 and 3 together with the atomic labels used in this study. As seen, the two molecules have almost identical conformations, they both adopt a kind of butterfly conformation. No short intermolecular distances indicating intermolecular hydrogen bonds were found. The three hydroxyl hydrogens in each molecule are all involved in strong intramolecular hydrogen bonds, giving O···O distances ranging from 2.4 to 2.7 Å. The hydrogen bond distances and angles, listed in Table 2, indicate that the bond strength increases in the order O(19)–H···O(11), O(17)–H···O(26) and O(13)···H···O(22) in consistence with the interpretation of the NMR and IR spectra reported by Forsén *et al.*[8] The very short O···O distance of 2.40 Å in the chelated O(13)···H···O(22) hydrogen bond is significantly shorter than those found in, *e.g.*, the dibenzoylmethanes [7] where a similar intramolecular hydrogen bond situation leads to O···O distances of about 2.47 Å. Distances of about 2.40 Å appears actually to represent the lower limit of O···O distances in hydrogen bonds found sofar.[8] The oxygen-hydrogen distances in the O(13)···H···O(22) hydrogen bond (*cf.* Table 2) suggests that the hydrogen is attached to different oxygen atoms in the two independent molecules in the crystal structure. This would be in agreement with the infrared spectroscopical studies on acetylacetones [9], which indicate that strong but unsymmetrical intramolecular hydrogen bonds would be a general feature of β-diketones. The existence of two different enolic tautomers, due to the differences of the O(13)···H···O(22) hydrogen bonds, would also be an explanation for the two molecules per asymmetric unit in the crystal structure. However, the standard deviations of the oxygen–hydrogen distances, 0.05 Å, and the expected systematic errors when locating hydrogens by X-ray diffraction techniques [10] does not permit any definitive decisions between the alternatives, symmetrical or unsymmetrical hydrogen bonds between O(13) and O(22). Further studies using single crystal neutron diffraction techniques have been initiated.

Fig. 3 shows the intramolecular bond scheme drawn in accordance with the observed bond distances. This bond scheme agrees largely with one of the tautomers of the earlier proposed bond scheme (Fig. 1a) and excludes the other tautomer (Fig. 1b). The only major difference being the complete delocalization of the double bonds over the region O(13)–C(3)–C(2)–C(12)–O(22), which leads to C–O bond lengths of 1.28 Å and C–C bond lengths of 1.44 Å.

Usnic acid of natural origin is represented by both its enantiomers which differ by the configuration around the quaternary carbon atom, C(1a), and is furthermore known to easily undergo thermal racemization in, *e.g.*, boiling xylene solution. The hitherto most widely accepted racemization mechanism is the one suggested by Stork [11] and involves a homolytic loosening of the C(1a)–C(1) bond to form a resonance stabilized diradical. Other suggestions,[12] on the other hand, include a homolytic loosening of the C(1a)–C(9a) bond. A striking support to any of these suggested mechanisms would of course be to observe a correspondingly elongated bond length, due to a weakened C–C bond, in the crystal structure. A certain support for Stork's mechanism might be gained from this study since the bond lengths obtained for the four bonds C(1a)–C(1), C(1a)–C(4a), C(1a)–C(9a), and C(1a)–C(10) around the quaternary carbon atom C(1a) are 1.54, 1.49, 1.51, and 1.57 Å, respectively. Thus, the C(1a)–C(1) distance of 1.54 Å is somewhat larger than the usual C(sp^3)–C(sp^2) bond distance [13] of 1.516 Å. No easy connection between the long (1.57 Å) C(1a)–C(10) distance and a racemization reaction can be formulated. It is noteworthy that the hydrogen bond O(19)–H···O(11), although the weakest of the three intramolecular hydrogen bonds, is of decisive importance for the thermal stability of usnic acid. Thus, the activation energy for racemization of (–)-usnic acid in decalin or toluene is some 4 kcal higher than in typical electron donor solvents as dioxane or tetrahydropyran, which evidently can break the O(19)–H···O(11) bond.[12,14] On the other hand, acetylation of (–)-usnic acid at the hydroxyl group containing O(19) gives a monoacetate [2] for which the corresponding low activation energy is largely independent of solvent type.[14]

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Kinetics and Other Characteristics of Diamine Oxidase of Pea Seedlings

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1. Spectrophotometric titration with phenylhydrazine has given evidence that diamine oxidase of pea seedlings contains one mol of reactive carbonyl groups per mol of enzyme.

2. The effect of variation in concentration of five amine substrates and oxygen, on the steady-state kinetic behaviour, have been studied in detail. The kinetics are found to conform to a Dalziel rate equation without ϕ_{12} term, and thus the enzymatic reactions are proposed to follow a ping-pong mechanism. Investigation of the pH dependence of the enzyme has given strong indication that for good binding the amine group in the substrate, which participates in the oxidation, must be uncharged, while the other amine group ought to be positively charged.

3. Electron paramagnetic resonance spectra showed that the protein-bound copper is not significantly reduced in the presence of substrate under anaerobic conditions, but a distinct shift in the signal gives evidence for a change in the way that the copper is bound.

Diamine oxidase of pea seedlings, like kidney diamine oxidase and plasma amine oxidase (benzylamine oxidase), contains copper [1–4] and a functional carbonyl group, which in the case of the last two enzymes is almost certainly pyridoxal phosphate.[5–6] However, attempts to demonstrate pyridoxal phosphate in pea seedling diamine oxidase have been unsuccessful.[7]

The presence and requirement of Cu^{2+} in the enzyme is well established,[1–4] but its function is not understood. Cupric ions are required to activate copper-free oxidase of pea seedlings,[4] bovine plasma,[8] and pig kidney.[9] Electron paramagnetic resonance studies have provided strong evidence that no change in valence state of copper occurs in benzylamine oxidase of blood plasma during catalysis.[10] The situation in the case of pig kidney diamine oxidase is more obscure, and evidence for reduction of copper has been reported.[11] The present investigation gives evidence for no reduction of copper in diamine oxidase of pea seedlings in the presence of substrate under anaerobic conditions.

The catalytic mechanism for the enzyme has not been well clarified. For some other amine oxidases strong evidence has been given for a ping-pong mechanism.[12–14] The present study attempts to clarify some characteristic kinetic behaviour of pea seedling diamine oxidase, and to see whether a mechanism similar to that found with other amine oxidases can be applied to this enzyme with its much higher specific activity.

MATERIALS AND METHODS

Diamine oxidase was prepared from pea seedling as described by Hill and Mann,[4] followed by column chromatography on Sephadex G-200. The enzyme thus obtained was 80 – 90 % pure according to specific activity tests, and was used for most of the experiments. For determination of the amino acid composition, the enzyme was further purified by analytical gel electrophoresis at pH 7.8. Preparations thus obtained had a specific activity of 85 U based on protein estimations with the Folin-Ciocalteau method as modified by Lowry *et al.*[15] and oxygen consumption measurements with a Clark electrode combined to an Eschweiler Combi-analysator U. The electrophoretically purified enzyme was used as a reference for almost pure enzyme. The specific activity was well above that reported by Hill and Mann [4] for an almost pure enzyme preparation. However, they used somewhat different

estimation procedures which may account for the discrepancy.

Other chemicals used in this investigation were of highest available purity. ^{14}C-Putrescine was obtained from Amersham, and catalase (2 times crystallized from bovine liver) was obtained from Sigma.

Titration with phenylhydrazine. Titrimetric experiments with phenylhydrazine at pH 7.2 in 0.2 M phosphate buffer were carried out according to methods previously described.[16] The concentration of the inhibitor hydrazine was such that 2 – 6 portions were required to reach the equivalence point. The enzyme selected for these experiments was more than 85 % pure according to specific activity determinations. In contrast to what has been found with other hydrazines,[4,17] the equilibration between phenylhydrazine and enzyme was very rapid. However, to ensure that equilibrium was attained, the reaction was allowed to proceed for 2 min after each addition of phenylhydrazine before measurements were made. 15 μl of this enzyme suspension were then assayed in the presence of 4 mM putrescine.

Steady-state kinetic methods. The enzymatic activity of diamine oxidase of pea seedlings was determined from the initial oxygen consumption at various concentrations of oxygen and amine substrate. The 2.5 ml reaction vessel contained enzyme, substrate and 50 μg catalase in 0.2 M potassium phosphate buffer. Above pH 8.0, 0.15 M potassium phosphate and 0.05 M potassium borate was used as the buffer. Estimations of initial activity were not possible at very low oxygen concentration. However, as the product inhibition is very low with all the substrates examined, except tryptamine, the kinetics at various oxygen concentrations could be determined by following oxygen consumption continuously until all the oxygen was consumed. The same procedure was used with tryptamine as substrate, but initial activity estimations were made down to an oxygen concentration of 5 μM, so that the product formed never exceeded 10 μM and the product inhibition could be neglected.

Transient-state kinetic methods. Transient-state kinetic experiments were performed in the Aminco-Morrow stopped-flow apparatus described by Lindström *et al.*[10] Anaerobic conditions were attained by the pumping-flushing technique described by Carrico *et al.*[18]

Electron paramagnetic resonance spectra of diamine oxidase. EPR spectra of diamine oxidase (about 80 % pure) were recorded at pH 8.0 in a Varian E-3 spectrometer at 77 K and 9.2 GHz. Spectra of the substrate-reduced form of the enzyme were determined in presence of 4 mM putrescine. The substrate was added as a solution directly into the EPR tube in an amount that diluted the enzyme by 20 %, and made the enzyme completely reduced and the solution anaerobic within a few seconds.

Table 1. Amino acid composition found in acid hydrolysate from electrophoretically purified diamine oxidase of pea seedling. Values are expressed as mol of amino acid per mol of methionine found in the hydrolysate.

Aspartic acid	16.8
Threonine [a]	9.2
Serine [a]	26.5
Glutamic acid	24.3
Proline	Not determined because of interference
Glycine	22.1
Alanine	11.2
Half-cystine	–
Valine	7.6
Methionine	1.0
Isoleucine	7.0
Leucine	8.2
Tyrosine	4.3
Phenylalanine	4.9
Histidine	4.7
Lysine	6.6
Tryptophan	–
Arginine	4.5
Ornithine	5.8

[a] Compensated for degradation during 24 h hydrolysis in 6 M HCl.

RESULTS

General properties. The general properties of the enzyme preparations used in the present investigation agree in all essential features with those reported by Hill and Mann.[2,4,20] The amino acid composition of the electrophoretically purified enzyme (Table 1) shows an unusually high content of ornithine, and as no half-cystine was observed, sulfur bridges seem to be absent in the enzyme.

Active site titration with phenylhydrazine. Pea seedling diamine oxidase like amine oxidase from pig kidney [9] and blood plasma,[19] shows a broad and fairly weak absorption in the visible region. On addition of phenylhydrazine the enzyme solution turns yellow due to the formation of an absorption band centered around 435 nm. Equilibrium is rapid and essentially completed within 15 s after each addition of phenylhydrazine at an enzyme concentration of 15 μM. No further change in absorption could be observed after addition of a threefold excess of phenylhydrazine, but the enzyme still showed

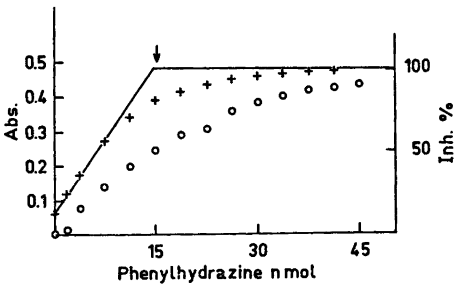


Fig. 1. Spectrophotometric titration of pea seedling diamine oxidase with phenylhydrazine. (+) shows the titration of 15.0 nmol enzyme in 1 ml 0.1 M phosphate buffer, pH 7.0, with 5 μl or 10 μl portions of 375 μM phenylhydrazine. measured at 435 nm; (O) shows the inhibition found simultaneously. Absorbancies and inhibitions have been corrected for dilution effects, and refer to a volume of 1 ml. Arrow indicates point of equimolar amounts of enzyme and inhibitor.

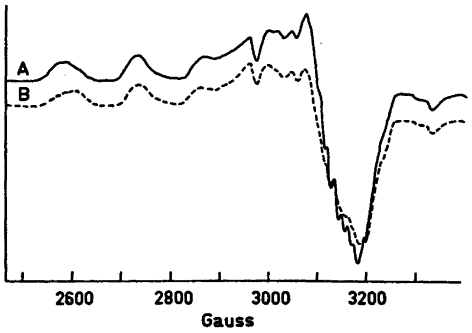


Fig. 2. Electron-paramagnetic-resonance spectra of 80 % pure diamine oxidase at 77 K and 9.2 GHz. Spectra were recorded at pH 8.0 in 0.4 ml aerobic 0.2 M phosphate buffer (A) and in an anaerobic solution after addition of 80 μl of a 24 mM solution of putrescine (B).

a low activity if measured in the presence of 4 mM putrescine (Fig. 1). The residual activity may be explained by the competition between substrate and hydrazine, as has been found with other hydrazines.[17] From Fig. 1, the number of active sites per molecule of enzyme is estimated graphically to be close to one, assuming a molecular weight of 96 000.[4] In an attempt to provide evidence for the presence of pyridoxal phosphate, indicated by the binding of hydrazines, the enzyme was reacted with ^{14}C-putrescine in an anaerobic solution, and reduced with $NaBH_4$, as described by Buffoni.[5] The treatment led to a significant incorporation of radioactivity into the protein, but no product which would indicate pyridoxal phosphate in the native enzyme was identified from paper electrophoresis at pH 3.6 after acid hydrolysis.

Electron paramagnetic resonance spectra of the enzyme. Fig. 2A shows the EPR spectrum of an 80 % pure preparation of pea seedling diamine oxidase as such, and Fig. 2B shows the spectrum recorded after reduction of the enzyme with an excess of substrate. The main features of the spectra indicate that the EPR signal arises largely from protein-bound Cu^{2+}. However, the contribution from an overlapping signal is possible on the basis of comparison with the EPR spectra reported for pig kidney diamine oxidase[11] and benzylamine oxidase.[10] This might be related to the presence of small amounts of Mn^{2+}, which has been reported as an impurity in pea seedling diamine oxidase preparations which are not homogeneous.[20] Integration of the spectra in Fig. 2 showed that there is no reduction of copper in the presence of a large excess of substrate, but the minor shifts observed for the two low-field hyperfine lines indicate a significant change in the binding situation of copper.

Kinetic experiments. Several amine oxidases have been reported to conform to a ping-pong mechanism.[12,14] As for pea seedling diamine oxidase, the ping-pong mechanism has been proposed from kinetic experiments with tryptamine by Yamasaki *et al.*[17] Attempts to verify their results showed that the strong product inhibition observed with tryptamine as substrate could substantially affect the results. The investigations described in this paper are therefore necessary for a more careful evaluation of the mechanism.

Preliminary experiments with different substrates showed that the apparent K_m for O_2 was always very low and was dependent on the nature and the concentration of the amine substrate. With poor substrates such as benzylamine the apparent affinity for oxygen was too high for a determination of the apparent K_m for O_2 (< 1 μM). Detailed kinetic studies of steady-state rate behaviour of the enzyme were carried out using the substrates: putrescine, cadaverine, histamine, tryptamine, and lysine. Each substrate was found to give linear and

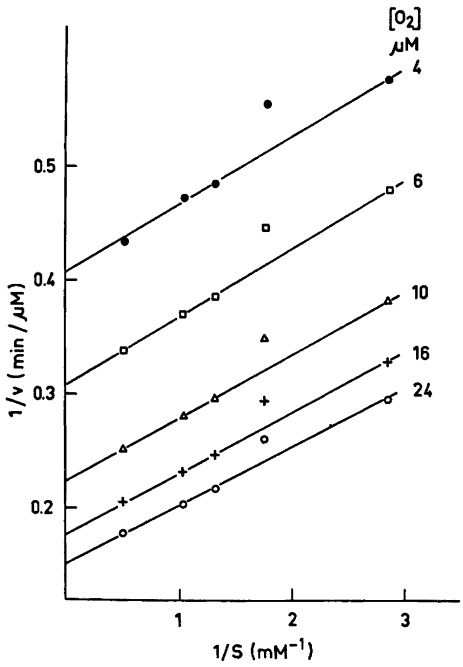


Fig. 3. Lineweaver-Burke plots of the effect of putrescine concentration on the rate of oxidation at different fixed concentrations of oxygen. Reaction solutions contained the diamine oxidase, catalase, and the substrates at 25 °C in 0.2 M phosphate buffer, pH 7.0.

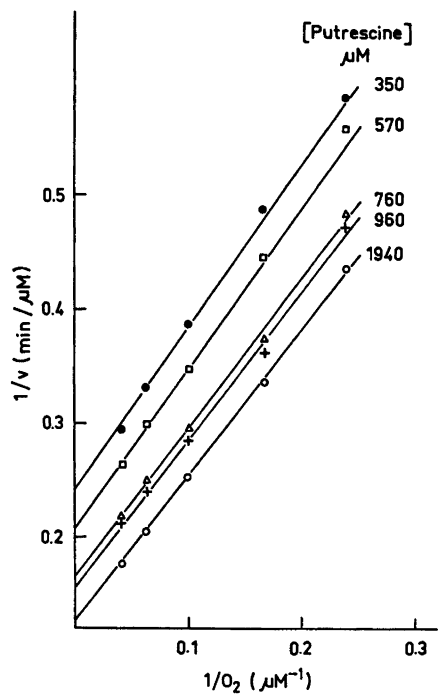


Fig. 4. Lineweaver-Burke plots of the effect of oxygen concentration on the rate of oxidation at various fixed concentrations of putrescine. Conditions as in Fig. 3.

parallel reciprocal plots when the oxygen concentration was varied at different constant substrate concentrations or *vice versa*, as is exemplified with putrescine (Figs. 3 and 4) and histamine (Figs. 5 and 6) at pH's 7.0 and 7.2, respectively. These data are consistent with the rate equation:

$$C_e/v = \phi_0 + \phi_1/\phi_2 + \phi_2/S \qquad (1)$$

As can be seen from Fig. 7 estimates of ϕ_1 obtained for the different substrates, except histamine, exhibit no significant variation with pH over the range 6 – 9. In reality the small variation observed may be ascribed to limitations in the function of the pO_2 apparatus at oxygen concentrations below 3 μM. Figs. 8 and 9 show that both ϕ_0 and ϕ_2 are strongly dependent on pH and vary in magnitude for the different substrates. Slopes of the straight lines obtained in plots of log ϕ_2 *vs* pH (Fig. 8) are essentially independent of the substrate, but the magnitude of ϕ_2 is obviously quite dependent on the nature of the substrate. ϕ_0 shows a minimum between pH 7.5 and 8.3 for all the substrates except histamine. Thus histamine deviates markedly in the behaviour of both ϕ_0 and ϕ_1 compared with the other substrates. In order to exclude the possibility that histamine reacts with a second active site in the enzyme or with some other enzyme present in the preparation, the influence of histamine on oxygen consumption was determined in the presence of cadaverine. The Lineweaver-Burke plots in Fig. 10 show that there is a strict competition between the two substrates for the same site in the enzyme.

Anaerobic-transient-state-kinetic experiments. If diamine oxidase of pea seedling is reduced by substrate under anaerobic conditions, the absorption band centered around 500 nm is replaced by bands with maxima at 466, 437.5, and 350 nm.[4] The formation of these bands and the disappearance of the 550 nm band was found to be very fast in the presence of 2 mM

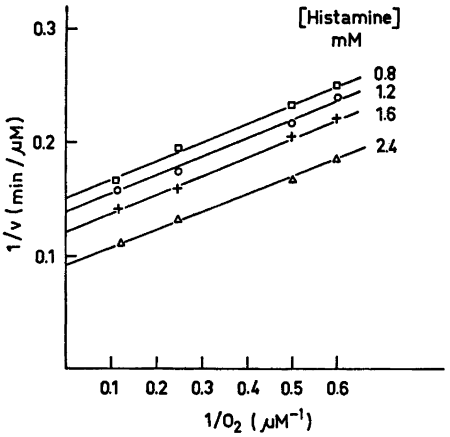


Fig. 5. Lineweaver-Burke plots of the effect of oxygen concentration on the rate of oxidation at various fixed concentrations of histamine. Conditions as in Fig. 3 except that pH was 7.2.

histamine. At 25 °C and pH 7, most of the absorption changes were within the dead-time (5 ms) of the instrument, but from what was registered all the chromophore changes seemed to be simultaneous.

DISCUSSION

Pea seedling diamine oxidase, pig kidney diamine oxidase and benzylamine oxidase are closely related in many respects. All these enzymes catalyze the oxidation of various amines

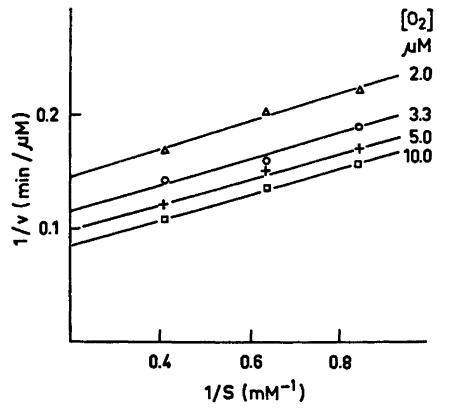


Fig. 6. Lineweaver-Burke plots of the effect of histamine concentration on the rate of oxidation at various fixed concentrations of oxygen. Conditions as in Fig. 5.

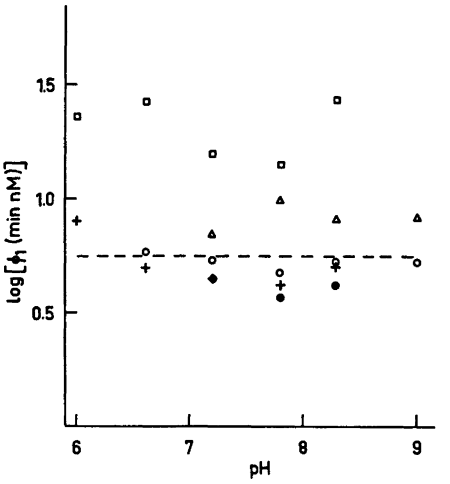
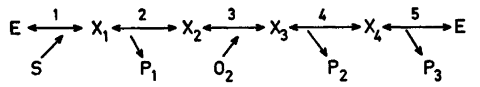


Fig. 7. Logarithmic plots of ϕ_1 for various substrates *vs.* pH For conditions see text. The symbols used are for histamine (□), lysine (△), tryptamine (●), cadaverine (+), and putrescine (○).

by molecular oxygen with the formation of the corresponding aldehydes, ammonia, and hydrogen peroxide. In this paper evidence is given that diamine oxidase of pea seedlings, like two other amine oxidases, conform to the rate equation (1), which for the other enzymes, has been taken as evidence for a ping-pong mechanism.[12,14] The results in this paper are in accordance with the mechanism in Scheme 1, suggested for benzylamine oxidase by Taylor *et al.*[14] The kinetic investigation cannot, however, provide any information as to the order in which the different products are released. If the kinetics are estimated under circumstances where no product inhibition is observed, steps 2, 4, and 5 can be regarded as irreversible, and thus:
$\phi_0 = 1/k_2 + 1/k_4 + 1/k_5$, $\phi_1 = (k_{-3} + k_4)/k_3k_4$, and $\phi_2 = (k_{-1} + k_2)/k_1k_2$. An examination of the different ϕ_i terms (Figs. 7, 8, and 9) shows that,



Scheme 1. The ping-pong mechanism suggested by Taylor *et al.*[14] for reactions catalyzed by benzylamine oxidase. S stands for amine substrate and P_i for the formed products. X_i denotes intermediately formed enzymatic species.

contrary to what is found with benzylamine oxidase,[10] the maximum activity under physiological conditions is largely independent of the oxygen concentration (ϕ_1).

For benzylamine oxidase a mechanism has been proposed, where the unprotonated amine is the real substrate.[21,22] A similar situation seems very likely for diamine oxidase of pea seedlings, as log (ϕ_2) decreases almost linearly with pH. All the substrates give slopes close to -1 (-0.6 to -0.9), which would be expected if only the substrates with uncharged amine groups can be oxidized by the enzyme, as the amine groups that participate in the oxidation always have pK_a's above 9.5. If this assumption is taken into account, the linear decreases of log (ϕ_2) *vs.* pH would largely reflect the increasing substrate concentration with increasing pH. The magnitude of ϕ_2, and most likely the binding of the substrate to the enzyme is, however, strongly dependent on the structure and the ionization of the substrate. All substrates of pea seedling diamine oxidase have one primary amine group which participates in the oxidation procedure, one hydrophobic region in the middle, and one specific region at the other end of the molecule. According to the nature of the specific region, the substrates can be divided into three main groups, which are characterized by different ϕ_2 values. Low ϕ_2 values are found in the first group, which contains substrates with a positively charged primary amine in the specific part of the substrate. Substrates in the second group are mainly uncharged in the specific part and are characterized by intermediate ϕ_2 values. High ϕ_2 values are found in the third group, which contains substrates with both a positively and negatively charged group in the specific part of the substrate.

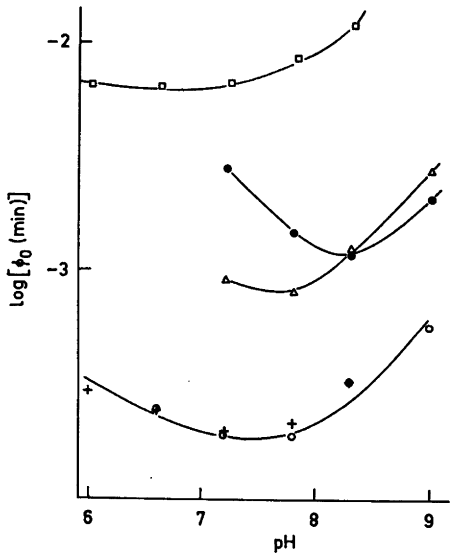


Fig. 9. Logarithmic plots of ϕ_0 for various substrates *vs.* pH. Symbols and conditions as in Fig. 7.

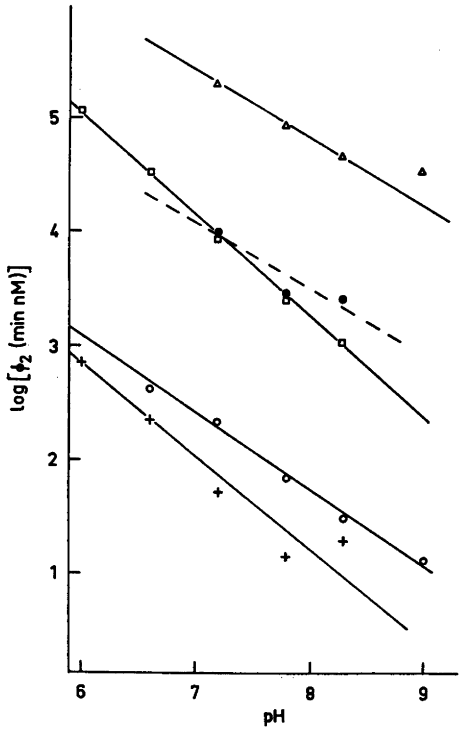
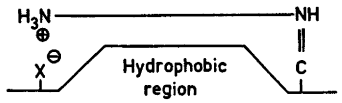


Fig. 8. Logarithmic plots of ϕ_2 for various substrates *vs.* pH. Symbols and conditions as Fig. 7.

The mode of enzyme-substrate interaction may, according to the discussion above, be described as in Scheme 2, proposed for pig kidney diamine oxidase.[22] The negatively charged group of the enzyme proposed in Scheme 2 is most likely a carboxyl group. Preliminary experiments in this laboratory have shown, that modification of carboxyl groups with carbodiimide causes strong inhibition of pea seedling diamine oxidase activity. This inhibition may



Scheme 2. The binding situation proposed for enzyme-substrate interaction with pig kidney diamine oxidase by Barsley *et al.*[22] (X) stands for a negatively charged group in the enzyme.

be due to modification of the negatively charged group proposed in Scheme 2.

From Figs. 7 and 9 it is clear that the behaviour of ϕ_0 and ϕ_1 differs markedly between histamine and all the other substrates used. The difference in ϕ_0 can be explained by the low pK_a for the imidazole moiety in histamine, which causes deprotonization mainly between pH 6 and 7, thus resulting in a poorer binding situation at high pH. The cause of the much higher ϕ_1 obtained with histamine is more difficult to explain but might reflect a negative co-operation between the imidazole moiety of the substrate and a group of the enzyme which participates in the binding of oxygen.

A valence change of copper could not be detected after reaction of diamine oxidase and substrate in an anaerobic solution. This does not exclude the possibility that reduction of copper may be an obligatory step in the catalytic mechanism, as has been pointed out by Mondovi *et al.*[11] A rapid redox equilibration between certain enzymatic species, favouring those containing copper in the oxidized state, would result in less than stoichiometric reduction of the electron paramagnetic resonance signal by substrate under anaerobic conditions. If such a situation exists in the case of diamine oxidase of pea seedlings, it may be concluded that the anaerobic equilibrium concentration of enzyme species containing copper in the reduced state is less than 5 % of the total enzyme concentration. The present investigation does not, however, give any indication of a valence change of copper during catalysis.

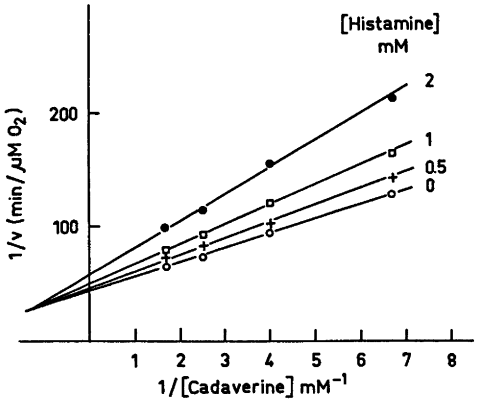


Fig. 10. Lineweaver-Burke plots of the effect of cadaverine concentrations on the rate of oxidation at various fixed concentrations of histamine. Conditions as in Fig. 3 except that pH was 7.2.

From this investigation it is clear that diamine oxidase of pea seedlings is closely related to other amine oxidases in many respects. Thus, all amine oxidases investigated show ping-pong mechanism. However, contrary to benzylamine oxidase,[10] the rate determining steps in catalysis with pea seedling diamine oxidase are independent of the oxygen concentration under physiological conditions. The function of copper must be quite similar for all the enzymes, even if it is doubtful that the cofactor is pyridoxal phosphate in pea seedling diamine oxidase.

Acknowledgement. The authors are indebted to Dr. G. Pettersson for his criticism and valuable discussions.

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Refinement of the Crystal Structure of L-DOPA Hydrochloride and a Comment on the Structure of α-Amino Acids

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The crystal structure of L-DOPA hydrochloride has been refined by X-ray crystallographic methods using 2122 observed reflections with $0.3 < \sin\theta/\lambda < 0.8$. The final conventional R-factor is 0.030, standard deviations in bond lengths are less than 0.002 Å and in angles about 0.1°. The conformation of the carboxyl group has been shown to deviate from what is usually found in that the carbonyl oxygen atom is in *anti* position relative to the nitrogen atom about the $C-C_\alpha$ bond. The geometry of this particular conformation in α-amino acids is discussed.

The crystal structure determination of L-mimosine sulfate hydrate [1] demonstrated the presence of a conformation of the carboxylic group not commonly found in α-amino acids, *i.e.* an *anti*-planar arrangement of the atoms in the N−C−C=O group. A brief search in the literature yielded only one further example of this conformation in protonized α-amino acids, namely histidine hydrochloride,[2] although more examples may be found in some peptides and in other types of carboxylic acids. However, an indication of the same conformation was found in the report on the structure of L-DOPA hydrochloride.[3] We have undertaken the refinement of the structure of the latter compound to an accuracy sufficient for establishing the geometry of the amino acid group.

EXPERIMENTAL AND REFINEMENT

Crystals of L-DOPA hydrochloride were formed by evaporation of a solution of the compound in hydrochloric acid. A single crystal ground to a sphere (radius 0.18 mm) was used in the X-ray experiments.

Unit cell dimensions were determined from diffractometer measurements of 12 general reflections using MoK_α radiation ($\lambda = 0.71069$ Å). In good agreement with the data given by Jandacek and Earle [3] they were found to be: $a = 6.271(0.002)$ Å; $b = 5.835(0.001)$ Å; $c = 15.660(0.003)$ Å; $\beta = 112.65(0.02)°$. The space group is $P2_1$.

Intensity data were recorded with the use of an automatic Picker diffractometer using graphite crystal monochromated MoK-radiation. The take-off angle was 4° and the temperature was 18 ± 1 °C during the data collection. The $\omega - 2\theta$ scan technique was used with a 2θ scan speed of 1° min^{-1} through the scan range from 0.6° below $2\theta(\alpha_1)$ to 0.6° above $2\theta(\alpha_2)$. Background counts were taken for 30 s at each of the scan range limits. The intensities of three standard reflections were measured at intervals during the intensity data measurements; they showed no systematic variations. The standard deviations were taken as $\sigma(I) = (C_T + (0.02C_N)^2)^{\frac{1}{2}}$ where C_T is the total number of counts and C_N the scan count minus background count. The measurements included 2408 unique reflections with $\sin\theta/\lambda$ less than 0.8. Of these 2265 had net intensity larger than 2.5 $\sigma(I)$ and were regarded as observed reflections whereas the remaining reflections were excluded from the further calculations.

The intensity data were corrected for Lorentz, polarization, and absorption ($\mu = 0.4$ mm^{-1}) effects.

Atomic form factors used were those of Doyle and Turner [4] for the chloride ion and oxygen, nitrogen, and carbon atoms, and of Stewart, Davidson and Simpson [5] for hydrogen. A description of the computer programs employed during the refinement procedure is given in Ref. 6.

The first attempt to refine the structure on the basis of the coordinates given by Jandacek and Earle was without success as it turned out that all negative signs for positional parameters were left out of their paper. The structure was redetermined using the position of the chlorine

Table 1. Fractional coordinates ($\times 10^5$ for heavy atoms and $\times 10^3$ for hydrogen atoms) and thermal parameters ($\times 10^5$ for heavy atoms, the temperature factor is given by $\exp-(B11h^2+B22k^2+B33l^2+B12hk+B13hl+B23kl)$). Standard deviations (in parentheses) apply to the least significant figures.

ATOM	X	Y	Z	B11, Å	B22	B33	B12	B13	B23
CL	40733(6)	41180(8)	5867(2)	1744(9)	1286(8)	250(1)	78(17)	261(5)	-6(7)
O1	-22002(21)	-17729(26)	65147(10)	1728(30)	1806(32)	391(6)	-366(53)	339(21)	324(24)
O2	-43179(20)	11788(30)	52106(9)	1307(25)	2244(38)	318(5)	290(54)	129(18)	117(24)
O3	96490(19)	52124(31)	81029(9)	1305(26)	3438(49)	346(5)	-638(62)	542(20)	423(28)
O4	91286(18)	46751(37)	93738(7)	1312(24)	3264(41)	258(4)	-356(75)	221(16)	449(33)
N	46815(17)	41793(31)	86688(7)	1229(24)	1462(27)	234(4)	-40(63)	473(16)	-115(27)
C1	26797(23)	21090(27)	67876(9)	1304(30)	1760(37)	178(4)	62(57)	366(19)	-167(22)
C2	14546(24)	2662(28)	69458(9)	1418(31)	1581(36)	216(5)	369(57)	221(21)	4(23)
C3	-8944(24)	-2(28)	64119(9)	1466(32)	1485(33)	236(5)	67(57)	428(21)	-95(23)
C4	-20311(22)	15943(28)	57179(9)	1262(30)	1728(36)	200(5)	354(57)	312(20)	-86(22)
C5	-8359(26)	34356(31)	56704(10)	1627(35)	1971(39)	214(5)	224(63)	309(22)	223(24)
C6	15138(26)	36951(28)	61838(10)	1610(34)	2003(54)	228(5)	-284(60)	417(21)	178(23)
C7	52518(23)	23818(31)	73386(10)	1175(29)	2081(43)	227(5)	232(60)	373(21)	-254(26)
C8	58175(20)	44344(26)	79981(9)	1037(25)	1557(45)	209(4)	-43(51)	347(17)	22(22)
C9	84246(22)	46348(25)	85851(10)	1165(28)	1594(40)	248(5)	-234(50)	351(20)	8(21)
HC2	219(5)	-84(7)	746(2)	3,1(,3)					
HC5	-165(4)	443(6)	503(2)	3,1					
HC6	247(5)	496(6)	598(2)	3,1					
H1C7	607(4)	266(6)	695(2)	2,9(,3)					
H2C7	567(5)	96(6)	768(2)	2,9					
HC8	535(5)	584(6)	767(2)	2,9					
H1N	514(6)	289(7)	981(2)	3,8(,3)					
H2N	319(5)	417(7)	845(2)	3,8					
H3N	499(6)	528(7)	903(3)	3,8					
HO1	-150(7)	-248(9)	694(3)	4,3(,4)					
HO2	-479(9)	213(9)	493(4)	4,3					
HO4	1046(7)	422(10)	964(3)	4,3					

atom as the heavy atom, and the model was refined by the usual methods. The full-matrix least-squares refinement program applied minimizes $\sum w(F_o-F_c)^2$ where w is the inverse of the variance of the structure factor. The parameters varied were all positional coordinates (except for one origin defining y coordinate), anisotropic thermal parameters for non-hydrogen atoms and isotropic thermal parameters for hydrogen atoms. The latter were constrained to be equal for groups of hydrogen atoms with presumably equal vibrations, *i.e.* hydrogen

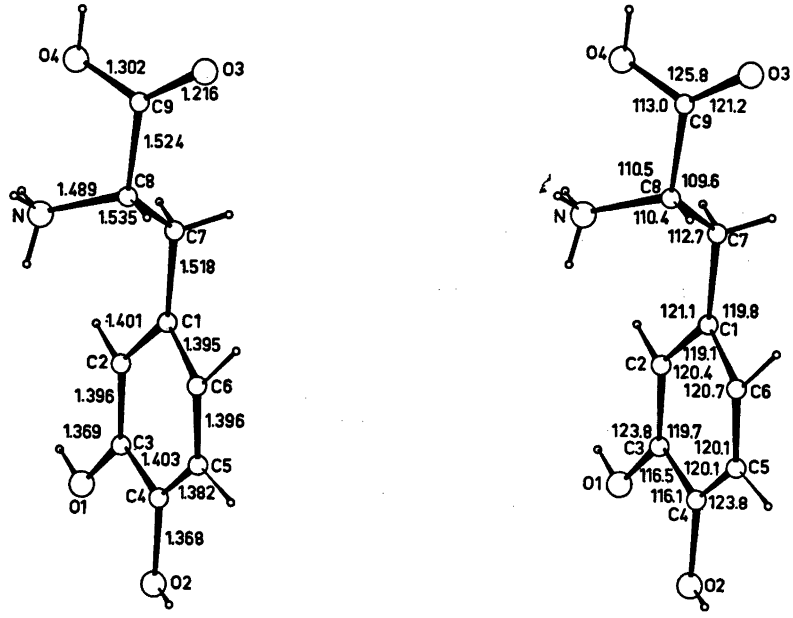

Fig. 1. a. Bond lengths (Å); b. bond angles (°) in L-DOPA hydrochloride.

Table 2. Observed and calculated structure factors (× 10).

H	K,L	Fo	Fc	H	K,L	Fo	Fc	H	K,L	Fo	Fc	H	K,L	Fo	Fc	H	K,L	Fo	Fc	H	K,L	Fo	Fc
1	18	2	9	118	115	7	16	18	2	47	45	17	34	31	8	6	5	-5	102	97	-18	64	63
2	70	75	10	100	99	8	19	20	3	121	117	16	66	62	7	66	66	-7	96	96	-19	42	40
3	63	65	11	125	129	9	31	33	4	123	120	15	12	13	6	72	65	-8	227	221	-20	37	37
4	186	199	12	25	25	4	22	23	5	143	144	14	14	15	5	75	76	-9	186	186	-21	22	20
5	121	124	13	24	22	3	38	38	6	103	101	13	101	103	4	196	192	-10	235	233	-22	17	14
6	49	43	14	52	53	2	46	46	7	48	49	12	106	107	3	178	160	-11	162	164	-23	15	18
7	240	239	15	86	97	1	51	52	8	67	67	11	167	166	2	256	249	-12	139	128	H	2,K	4
8	300	288	16	72	70	0	47	48	9	36	38	10	69	66	1	434	483	-13	98	96	-22	27	30
9	190	176	17	40	38	-1	43	44	10	71	69	9	67	65	0	91	108	-14	121	120	-21	39	38
10	179	178	18	60	58	-2	35	34	11	15	16	8	141	138	-1	34	34	-15	86	85	-20	13	14
11	88	89	19	55	53	-3	28	29	12	82	82	7	123	120	-2	55	55	-16	70	71	-19	18	18
12	60	61	20	14	9	-4	61	49	13	79	79	6	63	61	-3	451	523	-17	39	36	-18	18	20
13	28	27	H	0,K	5	-5	51	50	15	46	43	5	31	36	-4	314	308	-18	40	39	-17	53	54
14	94	94	19	43	42	-6	55	53	16	102	99	4	165	157	-5	463	498	-19	63	62	-16	31	32
15	124	121	18	15	15	-7	31	30	17	21	18	3	87	84	-6	283	281	-20	94	92	-15	92	93
16	143	142	17	20	22	-8	25	27	H	1,K	4	2	334	330	-7	239	237	-21	24	23	-14	102	103
17	30	29	16	38	38	-10	40	38	19	36	37	1	421	427	-8	222	216	-22	37	34	-13	76	75
18	15	17	15	31	33	-12	13	13	18	29	26	0	120	110	-9	162	159	-23	24	26	-12	75	75
19	57	56	14	44	46	H	1,K	7	17	74	72	-1	199	199	-10	46	42	-24	36	37	-11	76	76
20	35	34	13	23	22	-13	16	20	16	37	36	-2	203	191	-11	71	66	H	2,K	2	-10	47	47
21	11	9	12	56	56	-12	38	39	15	44	41	-3	98	90	-12	85	82	-23	14	13	-9	66	68
22	18	19	11	33	32	-11	60	59	13	63	66	-4	379	381	-13	116	114	-22	32	33	-8	68	68
H	0,K	1	10	71	70	-10	67	65	12	108	111	-5	35	19	-14	112	112	-21	21	22	-7	123	124
22	20	20	9	37	37	-9	58	58	11	85	85	-6	225	224	-15	66	65	-20	29	32	-6	155	155
21	21	21	8	17	19	-8	25	25	10	86	87	-7	182	171	-16	69	69	-19	21	22	-5	139	136
20	70	69	7	55	58	-7	27	26	9	104	107	-8	82	81	-17	14	11	-18	24	22	-4	75	72
19	34	34	6	61	60	-6	64	53	8	57	51	-9	45	46	-18	18	15	-17	22	23	-3	57	52
18	66	64	5	191	189	-5	52	53	7	24	28	-10	10	9	-19	47	46	-16	104	101	-2	77	73
17	35	36	4	43	44	-4	105	103	6	63	62	-11	66	66	-20	40	38	-15	115	116	-1	57	57
16	142	143	3	122	121	-3	21	22	5	120	122	-12	35	32	-21	40	39	-14	111	111	0	64	66
15	11	10	2	137	136	-2	68	68	4	63	62	-13	52	49	-22	29	28	-13	80	49	1	116	112
14	36	35	1	83	83	-1	66	63	3	64	62	-14	94	95	-23	14	15	-12	22	24	2	113	110
13	49	47	H	0,K	6	0	22	23	2	108	106	-15	87	86	H	2,K	0	-11	79	76	3	103	102
12	66	66	0	63	64	1	49	52	1	106	102	-16	58	55	-24	45	45	-10	46	42	4	28	29
11	101	99	1	162	161	2	19	19	0	207	201	-17	56	56	-21	90	93	-9	198	193	5	94	95
10	57	57	2	60	60	3	62	64	-1	72	74	-18	54	53	-20	17	18	-8	247	248	6	134	137
9	62	59	3	72	71	4	78	76	-2	124	121	-19	30	31	-19	26	25	-7	292	296	7	23	19
8	119	113	7	56	57	5	53	53	-3	116	115	-20	34	33	-17	49	48	-6	377	386	8	36	37
7	88	92	8	56	55	6	121	120	-4	94	88	-21	23	25	-16	79	79	-5	112	114	9	69	68
6	272	264	9	57	57	7	24	22	-5	173	173	-22	46	48	-15	173	172	-4	281	283	10	112	112
5	233	234	3	77	76	8	19	22	-6	123	122	-23	46	46	-14	115	112	-3	55	43	11	67	66
4	528	543	4	46	47	9	25	24	-7	180	175	H	1,K	1	-13	70	70	-2	62	56	12	61	64
3	101	92	9	107	104	11	37	38	-8	157	153	-23	36	35	-12	65	62	-1	155	150	13	67	66
2	465	484	10	43	42	13	29	31	-9	24	25	-22	50	48	-11	98	95	0	231	226	14	36	38
1	315	315	11	63	65	14	36	37	-10	49	47	-21	68	67	-10	96	91	1	160	177	15	14	10
H	0,K	2	12	26	26	H	1,K	8	-13	44	42	-20	21	18	-9	73	79	2	126	126	17	19	17
0	838	856	13	25	23	16	38	38	-14	89	89	-19	75	75	-8	80	49	3	72	66	18	18	16
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5	103	95	H	0,K	7	10	102	102	-21	35	35	-14	56	57	-3	273	264	8	113	114	13	33	31
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Table 2. Continued.

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- 4	72	73	-14	42	41	16	39	38	- 2	194	202	-24	40	40	3	26	26	- 1	37	37	-10	54	67						
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- 2	18	20	-12	31	33	Hs	3,Ks	2	- 4	230	234	-24	43	45	5	43	44	1	24	25	-12	47	48						
- 1	24	23	-11	41	44	18	15	12	- 5	426	404	-23	20	21	6	84	85	3	20	23	-14	21	24						
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Hs	3,Ks	7	3	59	59	-20	37	38	- 6	88	84	15	35	34	-18	19	21	- 6	13	12	8	47	46						
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-15	30	28	1	121	123	-22	39	37	- 4	63	66	Hs	4,Ks	3	Hs	4,Ks	6	- 8	54	52	10	57	58						
-14	35	36	0	18	18	-23	13	13	- 3	78	74	15	25	23	-18	27	25	- 9	42	43	11	42	40						
-13	25	24	- 1	58	58	Hs	3,Ks	1	- 2	158	154	14	18	18	-17	21	22	-10	53	55	12	58	59						
-12	27	29	- 2	70	67	-24	22	23	- 1	122	123	13	24	27	-16	17	16	-11	15	18	13	98	88						
-11	37	36	- 3	179	185	-22	12	9	0	81	80	12	35	36	-15	27	27	-12	38	39	Hs	5,Ks	2						
-10	56	55	- 4	206	209	-21	17	17	1	38	34	11	70	68	-13	37	35	-13	23	22	14	19	16						
- 9	32	33	- 5	95	93	-20	14	12	2	125	121	10	71	72	-12	16	16	-14	16	15	13	14	13						
- 8	53	54	- 6	135	137	-19	27	27	3	17	16	9	51	53	-11	31	31	-16	21	22	12	16	17						
- 7	21	18	- 7	86	84	-18	45	45	4	32	35	8	51	49	-10	70	69	-17	14	20	11	34	34						
- 6	32	33	- 8	54	55	-17	108	110	5	93	88	7	22	21	- 9	47	46	-18	17	16	10	14	19						

Table 2. Continued.

9	36	36	2	108	114	-20	34	35	- 5	69	67	-17	31	31	- 6	74	72	- 4	46	45	- 6	13	14
8	30	30	1	48	45	-23	23	19	- 4	43	42	-16	16	15	- 7	37	37	- 3	51	51	- 7	15	15
7	90	92	0	88	88	-24	24	21	- 3	55	53	-15	17	16	- 8	38	39	- 2	97	96	- 6	17	18
6	84	82	- 1	130	138	Hm	6,Ka	2	- 2	39	38	-14	94	90	- 9	47	45	0	30	29	- 5	32	32
5	22	23	- 4	20	22	-23	35	31	- 1	63	63	-13	36	35	-10	16	16	1	20	15	- 4	39	39
4	47	48	- 5	166	167	-21	21	24	0	75	74	-12	15	13	-11	26	31	2	136	139	- 3	28	30
3	45	43	- 6	106	106	-20	37	36	1	66	66	-11	52	52	-12	23	24	3	27	27	- 2	29	27
2	22	20	- 7	49	51	-19	38	36	2	43	42	-10	64	66	-13	50	50	4	19	14	- 1	35	33
1	73	76	- 8	169	170	-18	14	9	3	69	67	- 9	15	17	-14	78	78	Hm	6,Ka	1	0	20	21
0	48	49	- 9	121	120	-17	52	58	4	46	43	- 8	49	49	-15	61	49	5	24	22	1	29	32
- 1	129	128	-10	124	125	-16	46	47	5	25	24	- 7	13	16	-17	35	37	4	24	32	Hm	8,Ka	5
- 2	80	82	-12	35	40	-15	65	66	6	27	31	- 5	12	9	-19	13	11	5	44	46	- 2	16	17
- 3	139	141	-13	60	60	-14	104	107	7	59	61	- 4	58	57	-20	14	9	2	80	80	- 3	59	53
- 4	109	109	-14	17	22	-13	35	37	8	52	50	- 3	69	68	-21	15	14	1	35	36	- 4	24	22
- 5	23	22	-16	10	6	-12	37	38	9	33	33	- 2	40	39	-22	24	27	0	59	54	- 5	16	18
- 6	107	108	-17	65	69	-11	14	11	Hm	6,Ka	5	- 1	28	27	Hm	7,Ka	1	- 2	70	69	- 6	19	20
- 7	141	141	-18	29	28	-10	48	46	7	22	24	0	11	7	-20	44	41	- 3	59	60	- 7	40	39
- 8	125	128	-19	38	36	- 9	96	96	6	22	23	1	17	16	-19	76	76	- 4	54	55	- 8	40	39
- 9	188	190	-20	34	31	- 8	122	124	5	32	35	Hm	7,Ka	4	-18	29	31	- 5	34	32	- 9	34	35
-10	45	42	-21	101	100	- 7	105	104	3	39	40	6	35	39	-17	40	44	- 6	103	100	-10	28	27
-11	67	66	-22	58	64	- 6	107	106	2	19	19	5	32	30	-16	24	21	- 7	56	56	-12	15	16
-12	16	15	-23	11	11	- 5	35	35	1	34	32	4	22	24	-15	43	44	- 8	53	52	-13	18	16
-13	24	24	Hm	6,Ka	0	- 3	57	60	0	25	25	3	27	25	-13	69	68	- 9	48	47	Hm	9,Ka	3
-14	36	37	-24	39	39	- 2	31	30	- 1	32	32	2	66	64	-12	76	76	-10	44	43	-14	23	23
-15	15	13	-23	37	40	- 1	66	65	- 2	42	40	1	42	42	-11	62	60	-11	24	25	-13	21	24
-16	29	31	-22	21	20	0	74	74	- 3	32	33	0	37	38	-10	49	48	-13	49	47	-12	27	26
-18	46	45	-20	41	40	1	105	107	- 4	51	50	- 1	43	42	- 9	76	76	-14	25	21	-11	18	15
-19	52	53	-19	37	36	2	83	83	- 5	101	101	- 2	63	61	- 8	49	47	-15	23	27	-10	17	16
-20	52	50	-18	13	11	3	66	67	- 6	81	79	- 3	28	27	- 7	28	26	-16	53	53	- 8	15	15
-21	27	23	-17	22	23	4	46	45	- 7	33	34	- 4	35	33	- 6	24	24	-17	35	34	- 7	34	33
-22	11	10	-16	67	69	5	43	40	- 9	29	30	- 5	24	23	- 5	108	107	-18	84	83	- 6	17	13
-23	23	29	-15	89	89	6	62	64	-10	30	28	- 6	19	21	- 4	79	79	-19	34	31	- 5	25	23
-24	24	21	-14	70	70	7	75	76	-11	24	23	- 7	76	75	- 3	81	79	-21	20	19	- 4	23	20
Hm	9,Ka	1	-13	13	12	8	94	96	-12	42	42	- 8	36	34	- 2	35	37	Hm	8,Ka	2	Hm	9,Ka	2
-24	37	37	-12	18	16	9	44	42	-13	49	47	- 9	34	32	- 1	21	21	-20	13	12	- 1	36	37
-23	51	53	-10	144	141	10	66	66	-14	30	27	-11	33	31	0	46	44	-18	31	27	- 2	61	59
-22	26	27	- 9	53	50	11	30	30	-15	20	19	-12	16	16	1	43	42	-17	38	36	- 3	21	20
-21	33	38	- 8	143	144	Hm	6,Ka	3	-18	21	19	-13	37	37	2	35	36	-16	47	46	- 4	42	37
-20	34	37	- 7	235	237	11	30	28	-19	34	33	-14	55	57	4	53	59	-15	37	36	- 5	31	30
-19	50	51	- 6	58	58	10	17	17	Hm	6,Ka	6	-15	62	58	5	82	85	-14	54	52	- 6	23	22
-18	57	61	- 5	18	23	9	42	38	-16	18	19	-16	39	40	6	22	25	-13	46	44	- 9	33	28
-17	45	42	- 4	29	29	8	63	64	-15	39	40	-17	26	25	7	50	59	-12	25	23	-10	36	35
-16	21	20	- 2	112	112	7	50	54	-14	12	11	-18	19	20	8	28	38	-11	29	29	-11	22	21
-15	53	49	- 1	41	45	5	22	21	-13	29	30	Hm	7,Ka	3	Hm	7,Ka	0	-10	29	29	-12	23	22
-14	117	121	0	124	126	4	62	61	-12	45	46	-21	12	3	9	26	25	- 9	13	14	-13	46	43
-13	112	119	1	102	107	3	56	54	-11	53	49	-20	38	39	8	27	25	- 8	47	44	-14	25	25
-12	110	114	2	89	87	2	14	13	-10	27	29	-19	46	45	7	12	13	- 7	45	43	-15	33	29
-11	51	53	3	88	89	1	13	13	- 9	68	67	-18	47	46	6	41	42	- 6	16	15	-16	26	25
-10	58	59	4	44	41	0	33	33	- 8	64	64	-17	37	38	4	63	70	- 4	35	36	-17	16	15
- 9	32	32	5	44	47	- 1	39	37	- 7	33	34	-16	26	26	3	56	58	- 2	84	80	Hm	9,Ka	1
- 8	44	44	7	87	88	- 2	63	63	- 6	32	31	-15	44	43	2	25	26	- 1	44	43	-18	16	15
- 7	45	47	8	78	82	- 3	60	60	- 4	23	20	-13	14	14	1	31	32	0	12	15	-17	30	29
- 6	53	51	9	50	51	- 4	93	93	- 3	12	14	-12	19	21	0	46	44	1	64	67	-16	11	6
- 5	280	284	10	14	13	- 5	34	36	- 1	64	62	-11	79	78	- 1	22	24	2	35	32	-14	12	12
- 4	235	238	11	12	16	- 6	40	41	0	29	31	-10	25	23	- 2	41	39	3	16	20	-13	15	14
- 3	153	153	12	24	22	- 7	36	35	1	51	51	- 9	47	44	- 3	126	119	4	12	19	-12	27	28
- 2	57	64	Hm	6,Ka	1	- 8	62	58	2	55	55	- 8	35	33	- 4	48	46	Hm	8,Ka	3	-11	14	15
- 1	32	29	12	40	42	- 9	19	18	3	20	21	- 7	29	29	- 5	55	54	3	36	40	-10	23	20
0	49	53	11	32	36	-10	17	18	4	25	25	- 6	19	18	- 6	10	12	2	45	44	- 9	31	27
1	106	109	10	15	13	-11	85	89	5	17	15	- 5	32	32	- 7	28	30	1	34	34	- 8	32	31
2	86	86	9	66	62	-12	68	68	Hm	6,Ka	7	- 4	71	72	- 8	17	16	0	40	39	- 7	22	21
3	150	154	8	52	52	-13	25	25	- 2	32	30	- 3	68	67	- 9	90	88	- 1	17	17	- 6	14	14
4	89	90	6	54	52	-14	13	11	- 3	37	37	- 2	81	81	-10	72	73	- 2	58	56	- 5	55	52
5	39	39	5	18	18	-15	43	44	- 4	45	44	- 1	21	20	-11	44	44	- 3	49	48	- 4	44	40
6	81	81	4	29	29	-16	17	19	- 5	31	31	0	50	47	-12	27	31	- 4	27	27	- 3	18	19
7	64	62	3	15	11	-17	11	11	- 7	12	12	1	37	33	-13	162	163	- 5	26	25	- 2	34	32
8	51	50	2	22	23	-18	28	30	- 8	17	15	2	37	35	-14	18	20	- 6	34	34	- 1	33	34
9	38	38	1	27	29	-19	29	30	- 9	28	29	3	23	25	-15	57	57	- 7	73	69	0	27	30
10	60	62	0	20	18	-20	20	20	-10	12	16	4	49	51	-16	30	28	- 8	47	48	Hm	9,Ka	0
11	115	117	- 1	52	51	-21	26	23	-11	23	22	5	26	27	-18	47	48	- 9	61	61	1	11	11
12	60	59	- 2	46	45	-22	11	12	Hm	7,Ka	6	6	28	32	-19	20	17	-10	18	19	0	12	15
13	33	34	- 3	152	155	Hm	6,Ka	4	0	34	32	7	36	41	-21	11	15	-11	32	30	- 1	68	64
14	16	17	- 4	88	89	-21	14	11	- 1	19	17	Hm	7,Ka	2	-23	25	26	-12	36	33	- 2	23	20
Hm	9,Ka	0	- 5	58	59	-20	16	15	- 2	27	25	8	13	19	Hm	8,Ka	0	-13	23	23	- 3	79	77
15	31	30	- 6	50	49	-18	34	34	- 3	30	31	7	21	23	-20	17	18	-14	14	18	- 4	19	22
14	40	39	- 7	104	104	-17	28	28	- 4	25	26	6	75	74	-19	25	22	-15	22	22	- 5	29	26
13	14	12	- 8	84	82	-16	39	38	- 5	14	11	4	52	56	-18	22	26	-16	23	23	- 6	12	17
12	17	19	- 9	86	87	-15	48	50	- 6	23	19	3	55	53	-17	11	8	-17	50	48	- 7	42	41
11	53	56	-10	102	102	-14	35	35	- 7	45	44	2	28	29	-15	11	6	-18	54	51	- 8	45	43
10	49	62	-11	75	77	-13	39	42	- 8	37	37	1	92	90	-14	84	82	-19	16	14	- 9	41	40
9	13	13	-12	36	38	-12	11	8	- 9	21	23	0	52	52	-13	18	14	Hm	8,Ka	4	-10	17	19
8	44	44	-13	51	50	-11	33	34	-10	34	34	- 1	26	24	-12	40	39	-16	24	23	-11	34	32
7	100	104	-14	26	23	-10	41	42	-11	16	15	- 2	39	35	- 8	34	32	-14	38	37	-12	51	49
6	69	71	-15	31	31	- 9	79	81	-12	38	38	- 3	78	74	- 7	56	53	-13	41	41	-13	34	35
5	43	41	-16	44	40	- 8	128	130	-13	25	24	- 4	72	70	- 6	36	34	-12	27	25	-14	36	36
4	23	23	-18	37	37	- 7	76	77	Hm	7,Ka	5	- 5	29	29	- 5	17	18	- 9	28	28	-18	24	22
3	25	36	-19	46	48	- 6	30	29															

atoms bonded to the benzene nucleus, those bonded to the carbon atoms of the side chain, those bonded to nitrogen, and the hydroxyl hydrogen atoms.

The analyses of $w\Delta^2$ as functions of F_o and of sin θ/λ showed that the variances of the structure factors had been underestimated at low scattering angles; thus, during the last stages of the least-squares refinement structure factors with sin θ/λ less than 0.3 were ignored, leaving 2122 observations for the determination of 175 parameters. The refinement converged with a conventional R-factor of 0.030 for the data actually utilized (0.032 for the total data set), the final R_w was 0.035.

The atomic parameters and their standard

Table 3. Bond lengths (Å), bond angles (°) and hydrogen bond data for L-DOPA hydrochloride. Standard deviations in distances and angles involving only heavy atoms are 0.002 Å and 0.1°, respectively.

Bond		Corrected	Angle		Angle	
C1–C2	1.398	1.401	C6–C1–C2	119.1	C7–C8–N	110.4
C2–C3	1.395	1.396	C1–C2–C3	120.4	N–C8–C9	110.5
C3–C4	1.399	1.403	C2–C3–C4	119.7	C8–C9–O3	121.2
C4–C5	1.379	1.382	C3–C4–C5	120.1	C8–C9–O4	113.0
C5–C6	1.394	1.396	C4–C5–C6	120.1	O3–C9–O4	125.8
C6–C1	1.391	1.395	C5–C6–C1	120.7	C2–C3–O1	123.8
C1–C7	1.516	1.518	C2–C1–C7	121.1	O1–C3–C4	116.5
C7–C8	1.531	1.535	C6–C1–C7	119.8	C3–C4–O2	116.1
C8–C9	1.522	1.524	C1–C7–C8	112.7	O2–C4–C5	123.8
C3–O1	1.367	1.369	C7–C8–C9	109.6		
C4–O2	1.367	1.368	Dihedral angle			
C9–O3	1.213	1.216	C4–C3–O1–H	174		
C9–O4	1.300	1.302	C3–C4–O2–H	171		
C8–N	1.485	1.489	C6–C1–C7–C8	−70		
			C1–C7–C8–C9	178		
			N–C8–C9–O4	−13.3		

Hydrogen bond		D···A	H···A	∠D–H···A
O1–HO1···O3	$(-1+x, -1+y, z)$	2.899	2.16	166
O2–HO2···O1	$(-1-x, \frac{1}{2}+y, 1-z)$	2.992	2.41	142
O2–HO2···O2	$(-1-x, \frac{1}{2}+y, 1-z)$	3.040	2.42	150
O4–HO4···Cl	$(1+x, y, 1+z)$	2.947	2.18	169
N–H1N···Cl	$(1-x, -\frac{1}{2}+y, 1-z)$	3.161	2.29	161
N–H2N···O3	$(-1+x, y, z)$	2.993	2.16	162
N–H3N···Cl	$(1-x, \frac{1}{2}+y, 1-z)$	3.094	2.33	152

deviations are given in Table 1, observed and calculated structure factors are listed in Table 2.

A rigid-body analysis showed that the thermal motion of the molecule to a fair approximation could be interpreted in terms of translational and librational oscillations; bond lengths were corrected for thermal libration.

Standard deviations in interatomic distances and bond angles were calculated from the correlation matrix ignoring uncertainties in unit cell parameters. Standard deviations in distances between non-hydrogen atoms are 0.002 Å and in angles 0.1°. If a hydrogen atom is involved the figures are 0.05 Å and 3–5°, respectively.

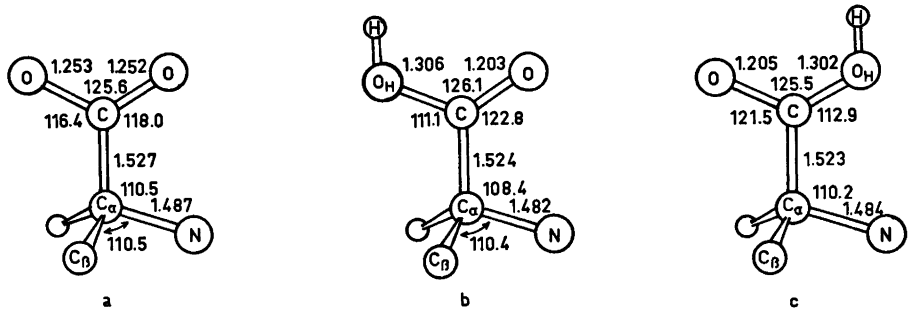

Fig. 2. Structural data for α-amino acids. a. The zwitterion (Marsh and Donohue [8]). b. Protonized group, carbonyl group *syn*-planar to nitrogen (Sundaralingam and Putkey [9]). c. Protonized group, carbonyl group *anti*-planar to nitrogen (present work).

Table 4. Structural data for three α-amino acids with the hydroxyl group *syn*-planar with respect to the nitrogen atom. Standard deviations in bond lengths are 0.002 Å, 0.005 Å, and 0.003 Å, respectively.

	DOPA.HCl	Mimosine. H_2SO_4	Histidine.HCl	Weighted mean
C=O	1.213 Å	1.202 Å	1.196 Å	1.205 Å
C−O(H)	1.300 Å	1.289 Å	1.313 Å	1.302 Å
C_α−C	1.522 Å	1.524 Å	1.525 Å	1.523 Å
C_α−N	1.485 Å	1.487 Å	1.482 Å	1.484 Å
O=C−O	125.8°	126.3°	124.9°	125.5°
O=C−C	121.2°	119.1°	123.4°	121.5°
O−C−C	113.0°	114.7°	111.6°	112.9°
C−C−N	110.5°	110.9°	109.2°	110.2°
Dihedral angle N−C−C−O(H)	−13°	6°	25°	

DISCUSSION

Bond lengths and angles are given in Table 3 and in Fig. 1, *a* and *b*. The numbering of the atoms is not the same as that given by Jandacek and Earle [3] but corresponds to that given for L-DOPA.[7] Compared to the original structure determination of L-DOPA. HCl the standard deviations of the present refinement are improved by factors between 7 and 10.

The geometry of the L-DOPA ion is nearly identical to that of the L-DOPA molecule as found in the crystals of the pure compound,[7] the obvious deviation being that of the alanine moiety which contains a carboxylate group in L-DOPA and a carboxyl group in the hydrochloride. The similarities apply to both bond lengths and angles as well as to the conformation.

The C8−C9 bond is slightly shorter in the protonized group than in the carboxylate group. The difference is highly significant even if no such difference exists between the mean value of the corresponding bond length (1.527 Å) in α-amino acids given by Marsh and Donohue [8] as compared to the value for protonized α-amino acids (1.524 Å) given by Sundaralingam and Putkey [9] (*cf.* Fig. 2).

The main reason for taking up the present refinement was to determine the structure of the carboxyl group with this special conformation about the C−Cα bond. As pointed out by several authors (*e.g.* Refs. 9, 10, 11) the conformation about this bond in α-substituted carboxylic acids is such as to bring the carbonyl oxygen atom in a *syn* planar arrangement with respect to the α-substituent. As far as α-amino acids are concerned no exception to this rule has been reported until recently. However, in the present structure it is the hydroxyl group which is found *syn* relative to the nitrogen atom; the dihedral angle N−C−C−O(H) is equal to −13.3°. The same conformation has been found in mimosine sulfate [1] (dihedral angle 5.6°) and in histidine hydrochloride.[2]

The structural data for the α-amino acid group (not corrected for thermal motion) for the three compounds found to have this uncommon conformation are given in Table 4. The weighted mean values are given in Fig. 2 together with the corresponding values given by Marsh and Donohue [8] for a zwitterionic group and those given by Sundaralingam and Putkey [9] for protonized α-amino acids with the carbonyl group in the *syn*-planar conformation.

From the data given in Fig. 2 it appears that the 180° rotation about the C−Cα bond has only a small, if any, influence on the Cα−C−O angles. However, Table 4 indicates a consistent variation of the C−C−O angles with the torsion angle N−C8−C9−O as would be expected if a repulsion between the carboxyl oxygen atoms and the substituents on the Cα carbon atom is postulated. Mean values such as those given in Fig. 2 should thus be used with caution.

The packing of ions in the crystals of L-DOPA hydrochloride has been discussed in detail by Jandacek and Earle.[3] The hydrogen bond lengths found in the present refinement are listed in Table 3; they are in good agreement with those given in the original paper. It was

suggested, however, that the chlorine ion is hydrogen acceptor in *four* hydrogen bonds in a way involving a set of bifurcated hydrogen bonds. After having localized the hydrogen atoms of the ammonium group we believe that only *three* of the interactions referred to may be described as proper hydrogen bonds; the remaining short N – Cl contact is 3.171 Å, whereas the hydrogen atoms bonded to this nitrogen atom are situated 2.80 Å or more from the chlorine ion. This short contact is thus better classified as a short van der Waals contact rather than a weak hydrogen bond, in which case the bifurcated hydrogen bonds reduce to one normal N – H···Cl hydrogen bond.

REFERENCES

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Isolation of ^{32}P-Labeled Phosphorylserine and Phosphorylthreonine from Ehrlich Mouse Ascites Tumor Cells Suspended in Different Isotonic Media Containing ^{32}P-Labeled Adenosine Triphosphate

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Intact Ehrlich tumor ascites cells were handled under physiological conditions and incubated for short periods with [^{32}P]ATP in isotonic media of varying cationic composition. Labeled phosphorylserine, phosphorylthreonine as well as three additional labeled fractions were isolated from a partial hydrolysate of the phosphoproteins in the outer layer of the plasma membrane.

The specific ^{32}P radioactivity was about the same for the phosphorylserine and the phosphorylthreonine fractions as well as for an additional one (peak 1, Fig. 1).

A complete amino acid analysis was carried out on the five fractions. As expected serine and threonine were most abundant in the corresponding phosphorylated fractions. Surprisingly, a high amount of proline was found in the sample corresponding to peak 1, having the same high specific radioactivity as found for phosphorylserine and phosphorylthreonine.

Cyclic AMP even in as high a concentration as 1×10^{-4} M did not stimulate a further phosphorylation. Maximal incorporation occurred in the presence of both Na^+ and K^+ as well as Mg^{2+}. If Mg^{2+} was stoichiometrically exchanged for Ca^{2+}, the incorporating activity decreased by more than 80 %. If half the amount of Mg^{2+} was excanged for Ca^{2+}, the corresponding activity was 50 % lower. With only Mg^{2+} and a monovalent cation present choline stimulated more than Na^+, which in its turn was more stimulatory than K^+. Possible functions of the labeled phosphoproteins are discussed.

Previous work from this laboratory has shown that [^{32}P]-labeled phosphorylserine as well as phosphorylthreonine can be isolated from Ehrlich mouse ascites tumor cells incubated under isotonic conditions with [γ^{32}P]ATP or [γ^{32}P]GTP. The extent of phosphorylation is much lower when either of these two nucleotides is replaced by CTP, UTP or pyrophosphate and is negligible when replaced by [^{32}P] orthophosphate. [1–3] It was concluded that the reaction between [γ^{32}P]ATP and the cells took place at the cell surface because ATP does not penetrate the intact cell membrane [4] and the cells remained intact throughout the experiments.

The labeled phosphoryl groups in the two phosphorylated amino acid residues represented only a small fraction of the total amounts of labeled orthophosphate liberated from [γ^{32}P] ATP at the cell surface. This type of phosphorylation may represent either an intermediary phosphorylation of an enzyme or a regulatory phosphorylation of a protein in the membrane. The latter type of reaction is catalyzed by a protein kinase. Furthermore, an intracellular protein kinase has been shown to be sensitive to cyclic AMP stimulation.[5,6]

In the present investigation, we have studied the effect of adding cyclic AMP to the incubation medium and of varying the cationic composition of the isotonic medium on the transfer of phosphate from external ATP to seryl- and threonyl residues of proteins presumably located at the cell surface.

MATERIAL AND METHODS

The Ehrlich mouse ascites tumor cells were grown for 7 – 8 d in 5 week old Swiss albino

mice obtained from the Anticimex breeding farm, Norrviken, Stockholm. The tumor cells were separated by centrifugation of the ascitic fluid, which had been diluted without delay several-fold with icecold Krebs-Ringer bicarbonate medium in order to diminish the tendency of cell agglutination. The cells were washed once in the Krebs-Ringer bicarbonate medium and the final washing was carried out in a medium with the same buffer and cationic composition as the incubation medium. The [γ^{32}P]ATP used was prepared as previously described.[3]

The cells were incubated in a medium containing either 130 mM sodium chloride and 25 mM potassium chloride or 155 mM of one single cationic salt (NaCl, KCl, choline Cl) to maintain isotonicity. The incubation procedure as well as the isolation of the phosphorylated amino acid residues have been described earlier.[1,2] The Schneider protein fraction was isolated from the cell [7] and partially hydrolyzed according to Lipmann. [8] Radioactivity was determined in a Nuclear Chicago Scintillation counter by measuring the Cherenkov radiation.

RESULTS

Fig. 1 illustrates the elution pattern from a typical experiment where 500 mg of a partial hydrolysate of the Schneider protein from incubated Ehrlich cells was separated on a Dowex 50 column. Five peaks can be distinguished. The two main peaks contain [^{32}P] phosphorylserine and [^{32}P]phosphorylthreonine residues. In addition three smaller labeled peaks are distinguished, the first in position

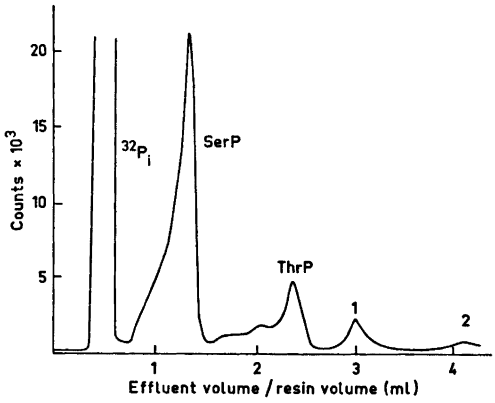

Fig. 1. Elution curve from a partial hydrolysate of the Schneider protein from incubated intact Ehrlich cells. Chromatography was performed on a 50 ml Dowex 50 (8 % DVB) column with 0.01 M HCl as eluent.

Table 1. Total radioactivity of the five fractions collected during chromatography on Dowex 50 (Fig. 1) together with their relative specific activities.

Fraction obtained[a]	Total ^{32}P-radio-activity[b]	Fraction weight[c]	Specific ^{32}P-radio-activity[d]
SerP	8 317	12	100
Peak between SerP and ThrP	630	6	15
ThrP	2 384	3	115
Peak 1	674	1	97
Peak 2	177	3	8

[a] Peak 3, occasionally found, is not included because of its very low specific activity. [b] Activity expressed in counts per min. [c] Schneider protein, mg [d] Expressed as percentage of the specific activity of SerP.

between phosphorylserine and phosphorylthreonine and the other two after the phosphorylthreonine peak. Occasionally a third peak after phosphorylthreonine could be observed.

Table 1 summarizes the total amount of material corresponding to each peak as well as total ^{32}P-activity. It is evident from the table that the specific activity of the [γ^{32}P]-phosphoryl group of ATP incorporated into the five fractions was about the same for the phosphorylserine and phosphorylthreonine fractions and the first fraction after phosphorylthreonine while it was significantly lower for the two other peaks.

Each fraction collected from the column was submitted to total acid hydrolysis and the amino acid content was analyzed. The amino acid content of each of the five fractions are given in Table 2. Serine residues were predominant in the [^{32}P]phosphorylserine fraction as is seen in the table.

Only slight amounts of threonine were detected in that fraction. Glutamic acid was present in a higher amount than any other amino acids with the exception of serine. Likewise, in the small fraction eluted between phosphorylserine and phosphorylthreonine, glutamic acid and serine were predominant.

The threonine content of the phosphorylthreonine fraction was high compared with the amount of serine. Even here as was the case for the phosphorylserine fraction glutamic

Table 2. Amino acid composition of the five radioactive fractions collected during chromatography on Dowex 50 of the partially hydrolyzed Schneider protein from Ehrlich cells.

Amino acid composition	Serine[a]	Threonine	Glutamic acid	Leucine	Isoleucine	Proline	Glycine	Valine	Aspartic acid	Alanine	Cystine	Phenylalanine[c]
SerP	216[b]	5	39	12	13	5	10	6	6	5	<5	<5
Peak between SerP and ThrP	30	9	34	8	5	11	9	12	<5	7	<5	5
ThrP	8	27	17	<5	<5	12	7	<5	<5	<5	<5	<5
Peak 1	13	5	24	8	10	126	6	14	<5	<5	5	6
Peak 2	9	<5	11	5	5	5	5	<5	<5	<5	<5	<5

[a] Amino acid analysis was carried out according to Stein and Moore using a Biocal BC 200 analysator. [b] Figures denote nmol per 100 mg Schneider protein from Ehrlich cells. [c] Tyrosine, methionine, arginine, histidine, and lysine were all less than 5 nmol per 100 mg Schneider protein in all fractions.

Table 3. Incubation of Ehrlich cells for 30 s at 37 °C with [γ^{32}P]ATP in a medium made isotonic by 130 mM NaCl, 25 mM KCl, and 2 mM $MgCl_2$.

Medium[a]	SerP	ThrP	Peak 1
1. Na^+, K^+, Mg^{2+}	100[b]	100	100
2. As in 1 plus 3′,5′-AMP (10^{-4} M)	90	90	84
3. As in 1 plus 3′,5′-AMP (10^{-6} M)	87	92	83

[a] Unlabeled orthophosphate concentration was 1×10^{-3} M in all media. The incubation was terminated with trichloroacetic acid giving a final conc of 5 %. [^{32}P]phosphorylserine (SerP), [^{32}P]phosphorylthreonine (ThrP), and [^{32}P]-labeled peptide material (Peak 1) were isolated from the acid insoluble material as described in Methods. [b] The figures given denote percentage values of phosphoryl group incorporation obtained in a medium containing Na^+, K^+ and Mg^{2+} but lacking 3′,5′-AMP. All figures are average values from two experiments.

acid was present in a comparatively high amount. The fraction corresponding to peak No. 1, Fig. 1, contained both serine and threonine in a relationship of 2.6 to 1. The amounts of glutamic acid in this fraction were again relatively high. Proline, however, was the predominant amino acid of this fraction exceeding by nearly ten times the amount of serine.

In order to elucidate the effect of cyclic AMP on the phosphorylation of the different membraneous protein fractions, experiments with [γ^{32}P]-labeled ATP were carried out with the addition of two different concentrations of cyclic AMP (10^{-6} M and 10^{-4} M, respectively) to the isotonic medium. As is seen in Table 3 cyclic AMP, even at the higher concentration, does not stimulate the phosphorylation by [γ^{32}P]ATP.

The effects of different cations on the phosphorylation of the phosphorylserine and phosphorylthreonine residues are given in Table 4.

Table 4. Incubation of Ehrlich cells for 1 min at 37 °C with [γ^{32}P]ATP in an isotonic medium with variation of the cationic composition.

Medium[a]	SerP	ThrP	Peak 1	Preak 2
1. Na^+, K^+, Mg^{2+}[b]	401.6[c] (100)[d]	100.9 (100)	31.2 (100)	13.8 (100)
2. Na^+, K^+, Mg^{2+}, Ca^{2+}	210.3 (52.4)	39.3 (39.9)	11.4 (36.5)	0
3. Na^+, K^+, Ca^{2+}	71.2 (17.7)	14.0 (13.9)	0	0
4. Na^+, Mg^{2+}	189.8 (47.3)	47.8 (47.4)	14.7 (47.1)	5.4 (39.5)
5. K^+, Mg^{2+}	142.2 (35.4)	32.0 (31.7)	12.2 (39.2)	0
6. $Choline^+$, Mg^{2+}	293.3 (73.0)	69.1 (68.5)	21.7 (69.7)	0

[a] Unlabeled orthophosphate concentration was 1×10^{-3} M in all media. The incubation was terminated with trichloroacetic acid giving a final conc of 5 %. [^{32}P]Phosphorylserine (SerP), [^{32}P]phosphorylthreonine (ThrP) and [^{32}P]-labeled peptide material (Peak 1 and Peak 2) were isolated from the acid insoluble material as described in Methods. [b] 1. 130 mM Na^+, 25 mM K^+ and 2 mM Mg^{2+}; 2. Na^+ and K^+ as in 1, with 1 mM Mg^{2+} and 1 mM Ca^{2+}; 3. Na^+ and K^+ as in 1 with 2 mM Ca^{2+}; 4. 155 mM Na^+ and 2 mM Mg^{2+}; 5. 155 mM K^+ and 2 mM Mg^{2+}; 6. 155 mM $choline^+$ and 2 mM Mg^{2+}. [c] Figures denote [^{32}P]phosphoryl groups incorporated in pmol per 100 mg Schneider protein from Ehrlich cells. [d] Figures in brackets denote percentage value of that obtained under standard incubation conditions (1. Na^+, K^+, Mg^{2+}).

When the Mg^{2+} concentration and the ATP/-Mg^{2+} ratio are kept constant, the phosphorylation is greatest when both Na^+ (130 mM) and K^+ (25 mM) are present. If all Mg^{2+} is replaced stoichiometrically by Ca^{2+} under otherwise similar experimental conditions the phosphorylation observed represents only 18 % of the maximum phosphorylation. This phosphorylation can be increased by replacing half the amount of Ca^{2+} by Mg^{2+}.

Experiments were also made in the presence of Mg^{2+} and [γ^{32}P]ATP as described above, but in the presence of only *one* monovalent cation (155 mM). The monovalent cations tested were: sodium, potassium, and choline. Most phosphorylation was obtained with choline (73 % of maximum value), whilst with sodium and potassium phosphorylation proceeded to the extent of 47 % and 35 %, respectively, of the maximum value.

The amount of labeled phosphorylthreonine residue was in all experiments 4 – 5 times smaller than that of the phosphorylserine residue [3] regardless of extracellular ionic conditions.

DISCUSSION

The time interval between the removal of the tumor cells from the donor animals and the incubation never exceeded 50 min. In addition, the cells were handled in a physiological buffer at 4 °C during preparation. We therefore have every reason to believe that the cells were intact during the incubation. In addition, no macroscopic signs of cell damage, *e.g.* any tendency of agglutination, were observed during the incubation. Since [γ^{32}P]ATP does not penetrate an intact plasma membrane [4] we conclude that the phosphorylation reactions observed occur at the cell surface.

Phosphorylserine and phosphorylthreonine residues from a protein hydrolysate are indicative of either an intermediate phosphorylation of certain enzymes [9,10] or a regulatory phosphorylation catalyzed by protein kinase.[6]

Alkaline phosphatase, an enzyme forming an intermediate which is phosphorylated at a phosphorylserine residue,[3] has been shown by de Thé [11] to be associated with the outer surface of the plasma membrane of thymomas of leukemic mice. Thus, alkaline phosphatase activity might also be associated with the outer surface of the plasma membrane of Ehrlich tumor cells. However, Engström [8] has shown that alkaline phosphatase is also phosphorylated with [^{32}P]orthophosphate as substrate. But since [^{32}P]orthophosphate does not act as a phosphoryl donor at the Ehrlich cell surface [3] and labeled phosphorylthreonine has never been isolated from alkaline phosphatase (Engström, personal communication 1974), we consider it unlikely that the observed labeling involves an alkaline phosphatase on the cell surface. In addition, although the experimental conditions have been varied as regards substrates,[3] ionic composition of the incubation medium as well as the incubation time the ratio between phosphorylserine and phosphorylthreonine has remained constant. This fact favors the view that one single entity of protein(s) is phosphorylated with [γ^{32}P]ATP.

Other enzymes (*e.g.* phosphoglucomutase) are probably also phosphorylated exclusively at a serine residue in the active centre. The hypothesis of an intermediate enzymatic phosphorylation would therefore appear untenable.

The phosphorylation studied here might well represent a regulatory phosphorylation catalyzed by one or more protein kinases localized at the surface of the plasma membrane. In this connection it may be mentioned that Matsumura and Takedo [12] have observed that a phosphorylation probably representing a regulatory function occurs in the cytosol of rat liver cells. Furthermore, they were able to separate two main fractions one catalyzing mainly the phosphorylation of a seryl residue, the other mainly a threonyl residue. The importance of the phosphorylation of the threonyl residue in our study is emphasized by the fact that the specific activities of the phosphorylserine and phosphorylthreonine residues were about the same (Table 1).

We made the interesting observation that cyclic AMP even at high concentrations (1×10^{-4} M) did not stimulate the assumed protein kinase reaction. This does not exclude the possible existence of a regulatory phosphorylation system localized at the surface of the cell membrane. This behavior might reflect an adaptation to the physiological conditions existing in the plasma membrane architecture when adenyl cyclase is believed to be located

on the inner surface of the plasma membrane.[13] Adenyl cyclase activity has never been possible to demonstrate at the outer surface of several types of tumor cells.[14] The possible existence of external ATP as a substrate in a "membraneous metabolic pool" has been discussed earlier.[15] Recent reports concerning the effects of *extracellular* ATP on different cells [16–19] might be explained by protein kinase action at the outer surface of the cell.

Our results demonstrate the presence of metabolically active proteins on the outside of the plasma membrane. In accordance with this observation it is interesting to note the recent finding of the occurrence of surface peptides on intact Ehrlich cells.[20]

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Studies on Orchidaceae Alkaloids. XL.* Biosynthetic Studies of (−)-Cryptostyline I in *Cryptostylis erythroglossa* Hayata

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The biosynthesis of (−)-cryptostyline I (I) has been studied using radioactive precursors, the position of the radiolabel being determined by subsequent degradation.

The biosynthetic results show that 3,4-dimethoxyphenethylamine, *N*-(3-hydroxy-4-methoxybenzyl)-3-hydroxy-4-methoxyphenethylamine (XI) and 1-(3,4-methylenedioxyphenyl)-6,7-dimethoxy-2-methyl-3,4-dihydroisoquinolinium bromide (II) are specifically incorporated. Preliminary results indicate that dopamine is a precursor of the 1-phenyl group and the C-1 carbon atom. Vanillin is shown to be better incorporated than isovanillin.

* For number XXXIX, see Ref. 1.

In a previous report [1] we showed that tyrosine and 3,4-dihydroxyphenylalanine as well as tyramine and dopamine are specifically incorporated into the phenethylamine portion of (−)-cryptostyline I (I). It was also evident that 3-hydroxy-4-methoxyphenethylamine was incorporated to a greater degree than 4-hydroxy-3-methoxyphenethylamine. We now report further experiments on the biosynthesis of (−)-cryptostyline I (I). The specificity of the incorporation of the precursors into (−)-cryptostyline I (I) has been established by degradation studies (Table 1 and Fig. 1).

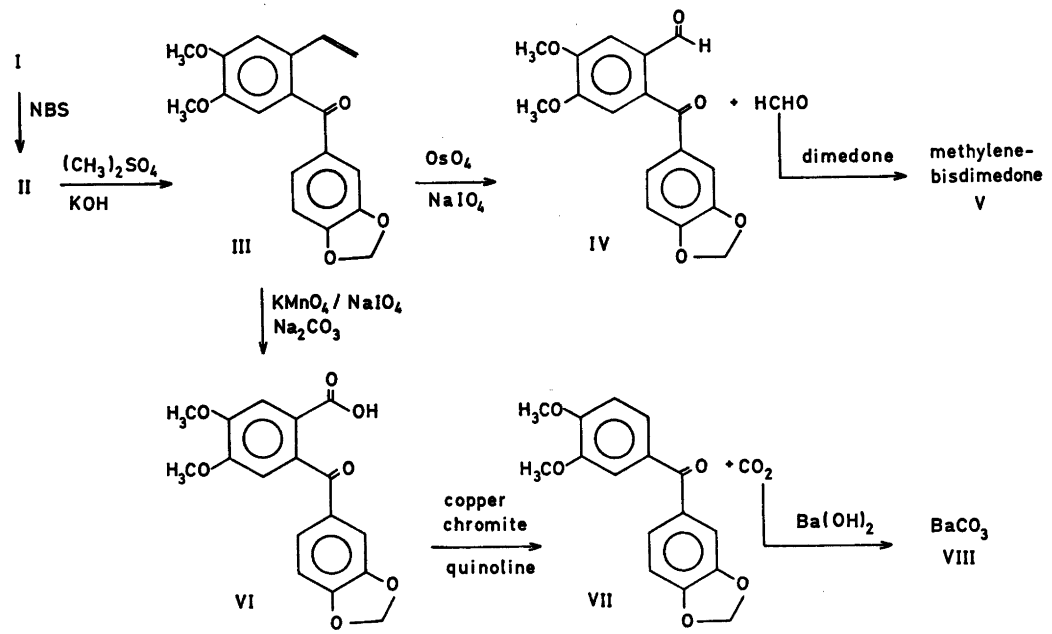

Fig. 1. Degradation of cryptostyline I.

Table 1.

Labelled compounds	Amount fed		Isolated (−)-cryptostyline I	Added carrier
	mg	μCi	mg	mg
Dopamine-β-^{14}C	0.26	100	60	240
3,4-Dimethoxyphenethylamine-α-^{14}C	2.4	100	30	60
N-(3-Hydroxy-4-methoxy-1′-^{3}H-benzyl)-3-hydroxy-4-methoxyphenethylamine-α-β-^{3}H (XI)[a]	5	325	55	40
1-(3,4-Methylenedioxyphenyl)-6,7-dimethoxy-2-methyl-3,4-dihydroisoquinolinium bromide-^{3}H	5	24	25	25
Vanillin-^{3}H	5	105	35	30
Isovanillin-^{3}H	5	150	40	35

Spec. act. in isol. (−)-cryptostyline I μCi/mmol × 10^{-3}	Recovered radioactivity % × 10^{-3}	Radioactivity % I	II	III	IV	V	VI	VII	VIII
77.0	14.0	100					96	57	35
1.7	0.16	100		98	12	91			
5.7	0.29	100[b]	81[b]				16[b]		
4700	1500	100[c]							
2.3	0.23	100[c]							
0.36	0.029	100[c]							

[a] The tritium-ratio between the C_1 and C_3-C_4 positions was 2.7 asuming non-exchange during debenzylation.

[b] The tritium-ratio between the C_1 and C_3-C_4 positions was 0.29. This figure was calculated by dividing the 19 % label shown by degradation to have occurred at the C_1 position by 65 % calculated by substracting the 16 % label in VI from the 81 % present in compound II. On the assumption that the losses of tritium and hydrogen at C-1 during the cyclization to (−)-cryptostyline I (I) are equal the ratio 0.29 in I should be doubled when I is compared with the precursor XI.

[c] Boiling with concentrated hydrochloric acid gave an unlabelled (−)-cryptostyline I (I).

As shown in Table 1, 3,4-dimethoxyphenethylamine is incorporated into (−)-cryptostyline I (I) but considerably less efficiently than dopamine. The latter was, however, administered in smaller amounts. It therefore appears probable that 3,4-dimethoxyphenethylamine is demethylated to the known precursor 3-hydroxy-4-methoxyphenethylamine before incorporation into I. An analogous reaction is known [2] for cactus species, where 3,4-dimethoxyphenethylamine was demethylated to 4-hydroxy-3-methoxyphenethylamine before further biosynthetic reactions.

Dopamine, labelled in the β-carbon, was administered to the plants to elucidate whether it is a progenitor of only one or of both the aromatic rings in I. The results suggest that dopamine is a precursor of both the aromatic parts of the molecule, since only about half of the radioactivity from dopamine-β-^{14}C was located at carbon C-4 of (−)-cryptostyline I (I). We have previously shown [1] that dopamine-α-^{14}C is incorporated into (−)-cryptostyline I (I) with only a slight scrambling of label and hence, it seems likely that the remaining label is present at C-1.

The origin of the 1-phenyl group was further investigated. Vanillin (Table 1) was decidedly better incorporated into I than was isovanillin. Incorporation rates were low but due to their minimal water solubilities both aldehydes had to be applied to the leaves as solutions in poly-

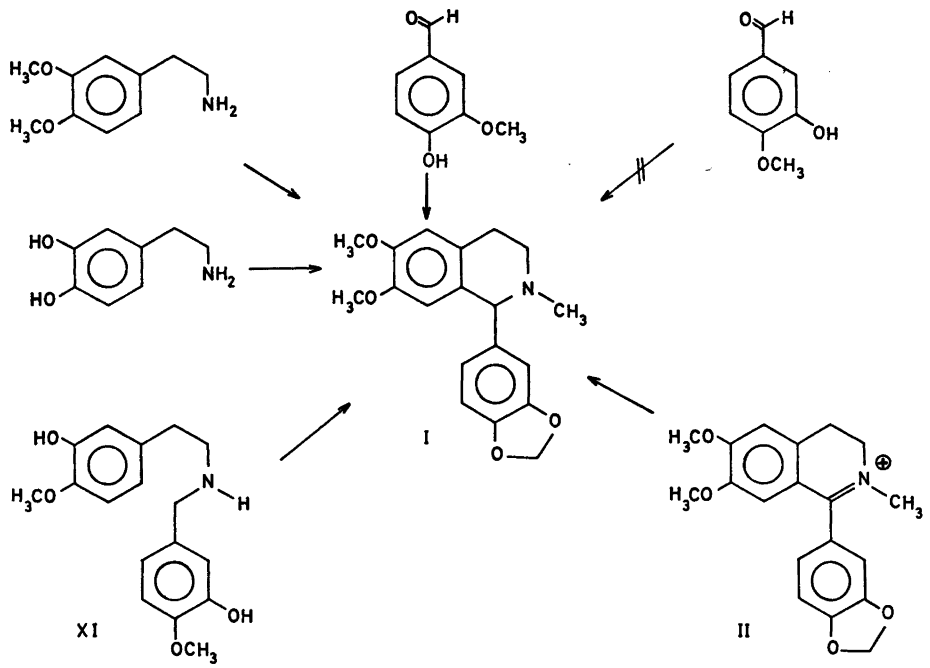

Fig. 2. Suggested precursors for the biosynthesis of (−)-cryptostyline I.

ethyleneglycol and thus may not be available for biosynthesis to the same extent as the amines. Barton *et al.*[3] have earlier found that isovanillin is poorly utilized in the biosynthesis of alkaloids owing to its poor absorption. We have not established which aromatic moiety became labelled from the aldehyde. The C_6-C_1 unit in some Amaryllidaceae alkaloids has previously been shown to be derived from protocatechualdehyde.[4]

N-(3-Hydroxy-4-methoxybenzyl)-3-hydroxy-4-methoxyphenethylamine (XI), labelled in both parts of the molecule, was incorporated in low yield. If we assume that XI, with the exception of the methyl groups, is incorporated intact into I it is clear from Table 1 that there has been a considerable loss of tritium from the position in XI which later appears at C_1 in (−)-cryptostyline I (I). The original tritium-ratio between the C_1 and the C_3-C_4 positions, which was 2.7 in XI, has decreased to 0.58 in I, as discussed in the footnotes to Table 1. This may be due to loss of tritium during passage through an intermediate such as the immonium salt of I (II). The indication that II is a main intermediate is supported by the efficient incorporation of II into (−)-cryptostyline I (I). Alternative explanations include metabolic degradation of XI to smaller fragments followed by re-incorporation. It should be noted that in XI the isovanillin substitution pattern is present in the 1-phenyl ring. In view of the results with vanillin and isovanillin it seems more probable that the isomer of XI, *N*-(4-hydroxy-3-methoxybenzyl)-3-hydroxy-4-methoxyphenethylamine, is the true precursor of II and (−)-cryptostyline I (I).

EXPERIMENTAL

The general methods described in Ref. 1 were used. 3,4-Dimethoxyphenethylamine-1-^{14}C and dopamine-2-^{14}C were obtained from the Radiochemical Centre, Amersham, UK and New England Nuclear Corp., Boston, USA, respectively.

Feeding experiments. The labelled amines were dissolved in small amounts of dilute acetic acid and the solutions were injected into the leaves of *C. erythroglossa.* The labelled vanillin and isovanillin were dissolved in small amounts of polyethyleneglycol and the solutions were applied to the leaves of the plants. (−)-Cryptostyline I (I) was isolated as described earlier.[1]

Unlabelled carrier was added to the isolated alkaloid in amounts specified in Table 1.

3,4-Dimethoxy-3',4'-methylenedioxybenzophenone (VII). A mixture of 2-(3,4-methylenedioxybenzoyl)-4,5-dimethoxybenzoic acid (VI, 70 mg), quinoline (1 ml) and copper chromite (20 mg) was refluxed for 10 min. Chloroform (10 ml) was added and the copper chromite filtered off. The chloroform phase was washed with hydrochloric acid (2 %) until all quinoline was removed, dried (Na_2SO_4) and concentrated. The residue was chromatographed on a silica gel column (2 × 5 cm) using chloroform as eluent. The fraction containing VII was further purified by preparative thin-layer chromatography on silica gel using chloroform as eluent ($R_F = 0.5$). Recrystallization from toluene gave VII (40 mg), m.p. 164–165 °C (Lit.[9] m.p. 164–165 °C). The carbon dioxide formed in the reaction was trapped in a saturated aqueous solution of barium hydroxide. The precipitate was filtered off and immediately dried under a high vacuum to give barium carbonate (VIII, 20 mg).

Vanillin labelled in the aromatic ring. Concentrated hydrochloric acid (0.03 ml) and tritiated water (0.04 ml, 0.2 Ci) were added to a solution of vanillin (46 mg) in dioxane (0.10 ml). The solution was heated in a sealed ampoule at 115 °C. After 3 h, water (4 ml) was added and the aqueous solution extracted with chloroform (4 × 5 ml). The combined chloroform solutions were dried (Na_2SO_4) and evaporated to dryness. The residue was dissolved in methanol and the solvent evaporated. This procedure was repeated five times. The residue was dissolved in chloroform and filtered through a short silica gel column. Evaporation of the eluate to dryness and recrystallization of the residue from toluene gave the tritiated vanillin, m.p. 80–81 °C (Lit.[5] m.p. 83–84 °C) with a specific activity of 3.2 mCi/mmol.

Tritiated isovanillin was prepared as described for vanillin, m.p. 116–117 °C (Lit.[6] m.p. 114–116 °C) with a specific activity of 4.6 mCi/mmol.

(±)-Cryptostyline I labelled with tritium in the aromatic rings was obtained by heating (±)-cryptostyline I (I, 34 mg) dissolved in dioxane (0.1 ml) with concentrated hydrochloric acid (0.03 ml) and tritiated water (0.05 ml, 0.25 Ci) in a sealed ampoule at 120 °C for 5 h. Water was added (2.5 ml) and the aqueous solution was washed with ether (5 ml), made alkaline (pH 13) with sodium hydroxide (1 M) and extracted with ether (2 × 5 ml). The combined ether solutions were dried (Na_2SO_4) and evaporated to dryness. Recrystallization of the residue from ether gave (±)-cryptostyline I (I, 21 mg), m.p. 117–118 °C (Lit.[7] m.p. 117–118 °C) with a specific activity of 2.1 mCi/mmol. Oxidation of this sample with *N*-bromosuccinimide [1] gave 1-(3,4-methylenedioxyphenyl)-6,7-dimethoxy-2-methyl-3,4-dihydroisoquinolinium bromide with a specific activity of 1.9 mCi/mmol.

N-(3-Benzyloxy-4-methoxy-1'-^{3}H-benzyl)-3-benzyloxy-4-methoxy-α,β-^{3}H-phenethylamine hydrochloride (X). 3-Benzyloxy-4-methoxybenzaldehyde (96 mg) and 3-benzyloxy-4-methoxy-α,β-^{3}H-phenethylamine [8] (103 mg, 5.38 mCi/mmol) were dissolved in ether (20 ml) and the solution was allowed to stand for 20 h at room temperature. Evaporation of the solvent gave the crude imine IX, which was dissolved in dioxane:methanol 3:1 (27 ml) and reduced with sodium borotriteride (11.8 mg, 62 mCi). After

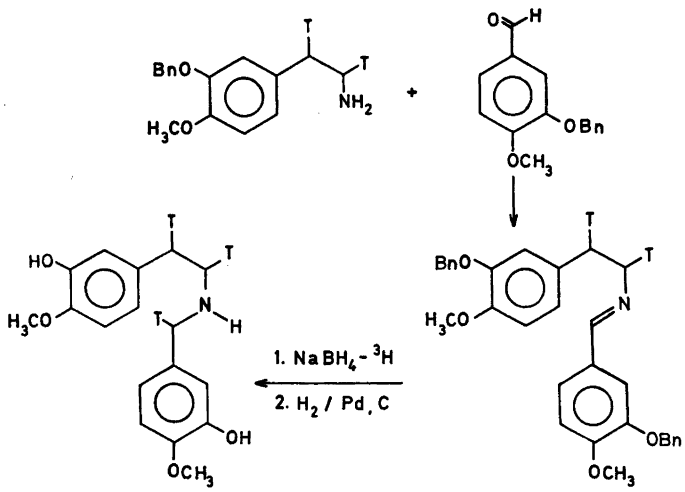

Fig. 3. Synthesis of the labelled compound XI.

stirring for 1.5 h an excess of sodium borohydride (120 mg) was added and the stirring continued for 2 h. The solution was diluted with water (25 ml) and extracted with ether (4 × 40 ml). The combined ether solutions were dried (Na_2SO_4) and evaporated to dryness leaving the crude phenethylamine derivative, which was converted to the corresponding hydrochloride. Recrystallization from ethanol gave X in the form of needles (150 mg), m.p. 148–150 °C. (Found: C 71.6; H 6.6; N 2.6. Calc. for $C_{31}H_{34}NO_4$: C 71.6; H 6.6; N 2.7.) UV spectrum, nm (ε): λ_{max} (ethanol) 279 (5600), 231 (16 900). NMR spectrum ($CDCl_3$): δ 2.98 (m, 4 H), 3.68 (s, 3 H), 3.78 (s, 3 H), 3.92 (s, 2 H), 5.05 (s, 2 H), 5.24 (s, 2 H), 6.65–7.65 (m, 16 H).

N-(3-Hydroxy-4-methoxy-1'-^{3}H-benzyl)-3-hydroxy-4-methoxy-α,β-^{3}H-phenethylamine (IX). A solution of X (150 mg) in acetic acid (15 ml) was hydrogenated over palladium on carbon (10 %, 10 mg) at room temperature and atmospheric pressure. When the theoretical amount of hydrogen had been consumed (40 min) the catalyst was filtered off and water (50 ml) was added. After washing with ether (2 × 25 ml) the pH of the solution was adjusted to 8.0–8.5 with sodium hydroxide (1 M) and extracted with ether (5 × 40 ml). The combined ether extracts were dried (Na_2SO_4) and the solvent evaporated. The residue was recrystallized from ethanol giving XI, m.p. 140–143 °C. (Found: C 66.8; H 6.5; N 4.4. Calc. for $C_{17}H_{21}NO_4$: C 67.3; H 7.0; N 4.6). NMR spectrum (pyridine-d_5): δ 2.92 (m, 4 H), 3.73 (s, 6 H), 3.84 (s, 2 H), 6.10–7.45 (m, 9 H). Specific activity: 19.7 mCi/mmol.

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Studies on the Smith Degradation

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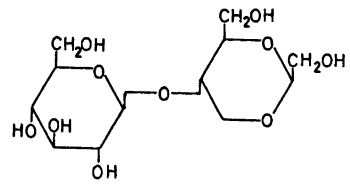
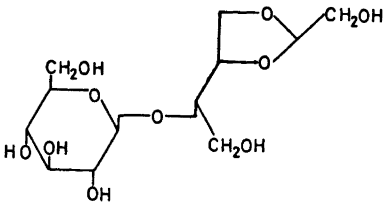
Two oligosaccharide derivatives, methyl 6-*O*-(β-D-glucopyranosyl)- 3-*O*-methyl-α-D- glucopyranoside (III) and *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-galactopyranosyl-(1→2)-glycerol (IV) have been subjected to the Smith degradation. Only the normal degradation products, methyl 3-*O*-methyl-α-D-glucopyranoside (IX) and 2-*O*-α-D-galactopyranosyl-glycerol (X), respectively, were obtained. The results indicate that acetal migration with the formation of cyclic acetals involving sugar moieties is insignificant in the Smith degradation.

Following Smith degradation of polysaccharides,[1] that is periodate oxidation, borohydride reduction, and mild acid treatment, glycosidic linkages of unoxidized sugar residues are not hydrolyzed while non-cyclic acetals in modified sugar residues are cleaved. Investigation of the resultant products, therefore, reveals structural features of the original polysaccharide.

One complication, observed by Smith and his coworkers,[1] is acetal migration during the mild acid treatment. Cyclic acetals formed by such migration are considerably more stable to acid hydrolysis than the non-cyclic acetals; consequently compounds containing cyclic acetal structures may be present in the product. Thus on Smith degradation of oat glycan,[1] not only 2-*O*-β-D-glucopyranosyl-D-erythritol but also 1,3-*O*-(2-hydroxyethylidene)-2-*O*-β-D-glucopyranosyl-D-erythritol (I) was obtained. Gorin and Spencer [2] observed that smaller amounts of analogous 3,4-*O*-(2-hydroxyethylidene)-2-glycopyranosyl derivatives of erythritol (*e.g.* II) were also formed from polysaccharides containing β-(1→4)-linked D-glucose or D-mannose residues. Such acetals became the main byproducts when polysaccharides containing



I



II

Fig. 1.

α-(1→4)-linked D-glucose or D-mannose residues were degraded.

It seemed possible that cyclic acetals, involving sugar moieties of the degraded product, could also be formed by acetal migration during the Smith degradation. This possibility has now been investigated, using suitable model substances.

RESULTS AND DISCUSSION

Two model substances were investigated. One, methyl 6-*O*-(β-D-glucopyranosyl)-3-*O*-methyl-α-D-glucopyranoside (III), was prepared by conventional methods from 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and methyl 2,4-di-*O*-acetyl-3-*O*-methyl-α-D-glucopyranoside. Fully trideuteriomethylated III gave the ex-

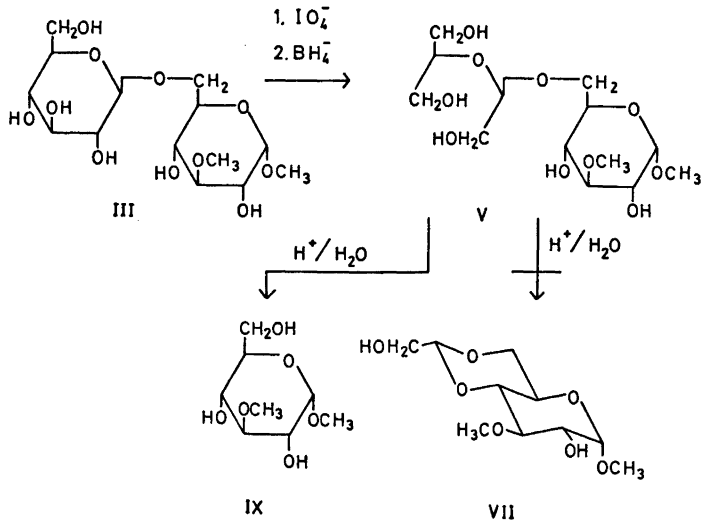


Fig. 2.

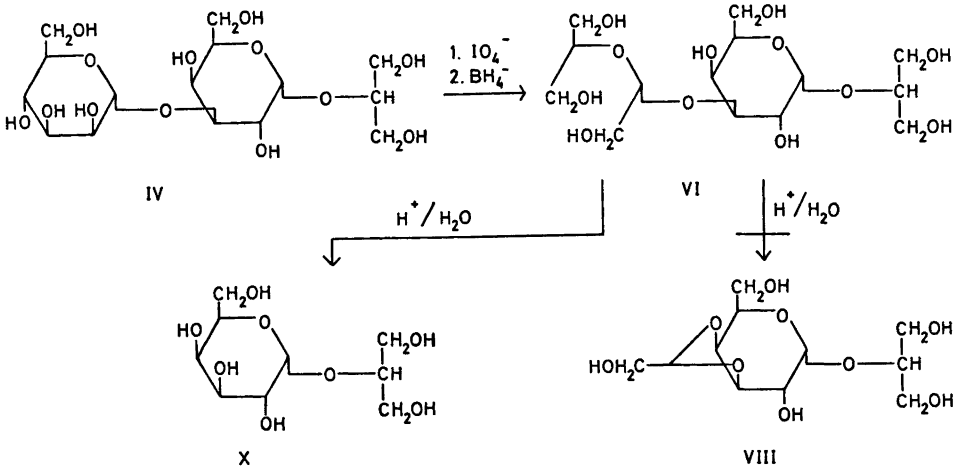


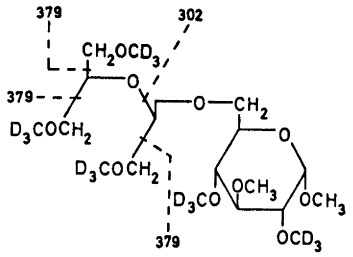
Fig. 3.

pected mass spectrum.[3] The other substance *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-galactopyranosyl-(1→2)-glycerol (IV) was previously isolated from the marine red alga *Furcellaria fastigiata*.[4] It seemed possible that the polyalcohols (V, VI) obtained from these should yield some 4,6- and 3,4-(2-hydroxyethylidene)-acetal (VII and VIII), respectively, on mild acid treatment.

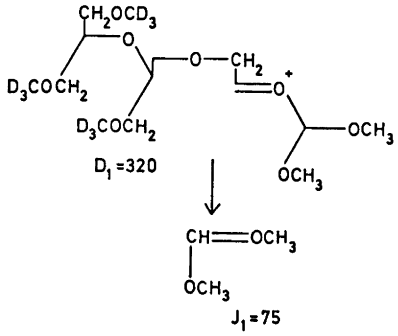
Each disaccharide derivative (III and IV) was oxidized with sodium metaperiodate in aqueous methanol and reduced with sodium borohydride. The product was isolated by chromatography on Sephadex G25 and its purity checked by TLC and, after permethylation, by GLC. From III a single product was obtained. The MS of its pertrideuteriomethylated derivative (XI) was in agreement with the postulated structure. The origins of some major fragments are indicated in the formula.

Two components were obtained from IV, probably due to preferential cleavage between

C-2 and C-3 and formation of a cyclic hemiacetal in equilibrium with the open structure, whereby further oxidation is slowed down. By subjecting the product to borohydride reduction and a second oxidation reduction, a single component was obtained. The MS of the permethylated derivative (XII) of polyalcohol VI indicated

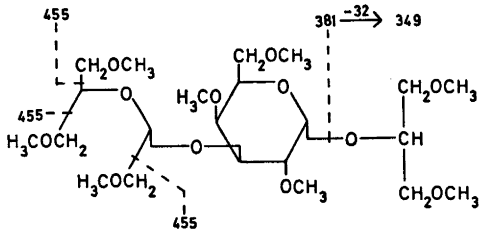


XI Mw 427



that it was the expected product. The origins of some major fragments are indicated in the formula.

The polyalcohols V and VI were hydrolysed at different temperatures. The rate constants



XII Mw 500

Table 1. Rate constants and activation energies for the acid hydrolysis of the mixed acetals V, VI, and XIII.[5]

Compound	Temp. °C	$k \times 10^4$ s^{-1}	E kJ/mol
	20	0.25	
V	30	0.99	114.0
	40	4.9	
	20	0.47	
VI	30	2.2	108.5
	40	8.1	
	20	0.89	
XIII	30	3.62	98.5
	40	11.30	

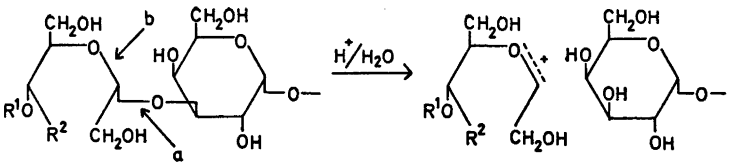
were of the same order of magnitude as those for XIII,[5] obtained on oxidation-reduction of methyl α-D-glucopyranoside (Table 1).

During the hydrolyses of V and VI, samples were withdrawn, borohydride reduced, and analysed by TLC and, after permethylation, by GLC. Only unchanged starting material and the expected degradation product, methyl 3-*O*-methyl-α-D-glucopyranoside (IX) and 2-*O*-α-D-galactopyranosyl-glycerol (X), respectively, were observed and not even traces of cyclic acetals, such as VII and VIII. The results of the present study therefore indicate that the formation of cyclic acetals involving unoxidized sugar residues is insignificant during Smith degradation. One explanation for the findings that cyclic acetals involving erythritol occur, but those involving sugar residues do not, is that the first linkage cleaved when the non-cyclic acetal (*e.g.* XIV) is hydrolysed is the one to the sugar residue (a) and not the other linkage to the alditol residue (b).

Although an explanation is not obvious, perhaps there is strain due to crowding of substituents in the solvated ion formed by protonation of the oxygen at *b*, which should precede the fission, and less strain in the corresponding ion formed by protonation at *a*, and that this is responsible for the observed effect.

EXPERIMENTAL

General methods. Melting points are corrected. Concentrations were performed at reduced pressure at a bath temperature not exceeding 40 °C. Optical rotations were determined using a Perkin-Elmer 141 instrument. GLC was per-



XIV

R^1 = H, sugar residue
R^2 = H, CH_2OH

Fig. 4.

formed using a column (0.15 × 180 cm) of 3 % OV 1 on Gaschrom Q at 210 °C and a Perkin-Elmer 900 instrument. Permethylated melibiitol ($T_M = 1$) was used as standard. GLC-MS was performed using Perkin-Elmer 270 and Varian Mat 311 instruments. TLC was performed on precoated plates with Silica Gel 60 F_{254} (Merck), 0.25 mm.

Methyl 3-O-methyl-α-D-glycopyranoside was obtained together with the 2-*O*-methyl derivative by partial methylation of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (7.6 g), fractionation of the product by silicic acid chromatography and catalytic hydrogenation, as previously described.[5] Methyl 4,6-*O*-benzylidene-3-*O*-methyl-α-D-glucoside (2.1 g) was eluted as the second component and showed, after crystallization from ethanol, m.p. 149–150 °C and $[\alpha]_{578}^{20}$ +116° (*c* 1.0, $CHCl_3$), in good agreement with published values. The title compound was obtained as a chromatographically pure syrup (1.25 g). The MS of the permethylated (CD_3I) product agreed with the postulated structure.[3]

Methyl 2,4-di-O-acetyl-3-O-methyl-6-trityl-α-D-glucopyranoside. Triphenylmethyl chloride (3.9 g) was added to a solution of methyl 3-*O*-methyl-α-D-glucopyranoside (3.0 g) in dry pyridine (100 ml). The solution was heated on a steam bath for 20 min, acetic anhydride (80 ml) was added and the heating continued for further 20 min. The solution was cooled and poured into a mixture of ice (300 g) and water (100 ml), which was then extracted with chloroform (4 × 100 ml). The chloroform solution was washed with 8 % aqueous sodium hydrogen carbonate (4 × 100 ml) and water (3 × 100 ml), dried (Na_2SO_4) and concentrated. The product (3.5 g) crystallized from ethanol and showed m.p. 158–159 °C and $[\alpha]_{578}^{20}$ +90° (*c* 1.9, $CHCl_3$). (Found: C 69.8; H 6.23. $C_{31}H_{34}O_8$ requires: C 69.7; H 6.41).

Methyl 2,4-di-O-acetyl-3-O-methyl-α-D-glucopyranoside. A saturated (at 0 °C) solution (1.5 g) of hydrogen bromide in acetic acid was added to a solution of the above trityl compound (3.0 g) in acetic acid (12 ml), kept at 10 °C. The mixture was shaken for 60 s and then rapidly filtered into ice and water (50 g). The trityl bromide was washed with acetic acid (10 ml) and the combined filtrate and washings extracted with chloroform (4 × 20 ml). The chloroform solution was washed with ice-water (4 × 20 ml) and dried (Na_2SO_4). Concentration of the solution yielded a syrup (1.4 g), used without further purification in the following step.

Methyl 6-O-β-D-glucopyranosyl-3-O-methyl-α-D-glucopyranoside (III). The above syrup (0.8 g), silver oxide (0.7 g), Drierite (2.5 g) and dry ethanol-free chloroform (10 ml) were stirred in the dark for 1 h and iodine (0.2 g) was added. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (1.0 g) in chloroform (10 ml) was added in 1 ml portions over 2 h and stirring continued for further 24 h. The mixture was filtered, concentrated and fractionated on a silica gel column (5 × 40 cm), using chloroform – acetone (5:1) as irrigant. The fractions were investigated polarimetrically and by TLC. The main component was obtained as a syrup (1.0 g) that crystallized from ethanol, m.p. 126–128 °C, $[\alpha]_{578}^{20}$ +49° (*c* 0.4, $CHCl_3$). (Found: C 50.1; H 6.09. $C_{26}H_{38}O_{17}$ requires: C 50.2; H 6.15). This product (0.9 g) was dissolved in methanol (10 ml) and methanol saturated (room temperature) with ammonia (50 ml) was added. The solution was kept overnight at room temperature, concentrated and the resulting syrup (0.5 g) purified by chromatography on a Sephadex G25 column to give the title compound as a chromatographically pure syrup.

α-D-Mannopyranosyl-(1→3)-α-D-galactopyranosyl-(1→2)-glycerol. The nonaacetate of the title compound was deacetylated and the product purified as above, yielding a chromatographically pure syrup.

The mixed acetal V. To the disaccharide glycoside III (0.2 g) in methanol (20 ml) was added 0.23 g sodium metaperiodate in water (5 ml). The mixture was stirred for 2 h at room temperature in the dark, ethylene glycol (0.1 g) was added and stirring continued for 15 min. Methanol (30 ml) was then added and the mixture filtered, concentrated and dissolved in water (10 ml). Sodium borohydride (0.2 g) was added, the solution kept at room temperature

for 12 h, excess borohydride was decomposed by addition of acetic acid and the solution was concentrated. Boric acid was removed by codistillations with methanol and the product purified by chromatography on Sephadex G25, $[\alpha]_{578} \simeq +117°$. TLC (ethanol—chloroform—water, 15:5:1) and GLC after permethylation ($T_M = 0.54$) showed that the product (0.15 g) was pure. The MS of the permethylated (CD_3I) product showed, *inter alia*, the following peaks (relative intensities in brackets): 48(100), 74(39), 75(21), 76(10), 91(51), 92(12), 104(9), 107(14), 109(12), 134(8), 151(2), 176(1.5), 179(1.6), 193(2.4), 211(1.2), 225(1.8), 301(0.6), 302(0.1), 320(0.2), 321(0.1), 357(0.1), 379(0.6).

The mixed acetal VI, $[\alpha]_{578} \simeq +94°$ was prepared as described above for V, except that the oxidation-reduction had to be repeated. The permethylated product showed $T_M = 1.23$, compared to $T_M = 1.89$ for the component that disappeared after the second oxidation-reduction. MS of the former showed, *inter alia*, the following peaks: 45(100), 71(41), 73(10), 101(12), 103(14), 127(6.1), 145(2.9), 159(2.0), 163(1.3), 177(3.0), 187(1.2), 207(0.4), 215(0.4), 233(0.4), 247(0.2), 275(0.2), 349(0.1), 380(0.1), 381(0.2), 455(0.1).

Acid hydrolysis studies. The mixed acetal (V or VI) (about 10 mg) in 0.125 M sulfuric acid (1 ml) was transferred to a jacketed polarimeter tub (10 mm) maintained at the required temperature. The optical rotation, at 365 nm, was determined at intervals and the reaction constant calculated, assuming first order kinetics. Samples from a parallel experiment were withdrawn at intervals, borohydride reduced and analysed by TLC and, after permethylation, by GLC and GLC-MS. In addition to unchanged starting material, methyl 3-*O*-methyl-α-D-glucopyranoside (T_M too low to be determined accurately) and 2-*O*-α-D-galactopyranosyl-glycerol ($T_M = 0.24$), respectively, were observed but no components indicative of acetal migration.

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Thermolytic Fragmentation of 3-Alkylthio- and 3-Arylthio-1,2-dithiolylium Iodides in the Ion Source of the Mass Spectrometer

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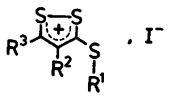
A series of 3-alkylthio- and 3-arylthio-1,2-dithiolylium iodides have been thermolysed in the ion source of the mass spectrometer. The mass spectra obtained from the thermolysis products of the alkylthio substituted compounds can be rationalized by assuming the primary formation of a 1,2-dithiolyl radical. The radical may stabilize either by loss of the thioalkyl alkyl group or by loss of a hydrogen atom.

The 3-arylthio substituted compounds form a tetrathiafulvalene, probably by dimerization of an intermediately formed carbene.

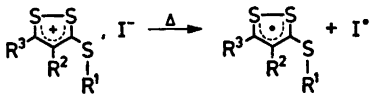
A mass spectrometric study of the thermolysis products of alkyl- and aryl substituted 1,2-dithiolylium salts has recently been reported.[1] Although some of the thermolysis products are not stable enough to be isolated, they are sufficiently long lived to allow ionization by electron impact when formed directly in the ionization chamber. Thus mass spectra obtained in this way make it possible to study the primary thermolysis products or the primary rearrangements products.

In this investigation salts of the type (A) (Table 1) were studied under the same conditions as before.[1]

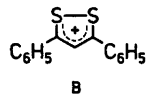


A

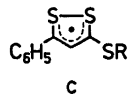
The mass spectra of the various thermolysis products may be rationalized by assuming the primary formation of a 1,2-dithiolyl radical by a sort of homolytic cleavage (I – XIII).



An analogous process was proposed as the primary process in the thermolysis of alkyl- and aryl substituted 1,2-dithiolylium salts,[1] where the thermolysis in some cases, *e.g.* 3,5-diphenyl-1,2-dithiolylium bromide, gave rise to the formation of stable radicals. Subsequent ionization then yielded the stable aromatic 1,2-dithiolylium cation (B) as the very abundant parent ion in the mass spectrum.



B



C

Similar abundant ions corresponding to radicals as (C) are not observed in any of the mass spectra from the salts studied in this investigation. However, the spectra from alkylthio dithiolylium salts bearing an aromatic substituent (IX – XIII) exhibit, besides the spectrum of the thermolysis product, an ion of low abundance (0.1 – 2 %) at a *m/e* value corresponding to the appropriate radical. Such peaks could not be detected in the spectra of I to VIII since the M + 1 isotope peaks of the abundant molecular

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Table 1. Salts of type (A).

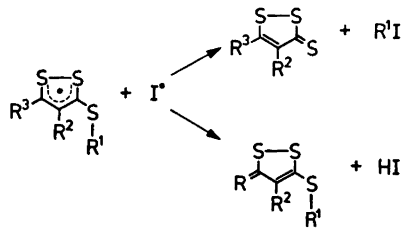
	R^1	R^2	R^3	M.p., °C [a]
I	CH_3	H	CH_3	
II	CD_3	H	CH_3	
III	CH_3	CH_3	CH_3	
IV	C_2H_5	CH_3	CH_3	153–155
V	CH_3	$-CH_2-CH_2-CH_2-$		
VI	CD_3	$-CH_2-CH_2-CH_2-$		162–165
VII	CH_3	$-CH_2-CH_2-CH_2-CH_2-$		148–149
VIII	CD_3	$-CH_2-CH_2-CH_2-CH_2-$		147–148
IX	CH_3	C_6H_5	H	
X	C_2H_5	C_6H_5	H	174–176
XI	CH_3	H	C_6H_5	
XII	C_2H_5	H	C_6H_5	
XIII	CH_3	C_6H_5	C_6H_5	
XIV	C_6H_5	H	C_6H_5	

[a] All compounds melt with decomposition.

ion of the decomposition product appear at the same nominal mass number.

The observations described above are in accordance with the electrochemical behaviour of the two groups of salts. The 3,5-diphenyl-1,2-dithiolylium perchlorate gave reversible electrochemistry at room temperature and the corresponding stable radical was characterized by means of ESR spectroscopy.[2] 3-Methylthio-5-phenyl-1,2-dithiolylium methosulfate showed reversible electrochemistry only at −70 °C [3] indicating the lower stability of the radical.

The primary rearrangement process of the radicals initially formed from the alkylthio substituted salts could proceed in two different ways as suggested in Scheme 1.



Scheme 1.

Loss of the thioalkyl group R^1

The mass spectra exhibited by IX to XIII show abundant molecular ions corresponding to alkyl iodides and the appropriate 1,2-dithiol-3-thiones. The fragmentation patterns of the latter are virtually identical with those previously reported for these compounds.[4] This process accounts for all significant peaks observed in the spectra.

Loss of the alkyl group R^1 is also observed in the spectra of I to VIII, but to a much smaller extent.

Loss of a hydrogen atom

The major rearrangement process of the initially formed radicals in the case of compounds I to VIII corresponds to the initial loss of a hydrogen atom (Scheme 2). The mass spectra show the resulting $[HI]^{+\cdot}$ ion and abundant molecular ions corresponding to loss of hydrogen iodide from the parent salt (*cf.* Figs. 1 and 2). The hydrogen atom involved in this process do not originate from the alkylthio group R^1, as demonstrated by the spectra obtained from the deuterated salts II, VI, and VIII. In these cases all labelled hydrogens are retained in the molecular ions.

The mass spectrum exhibited by VII is shown in Fig. 1. A reasonable structure for the molecular ion appearing at *m/e* 202 may be *a*. This is in accordance with the structure proposed for the thermolysis product obtained from 3-methyl-4,5-tetramethylene-1,2-dithiolylium bromide.[1] Similarly the [M−H] ion is

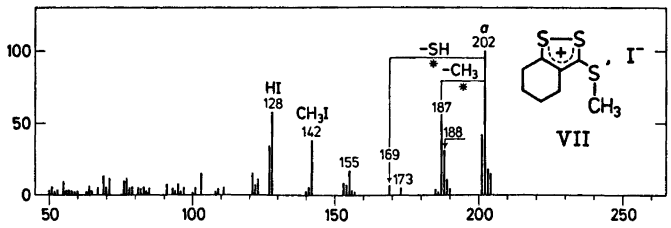
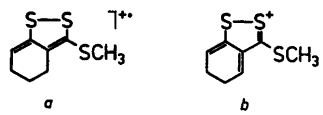


Fig. 1.



a *b*

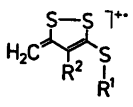
designated structure *b*, which is consistent with the spectrum of the deuterium labelled compound VIII.

The ion corresponding to the loss of $\cdot CH_3$ from the molecular ion gives rise to the abundant peak at *m/e* 187. Loss of methyl from the condensed ring system leading to a ring contraction as suggested for 3-methyl-4,5-tetramethylene-1,2-dithiolylium bromide [1] may also take place here. However, the alkyl group R^1 is lost preferentially, since in the spectrum of VIII the peak corresponding to the loss of CD_3 is five times as abundant as that corresponding to the loss of CH_3.

The hydrogen atom involved in the ·SH loss preferentially originates from the condensed system.

The peak at *m/e* 188 corresponds to the molecular ion of 4,5-tetramethylene-1,2-dithiol-3-thione most likely formed by loss of the alkyl group R^1 from the primary radical, Scheme 1. Loss of $\cdot CH_3$ and ·SH from *m/e* 188 give rise to the ions at *m/e* 173 and 155, respectively, as was also observed for an authentic sample of 4,5-tetramethylene-1,2-dithiol-3-thione.[5]

The structure of the molecular ion observed in the mass spectra obtained from I to IV corresponds to the loss of a hydrogen atom from the methyl group in the 5 position of the initially formed radical. This process yielding *c* is analogous to the formation of *a*, and is in



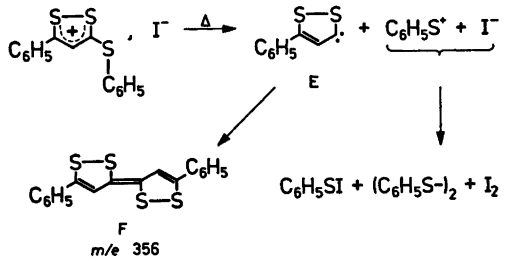
c

accordance with calculations of the spin densities in the dithiolyl radical.[6,7] These calculations suggest a high spin density in the 3 and 5 positions which will facilitate the loss of a hydrogen atom from the methyl group in the 5 position.

The mass spectrum of compound I is typical for this group and is shown in Fig. 2. Loss of R^1 and SR^1 from the molecular ion are characteristic processes. If the salt is unsubstituted in the 4 position, *e.g.* I, the molecular ion shows the loss of $\cdot S_2H$ whereas III and IV with a methyl group in the 4 position expel ·SH from the molecular ions. These differences in fragmentation are the same as found for 4-methyl-1,2-dithiol-3-thione and 5-methyl-1,2-dithiol-3-thione.[4] The peaks at *m/e* 83 and 71 are due to the loss of S_2 and CS_2, respectively, from the $[M-R^1]$ ion. Corresponding peaks are found in the other cases too.

The mass spectrum of the phenylthio compound XIV indicates a somewhat different thermolytic behaviour than that observed for the alkylthio substituted compounds.

The spectrum of XIV exhibits the base peak at *m/e* 109, which corresponds to C_6H_5S. This fragment is probably formed by a heterolytic cleavage (Scheme 2) and before ionization C_6H_5SI and $C_6H_5S-SC_6H_5$ are formed by nu-



Scheme 2.

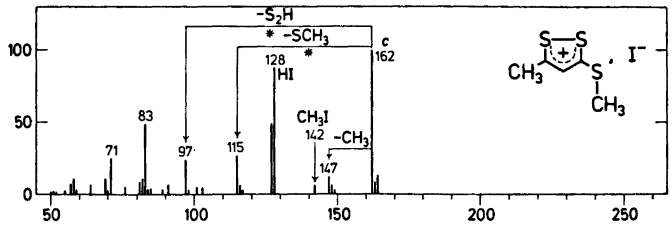
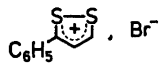


Fig. 2.

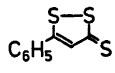
cleophilic attack of the iodide ion on $C_6H_5S^+$ and by reduction of this ion by I^-. The phenyl sulfenyl iodide and the diphenyl disulfide give rise to intense peaks at *m/e* 236 and *m/e* 218, respectively. Intense peaks corresponding to $[I]^{+\cdot}$, $[HI]^+$, and $[I_2]^{+\cdot}$ are also present.

The carbene formed by loss of $C_6H_5S^+$ dimerize prior to ionization accompanied by the formation of the tetrathiafulvalene (F). This behaviour is analogous to that previously reported for 5-phenyl-1,2-dithiolylium bromide (G),[1] and the spectra obtained from XIV and (G) are very similar (apart from the iodine and C_6H_5S containing ions).

The fragmentation pattern of 5-phenyl-1,2-dithiol-3-thione (H) gives rise to abundant peaks in the spectrum of XIV. (H) may arise by a mechanism analogous to that depicted for the formation of 1,2-dithiol-3-thiones in Scheme 1. However, since no peak corresponding to $[C_6H_5I]^{+\cdot}$ is observed, a thermal mechanism similar to that proposed in the case of (G) [1] is considered to be more likely.



G



H

EXPERIMENTAL

The mass spectra were obtained on a MS 902 mass spectrometer using the direct sample insertion system with a sample probe of pyrophylite and the lowest feasible ion source temperature (120 – 250 °C). 70eV electrons were used. Peaks corresponding to doubly charged ions appearing at half mass numbers and peaks of abundance lower than 2 % were omitted in the spectra shown.

*3-Alkylthio-1,2-dithiolylium iodides** were prepared from the appropriate 1,2-dithiol-3-thiones and methyl iodide either in refluxing butyl acetate [8] or in chloroform at room temperature.[9]

*3-Phenylthio-1,2-dithiolylium iodide** was prepared as described by Faust *et al.*[10]

Acknowledgement. The authors are indebted to the Danish Government for a grant to one of them (N.L.H.).

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* Satisfactory elemental analysis was obtained.

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Chlorinated Long-chain Fatty Acids. Their Properties and Reactions. VIII. The Kinetics and Stereochemistry of the Alkaline Dehydrochlorination of Diastereoisomeric Sodium 9,10-Dichlorooctadecanoates

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The base-promoted removal of the first chlorine of sodium *erythro*-9,10-dichlorooctadecanoate in aqueous ethylene glycol was found to produce sodium 9(10)-chloro-*cis*-octadecenoate. The corresponding *threo* isomer gave 9(10)-chloro-*trans*-9-octadecenoate by a 2.5 times higher rate (363 K) in identical circumstances.

When kept in aqueous alkaline ethylene glycol (5.4 wt. % of water) at 418 K *threo*-dichloride lost all of its chlorine within 24 h, whereas *erythro*-dichloride lost only 68 % of its chlorine content within 825 h in agreement with the fact that intermediate vinylic *cis*-monochlorides react much slower than the corresponding *trans*-monochlorides.

Stereochemically the first reaction step is a *trans*-elimination process in which the different reaction rates of the diastereoisomers were shown to be mainly due to the differences in the ground state energies of their reactive conformations [$k_{threo}/k_{erythro} = 5.0$ (obs.) and 6.5 (calc.) at 298 K].

Diastereoisomeric compounds, such as *threo*- and *erythro*-isomers, may have different reaction rates.[1] For example, the base-promoted dehydrochlorinations of sodium *threo*- and *erythro*-9(10)-chloro-10(9)-hydroxyoctadecanoates have been found to occur at different rates.[2]

The kinetics and mechanisms of the removal of HCl from sodium *threo*-9,10-dichlorooctadecanoate have been studied earlier.[3,4] To complete these studies and to clarify the stereochemical course of the reaction, sodium *erythro*-9,10-dichlorooctadecanoate (*1*) was also prepared and subjected to alkaline dehydrochlorination.

EXPERIMENTAL

GLC analyses were carried out on a Perkin Elmer F 11 gas chromatograph equipped with a hydrogen flame ionization detector. The stainless steel columns were packed with Chromosorb G (60/80 mesh) coated with a polar (5 % XE-60, 200 cm × 3 mm) or non-polar silicone grease (3 % SE-30, 300 cm × 3 mm). The chromatograms were run under isothermal conditions at 160 °C with the former column and at 210 °C with the latter. Before analyses the acids were esterified with diazomethane in diethyl ether containing 10 % of methanol (v/v). The methyl esters of stearic (C18), arachidic (C20), 9-octadecynoic (C18) and 9(10)-chloro-*trans*-9-octadecenoic acids were used as reference compounds.

Proton resonance spectra were recorded on a Perkin Elmer R10 spectrometer (60 MHz) using CCl_4 as solvent and tetramethyl silane (TMS) as internal reference. The sample concentrations varied from 5 to 10 % (w/w). A Perkin Elmer Model 180 spectrophotometer was used to record IR absorption spectra using a thin film of sample between KBr windows. UV absorption spectra were run on a Unicam SP 800 spectrophotometer using absolute ethanol (the State Alcohol Monopoly, Grade AaS, for Spectrophotometry) as solvent. Mass spectra of the prepared methyl esters of the acids were determined with GLC-MS [0.9 m × 3 mm stainless steel column filled with silicone oil OV-17 (2 %; on Chromosorb G 60/80 mesh; 220 °C) connected to a Perkin Elmer M 270 mass spectrometer (ionizing potential 70 eV)].

The chlorine contents of *erythro*-9,10-dichlorooctadecanoic acid and its dehydrochlorination products were determined by the method described previously.[5]

Kinetic measurements and calculation of the reaction rate coefficients were accomplished as shown earlier.[3,4]

Syntheses. Elaidic acid was prepared by isomerization of oleic acid (Fluka AG, 96 % by GLC) by nitrogen oxide.[6] Its melting point after consecutive recrystallizations from diethyl ether and acetone at −17 °C was 42.5 °C (lit.[6] 43−44 °C).

erythro-9,10-Dichlorooctadecanoic acid was obtained by chlorination of elaidic acid (12 g) in CCl_4 solution (150 ml) saturated with chlorine.[5] After evaporation of solvent and excess chlorine the oily residue was treated with urea (12 g) in 100 ml of chloroform containing 10 ml of methanol to remove saturated acids present as impurities in the starting material. The mixture was stirred at 22 °C for 44 h. The excess urea and its fatty acid complexes were then filtered off. Recrystallization of the crude dichloride (about 17 g, after removal of solvent) twice from hexane (4 g of acid per 1 ml of solvent) at −17 °C yielded 7.2 g of acid, m.p. 44.5−45.5 °C (lit.[7] 47.5 °C). The chlorine content based on six determinations was 20.39±0.11 % (calc. 20.07 %). ^{1}H NMR (CCl_4): δ 0.89 (CH_3-), 1.33 [$-(CH_2)_n-$], 2.22−2.31 ($-CH_2COOH$), 1.86 ($-CH_2CHCl-$), 3.9 [$-CH(Cl)-$], and 12.1 ($COOH$). IR, ν_{max}: 1710 (COOH) and 646 cm^{-1} (C−Cl). The acid was converted to its sodium salt (*1*) by methanolic alkali.

Separation of the reaction products. The products formed in the first dehydrochlorination step were obtained by keeping a sample of sodium *erythro*-9,10-dichlorooctadecanoate (*1*) in aqueous alkaline ethylene glycol at 110 °C for 100 min. For the further removal of HCl from the vinylic monochloride formed from *1* more drastic conditions were used: the reaction vessel was kept at 145 °C for 343 and 825 h. The reaction mixture included 1.5 g of *1* and 0.3 mol of NaOH in 400 g of ethylene glycol, which contained 5.4 % of water by weight. The dehydrochlorination products were separated from the reaction mixtures by extraction with CCl_4 as described in an earlier paper.[8] The amount of the viscous residues was about 1 g in each case.

RESULTS AND DISCUSSION

Analyses of the reaction products. After esterification with diazomethane the dehydrochlorination products of *1* separated from the reaction mixtures were analyzed by GLC, NMR, IR, UV, and mass spectroscopy.

The product, after the first dehydrochlorination step, contained 10.1 % of chlorine (10.0 % in theory). IR ν_{max}: 3030 (CH=), 1710 (COOH), 1650 (CH=CCl) and 650 cm^{-1} (C−Cl), ^{1}H NMR (CCl_4): δ 0.89 (CH_3-), 1.96 ($-CH_2CH=$), 2.08 ($-CH_2CCl=$ or $-CH_2C=$), and 5.51 ($-CH=CCl-$; a triplet; J=7.5 Hz). The weak signal at about δ 3.6 may be due to $-CH(OR)-$, where R may be H or the anion of ethylene glycol, or to the alkoxy protons of $-CH(OCH_2CH_2OH)=CH-$. These systems may be formed through the addition of water or ethylene glycol to a double or triple bond. The UV spectrum of the first-step product had a maximum at 218 nm and an inflexion at 232 nm, which may be due to the formation of some conjugated dienes.

Because of the low reactivity of vinylic chloride formed in the first dehydrochlorination step of *1* the removal of the second mol of HCl required more vigorous reaction conditions: a sample of *1* was treated with alkaline reagent for 343 and 825 h at 145 °C. The dehydrochlorination products separated contained 8.3 and 6.4 % of chlorine, respectively. The IR spectrum of the product obtained after the shorter dehydrochlorination period had a new band at 1960 cm^{-1} (C=C=C), which is weakened by the prolonged alkali treatment. The product after 825 h showed also a weak IR band at 2280 cm^{-1} (C≡C). The presence of the allene group is confirmed by a multiplet at δ 5.0 in the NMR spectra of the dehydrochlorination products separated after 343 and 825 h. The product after 343 h had UV maxima at 228 and 255 nm, but after 825 h, only at 255 nm.

The GLC data relative to methyl stearate for the 'methylated' dehydrochlorination products of *1* are collected in Table 1. The results show, that on the polar XE-60 column, the main component (peak 2) of the first dehydrochlorination product is eluted after methyl 9(10)-chloro-*trans*-9-octadecenoate (peak 11). On the non-polar SE-30 column these compounds (peaks 2 and 11) have similar relative retention times. These results are in accordance with those of Stein [9] and Gunstone [10] for the corresponding bromo derivatives, methyl 9(10)-bromo-*cis*-9- and 9(10)-bromo-*trans*-9-octadecenoates. The mass spectrum of peak 2 showed the parent peak (M^+) at *m/e* 331 in agreement with the molecular weight (330.9) of $C_{19}H_{35}O_2Cl$ and the fragment peaks at *m/e* 299, 294, and 263, probably due to $[M-CH_3OH]^+$, $[M-HCl]^+$, and $[M-CH_3OH-HCl]^+$, respectively. Thus the principal product

Table 1. Gas chromatographic retention data for some model compounds and for dehydrochlorination products of sodium *erythro*-9,10-dichlorooctadecanoate (*1*). Retention times are given relative to the retention times for methyl stearate [1.00≡5.24 min (XE-60) and 1.00≡ 6.5 min (SE-30)].

Peak No.	Compound	Relative retention times XE-60	SE-30
Methyl esters of the dehydrochlorination products			
110 °C; 100 min			
1		0.97	0.86
			1.01
			1.18
2		2.56[a]	1.60[a]
145 °C; 343 h			
3		1.11	
4		1.21	1.00
2		2.56[a]	1.63[a]
145 °C; 825 h			
3		1.09	
4		1.21	1.01
2		2.52[a]	1.63[a]
Methyl esters of model acids			
5	Alkali-conjugated linoleic acid	1.01	0.93
6		1.36[a]	1.05[a]
7		1.53	1.18
8	Stearic acid	1.00	1.00
9	9-Octadecynoic (stearolic) acid	1.21	1.00
10	Arachidic acid (C20)	2.26	1.91
11	9(10)-Chloro-*trans*-9-octadecenoic acid	2.36	1.61

[a] The major peak in the chromatogram.

in the first dehydrochlorination step seems to be sodium 9(10)-chloro-*cis*-9-octadecenoate.

Two compounds formed after the further dehydrochlorination (peaks 3 and 4 in Table 1) gave the molecular ion peaks at *m/e* 294 indicating the presence of dienic, allenic, or ynoic esters with the molecular weight (294.4) of $C_{19}H_{34}O_2$. According to the GLC data peak 4 may be an acetylenic ester since its relative retention time on the XE-60 column is equal to that of an authentic sample of methyl 9-octadecynoate (peak 9). Gunstone *et al.*[10] observed that on a polar column the long-chain allenic C_{18}-ester was eluted before the corresponding acetylenic one. Thus the relative retention times 1.1 (peak 3) and 1.2 (peak 4) may also indicate that the former is an allenic intermediate. Moreover, after 343 h the area of the peak of the allenic ester (peak 3 in Table 1) was larger than that of the ynoic ester (peak 4), while after the dehydrochlorination for 825 h, the order was reversed.

GLC and UV spectroscopy pointed out that the first-step dehydrochlorination product contained only small amounts of conjugated dienes, which moreover, were not among the products formed during the further dehydrochlorination of *1*. In the latter case the equilibrium reached was in favour of allenes and acetylenes, but not of conjugated dienes (Scheme 1).

Kinetics and mechanisms of dehydrochlorination. The values of the rate coefficients at five temperatures are collected in Table 2 and the values of thermodynamic functions of activation obtained from them by the method of least squares, in Table 3. Accordingly, the first chlorine from sodium *erythro*-9,10-dichlorooctadecanoate (*1*) is removed as HCl rather easily, although the rate of this reaction is about 2.5 times higher for the corresponding *threo* isomer under identical conditions (Table 3). This observation is in accordance with that reported by Gunstone *et al.*[10] for the corresponding dibromooctadecanoates. The removal of HCl from the vinylic *cis*-monochloride under the conditions used was too slow for determination. By heating of *1* with alkali for 825 h at 145 °C 36 % of the second chlorine was reacted,

Table 2. Rate coefficients for the first dehydrochlorination step of sodium *erythro*-9,10-dichlorooctadecanoate in aqueous alkaline ethylene glycol ($x_{H_2O}=0.165$) at different temperatures.[a]

Temperature °C	10^5k (s^{-1})	10^5k_{OH} (kg mol^{-1} s^{-1})
80	7.02 ± 0.09[b]	4.54 ± 0.06[b]
90	20.5 ± 0.3	13.3 ± 0.2
90	19.9 ± 0.3	12.9 ± 0.2
95	32.4 ± 0.3	20.5 ± 0.2
100	51.0 ± 0.6	33.0 ± 0.4
100	48.0 ± 0.6	31.1 ± 0.4

[a] The substrate concentration was 0.01 mol per kg of solvent, sodium hydroxide concentration was 0.773 mol per kg of solvent. [b] Standard deviation.

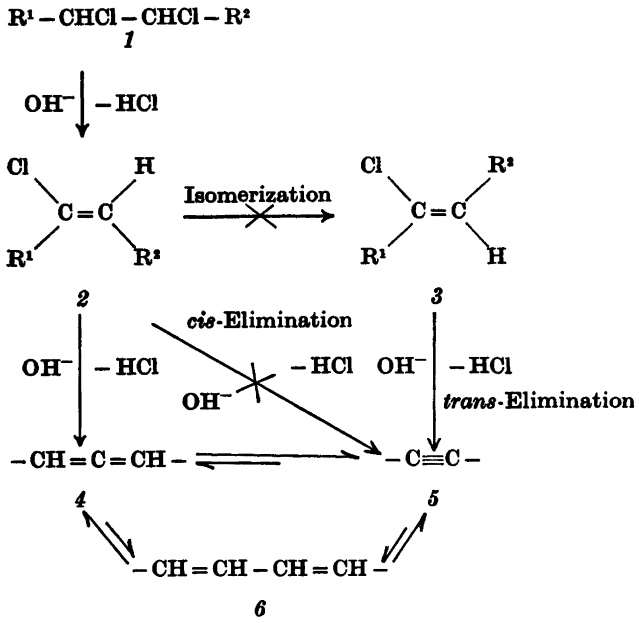
Table 3. Values of the thermodynamic functions of activation and relative rates at 90 °C for the alkaline dehydrochlorination of sodium *threo*- and *erythro*-9,10-dichlorooctadecanoates in aqueous alkaline ethylene glycol containing 16.5 mol % of water.

	Reaction step	$\Delta H^{\ddagger}$ kJ mol^{-1}	$\Delta S^{\ddagger}$ J mol^{-1} K^{-1}	$\Delta G^{\ddagger}$ kJ mol^{-1}	Rel. rate
erythro	I	103.2 ± 2.4[a]	-36.9 ± 6.6[a]	116.6 ± 0.05[a]	140
threo [b]	I	93.8 ± 0.4	-55.3 ± 1.2	113.9 ± 0.02	340
	II	108.1 ± 1.8	-64.3 ± 4.3	131.5 ± 0.2	1

[a] Standard deviation. [b] Refs. 3, 4.

whereas from *threo*-9,10-dichlorooctadecanoate all chlorines were removed after 24 h at 145 °C. This result confirms that the dehydrochlorination of *1* also occurs through *trans*(*anti*)-elimination (E2) where 9(10)-chloro-*cis*-9-octadecenoate is the first step product. The difficulty of the further dehydrochlorination of this *cis*-monochloro-ene (*2*) is mainly due to the great steric hindrance for the *cis*-(*syn*)-elimination.

According to Staley and Doherty [11] the formation of allene from 4-bromo-*cis*-4-octene is very much faster than that of the triple bond through *cis*(*syn*)-elimination. Their results show also that *trans*(*anti*)-elimination of HBr from 4-bromo-*trans*-4-octene yields the triple bond about forty times faster than the corresponding *cis*-isomer forms the allene. Thus the rates of dehydrobromination of monobromooctenes decrease in the sequence $k_{anti} > k_{allene} > k_{syn}$. Vigorous treatment of *1* with alkali yielded acetylenic and allenic intermediates but very little, or no conjugated dienes in accordance with the above results and those of Gunstone



$R^1 = CH_3(CH_2)_7 -$
$R^2 = -(CH_2)_7COONa$

Scheme 1. The possible reaction pathways in the alkaline dehydrochlorination of *1*.

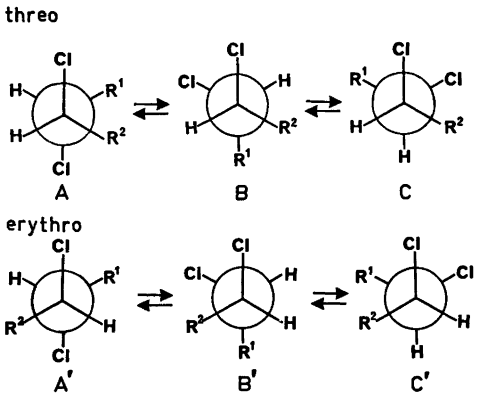


Fig. 1. The minimum energy conformations of sodium *threo*-(A – C) and *erythro*-9,10-dichlorooctadecanoates (A′ – C′).

et al.[10] for the corresponding bromo octadecanoates. Consequently, the base-promoted dehydrochlorination of *1* may be proposed to occur through the following steps (Scheme 1): in the first reaction step *trans*(*anti*)-elimination produces 9(10)-chloro-*cis*-9-octadecenoate (*2*) which during prolonged dehydrochlorination gives allenic intermediates (*4*) and octadecynoates (*5*) both of which are in equilibrium with dienic derivatives (*6*). The formation of *5* through *cis*(*syn*)-elimination or through isomerisation of *2* to *trans*-chloro-ene (*3*) followed by *trans* (*anti*)-elimination is only of minor importance. Secondary isomerizations of *4* and *5* may, of course, give other allenic and acetylenic derivatives,[12,13] which may also be formed from the vinylic *cis*-chloro alkene *2* (Scheme 1) through elimination of HCl preceded by a double bond shift.

Stereochemistry of the first dehydrochlorination step. The basepromoted dehydrochlorination proceeds under the conditions used by E2 mechanism.[14] A stereochemical requirement for this mechanism is that the leaving groups H and Cl are in an *anti* (or *trans*) periplanar conformation.[3,4,14,15]

Both sodium *threo*- and sodium *erythro*-9,10-dichlorooctadecanoates have three minimum energy conformations (Fig. 1) from which only C (*threo* isomer) and B′ and C′ (*erythro* isomer) can undergo the E2 elimination.

The rate of a given reaction of a conformationally heterogeneous system may be presented by the relation [15]

$$k = \sum x_i k_i$$

where x_i is the mol fraction and k_i the rate coefficient of the reaction studied for the ith conformation. Hence the total rates for the first dehydrochlorination steps of sodium *threo* (k_t) and *erythro*-9,10-dichlorooctadecanoates (k_e) may be written

$$k_t = k_A x_A + k_B x_B + k_C x_C$$

$$k_e = k_{A'} x_{A'} + k_{B'} x_{B'} + k_{C'} x_{C'}$$

If the dehydrochlorination reaction occurs predominantly as an *anti*-elimination $k_C \gg k_A \approx k_B \approx 0$ and $k_{C'} \approx k_{B'} \gg k_{A'} \approx 0$. Accordingly,

$$k_t \approx k_C x_C \text{ and}$$

$$k_e = k_{B'} x_{B'} + k_{C'} x_{C'}$$

Moreover, it is reasonable to assume that $k_C = 2k_{B'} = 2k_{C'}$ and $x_{B'} = x_{C'}$ where the factor two is due to the double *anti*-arrangement of the conformation C.[14] Consequently,

$$k_t/k_e = x_C/x_{C'}$$

If the *gauche* interactions due to the groupings R^1 and R^2 do not differ essentially from those of a methyl group the mol fraction of the different conformations may be estimated using the following values (in kJ mol^{-1}) for the various *gauche* interactions at 298 K: $R^1 - R^2$ (Me – Me) [16] + 2.7, Cl – Cl [17] + 5.0, and R^1 – Cl or R^2 – Cl (Me – Cl) [17] – 0.2.

The estimated interaction energies for the conformations of *threo*- and *erythro*-9,10-dichlorooctadecanoates are then (Fig. 1)

A + 2.3	B + 7.7	C + 4.6
A′ – 0.4	B′ + 7.5	C′ + 7.5

Since [15] $x_i/x_{i+1} = \exp\ [(H_i - H_{i+1})/RT]$ and $\sum x_i = 1$ we can estimate that $x_C \simeq 0.26$ and $x_{C'} = 0.04$ and thus $k_t/k_e \simeq x_C/x_{C'} \simeq 6.5$ at 298 K. Experimentally, the rate ratio for the *threo*- and *erythro*-9,10-dichlorooctadecanoates was found to be 2.5 at 363 K and 5.0 at 298 K.[14]

The calculated and experimental results being very close to each other support the postulation that the reaction is really preponderantly *trans*-elimination (E2) and that the difference in the reaction rates is mainly due to the different ground state energies of the reactive conformations of the *threo* and *erythro* isomers.

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Crystalline Leghemoglobin. XV. Effect of Urea on the Conformation of the Slow Component (Lb*a*)

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Soybean ferrileghemoglobin is reversibly unfolded by urea in phosphate buffer, pH 6.5. The unfolding is accompanied by extensive changes in the ultraviolet and visible spectra of the protein, which have been utilized in investigating the equilibrium and kinetics of the unfolding process at 25 °C. The studies were performed on component *a*, Lb*a*, of soybean leghemoglobin and, for comparison, on sperm whale myoglobin. The equilibrium denaturation data showed that the 50 % denaturation point is at 5.15 M urea for leghemoglobin and 7.60 M for sperm whale myoglobin.

The spectrum of denatured leghemoglobin (8.8 M urea) shows an increase in its low spin character, which is assumed to indicate that the heme group is still attached to the denatured protein in the protein concentrations used in this study. The denaturation of ferrileghemoglobin at high urea concentrations was found to be largely reversible upon diluting the reaction mixture. The kinetics of the unfolding reaction for leghemoglobin and for sperm whale myoglobin at pH 6.5 were investigated in the Soret band region. These studies indicate that leghemoglobin is a considerably less stable molecule than sperm whale myoglobin.

Urea denaturation has been extensively used to investigate the forces that maintain the protein molecule in its native form.[1-3] The effect of urea on myoglobin has recently received attention. Schlechter and Epstein [4] have reported a study of the denaturation equilibria of both whale and horse metmyoglobins, and Cann observations on the kinetics of the urea denaturation of metmyoglobin.[5-7] This investigation reports the effect of urea on the slow component of soybean leghemoglobin (Lb*a*). For comparison, some studies were also made on the effect of urea on sperm whale myoglobin.

MATERIALS AND METHODS

Leghemoglobin. The two main components (Lb*a* and Lbc) of soybean leghemoglobin were prepared as described previously.[8]

Sperm whale myoglobin was a commercial preparation (Type II) from Sigma Chemical Company (St. Louis, U.S.A.).

Urea, pure, from E. Merck AG (Darmstadt, Germany) was used. It was twice recrystallized from ethanol at 4 °C. Crystals were collected and washed with cold absolute ethanol, dried *in vacuo* and stored *in vacuo* over $CaCl_2$ and $CaSO_4$. Urea solutions were deionized immediately before use by passing the solutions through two ion exchange columns (2 × 8 cm containing 25 ml of Dowex 1 × 2 and 25 ml of Dowex 50 × 2, respectively).

Measurements of absorption spectra. Most of the spectra were measured using a Cary 15 recording spectrophotometer equipped with a jacketed cell holder through which circulated water from a constant temperature bath.

For the absorption spectra measurements at a fixed wavelength a Beckman DU-2 instrument was used without recording attachment.

Procedure for measuring kinetics. A cell containing the buffered urea solution (2.475 ml) was placed in the sample compartment of a Cary 15 recording spectrophotometer. 25 µl of a concentrated solution of Lb*a* or Mb was added to the cell using a cuvette-add-a-mixer. The change in absorption at the Soret band was monitored using the synchronous drive attachment of the spectrophotometer. The denaturation was followed until a very slow linear decline was obtained.

Circular dichroism measurements. Circular dichroism (CD) data for Lb*a* solutions were obtained using a Cary 61 recording spectropolarimeter, which was calibrated with D-10-camphorsulfonic acid. The circular dichroism data were reduced to mean residue ellipticities, $[\theta']_\lambda$, using the following formula:

$$[\theta']_\lambda = M_0[\theta]/10lc$$

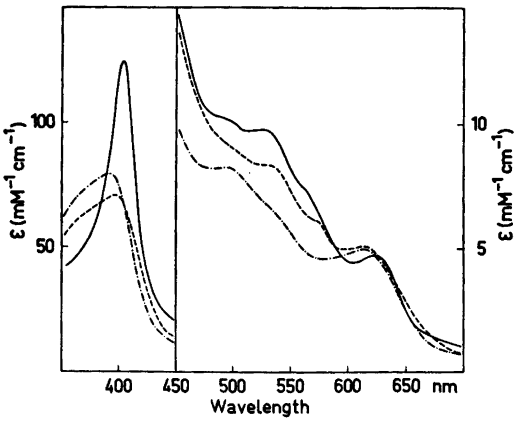

Fig. 1. Absorption spectra of soybean ferrileghemoglobin, Lb*a*, in 0.05 M sodium phosphate buffer, pH 6.5 at 25 °C. (———) native Lb*a*, (– – –) Lb*a* in 8.8 M. For comparison, the spectrum of protohemin IX (– · –) in 8.8 M urea and the same buffer is shown. The actual concentration of Lb*a* and of free protohemin were 7.2×10^{-5} M.

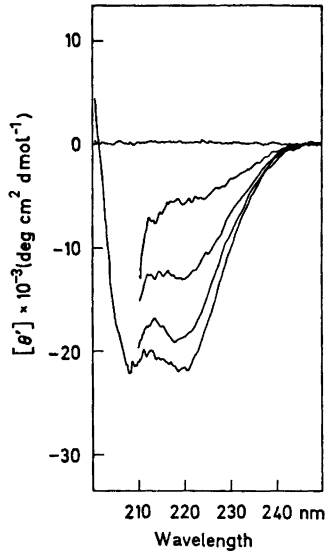

Fig. 2. The effect of urea on circular dichroism spectra of soybean ferrileghemoglobin, Lb*a*, in the spectral region below 250 nm. The measurements were made in 0.05 M sodium phosphate buffer, pH 6.5 at 25 °C, using a silica cell with 1 mm light path. Urea concentrations: 0, 5.0, 6.0, and 8.0 M. The value of mean residue ellipticity increases with increasing urea concentration. The actual protein concentration was 1.02×10^{-5} M.

where M_0, l, c are the mean residue weight, the length of the light path in cm, and concentration of the protein in g/ml, respectively. $[\theta]$ is the ellipticity in degrees recorded on the chart paper. The above formula gives the $[\theta']$ value, expressed in degree cm²/decimol of residues, which satisfies the relationship

$$[\theta]_\lambda = 2.303\ (4.500/\pi)(\varepsilon_L - \varepsilon_R)$$
$$= 3.300(\varepsilon_L - \varepsilon_R)$$

where $(\varepsilon_L - \varepsilon_R)$ is the difference in the molar extinction coefficients for left and right polarized light. The mean residue weight employed for Lb*a* was 106.5.[9]

Pyridine hemochrome was determined according to Paul *et al.*[10]

pH Measurements were carried out at 20 °C with a Radiometer PHM 3 pH-meter, which had been standardized against phthalate and borate buffers. The pH-values given in the experimental section refer to the pH-values of the original buffer.

RESULTS

The absorption spectrum of native Lb*a* and that of the protein in 8.8 M urea at pH 6.5 are shown in Fig. 1. At high urea concentrations spectral changes occur indicating conformational changes at the heme site. The typical ferrihemochrome spectrum with maxima at 530 nm and 565 nm shows that the low spin character of Lb*a* has increased in 8.8 M urea compared to that of protohemin IX in 8.8 M urea (Fig. 1). This is assumed to indicate that the heme moiety is not dissociated, but is attached to the denatured protein to a great extent at the protein concentrations used in this study.

CD spectra of Lb*a* in urea solutions were also measured in the ultraviolet region, as shown in Fig. 2. An increase of the ellipticity in the region of 200 – 250 nm at 8.0 M urea indicates extensive unfolding of the α-helix. It is evident that concomitant changes in the absorption and CD spectra occur with the denaturation by urea. Fig. 3 shows a plot of the fractional absorbance change at the Soret maximum as a function of the urea concentration.

The duration of incubation was found to have an influence on the extinction values; the largest decrease occurred within 30 s at the beginning of the incubation with the values

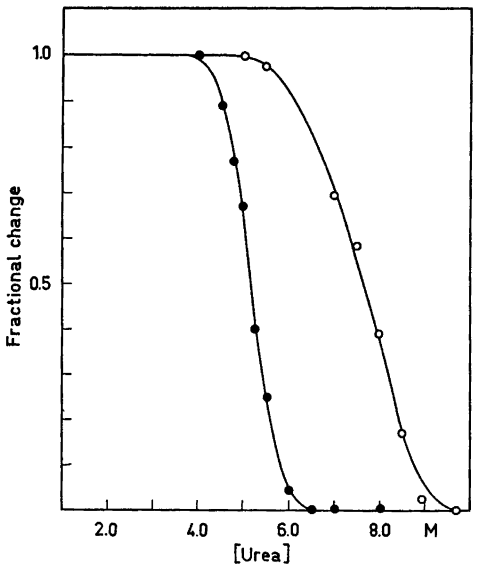

Fig. 3. Fractional absorption change at Soret maximum that occurs on denaturation of soybean ferrileghemoglobin, Lb*a*, (●), and sperm whale ferrimyoglobin, Mb, (○) at different urea concentrations in 0.05 M sodium phosphate buffer, pH 6.5 at 25 °C. The wavelengths used were 403.5 nm (Lb*a*) and 409.5 nm (Mb). The fractional change is equal to $(A_U - A_D)/(A_N - A_D)$, where A_N denotes the absorbance of the native protein, A_D that of the totally denatured protein, and A_U the absorbance at a given urea concentration. The actual protein concentration was 3.34×10^{-6} M.

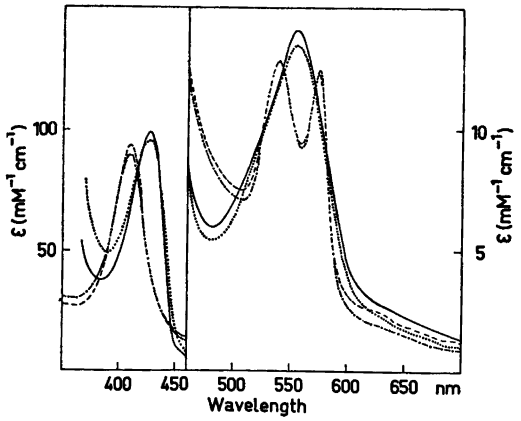

Fig. 4. Spectral properties of soybean leghemoglobin, Lb*a*, after reversal of the denaturation. FerriLb*a* (14.4×10^{-5} M) was denatured at 25 °C for 30 min in 0.05 M sodium phosphate buffer, pH 6.5, containing 8.0 M urea, after which the urea was diluted to 4.0 M with same buffer. (····) shows the spectrum of the reduced form (ferroLb*a*) produced by the addition of a small amount of dithionite. (— · —) represents the spectrum of the oxygen complex, obtained by bubbling air through the solution of the renatured ferroLb*a*. For comparison, the spectra of the native ferroLb*a* (———) and the oxygen complex of the native ferroLb*a* (— — —) in 0.05 M sodium phosphate buffer, pH 6.5, are shown.

decreasing more slowly thereafter. In the case of myoglobin the fast period in the beginning lasted about 10 min. The 50 % denaturation point of Lb*a* was found to be at 5.15 M urea and that of sperm whale myoglobin at 7.60 M urea.

The spectral changes accompanying denaturation of Lb*a* in urea were found to be largely reversible when the urea concentration was reduced by dilution. The recovery of the visible spectrum following denaturation is shown in Fig. 4 for Lb*a* incubated in 8.0 M urea for 1 h prior to dilution to 4.0 M urea. The recovery of the reduced form of Lb*a* was not limited to the return of a native-like spectrum only, the oxygen binding capability of the reduced form was also recovered. This is a strong indication that Lb*a* must have returned to a conformation closely resembling that of the biologically active native form.

The recovery of the intensity of the Soret band upon dilution was found to depend on the length of time a solution was kept in 8.0 M urea before diluting to the lower concentration. If the heme group is dissociated from the denatured protein, the irreversibility may be associated with nonspecific binding to the renatured globin.

These results indicate that an equilibrium exists between the native and unfolded states of ferrileghemoglobin in urea solutions, with the heme groups attached to both forms. It is known to be difficult to distinguish between a simple "two-state" unfolded equilibrium and a more complex, or "multi-state", one. We assume, therefore, a "two-state" mechanism in this particular case, for which the observed transition is

$$[NH] \rightleftharpoons [DH]$$

where [NH] and [DH] represent the heme-

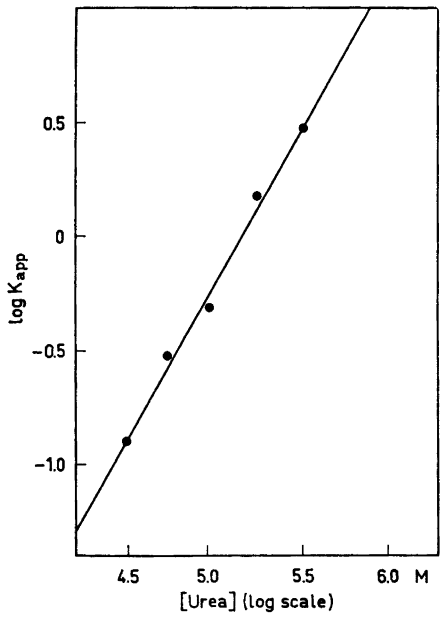

Fig. 5. Plot of log K_{app} *versus* logarithm of the urea concentration. The urea concentration when K_{app} is equal to unity is 5.15 M, and the slope of the line is about 16. The experimental conditions are identical to those in Fig. 3.

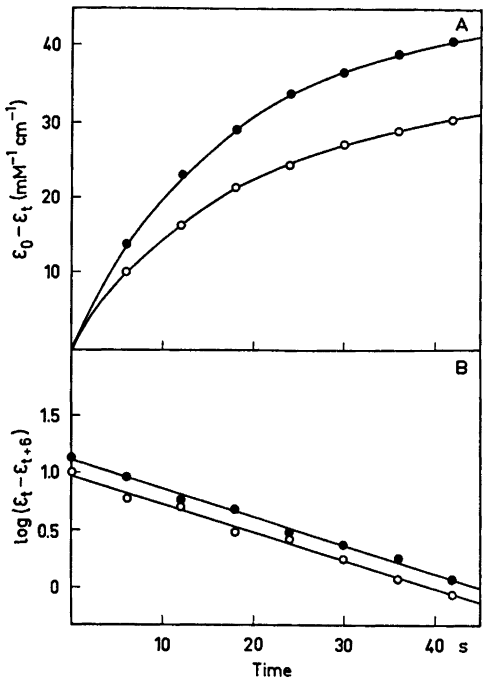

Fig. 6. The denaturation kinetics of soybean ferrileghemoglobin at urea concentrations of 5.25 and 5.50 M measured at 403.5 nm. The experimental conditions were identical to those in Fig. 3. *A*. The kinetics, recorded as differences between the extinction coefficients at zero time, and at the time t, (○) at 5.25 M and (●) at 5.50 M urea. *B*. Guggenheim plots for the denaturation kinetics. The ordinate refers to the logarithm of the difference between extinction coefficients separated by a constant time interval of 6 s, (○) at 5.25 M and (●) at 5.50 M urea.

protein complex in the native and denatured form, respectively. The equilibrium constant for the denaturation reaction is

$$K_D = [DH]/[NH]$$

and an apparent equilibrium constant can be calculated using an equation of the form [3]

$$K_{app} = A[U]^{\nu}$$

where [U] is the urea concentration and A and ν are constants. A plot of log K_{app} against log [U] is given in Fig. 5. The midpoint of the transition, which occurs when K_{app} is equal to unity, is found to be at 5.15 M urea. The slope was calculated to be about 16.

The effect of urea concentration on the course of denaturation was studied for Lb*a* and, for comparison, sperm whale myoglobin. In Fig. 6A the difference between the extinction coefficient at 403.5 nm at zero time and at time t is plotted against time. Fig. 7A shows a similar plot for myoglobin measured at 409.5 nm. It can be seen that the rate of denaturation of Lb*a* is much faster than that of myoglobin under the same conditions. Fig. 6B shows a plot according to the Guggenheim method,[11] which eliminates the need for a final absorbance value. The expression used is

$$\ln (A_t - A_{t+\Delta t}) = -(k_{+1} + k_{-1})t + \text{constant}$$

where A_t and $A_{t+\Delta t}$ are absorption values at a time t and $t+\Delta t$, respectively. A Δt of 6 s was chosen. It can be seen that the kinetics at the given urea concentrations are first order as for myoglobin (Fig. 7B).

DISCUSSION

The denaturation behaviour of soybean leghemoglobin (Lb*a*) was studied in urea solutions.

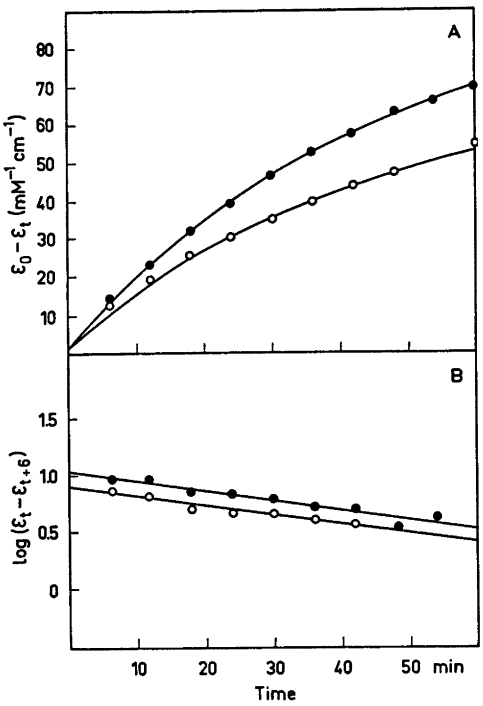

Fig. 7. The reaction kinetics of sperm whale ferrimyoglobin at urea concentrations of 7.5 and 8.0 M measured at 409.5 nm. The experimental conditions were identical to those in Fig. 3. *A.* The kinetics, recorded as differences between the extinction coefficients at zero time and at the time *t*, (○) at 7.5 M and (●) at 8.0 M urea. *B.* Guggenheim plots for the denaturation kinetics. The ordinate refers to the logarithm of the difference between extinction coefficients separated by a constant time interval of 6 min, (○) at 7.5 M and (●) at 8.0 M urea.

Comparative studies have also been performed on sperm whale myoglobin. The equilibrium denaturation data showed that the 50 % denaturation point is at 5.15 M urea for soybean leghemoglobin and 7.60 M urea for sperm whale myoglobin, the latter being close to the values reported in earlier studies.[4,12] The denaturation of the two proteins was found to be reversed by diluting the reaction mixture. It was shown that changes in the absorption spectra and circular dichroism accompanied the denaturation of Lb*a* by urea, but whether this reflects the same structural changes as those responsible for the changes in the heme absorption, or whether it concerns different areas of the molecule, is uncertain.

In the case of heme proteins, including leghemoglobin, the conformational properties of the globin are largely dependent upon the presence of the heme, the globin structure corresponding to a more unfolded state than the heme-complex.[13–17] It is therefore not surprising, that some detachment of the heme groups may accompany denaturation of the protein. However, with the present experimental conditions, it is assumed that no heme dissociation occurs to any large extent during the urea denaturation of leghemoglobin. It is evident that the renaturation of the denatured Lb*a* proceeds more completely when the heme moiety remains attached to the denatured protein. This additionally supports the belief that the heme group has an important role in maintaining the secondary and tertiary structures of the molecule.

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Phloroglucinol Derivatives of *Hagenia abyssinica*. II.* The Structure Determination of Kosotoxin and Protokosin**

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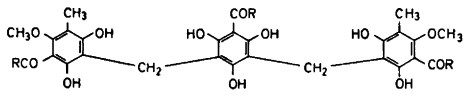
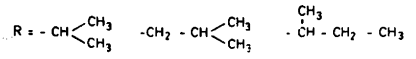
Phloroglucinol derivatives (kosins) from *Hagenia abyssinica* (Bruce) Gmel. have been reinvestigated. On the basis of reductive alkaline cleavages and spectroscopic evidence the structures (II) and (III) are proposed for kosotoxin and protokosin, respectively. The so-called β-kosin proved to consist of a mixture of isobutyryl (iB), isovaleryl (iV), and 2-methylbutyryl (2-MeB) side chain homologues of methylene-bis-pseudo-aspidinol (IV).

Les dérives phloroglucinoliques (kosines) isolés d'*Hagenia abyssinica* (Bruce) Gmel. ont été réexaminés. Sur la base des données des clivages réductifs alcalins et des preuves spectrales les structures (II) et (III) sont proposées pour la kosotoxine et la protokosine, respectivement. La prétendue β-kosine est en fait un mélange des homologues isobutyryles (iB), isovaléryles (iV) et méthyl-2 butyryles (2-MeB) des chaînes latérales du méthylène-bis-pseudo-aspidinol (IV).

In a recent publication [1] we reported the isolation of four phloroglucinol derivatives (kosins), designated K1 – 4, from kousso flowers (*Flos koso*).*** For one of these (K1), apparently identical with the earlier known kosidin,[2] we proposed the structure (I). Owing to its close structural similarity to the *Dryopteris* phloroglucinols, however, we preferred to give to kosidin the new name trispseudo-aspidinol.



(I)



We also isolated [1] substances with the properties recorded for kosotoxin (K2) [3,4] and protokosin (K3 and/or K4).[3,5,6] All kosins proved to be mixtures of isobutyryl (iB), isovaleryl (iV), and 2-methylbutyryl (2-MeB) side-chain homologues. Because of the small amounts isolated, the structures of kosotoxin (K2) and protokosin (K3 and/or K4) could not be fully elucidated. Through the kind cooperation of Professor T. Reichstein of Basle, however, we obtained a further commercial sample of Flos koso. As a result of extensive spectral analyses and examinations of degradative products, the structures (II) and (III) are now proposed for kosotoxin (K2) and protokosin (K4), respectively.†, ††

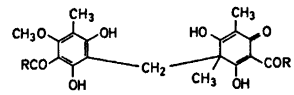
* Part I, Ref. 1.

** Part of this work was presented at the 4th Scandinavian Natural Products Chemistry Symposium held at Koli, Finland, June 12 – 17, 1973.

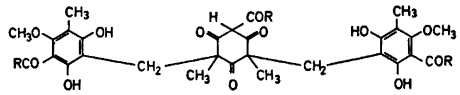
*** The dried female flowers (Flos koso) of *Hagenia abyssinica* (Bruce) Gmel. are known unde there names "kousso", "kosso" or "kusso" especially in Africa and the Near East. The kosins are presumably located in typical glandular hairs occurring on the epidermis.

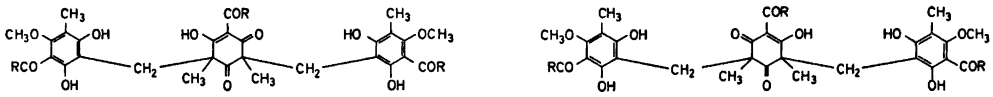
† It is plausible that even kosotoxin (II), for which only one formula is presented, can exist in several tautomeric forms.

†† K3, for which similar chemical and physical properties to those of K4 (protokosin) were reported,[1] proved to consist mainly of K4. The differences in their mass spectra turned out to be due to the fact that protokosin, at high temperatures, is transformed in the ionization chamber of the mass spectrometer to a mixture of compounds corresponding to $C_{41}H_{50}O_{11}$, $C_{40}H_{48}O_{11}$, $C_{39}H_{46}O_{11}$, and $C_{38}H_{44}O_{11}$.

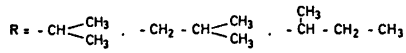


(II)

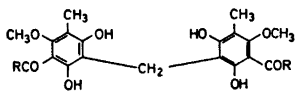




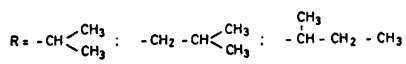
(III)



Moreover, we studied the so-called kosin, that is formed from kousso constituents by the action of alkali.[2–7] This "kosin", which was earlier considered to be a mixture of two isomeric dimethyl ethers of methylene-bis-methylphloroisobutyrophenone, assigned as "α-" and "β-kosin",[6,8] proved to be a mixture of compounds differing only in their acyl side chains. Thus, they all have the same "skeletal structure" as the earlier known "α-kosin" [IV, R = $-CH(CH_3)_2$]. Moreover, the acyl side chains proved to be the same as those found in the above mentioned naturally occurring phloroglucinol constituents of kousso: isobutyryl (iB), isovaleryl (iV), and 2-methylbutyryl (2-MeB). For the same reasons as advanced for trispseudo-aspidinol (I),[1] the name methylene-bis-pseudo-aspidinol is proposed for this mixture of "kosin" homologues (IV). Analogously, the earlier designated "α-kosin" [IV, R = $-CH(CH_3)_2$] should be called methylene-bis-pseudo-aspidinol iBiB.



(IV)



STRUCTURE DETERMINATIONS

Trispseudo-aspidinol (kosidin) (I). This tricyclic phloroglucinol derivative, for which the structure (I) was proposed earlier [1] and which was obtained as yellowish plates, m.p. 167 – 169 °C (methanol),[1] was not isolated in crystalline form in connection with the present work. According to semiquantitative TLC (*cf.* Ref. 1) it is a minor component (< 5 %) in the phloroglucinol mixture of kousso (crude kosin).

The mass spectrum of trispseudo-aspidinol (I) (Fig. 1) shows molecular peaks at *m/e* 710, 696, 682, and 668, corresponding to $C_{39}H_{50}O_{12}$, $C_{38}H_{48}O_{12}$, $C_{37}H_{46}O_{12}$, and $C_{36}H_{44}O_{12}$, respectively. These four peaks, as well as the peaks at *m/e* 653, 639, and 625, which can be assigned to the cleavages of C_4H_9- and C_3H_7- side chain units from the molecular ions, are in good agreement with the results of alkaline cleavages [1] and confirm that trispseudo-aspidinol (I) is a mixture of side chain homologues. The general fragmentation of trispseudo-aspidinol (I) is analogous to that found earlier for polycyclic *Dryopteris* phloroglucinols [9–11] and supports the proposed structure (I).

The NMR spectrum ($CDCl_3$) of trispseudo-aspidinol also supports the structure (I), in agreement with the NMR data reported earlier,[1]

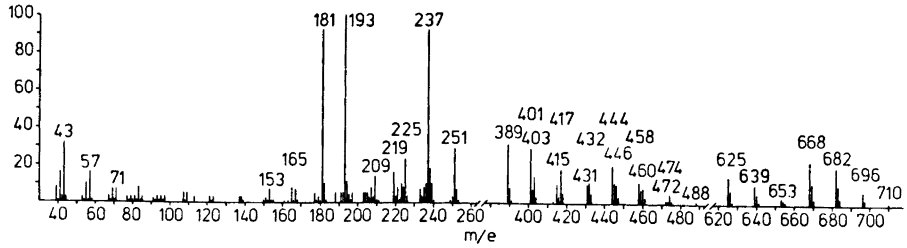


Fig. 1. Mass spectrum (70 eV) (*T* 200 °C) of trispseudoaspidinol (I).

showing the following signals:* δ 1.18 [18 H, d, *J* 7 Hz, mainly 3 $-CO-CH(CH_3)_2$], 2.12 (6 H, s, 2 CH_3-Ar), 3.72 (6 H, s, 2 CH_3O-Ar), 3.82 (4 H, s, 2 $-CH_2-$), ~3.9** [3 H, m, mainly 3 $-CO-CH(CH_3)_2$], 9.56 (2 H, s, 2 OH), 10.72 (2 H, s, 2 OH), 15.46 (1 H, s, OH), 15.56 (1 H, s, OH), 15.80 (1 H, br s, OH).

Kosotoxin (II). This bicyclic phloroglucinol derivative was isolated according to previously reported methods.[1] Repeated recrystallizations from hexane of the chromatographic fractions rich in kosotoxin gave yellowish plates, m.p. 119–122 °C (lit.[1] m.p. 110–112 °C). Kosotoxin is the main component (> 50 %) in the phloroglucinol mixture of kousso. The optical activity of kosotoxin (*cf.* Experimental Section) is in agreement with the asymmetric structure proposed (II). For the products obtained by reductive alkaline cleavage, which support well the structure (II); see Ref. 1 and Part III.

The mass spectrum of kosotoxin (II) (Fig. 2) shows molecular peaks at *m/e* 488, 474 and 460, corresponding to $C_{27}H_{36}O_8$, $C_{26}H_{34}O_8$ and $C_{25}H_{32}O_8$, respectively. The peaks at *m/e* 431 and 417 support the assumption that kosotoxin is a mixture of C_4H_9- and C_3H_7- side chain homologues and confirm the results of the alkaline cleavages. The general fragmentation pattern is similar to that found earlier for polycyclic *Dryopteris* phloroglucinols [9–11] and supports the proposed structure II.

The NMR spectrum ($CDCl_3$) of kosotoxin also supports the structure II, the following signals are shown: δ 1.12 [6 H, d, *J* 7 Hz, mainly $-CO-CH(CH_3)_2$], 1.18 (6 H, d, *J* 7 Hz, mainly $-CO-CH(CH_3)_2$), 1.28 (3 H, s, $CH_3-C\lesssim$), 1.92 (3 H, s, $CH_3-C\lesssim$), 2.20 (3 H, s, CH_3-Ar), 3.04 (2 H, br, s, $-CH_2-$), 3.76 (3 H, s, CH_3O-Ar), ~ 3.9 [2 H, m, mainly 2 $-CO-CH(CH_3)_2$], 9.66 (1 H, s, OH), 11.06 (1 H, s, OH), 15.54 (1 H, s, OH) 19.18 (1 H, s, OH).

Protokosin (III). This tricyclic phloroglucinol derivative was isolated as described previously,[1] crystallized from acetone, and obtained in the form of colourless needles, m.p. 181–183 °C (lit. m.p. 174–176 °C,[1] 176 °C,[2,3] 182 °C,[2,5]). Protokosin proved to be a major component (*ca.* 30–40 %) in the phloroglucinol mixture of kousso. Its optical activity (*cf.* Experimental Section) supports the asymmetric structure proposed (III). For the products obtained by reductive alkaline cleavage, which are in agreement with the structure III, see Ref. 1 and Part III.

The mass spectrum of protokosin (III) (Fig. 3) presents molecular peaks at *m/e* 738 (weak), 724, 710, and 696, corresponding to $C_{41}H_{54}O_{12}$, $C_{40}H_{52}O_{12}$, $C_{39}H_{50}O_{12}$, and $C_{38}H_{48}O_{12}$, respectively. The peaks at *m/e* 681, 667, and 653 support the assumption that protokosin is a mixture of C_4H_9- and C_3H_7-side chain homologues and confirm the results of the alkaline cleavages. The general fragmentation pattern is similar to that found earlier for polycyclic *Dryopteris* phloroglucinols [9–11] and supports the proposed structure (III). However, the very

* The signals assigned to the OH groups in the NMR spectra of trispseudo-aspidinol (I), kosotoxin (II), protokosin (III), "kosin" (IV) (m.p. 148–150 °C), and pseudo-aspidinol (VIII) (m.p. 65–66 °C) disappear after treatment with D_2O. The intensities given for acyl side chain proton signals should be regarded as approximative since trispseudo-aspidinol, kosotoxin, protokosin, and in smaller amount "kosin" (m.p. 148–150 °C) and pseudo-aspidinol (m.p. 65–66 °C) are mixtures of isobutyryl, isovaleryl, and 2-methylbutyryl side chain homologues. The weak signals due to protons in the isovaleryl side chains and γ-protons and secondary β-protons in the 2-methylbutyryl side chains are omitted.

** Owing to a typing error this signal was earlier reported as 3.0.[1]

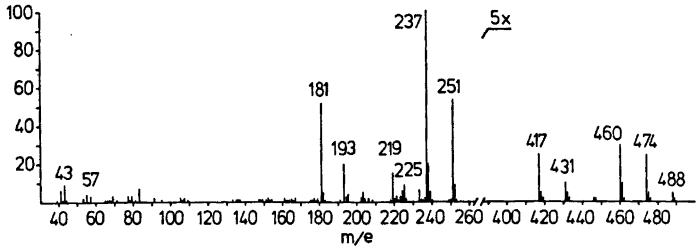


Fig. 2. Mass spectrum (70 eV) (*T* 150 °C) of kosotoxin (II).

weak intensities of the molecular peaks in the mass spectrum (Fig. 3) of protokosin are noteworthy.

The thermal rottlerone change, found earlier in connection with polycyclic *Dryopteris* phloroglucinols [9–11] and which takes place in the ionization chamber of the mass spectrometer, has to be taken into consideration in the interpretation of the fragmentation patterns of kousso constituents. In the case of protokosin, this phenomenon is clear, and among the compounds formed by this procedure, the "kosin" homologues are probably the most characteristic. They are partly responsible for example, for the formation of the ions assigned to *m/e* 488, 474, 460, 431, and 417.

The NMR spectrum ($CDCl_3$) of protokosin also supports the structure III showing the following signals: δ 1.16 [18 H, m, mainly 3 $-CO-CH(CH_3)_2$], 1.66 (6 H, br s, 2 $CH_3-C\leqslant$), 2.20 (6 H, s, 2 CH_3-Ar), 3.64 (4 H, s, 2 $\geqslant C-CH_2-C\leqslant$), 3.76 (6 H, s, 2 CH_3O-Ar), ~3.9 [3 H, m, mainly 3 $-CO-CH(CH_3)_2$], 6.02 (1 H, br s, OH), 10.40 (1 H, s, OH), 13.14 (1 H, s, OH), 14.56 (1, H br s, OH), 19.02 (1 H, s, OH). The intensities given for the signals should be regarded as approximate, owing to the existence of protokosin in several tautomeric forms and to the fact that protokosin is a mixture of isobutyryl, isovaleryl, and 2-methylbutyryl side chain homologues. The spectrum shows a weak signal at δ 3.46 which is probably due to the proton attached to the central ring in the fully ketonic form.

Methylene-bis-pseudo-aspidinol ("kosin") (pseudo-aspidin) (IV). In 1901 Lobeck [8] claimed that the commercial "kosin" of E. Merck (Darmstadt), apparently the first crystalline product isolated from kousso flowers, was a mixture of two closely related compounds, which he called "α-" and "β-kosin". Each of these compounds proved to contain two methoxyl groups. In 1952 Birch and Todd [6] suggested the formulae V and VI for "α-" and "β-kosin", respectively. On the basis of extensive synthetic work, Orth and Riedl [8] later rejected the proposed structures. As their synthetic 5,5′-methylene-bis-(3-methyl-phloroisobutyrophenone-2-methylether) [IV R = $-CH(CH_3)_2$] proved to be identical with the "α-kosin" of Birch and Todd,[6] the structure of "α-kosin" was settled. Moreover, Orth and Riedl [8] showed that "β-kosin" was not identical either with compound (VI) or any other synthetical dimethyl ether of 5,5′-methylene-bis-(3-methyl-phloroisobutyropheno-

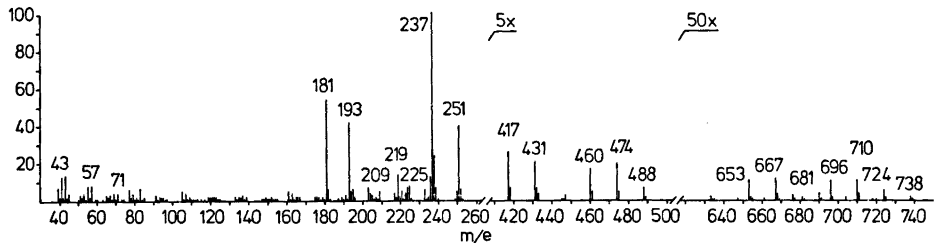
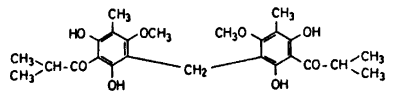
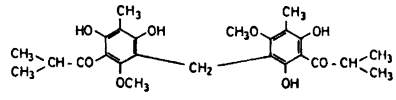


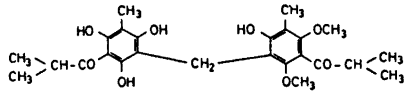
Fig. 3. Mass spectrum (70 eV) (*T* 220 °C) of protokosin (III).



(V)



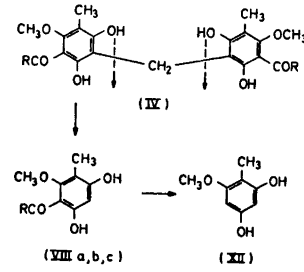
(VI)



(VII)

ne) containing the two methoxyl groups in different rings. Therefore, they proposed for "β-kosin" the tentative formula VII, where the two methoxyl groups are in the same ring.

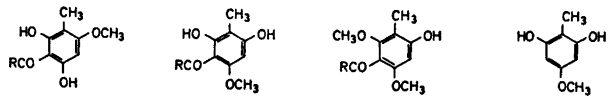
In the present work several "kosin" preparations with slightly varying melting points and crystalline forms * were isolated by column chromatography after treatment of crude kosin, kosotoxin (II), or protokosin (III) with alkali. In agreement with previous findings,[2-6] these "kosin" preparations proved to be very resistant against further break-down with alkali. "Kosin" was detected by TLC in the reaction mixture even after 24 h heating in 15 % NaOH on a water bath.** The sole decomposition products observed consisted of pseudo-aspidinols iB, iV, and 2-MeB (VIIIa, b, and c) (Scheme 1). As in our earlier work,[1] isomeric aspidinols such as (IX) and (X), were not detected by PC or TLC. Nor was there any sign of 3-methylphloroisobutyrophenone-2,6-dimethyl ether (XI) or other isomeric dimethyl ethers, which could be expected to appear among the decomposition products of (VII) or its isomer. On the other hand, some methylphloroglucinol-2-methyl ether (XII), resulting from the reductive alkaline cleavage of the acyl side chains of (VIIIa, b, and c), was recognized by TLC (Scheme 1). The same ether (XII) has previously been isolated by Lobeck [2] after strong reductive alkaline cleavage of his "α-kosin". Notably, no sign of methylphloroglucinol-4-monomethyl ether (XIII), which could be expected to be formed from compound X, was detected.



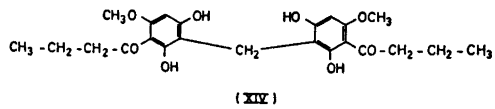
(IV) (VIII a,b,c) (XII)

R = -CH(CH3)2 (a), -CH2-CH(CH3)2 (b), -CH(CH3)-CH2-CH3 (c)

Scheme 1. Reductive alkaline cleavage of "kosin" (methylene-bis-pseudo-aspidinol) (IV)



(IX) (X) (XI) (XIII)



(XIV)

* According to Hems and Todd [5] "α-kosin" forms yellow needles, m.p. 158 °C, and "β-kosin" yellow prisms, m.p. 120 °C, when recrystallized from methanol.

** Even methylene-bis-pseudo-aspidinol BB (IV, R = $-CH_2-CH_2-CH_3$) (*cf.* Part III) and methylene-bis-*o*-desaspidinol BB (XIV), both of which contain methoxyl groups in *ortho* position to the acyl groups, are known to be very alkali stable compounds.[12,13]

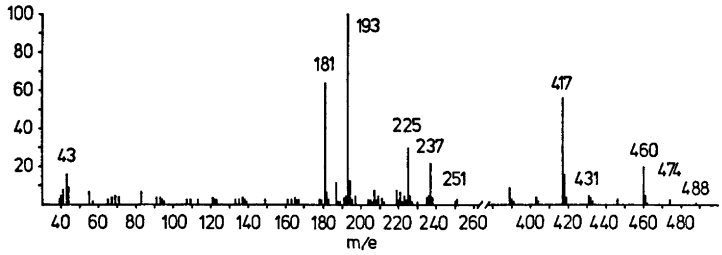


Fig. 4. Mass spectrum (70 eV) (*T* 130 °C) of "kosin" (methylene-bis-pseudo-aspidinol) (IV) (M.p. 148 – 150 °C).

In the light of these facts, it seems very probable that "α-" and "β-kosin" simply differ in their side chains, which consist of isobutyryl (iB), isovaleryl (iV), and 2-methylbutyryl (2-MeB) groups in different ratios.

The mass spectra of different "kosin" fractions (*cf.* Figs. 4 and 5) show molecular peaks at *m/e* 488, 474, and 460 corresponding to $C_{27}H_{36}O_8$, $C_{26}H_{34}O_8$, and $C_{25}H_{32}O_8$, respectively. The peaks at *m/e* 431 and 417 support the assumption that "kosin" fractions are mixtures of C_4H_9- and C_3H_7-side chain homologues and confirm the results of the alkaline cleavages. The general fragmentation pattern is similar to that found earlier for polycyclic *Dryopteris* phloroglucinols [9–11] and in agreement with the proposed structures (IV). The relative intensities of the molecular peaks in these spectra are in good agreement with the conclusion that "α-" and "β-kosin" differ only in their side chains. Thus the fraction melting at 148 – 150 °C shows a relatively strong molecular peak at *m/e* 460 and weak molecular peaks at *m/e* 474 and 488, supporting our conclusion that the "α-kosin" contains mainly isobutyryl side chains. On the other hand, the relative intensities of the molecular peaks at *m/e* 474 and 488 increase as the melting points of the different "kosin" fractions decrease, approaching the indicated melting point of "β-kosin" (m.p. 120 °C). Such changes point to the growing contribution of isovaleryl and 2-methylbutyryl side chains in the fractions.

The NMR spectrum (60 MHz) ($CDCl_3$) of the "kosin" fraction melting at 148 – 150 °C supports the structure (IV), showing the following signals: δ 1.18 [12 H, d, *J* 7 Hz, mainly 2 $-CO-CH(CH_3)_2$], 2.12 (6 H, s, 2 CH_3-Ar), 3.72 (6 H, s, 2 CH_3O-Ar), 3.84 (2 H, s, $-CH_2-$), ~3.9 [2 H, m, mainly 2 $-CO-CH(CH_3)_2$], 9.56 (2 H, s, 2 OH), 15.46 (1 H, s, OH) and 15.56 (1 H, s, OH).

The stereochemistry of both kosotoxin (II) and protokosin (III) has not yet been determined. However, it seems evident to us from the presence of optical activity, that the two chiral centres at positions 1 and 3 of the central ring of protokosin (III) (considered in the "triketonic" form) should have a like spatial arrangement of the groups. In this case the carbon atom at position 5 would not be asymmetric.

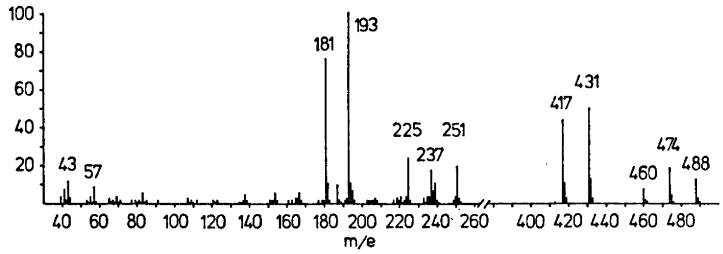
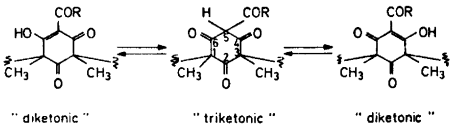


Fig. 5. Mass spectrum (70 eV) (*T* 130 °C) of "kosin" (methylene-bis-pseudo-aspidinol) (IV) (M.p. 125 – 127 °C).



If the two carbons at positions 1 and 3 should have an opposite spatial arrangement of the groups the whole molecule would have a plane of symmetry, the carbon atom at position 5 would be pseudo-asymmetric, and the compound would show no optical activity ("meso form").*

TLC of the kosins. The thin-layer chromatographic behaviour of the kosins was investigated on thin silica gel layers buffered to pH 4 – 8 using the gradient technique (Fig. 6).[14] Methylene-bis-pseudo-aspidinol (IV) and trispseudo-aspidinol (I) are easily separated at pH 4 – 7, but at pH 7 – 8 hardly at all. With increasing pH the R_F-value of methylene-bis-pseudo-aspidinol (IV) decreases, whereas that of trispseudo-aspidinol (I) increases. Protokosin (III) and kosotoxin (II) advance together at every pH examined, the former being a little faster than the latter. There is no distinct influence of the pH on the TLC-behaviour of these two substances. No separation of the different homologues of the different kosins was achieved by TLC.

EXPERIMENTAL

General. The UV spectra were measured with a Beckman DB-G grating spectrophotometer and the IR spectra with a Beckman IR-8 spectrophotometer. The NMR spectra have been taken with a Varian A-60 instrument using TMS as internal standard. The mass spectra of the natural products (I, II, and III) have been recorded on an A.E.I. MS-9 double-focusing mass spectrometer (70 eV) at the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France, through the courtesy of Dr. B. C. Das. The other mass spectra have been taken on a Perkin-Elmer 270 mass spectrometer. The optical rotations were measured on a Perkin-Elmer 141 Polarimeter. The melting points have been determined on a Reichert micro hot stage and are uncorrected. The thin-layer chromatographic methods were those previously reported.[1,14,15]

Material investigated. A 5 kg commercial sample of Flos koso "Siegfried" of unknown age and origin was investigated. By TLC it was shown to contain the same phloroglucinol derivatives as the material previously investigated (*cf.* Part I).[1]

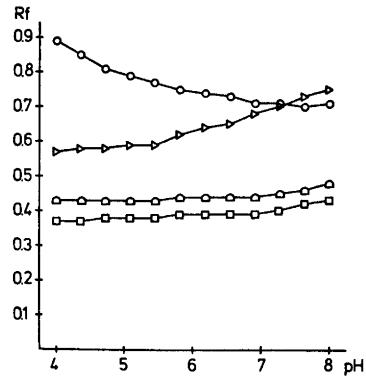


Fig. 6. Gradient TLC of trispseudo-aspidinol (I) (○, red), kosotoxin (II) (□, yellow), protokosin (III) (⌂, pale brown), and "kosin" (methylene-bis-pseudo-aspidinol) (IV) (△, pale brown) at pH 4 – 8. Chromatographed 3×. Solvent: hexane – chloroform 50:50. Colours with Fast Blue Salt B in parenthesis.

Preparation of ether extract and crude kosins. Preparations were made according to previously described methods.[1] The yields are listed in Table 1.

Column chromatography of crude Mg-kosin. Crude kosin (26.8 g) was suspended in benzene and chromatographed on 700 g of silica gel (Merck, particle size 0.05 – 0.2 mm) as previously described.[15] According to TLC, all fractions (10 ml each) were mixtures containing kosotoxin (II) and protokosin (III) in different ratios. The first fractions also contained some trispseudo-aspidinol (I). The four combined fractions (Table 2) were evaporated to dryness and separately dissolved in hot methanol, from which protokosin (III) started to crystallize as soon as cooling began. Kosotoxin (II), although present in large amounts (TLC), remained in solutions. After about one week, the mother liquors, containing only small amounts of protokosin (III),

Table 1. Yields of diethyl ether extract and crude kosins from Flos koso.

Flos koso (kg)	5.0
Material in diethyl ether extract (g)	324
(%)	6.5
Crude kosins MgO(g)	54
MgO(%)	1.1
$Ba(OH)_2$ (g)	70
$Ba(OH)_2$ (%)	1.4

* The 2-methylbutyryl group, which is one of the side chains present, has a chiral centre and this of course should cause some rotation.

Table 2. Melting points and yields of protokosin (III) from different fractions.

Fraction No. (10 ml each)	M.p. (°C)	Amount (mg)
1 – 32 (C_6H_6)	160 – 163	228
33 – 110 (C_6H_6)	166 – 171	1312
111 – 137 (C_6H_6)	171 – 175	593
138 – 410 (C_6H_6, C_6H_6-$CHCl_3$, 1:1)	175 – 178 [a]	870
Total		3003

[a] The melting point of the protokosin (III) of this fraction was raised to 181 – 183 °C by recrystallization from acetone.

were evaporated to dryness and the residues dissolved in hot hexane. The hexane solutions of the residues from fractions 33 – 410 (Table 3), which showed very similar TLC behaviour, were combined. On standing kosotoxin (II) slowly separated from the hexane solutions in several portions with slightly different melting points (Table 3). According to TLC the mother liquors still contained relatively large amounts of kosotoxin (II), which were not isolated in crystalline form.

Preparation of "kosins" (methylene-bis-pseudo-aspidinols) (IV), pseudo-aspidinols (VIIIa, b, c), and methylphloroglucinol-2-methyl ether (XII)

1. Mild reductive alkaline cleavage. Crude Ba-kosin (3.6 g) was mixed with 7.2 g of zinc powder, 50 ml of 5 % KOH was added, and the mixture heated on a water bath for 5 min. For details, see Part III, Cleavage A. The resulting product (3.6 g) was suspended in benzene and chromatographed on 100 g of silica gel. The fractions 1 – 7 (10 ml each) (benzene) gave 48.1 mg of "kosin" (IV) which, after crystallization from methanol, melted at 125 – 127 °C. The fractions 8 – 97 (benzene, benzene – chloroform, 1:1) gave another crop of "kosin" (IV) which, after crystallization from hexane, melted at 148 – 150 °C. The fractions 98 – 210 (chloroform) contained (TLC) pseudo-aspidinols (iB, IV, and 2-MeB acyl derivatives) (VIIIa, b, c) as well as some other compounds, the structures of which were not determined.

Table 3. Melting points and yields of kosotoxin (II) from the residues of different fractions.

Fraction No.	M.p. (°C)	Amount (mg)
1 – 32	100 – 110	315
	115 – 118	399
33 – 410	116 – 120	249
	119 – 122	121
	107 – 115	37
Total		1121

2. Strong reductive alkaline cleavage. Crude Ba-kosin (10 g) was mixed with 20 g of zinc powder, 100 ml of 15 % KOH was added and the mixture heated on a water bath for 24 h. For details, see Part III, Cleavage D. Part of the resulting product was used for determination of the organic acids formed. For details, see Part I. They consisted of isobutyric, isovaleric, and 2-methylbutyric acids in agreement with previous findings.[1] Another part of the resulting product (2.8 g) was used for column chromatography of the phloroglucinol derivatives formed. The fractions 1 – 12 (10 ml each) (benzene) gave 92 mg of "kosin" (IV), which, after crystallization from methanol, melted at 116 – 120 °C. The fractions 13 – 57 (benzene, benzene – chloroform, 1:1) contained only some unknown compounds, the structures of which were not determined. The fractions 58 – 112 (benzene – chloroform, 1:1, chloroform) contained some unknown compounds, as well as large amounts of pseudo-aspidinols (VIIIa, b, c). No crystalline compounds were obtained from hexane. Therefore, these fractions were rechromatographed on silica gel (hexane-benzene, 1:1). Seven fractions (50 ml each), containing pseudo-aspidinols (VIIIa, b, c), were collected and evaporated to dryness. These fractions, when combined and crystallized from hexane, gave three successive crystallizates of pseudo-aspidinols (VIIIa, b, c) (m.p. 59 – 61 °C, 275 mg), (m.p. 65 – 66 °C, 119 mg), (m.p. 62 – 65 °C, 98 mg). NMR ($CDCl_3$) (Fraction melting at 65 – 66 °C) : δ 1.18 [6 H, d, J 7 Hz, mainly $-CO-CH(CH_3)_2$], 2.12 (3 H, s, CH_3-Ar), 3.74 (3 H, s, CH_3-OAr), 3.86 [1 H, heptet, J 7 Hz, mainly $-CO-CH(CH_3)_2$] 6.22 (1 H, s, arom. H), 6.30 (1 H, s, OH), 13.06 and 13.12 (1 H, each s, OH). MS (fraction melting at 65 – 66 °C): M^+ at m/e 224 and 238 (weak) corresponding to $C_{12}H_{16}O_4$ and $C_{13}H_{18}O_4$. The fractions 113 – 127 (chloroform – ethanol, 95:5) gave 54 mg of methylphloroglucinol-2-methyl ether (XII), which, after crystallization from water, melted at 112 – 116 °C (lit.[12] 114 – 116 °C). MS: M^+ at m/e 154 corresponding to $C_8H_{10}O_3$.

Physical data of the identified natural products, as well as those of methylene-bis-pseudo-aspidinol ("kosin") (IV) obtained by the alkaline treatment of the crude kosins

1. Trispseudo-aspidinol (kosidin) (I.). Yellowish plates, m.p. 167 – 169 °C (methanol).

UV (cyclohexane) (ε values calculated on $C_{36}H_{44}O_{12}$):λ_{max} 229 (ε 39 000), 284 (ε 39 500) nm. λ_{min} 250 nm. IR (KBr): λ 3.06 (m), 3.34 (m), 3.38 (m), 3.46 (w), 6.22 (s), 6.90 (s), 7.10 (s), 7.24 (m), 7.38 (m), 7.60 (sh), 7.88 (s), 8.44 (s), 8.68 (s), 9.02 (s), 9.42 (w), 9.70 (w), 10.02 (w), 10.18 (w), 10.36 (w), 10.64 (w), 10.80 (w), 11.06 (w), 12.50 (w) μ. NMR ($CDCl_3$): See theoretical section. MS: See theoretical section.

2. Kosotoxin (II). Yellowish plates, m.p. 119–122 °C (hexane). $[\alpha]_D^{25} + 11.9 \pm 0.2°$ (*c* 2.335, $CHCl_3$). UV (cyclohexane) (ε values calculated on $C_{25}H_{32}O_8$): λ_{max} 226 (ε 19 700), 283 (ε 24 000) nm. λ_{min} 251 nm. IR (KBr): λ 3.06 (m), 3.34 (m), 3.38 (m), 3.46 (w), 3.66 (w), 3.76 (w), 6.00 (m), 6.24 (s), 6.88 (s), 7.08 (s), 7.22 (s), 7.40 (m), 7.52 (sh), 7.80 (s), 8.00 (m), 8.12 (s), 8.42 (s), 8.60 (s), 8.82 (m), 9.04 (s), 9.52 (w). 9.80 (w), 9.94 (w), 10.08 (m), 10.40 (w), 10.62 (w), 10.92 (w), 11.16 (w), 12.56 (m), 13.30 (w) μ. NMR ($CDCl_3$): See theoretical section. MS: See theoretical section.

3. Protokosin (III). Colourless needles, m.p. 181–183 °C (acetone). $[\alpha]_D^{25} + 13.9 \pm 0.5°$ (*c* 0.610, $CHCl_3$). UV (cyclohexane) (ε values calculated on $C_{39}H_{50}O_{12}$): λ_{max} 224 (ε 28 200), 285 (ε 36 400) nm. λ_{min} 249 nm. IR (KBr): λ 2.92 (m), 3.14 (w), 3.34 (m), 3.38 (m), 3.46 (w), 6.20 (s), 6.48 (m), 6.90 (s), 7.10 (s), 7.22 (m), 7.38 (m), 7.52 (sh), 7.84 (m), 8.14 (w), 8.46 (s), 8.68 (s), 8.98 (s), 9.10 (s), 9.40 (sh), 9.68 (m), 10.02 (m), 10.36 (m), 10.64 (w), 10.80 (w), 11.04 (m), 12.40 (w), 13.80 (w) μ. NMR ($CDCl_3$): See theoretical section. MS: See theoretical section.

4. Methylene-bis-pseudo-aspidinol ("kosin") (IV). Yellow needles, m.p. 148–150 °C (hexane). UV (cyclohexane) (ε values calculated on $C_{25}H_{32}O_8$): λ_{max} 229 (ε16 000), 286 (ε 22 100) nm. λ_{min} 252 nm. IR (KBr): λ 3.06 (m), 3.34 (m), 3.38 (m), 3.44 (w), 6.24 (s), 6.88 (s), 7.10 (s), 7.24 (m), 7.36 (m), 7.62 (w), 7.88 (s), 8.12 (w), 8.46 (s), 8.66 (s), 8.80 (m), 9.02 (s), 9.38 (w), 9.84 (m), 10.08 (m), 10.16 (w), 10.34 (w), 10.80 (w), 11.08 (w), 12.52 (w) μ. NMR ($CDCl_3$): See theoretical section. MS: See theoretical section.

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Phloroglucinol Derivatives of *Hagenia abyssinica*. III.* Reductive Alkaline Cleavages of Kosotoxin and Protokosin, and of Aspidin BB (*Dryopteris assimilis*)

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Products formed from kosotoxin (II), protokosin (III), and aspidin BB (VII) by reductive alkaline cleavages in different experimental conditions have been investigated. In all three cases, pseudo-aspidinol (IV), 3-methylfilicinic acid (X), methylphloroglucinol-2-monomethyl ether (XV), and methylene-bis-pseudoaspidinol (XVIII) were detected among the reaction products. Moreover, in the case of aspidin BB (VII), butyrylfilicinic acid (VIIIb), filicinic acid (IX), 3-methylbutyrylfilicinic acid (Vd), and albaspidine BB (XVII) were found.

Les produits formés à partir de la kosotoxine (II), de la protokosine (III) et de l'aspidine BB (VII) par clivages réductifs alcalins dans des conditions différentes expérimentales, ont été examinés. Dans les trois cas, le pseudo-aspidinol (IV), l'acide méthyl-3 filicinique (X), l'éther monométhylique-2 du méthylphloroglucinol (XV) et le méthylène-bis-pseudo-aspidinol (XVIII) ont été détectés parmi les produits réactionnels. De plus, dans le cas de l'aspidine BB (VII) l'acide butyrylfilicinique (VIIIb), l'acide filicinique (IX), l'acide méthyl-3 butyrylfilicinique (V d) et l'albaspidine BB (XVII) ont été trouvés.

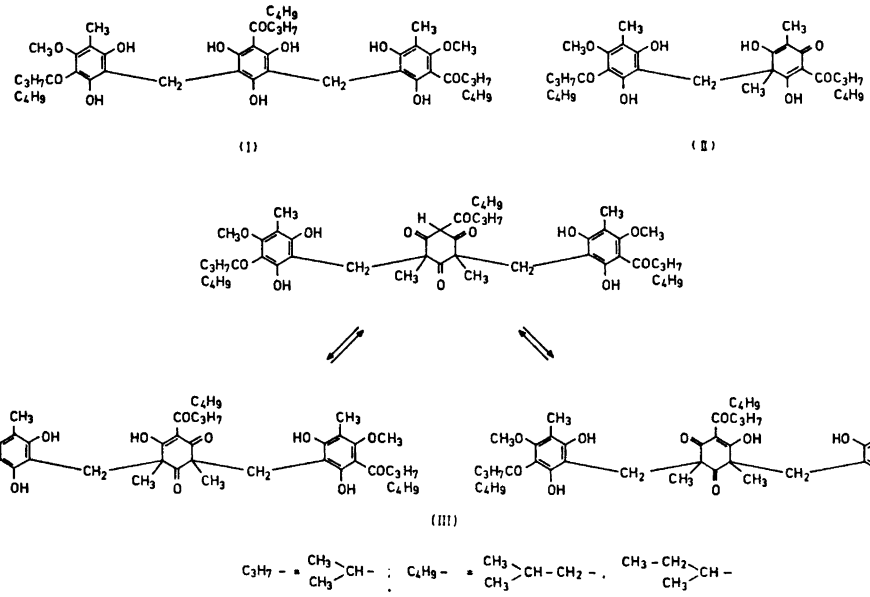
In previous papers [1,2] we have described the isolation and structure determination of three phloroglucinol derivatives (kosins) from female flowers of *Hagenia abyssinica* (Bruce) Gmelin. These are trispseudo-aspidinol (kosidin) (I), kosotoxin (II), and protokosin (III), each consisting of mixtures of isobutyryl (iB), isovaleryl (iV), and 2-methylbutyryl (2-MeB) acyl side chain homologues. The structure of trispseudo-aspidinol (I) was partly solved through an investigation of the monocyclic acylphloroglucinols formed by mild reductive alkaline cleavage of the molecule in the presence of metallic zinc.[1] This elegant cleavage method, originally reported by Boehm [3] and much used by him and later workers [4–6] for structure determinations of *Dryopteris* and *Hagenia* phloroglucinol derivatives, is here applied to the study of kosotoxin (II) and protokosin (III). Using the method in its original form, we were able to detect only the homologous pseudo-aspidinols iB, iV, and 2-MeB (IVa, b, and c) both from kosotoxin (II) and protokosin (III). No sign of 3-methylisobutyrylfilicinic acid (Va) or its iV (Vb) or 2-MeB (Vc) homologues was detected. Nor were 3,3-dimethylisobutyryl-filicinic acid (flavesone) (VIa), 3,3-dimethyl-isovalerylfilicinic acid (leptospermone) (VIb) or 3,3-dimethyl-(2-methylbutyryl)-filicinic acid (VIc) to be found among the products of protokosin (III).**

These results, as well as the absence of 3-methyl-butyrylfilicinic acid (fraginol ***) (Vd) from the decomposition products of *Dryopteris* phloroglucinol derivatives,[4,5,7] persuaded us to examine the products of reductive alkaline cleavage of kosotoxin (II) and protokosin (III) in more detail. Thereby, aspidin BB (VII)

* Part II, Ref. 2.

** Flavesone (VIa) and leptospermone (VIb) are known as naturally occurring compounds in the essential oils of certain *Leptospermum*, *Xanthostemon*, and *Eucalyptus* species (Myrtaceae).[8,9]

*** Fraginol (Vd) is reported as a naturally occurring compound in *Dryopteris fragrans* (L.) Schott.[10,11]



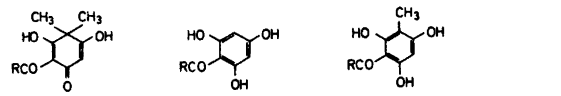
(I) (II)

(III)

(*Dryopteris assimilis* S. Walker) was used as a model compound. This phloroglucinol derivative, which frequently occurs in ferns of the *D. dilatata* complex,[11] is closely related to kosotoxin (II) in its chemical structure: *i.e.* the geminal dimethyl group found in aspidin BB (VII) is partly involved in the methylene bridge of kosotoxin (II).

Syntheses of isobutyrylfilicinic acid (VIIIa), 3-methylisobutyrylfilicinic acid (Va), and 3,3-dimethylisobutyrylfilicinic acid (VIa), and of their deacylated analogues (IX), (X), and (XI). For the examination of the decomposition products of kosotoxin (II) and protokosin (III) several synthetic model compounds were needed. Among these 3-methylisobutyrylfilicinic acid (Va), 3,3-dimethylisobutyrylfilicinic acid (VIa) and their deacylated analogues (X) and (XI) (syncarpic acid *) were of vital importance. The geminally substituted acylphloroglucinol derivatives were prepared in good yields by the C-methylation of isobutyrylphloroglucinol (XIIa), according to the methods of Riedl *et al.*[12,13] In all cases the resulting products were mixtures, containing isobutyrylfilicinic acid (VIIIa), 3-methylisobutyrylfilicinic acid (Va) and 3,3-dimethylisobutyrylfilicinic acid (VIa) in different ratios. In addition small amounts of substances such as unreacted isobutyrylphloroglucinol (XIIa), methylisobutyrylphloroglucinol (XIII), and their methyl ethers were also present. For the separation of isobutyrylfilicinic acid (VIIIa). 3-methylisobutyrylfilicinic acid (Va), and 3,3-dimethylisobutyrylfilicinic acid (VIa), obtained from the so-called carbonate fractions (see Refs. 12, 13), column chromatography on silica gel, with hexane – benzene in different ratios as eluent proved suitable. The first fractions consisted of mixtures of 3,3-dimethylisobutyrylfilicinic acid (VIa) and 3-methylisobutyrylfilicinic acid (Va),

* Syncarpic acid (XI) has been isolated from *Syncarpia laurifolia* Tenn. (Myrtaceae).[14]



(VIIIa) R = -CH(CH3)2 (XIIa) R = -CH(CH3)2 (XIII) R = -CH2-CH2-CH3

(XIIb) R = -CH2-CH2-CH3

Table 1. Products obtained by reductive alkaline cleavage of aspidin BB (VII) in different conditions (Cleavages A–C).

Compound	Cleavage A	B	C	R_F-values TLC [a]	PC [b]	Colour [c]
Aspidin BB (VII)	++	+	–	0.70	0.84 [d]	yellow
Albaspidin BB (XVII)	+	–	–	0.75	0.92 [d]	red
Methylene-bis-pseudo-aspidinol BB (XVIII)	+? [e]	+? [e]	+	0.73	0.92 [d]	pale brown
Pseudo-aspidinol B (IVd)	++	++	++	0.30	0.56 [d]	pale brown
Butyrylfilicinic acid (VIIIb)	++	++	++	0.23	0.41 [f]	red
3-Methylbutyrylfilicinic acid (Vd)	–	+	–	0.20	0.47 [f]	yellow
Filicinic acid (IX)	–	+	+	0.11	n.s.	blue
3-Methylfilicinic acid (X)	–	+	+	0.14	n.s.	orange yellow
Methylphloroglucinol-2-methyl ether (XV)	–	+	+	0.08	n.s.	dark brown

–, not detected; +, detected in traces or small amounts; ++, detected in large amounts; n.s., not studied. [a] At pH 6.0 in hexane–chloroform–ethanol 47.5:47.5:5.0.[1,5] [b] In cyclohexane–chloroform 1:1.[23] [c] Colour with Fast Blue Salt B. [d] At pH 8.6. [e] Could not be detected owing to overlapping of aspidin BB (VII) and albaspidin BB (XVII). [f] At pH 4.0.

from which the latter (Va) was separated by crystallization from ether. The subsequent fractions consisted of practically pure isobutyrylfilicinic acid (VIIIa).

The corresponding butyrylderivatives butyrylfilicinic acid (VIIIb) and 3-methylbutyrylfilicinic acid (Vd) were prepared by analogous procedures.[12]

The geminally substituted deacylated phloroglucinol derivatives (IX), (X), and (XI) were prepared by reductive alkaline cleavage of the corresponding isobutyryl compounds (VIIIa), (Va), and (VIa), respectively.

The thin-layer and paper chromatographic properties of the synthetically prepared acylfilicinic acids as well as their deacylated homologues appear in Tables 1 and 2. All other compounds except 3-methylisobutyrylfilicinic acid (Va) and 3,3-dimethylisobutyrylfilicinic acid (VIa) were easily separated in the chromatographic systems used.

Reductive alkaline cleavage of aspidin BB (VII). Aspidin BB (VII) was subjected to three different cleavages, A, B, and C, all being modifications of the original method of Boehm [3] (for details, see the Experimental section). The products isolated by column chromatography or detected by TLC and PC are listed in Table 1. The decomposition of aspidin BB (VII) is further depicted in Scheme 1.

The first step in the decomposition of aspidin BB (VII) by the reductive alkaline treatment is the cleavage of the methylene bridge, resulting in large amounts of pseudo-aspidinol B (IVd) and butyrylfilicinic acid (VIIIb). These are the main reaction products in all three cleavages (Cleavage A, B, and C). Of the other possible products, 3-methylbutyrylfilicinic acid (Vd) was detected in trace amounts, but only after cleavage B. On the other hand, no 3-methyl-pseudoaspidinol B (XIVd) could be found.

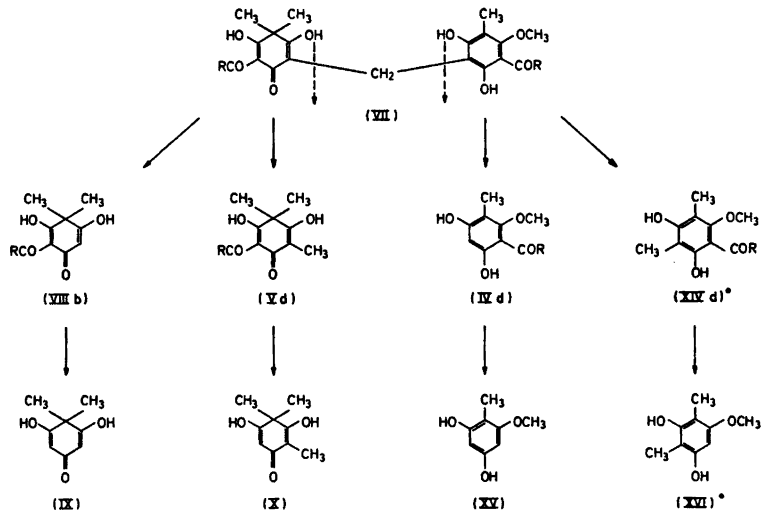
The second step in the decomposition of aspidin BB (VII) is the cleavage of the acyl side chains from the initially formed monocyclic compounds.* This second step, which leads mainly to the formation of filicinic acid (IX) and methylphloroglucinol-2-monomethyl ether (XV), is detectable after cleavage B and more clearly after cleavage C. In both cases, some 3-methylfilicinic acid (X) could also be de-

* To some extent, of course, the acyl side chain cleavage takes place directly from aspidin BB (VII).

Table 2. Products obtained by reductive alkaline cleavages of kosotoxin (II) and protokosin (III) in different conditions (Cleavages A – D).

Compound	Cleavage A	B	C	D	R_F-values in TLC [a]	in PC [b]	Colour [c]
Kosotoxin (II)	–	–	–	–	0.73	0.84 [e]	yellow
Protokosin (III)	–	–	–	–	0.73	0.84 [e]	pale brown
Methylene-bis-pseudo-aspidinol (XVIII)	+	+	+	+	0.75	0.92 [e]	pale brown
Pseudo-aspidinol (IV)	+ +	+ +	+ +	+ +	0.30	0.69 [e] 0.56	pale brown
Isobutyrylfilicinic acid (VIII a)	–	–	–	–	0.23	0.45 [f]	red
3-Methylisobutyrylfilicinic acid (Va)	–	–	–	–	0.20	0.50 [f]	yellow
3,3-Dimethylisobutyrylfilicinic acid (VIa)	–	–	–	–	0.20	0.50 [f]	yellow
3-Methylfilicinic acid (X)	–	–	–	+	0.14	n.s.	orange yellow
3,3-Dimethylfilicinic acid (XI)	–	–	–	+ ?[d]	0.20	n.s.	orange yellow
Methylphloroglucinol-2-methyl ether (XV) [g]	–	–	–	+	0.08	n.s.	dark brown

[a] At pH 6.0 in hexane – chloroform – ethanol 47.5:47.5:5.0.[1,5] [b] In cyclohexane – chloroform 1:1.[23] [c] Colour with Fast Blue Salt B. [d] From protokosin only. [e] At pH 8.6. The pseudo-aspidinol obtained from kousso constituents (see Experimental) separated in two spots, R_F 0.69 and 0.56. The latter was pseudo-aspidinol iB and the former a mixture of the pseudo-aspidinols iV and 2-MeB (see Ref. 1). [f] At pH 4.0. [g] Methylphloroglucinol-4-methyl ether (XXI) formed an orange spot with the R_F-value of 0.10.



Scheme 1. Reductive alkaline cleavage of aspidin BB (VII).

tected. 1,3-Dimethylphloroglucinol-4-monomethyl ether (XVI) was not found. We are the first to report the presence of 3-methylbutyrylfilicinic acid (Vd) among the decomposition products of aspidin BB (VII) (*cf.* Ref. 15–18). However, only traces were found, even here. 3-Methylfilicinic acid (X) on the other hand, was long ago isolated in small amounts by Boehm.[16] Earlier failures to detect 3-methylbutyrylfilicinic acid (Vd) or its acyl side chain homologues among the products of reductive alkaline cleavage of geminally disubstituted *Dryopteris* phloroglucinols, is possibly explained by the great instability of these products in the reaction conditions used (*cf.* also Riedl and Risse [7] and Aho [19]).

An alternative route in the decomposition of aspidin BB (VII) (*cf.* Ref. 17) leading to the formation of albaspidin BB (XVII) and methylene-bis-pseudo-aspidinol BB (XVIII $R = -CH_2-CH_2-CH_3$) is the so-called rottlerone change. This reaction, which proceeds according to the scheme

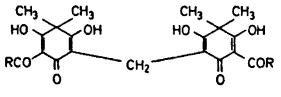
$$2R-CH_2-R' \rightleftharpoons R-CH_2-R + R'-CH_2-R'$$

is typical of the behaviour of methylene-bis-polyhydroxyphenols (polyhydroxydiphenylmethanes).[20] The formation of albaspidin BB (XVII) after cleavage A is clearly detected in its characteristic red spot (fast blue salt B) on TLC. The lightbrown spot corresponding to the other possible product, methylene-bis-pseudo-aspidinol BB (XVIII, $R = -CH_2-CH_2-CH_3$), cannot be detected with certainty because in the chromatographic systems used its R_F-values are very similar to those of the red spot of albaspidin BB (XVII) and the yellow spot of unreacted aspidin BB (VII). After more drastic reaction conditions, corresponding to cleavage B, there is no albaspidin BB (XVII) left, although the reaction mixture still contains some unreacted aspidin BB (VII). After cleavage C – the most drastic of the three variants used – the only of the bicyclic compounds present is methylene-bis-pseudo-aspidinol BB (XVIII, $R = -CH_2-CH_2-CH_3$), which is known to be a very alkali-stable substance.[18]

Reductive alkaline cleavage of kosotoxin (II) and protokosin (III). This was performed in the same three ways, as in the case of aspidin BB (VII). The products isolated by column chromatography or detected by TLC and PC are listed in Table 2, while decomposition is depicted in Schemes 2 and 3.

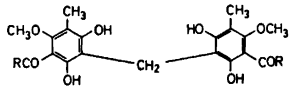
The first step in the decomposition of kosotoxin (II) and protokosin (III) is the cleavage of the methylene bridges, resulting in large amounts of pseudo-aspidinol iB, iV, and 2-MeB (IVa, b, c) in all three cases (cleavage A, B, and C). Although expected, no 3-methylisobutyrylfilicinic acid (Va), 1,3-dimethyl-5-isobutyrylphloroglucinol (XIX), or 3-methylpseudo-aspidinol iB (XIVa) or its isovaleryl or 2-methylbutyryl homologues were detected by TLC or PC. Neither were 3,3-dimethylisobutyrylfilicinic acid (VIa) or its isovaleryl and 2-methylbutyryl homologues observed in the case of protokosin (III).

The second step in the decomposition of both kosotoxin (II) and protokosin (III) could be expected to lead to 3-methylfilicinic acid (X), methylphloroglucinol-2-monomethyl ether (XV), 1,3-dimethylphloroglucinol-4-monomethyl ether (XVI), and 1,3-dimethylphloroglucinol (XX). Also some 3,3-dimethylfilicinic acid (XI) could be expected to be formed in the case of protokosin (III). However, none of these second step decomposition products were found after cleavage according to procedures A, B, or C. Rather, in all three cases, compounds of unknown structure were detected. Some of these showed R_F-values very similar to those of synthetical standards, but the colouration (fast blue salt B) was different. Owing to the presence of these unknown compounds in the reaction mixtures we tried a fourth, much more drastic variant of the reductive alkaline

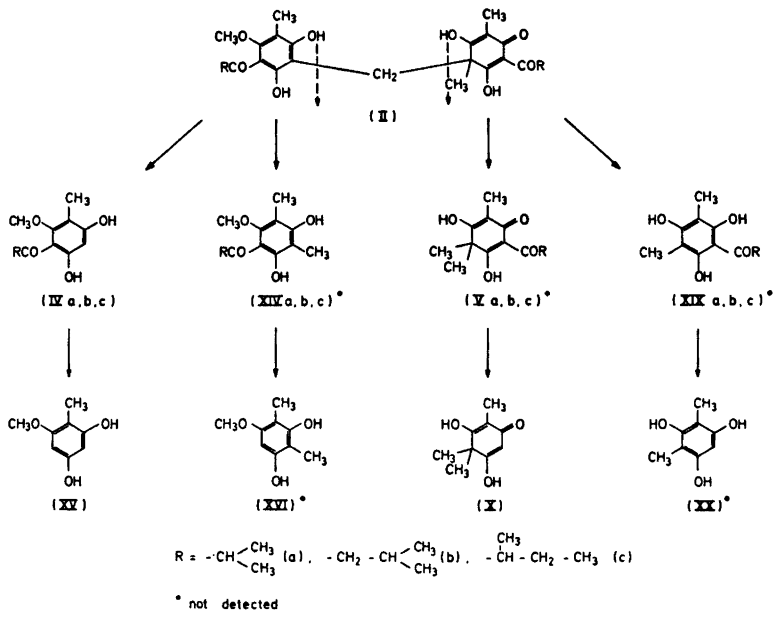


(XVII)

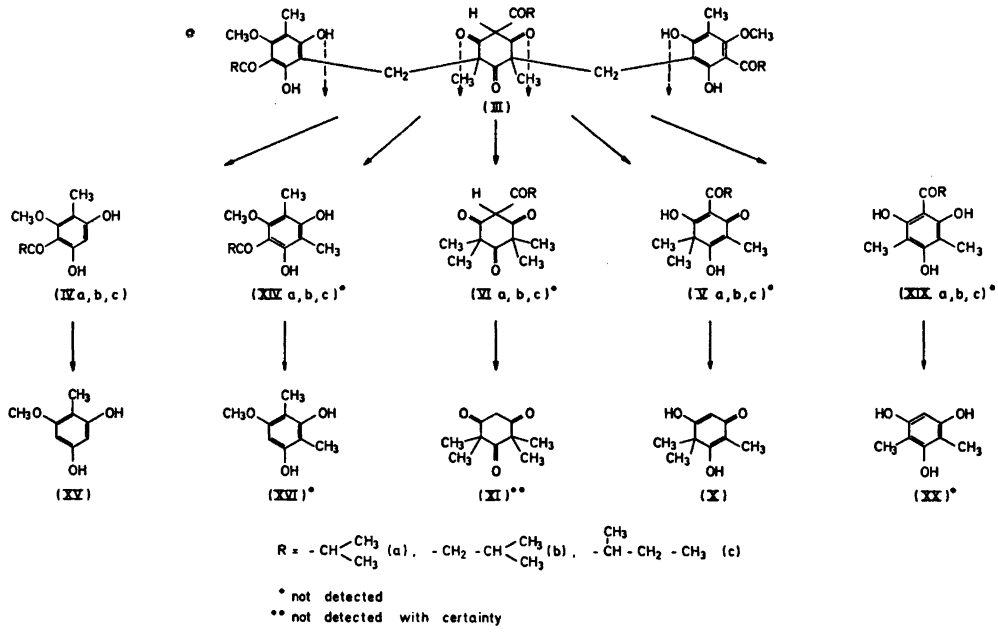
R = -CH2 - CH2 - CH3



(XVIII)

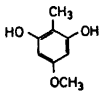


Scheme 2. Reductive alkaline cleavage of kosotoxin (II).



Scheme 3. Reductive alkaline cleavage of protokosin (III).

cleavage, called cleavage D (see the Experimental section). This time the unknown compounds were decomposed and large quantities of methylphloroglucinol-2-monomethyl ether (XV) * and trace amounts of 3-methylfilicinic acid (X) could be detected in the reaction mixture. The former was previously found by Lobeck [21] after reductive alkaline cleavage of kosotoxin (II) and "kosin" (XVIII).** However, the key degradation product expected from protokosin (III), 3,3-dimethylfilicinic acid (XI),*** could not be detected with certainty (*cf.* Table 2).



(XXI)

An alternative route in the decomposition of both kosotoxin (II) and protokosin (III) is the formation of iB, iV, and 2-MeB homologues of methylene-bis-pseudo-aspidinol ("kosin") (XVIII) ** by the rottlerone change. These products have been isolated earlier after reductive alkaline treatment of both kosotoxin (II) and protokosin (III), and they are known to be very alkali stable, as is the corresponding butyryl homologue (pseudo-aspidin BB) (XVIII R = $-CH_2-CH_2-CH_3$). Methylene-bis-pseudo-aspidinol ("kosin") (XVIII) ** could be detected after all three initial cleavage procedures (cleavage A, B, and C), but other products expected to be formed by the rottlerone change could not.

The structural similarity we have proposed here between aspidin BB (VII), kosotoxin (II), and protokosin (III) is supported by the fact that similar decomposition products, including methylene-bis-pseudo-aspidinol (XVIII),** pseudo-aspidinol (IV), methylphloroglucinol-2-monomethyl ether (XV), and 3-methylfilicinic acid (X), were obtained in all three cases.

EXPERIMENTAL

General. The IR spectrum was measured with a Beckman IR-8 spectrophotometer. The NMR spectra have been taken with a Varian A-60 instrument using TMS as internal standard. The mass spectra have been recorded on a Perkin-Elmer 270 mass spectrometer. The melting points have been determined on a Reichert micro hot stage and are uncorrected. The thin-layer chromatographic methods were those previously reported.[1,5] The paper chromatographic method was that of Penttilä and Sundman.[23]

Reference substances

1. Isobutyrylphloroglucinol.$2H_2O$ (XIIa) was prepared from phloroglucinol by the method of Riedl.[24] Colourless plates, m.p. 71–74 °C/134–136 °C (water) (lit.[24] m.p. 70 °C/138 °C). MS: M+ at *m/e* 196 corresponding to $C_{10}H_{12}O_4$. IR: C=O 6.24 μ.

2. Isobutyrylfilicinic acid (VIIIa) was obtained as a by-product in the synthesis of 3-methylisobutyrylfilicinic acid (Va) according to Riedl and Risse.[12] Slightly brownish plates, m.p. 152–154 °C (hexane) (lit.[23] m.p. 153–154 °C). MS: M+ at *m/e* 224 corresponding to $C_{12}H_{16}O_4$. NMR ($CDCl_3$): δ 1.16 [6 H, d, *J* 7 Hz, $-CO-CH(CH_3)_2$], 1.40 and 1.50 (6 H, each s, gem. dimethyl group of the ring), 3.96 [1 H, heptet, *J* 7 Hz, $-CO-CH(CH_3)_2$] and 5.54 (1 H, s, vinyl H only partly). The signals of OH-groups are omitted.

3. 3-Methylisobutyrylfilicinic acid (Va) † was prepared from isobutyrylphloroglucinol (XIIa) by the method of Riedl and Risse.[12] Colourless plates, m.p. 100–103 °C (ether). MS: M+ at *m/e* 238 corresponding to $C_{13}H_{18}O_4$. NMR ($CDCl_3$): δ 1.14 [6 H, d, *J* 7 Hz, $-CO-CH(CH_3)_2$], 1.42 and 1.50 (6 H, each s, gem. dimethyl group of the ring), 1.90 (3 H, s, $CH_3-C\ll$), 3.96 [1 H, heptet, *J* 7 Hz, $-CO-CH(CH_3)_2$] 7.40 (1 H, s, OH), and 18.30 (1 H, s, OH).

4. 3,3-Dimethylisobutyrylfilicinic acid (flavesone) (VIa) † was prepared from isobutyrylphloroglucinol (XIIa) by the method of Riedl and Risse.[12] Pale yellow oil (lit.[9] b.p. 134 °C/10 mmHg). MS: M+ at *m/e* 252 corresponding to

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* Noteworthy, no methylphloroglucinol-4-monomethyl ether (XXI) could be found.

** R = $-CH(CH_3)_2$; $-CH_2-CH(CH_3)_2$; $-CH(CH_3)-CH_2-CH_3$.

*** Recently Kashman *et al.*[22] have isolated from *Myrtus communis* L. (Myrtaceae) two new acylphloroglucinol derivatives which they called myrtucummulone A and B, and from which they were able to prepare 3,3-dimethylfilicinic acid (syncarpic acid) (XI) by alkaline treatment. However, owing to the fact that all four methyl groups in 3,3-dimethylfilicinic acid (XI) are already present as such in myrtucummulone A and B, and not partly involved in the methylene bridges as in protokosin (III), the cases are not quite analogous.

† 3-Methylisobutyrylfilinic (Va), and 3-3-dimethylisobutyrylfilicinic acid (VIa) decomposing almost completely after few months storage, are unstable substances compared with isobutyrylfilicinic acid (VIIIa).

$C_{14}H_{20}O_4$. NMR ($CDCl_3$): δ 1.16 (6 H, d, J 7 Hz, $-CO-CH(CH_3)_2$, 1.36 and 1.46 (12 H, each s, 2 gem. dimethyl groups of the ring), 3.80 (1 H, heptet, J 7 Hz $-CO-CH(CH_3)_2$, and 17.80 (1 H, s, OH) (*cf.* Ref. 9).

5. Filicinic acid (IX) was obtained from isobutyrylfilicinic acid (VIIIa) by the method of Riedl and Risse.[12] Colourless plates m.p. 215 °C (ethanol) (lit.[12] m.p. 214–215 °C). MS: M+ at *m/e* 154 corresponding to $C_8H_{10}O_3$. NMR (DMSO-d_6): δ 1.20 and 1.26 (6 H, each s, gem. dimethyl group), 5.20 and 5.48 (weak) (< 2 H, each s, vinyl H's only partly). The signals of OH-groups are omitted.

6. Butyrylfilicinic acid (VIIIb) was prepared by acylation of filicinic acid (IX).[7] Slightly brownish plates, m.p. 98–100 °C (hexane) (lit.[28] m.p. 98–99 °C). MS: M+ at *m/e* 224 corresponding to $C_{12}H_{16}O_4$. NMR ($CDCl_3$): δ 1.01 (3 H, t, J 7 Hz, $-CO-CH_2-CH_2-CH_3$), 1.40 and 1.50 (6 H, each s, gem. dimethyl group), 1.74 (2 H, m, $-CO-CH_2-CH_2-CH_2-CH_3$), 3.02 (2 H, t, J 7 Hz, $-CO-CH_2-CH_2-CH_3$) and 5.54 (< 1 H, s, vinyl H only partly). The signals of OH-groups are omitted (*cf.* Ref. 10).

7. 3-Methylbutyrylfilicinic acid (Vd) ** was prepared from butyrylphloroglucinol (XIIb) by the method of Riedl and Risse.[12] Colourless plates, m.p. 78–79 °C (ether) (lit.[7] m.p. 87 °C). MS: M+ at *m/e* 238 corresponding to $C_{13}H_{18}O_4$. NMR ($CDCl_3$): δ 0.98 (3 H, t, J 7 Hz, $-CO-CH_2-CH_2-CH_3$), 1.40 and 1.50 (weak) (6 H, each s, gem. dimethyl group), 1.68 (2 H, m, $-CO-CH_2-CH_2-CH_2-CH_3$), 1.90 (3 H, s. $CH_3-C\langle$) and 3.00 (2 H, t, J 7 Hz, $-CO-CH_2-CH_2-CH_3$). The signals of OH-groups are omitted (*cf.* Ref. 10).

8. 3-Methylfilicinic acid (X) was obtained by reductive alkaline cleavage of 3-methylisobutyrylfilicinic acid (Va).[12] Yellowish plates, m.p. 178 °C (benzene) (lit.[12] m.p. 179–180 °C). MS: M+ at *m/e* 168 corresponding to $C_9H_{12}O_3$. NMR: (DMSO-d_6): δ 1.22 and 1.30 (6 H, each s, gem. dimethyl group), 1.68 (3 H, s, $CH_3-C\langle$) and 5.36 (< 1 H, s, vinyl H only partly). The signals of OH-groups are omitted.

9. 3,3-Dimethylfilicinic acid (XI) was prepared by reductive alkaline cleavage of 3,3-dimethylisobutyrylfilicinic acid (VIa).[12] Colourless needles, m.p. 186–189 °C (benzene (lit.[12] m.p. 187–190 °C). MS: M+ at *m/e* 182 corresponding to $C_{10}H_{14}O_3$. NMR: (DMSO-d_6). δ 1.20 (weak) and 1.30 (12 H, each s, 2 gem. dimethyl groups) and 5.28 (1 H, s, vinyl H). The signal of OH-group is omitted.

10. Pseudo-aspidinol B (IVd) was prepared by alkaline treatment (Cleavage A) of aspidin BB (VII) according to the method of Aebi *et al.*[17] Colourless plates, m.p. 70–72 °C (hexane) (lit.[17] m.p. 70–72 °C). MS: M+ at *m/e* 224 corresponding to $C_8H_{10}O_3$. NMR ($CDCl_3$): δ 0.98 (3 H, t, J 7 Hz, $-CO-CH_2-CH_2-CH_3$), 1.74 (2 H, m, $-CO-CH_2-CH_2-CH_2-CH_3$) 2.10 (3 H, s, CH_3-Ar), 3.04 (2 H, t, J 7 Hz, $-CO-CH_2-CH_2-CH_3$), 3.74 (3 H, s, CH_3O-), 6.20 (2 H, two superposable s, OH and aromatic H) and 13.20 (1 H, s, OH) (*cf.* Ref. 25).

11. Methylphloroglucinol-4-monomethyl ether (XXI) was prepared by the method of Weidel.[26] Slightly brownish needles, m.p. 124 °C (xylene) (lit.[26] m.p. 124 °C). MS: M+ at *m/e* 154 corresponding to $C_8H_{10}O_3$.

12. Methylene-bis-pseudo-aspidinol BB (XVIII R = $-CH_2-CH_2-CH_2$) was prepared by alkaline treatment (Cleavage C) of aspidin BB (VII) according to the method of Aebi *et al.*[18] Colourless needles, m.p. 140–141 °C (acetone) (lit.[18] m.p. 141–143 °C) MS: M+ at *m/e* 460 corresponding to $C_{25}H_{32}O_8$.

13. For the preparation of methylphloroglucinol-2-monomethyl ether (XV) and for the isolation of kosotoxin (II) and protokosin (III), see Part II.[2]

14. Aspidin BB (VII), m.p. 124–125 °C, consisted of an old sample isolated from *D. assimilis* S. Walker.[27]

** This is an unstable substance, like its corresponding isobutyryl isomer (Va).

Reductive alkaline cleavages

1. Aspidin BB (VII). Cleavage A. 1.2 g of aspidin BB (VII) was thoroughly mixed with 2.4 g of zinc dust, and 100 ml of 5 % NaOH solution was added. The mixture was kept on a boiling water bath for 5 min. The warm green-yellow solution was then separated from Zn by filtering, and the filter paper was washed with water. After cooling, the solution was acidified (pH 2) with 33 % H_2SO_4, whereupon a yellow precipitate was formed. The acidic solution was shaken three times with 50 ml ether and the combined ether solutions were shaken twice with 50 ml of distilled water and dried over Na_2SO_4. The filtered ether solution was evaporated *in vacuo*, whereupon 1.2 g of an oil was obtained. The phloroglucinols contained in this oil were separated by column chromatography on 30 g of silica gel. The fractions 1–14 (10 ml each) (benzene-light petroleum 1:1) contained, according to TLC, a mixture of albaspidin BB (XVII) and aspidin BB (VII), that did not separate. The fractions 15–40 (benzene-light petroleum 1:1) gave on crystallization from hexane 44 mg of aspidin BB (VII), m.p. 121–123 °C. The fractions 41–67 (benzene) contained only traces of aspidin BB (VII) and the fractions 68–83 gave by crystallization from hexane 40 mg of pseudo-aspidinol B (IVd), m.p. 70–72 °C. The fractions 84–140 (benzene–ether 9:1) contained only butyrylfilicinic acid (VIIIb), which was obtained as an oil.

Cleavage B. A 100 mg sample of aspidin BB (VII) was mixed with 200 mg of zinc dust and 20 ml of 5 % NaOH solution was added. The mixture was boiled for 5 min over a bunsen flame and the phloroglucinols were

separated as explained above for cleavage A and investigated by TLC (*cf.* theoretical section).

Cleavage C. A 1.0 g sample of aspidin BB (VII) was mixed with 2.0 g of zinc dust and 100 ml of a 15 % solution of NaOH was added. The mixture was boiled for 5 min on a Bunsen flame and processed according to Cleavage A. Crystallization of the resulting oil (1.0 g) from acetone gave 20 mg of methylene-bis-pseudo-aspidinol BB (XVIII R= $-CH_2-CH_2-CH_3$), m.p. 140–141 °C. No further uniform compounds were obtained.

2. *Kosotoxin (II). Cleavage A.* 1.0 g of kosotoxin (II) was subjected to reductive alkaline cleavage in the same way as described for aspidin BB (VII). The phloroglucinols of the reaction mixture (1.0 g) were separated by column chromatography on 30 g of silica gel. The fractions 1–12 (10 ml each) (benzene) were crystallized from methanol to give 41 mg of "kosin" (XVIII),* m.p. 128–132 °C. The melting point of these crystals was raised to 148–150 °C by recrystallization from hexane. The fractions 13–32 (benzene, benzene–chloroform 1:1) failed to give crystalline compounds from methanol. The fractions 33–43 (benzene–chloroform 1:1) contained (TLC) pseudo-aspidinols (IVa, b, c) as well as one compound of unknown structure. No crystals were obtained from hexane. The fractions 44–52 (benzene-chloroform 1:1), which contained the same compounds as the fractions 33–43, were dissolved in ether and extracted 4 times with 10 % $NaHCO_3$ solution. The ether solution was evaporated, and the residue dissolved in hexane, whereupon 20.4 mg of pseudo-aspidinol (IVa, b, c,) m.p. 60–61 °C was obtained.

Cleavages B and C were performed as described for aspidin BB (VII) and the resulting products investigated by TLC.

Cleavages D. 100 mg of kosotoxin (II) was mixed with 200 mg of zinc dust and added to 20 ml of a 15 % solution of NaOH. The mixture was warmed for 24 h on a water bath and processed as described for aspidin BB (VII), and then investigated by TLC.

3. Protokosin (III). Cleavage A. 1.06 g of protokosin (III) was subjected to reductive alkaline cleavage as described for aspidin BB (VII). The resulting phloroglucinols (1.06 g) were chromatographed on 32 g of silica gel. The fractions 1–22 (10 ml each) benzene) gave 77.9 mg of "kosin" (XVIII),* m.p. 127–129 °C, when crystallized from hexane. Fractions 23–32 (benzene) contained an unknown compound, the structure of which was not determined.

Fractions 33–92 (benzene, benzene–chloroform 1:1) contained pseudo-aspidinols (IVa, b, c). The residue after evaporation of the solvents was dissolved in ether and extracted four times with a solution of $NaHCO_3$. The evaporated ether solution was dissolved in hexane, giving 27.6 mg of pseudo-aspidinols (IVa, b, c), m.p. 58–61 °C. Fractions 93–190 (benzene-chloroform 1:1, chloroform) gave only mixed crystals of pseudo-aspidinols (IVa, b, c) and unknown compounds which were not further separated.

Cleavages B, C and D were performed as described for aspidin BB (VII) and kosotoxin (II), and resulting products were investigated by TLC.

The authors thank Miss Leena Karppi for skillful technical assistance.

* R= $-CH(CH_3)_2$; $-CH_2-CH(CH_3)_2$; $-CH(CH_3)-CH_2-CH_3$.

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Bacterial Carotenoids. XLIV.* Zeaxanthin Mono- and Dirhamnoside

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The isolation of zeaxanthin (*1a*, 2 % of total carotenoid), zeaxanthin monorhamnoside (*3a*, 18 %) and zeaxanthin dirhamnoside (*2a*, 80 %) from a coryneform hydrogen bacterium strain 14 g (Institute of Microbiology, University of Göttingen) is reported.

The partial structures of these first, secondary, non-allylic, remarkably stable, carotenoid glycosides (*2a* and *3a*) were established by spectroscopic (visible light, IR, ^{1}H NMR and mass spectra) and chemical evidence. Acid hydrolysis provided rhamnose and strong alkaline hydrolysis zeaxanthin (*1a*) and modified dehydroaglycones.

The isolation and structure elucidation of carotenoid glycosides have recently been reviewed.[1] Also carotenoid glycosides subsequently isolated [2–5] belong to the same type of prim./sec. allylic or tert. glycosides.

We now report the first isolation of secondary, non-allylic carotenoid glycosides with remarkable stability.

The source of these rhamnosides is a yellow coryneform hydrogen bacterium strain 14 g under current investigation [6,7] at Institute of Microbiology, University of Göttingen.

RESULTS AND DISCUSSION

Strain 14 g contained *ca.* 0.074 % carotenoid per dry weight, comprising zeaxanthin (*1a*, 2 %), zeaxanthin dirhamnoside (*2a*, 80 %), and zeaxanthin monorhamnoside (*3a*, 18 %). Solubility properties, impurities and low recovery on acetylation of crude extracts complicated the isolation of the rhamnosides. Best results were obtained by purification of the free glycosides on acetylated polyamide columns.[3,4]

Zeaxanthin (*1a*, available 0.5 mg) exhibited visible spectrum and mass spectrum consistent with structure *1a*, was inseparable from authentic zeaxanthin (*1a*), and more polar than the 2,2′- or 4,4′-dihydroxy analogues.[8] Its diacetate *1b* was inseparable from authentic zeaxanthin diacetate (*1b*) and different from isozeaxanthin diacetate.

The major carotenoid (*2a*, available *ca.* 18 mg) had, according to the electronic spectrum, the same bicyclic chromophore as zeaxanthin (*1a*). Polarity and mass spectrometric data indicated its glycosidic character. The molecular ion of *2a* [*m/e* 860, consistent with $C_{40}H_{54}O_2(C_6H_{11}O_4)_2$] was shifted to *m/e* 1112 on peracetylation to *2b* and to *m/e* 944 on permethylation to *2c*, demonstrating the presence of six hydroxy groups. Molecular ions were generally supported by M − 79, M − 92, M − 106 and M − 158 ions caused by common in-chain fragmentations.[9]

At least one glycosidically bound methylpentose followed from the fragmentation pattern on electron impact. Thus *2a* itself had characteristic fragment ions M − 164, M − 163, M − 146 and *m/e* 147 and the hexaacetate *2b* diagnostically important M − 290, M − 289, M − 288, M − 272 and *m/e* 273 and 169 ions, typical of methylpentosides.[10,11] Corresponding fragment ions were also observed for the hexamethyl ether *2c*: M − 206, M − 188, *m/e* 189 and 167.[10] An *m/e* 331.1025 ($C_{14}H_{19}O_9$) ion of varying intensity in spectra of the hexaacetate *2b*, consistent with the tetraacetyl oxonium ion of a hexose,[10] could by other criteria (molecular ion

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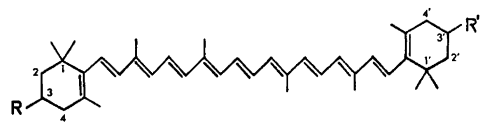
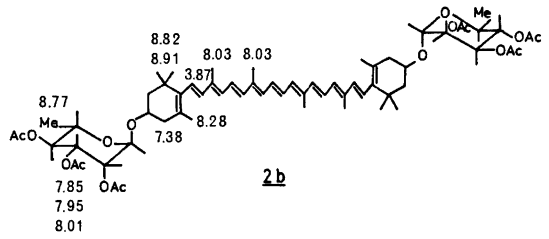


1a R=R'=OH
1b R=R'=OAc
1c R=R'=OMe

2a R=R'= Rhamnosyloxy
2b R=R'= Triacetylrhamnosyloxy
2c R=R'= Trimethylrhamnosyloxy

3a R = Rhamnosyloxy
R'=OH
3b R = Triacetylrhamnosyloxy
R'=OAc



2b

Scheme 1.

and glycoside hydrolysis) not be explained by a mixed hexoside, *cf.* Refs. 11–13. Contamination with an acetylated glucose polymer may offer an explanation, since this bacterium is known to produce polyglucose.[14] However, at this stage acetyl transfer within a disaccharide was also considered.

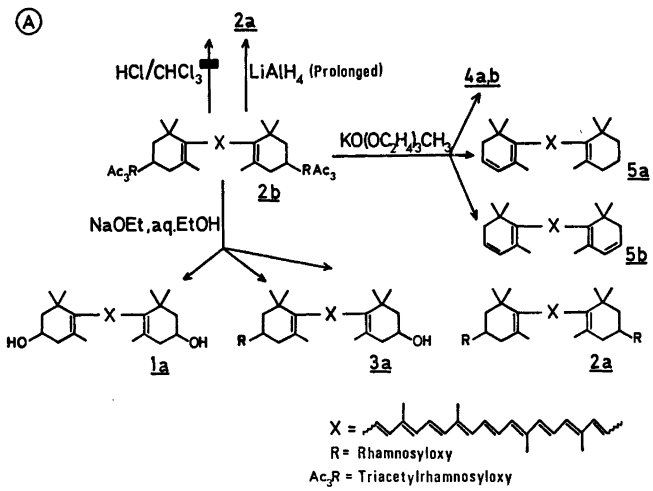
Methanolysis, followed by hydrolysis of the methyl glycosides thus formed gave no reducing sugars under conditions effective for secondary, allylic carotenoid glycosides.[11,12] Methanolysis under more vigorous conditions gave rhamnose as the only reducing sugar seen by paper chromatography. The identification was further confirmed by GLC of the derived rhamnose tetraacetate and rhamnitol pentaacetate in direct comparison with authentic standards. Evidence that zeaxanthin is the aglycone involved is given below, and it may therefore be concluded that the glycoside in question is a zeaxanthin dirhamnoside.

The possibility of the two rhamnose units being present in a disaccharide rather than separately bound to the diol aglycone *1a* was ruled out by ^{1}H NMR data: In the symmetrical zeaxanthin dimethyl ether (*1c*) the 18- and 18′-methyl groups cause one signal in deuteriopyridine at τ 8.24. In the monorhamnoside *3a* discussed below, two separate signals at τ 8.24 (18–Me) and τ 8.17 (18′–Me) are observed. The occurrence of only one signal at τ 8.24 (18,18′–Me) in the dirhamnoside therefore demonstrated the symmetrical nature of *2a*.

Evidence leading to identification of the aglycone will now be considered. The ^{1}H NMR spectrum of the dirhamnoside hexaacetate (*2b*, Scheme 1, including assignments) suggested that the aglycone involved was a β,β-carotenediol derivative.[9] Contrary to secondary, allylic carotenoid glycosides [11,12] the present rhamnoside gave no modified aglycones on treatment with $HCl/CHCl_3$ or on prolonged $LiAlH_4$ reduction.

Others [15,16] have reported that the aglycone and dehydroaglycone may be obtained from tertiary carotenoid glycosides on base treatment. The hexaacetate *2b* gave on treatment with sodium ethoxide in aqueous ethanol zeaxanthin (*1a*, 5 %) and the monorhamnoside (*3a*, 45 %) in addition to the dirhamnoside (*2a*), thus proving that zeaxanthin (*1a*) is the aglycone involved, Scheme 2. The hexamethyl ether *2c* suffered no such hydrolysis, indicating that the free glycoside is required as an intermediate.

Treatment of the hexaacetate *2b* with potassium methoxytriethyleneglycolate [17] in dioxane gave unidentified modified aglycone products



Scheme 2.

(*4a,b*, *5a,b*) with electronic and mass spectra compatible with 3,4(3′,4′)-dehydrogenation, Scheme 2.

These alkaline degradations are not well understood, although possible analogies have been considered.[18–22]

Turning now to the stereochemistry of zeaxanthin dirhamnoside (*2a*), the all-*trans* isomer was the predominant geometrical isomer. The chirality of the aglycone was not established. Zeaxanthin from other sources is known to have 3*R*,3′*R*-configuration.[23,24] Rhamnose sufficient for rotation measurements could not be isolated; L-rhamnosides are most common.[25–28] Regarding the stereochemistry of the glycosidic linkage no definite conclusions can be made from the available evidence. The preference for α-L-rhamnopyranoside [22] (Scheme 1) is tentative.

Zeaxanthin monorhamnoside (*3a*, available 4 mg) exhibited the same electronic spectrum as *1a* and *2a* and had intermediate polarity. Its molecular ion at *m/e* 714 (consistent with $C_{40}H_{55}O_2(C_6H_{11}O_4)$ shifted to *m/e* 882 on acetylation, demonstrating the formation of a tetraacetate *3b*. The monorhamnoside *3a* and its tetraacetate *3b* exhibited the same prominent fragment ions as the dirhamnoside (*2a* and *2b*) on electron impact and showed an *m/e* 331 ion in agreement with the methylpentoside formulation. The ^{1}H NMR spectrum was consistent with structure *3a* and zeaxanthin (*1a*) was obtained as 13 % of the recovered carotenoid on alkaline hydrolysis with sodium ethoxide.

EXPERIMENTAL PART

Biological material. Strain 14 g of a yellow, coryneform hydrogen bacterium, Institute of Microbiology, University of Göttingen, was cultivated in Göttingen (Batch 1) and in the Department of Biochemistry of this University (Batch 2) in a Na-lactate medium as described in detail elsewhere;[22] available *ca.* 2.5 kg fresh cells.

General methods and instruments used were those generally employed.[29] When not otherwise stated R_F-values refer to Schleicher & Schüll No. 287 circular kieselguhr paper, acetone in petroleum ether (APE). TLC refers to kieselgel G.

Isolation. Several chromatographic systems and isolation procedures were investigated,[22] two being referred here: A. Most satisfactory was extraction of centrifuged cells with acetone at room temperature, followed by transfer of the concentrated pigment extract to ether with 5 % aqueous NaCl, drying of the pigment by azeotropic distillation with benzene and chromatography on acetylated polyamide.[3,4] The crude pigment, insoluble in petroleum ether, ether, acetone, chloroform, benzene, methanol, and CS_2, but soluble in pyridine or benzene-methanol (*ca.* 1:1) was dissolved in the latter system and diluted with benzene for chromatography. *1a* required benzene, *3a* 2 % methanol in benzene, and *2a* 6 % methanol in benzene for elution from acetylated polyamide; pigment recovery 79 %.

B. Partition of the crude pigment, dissolved in pyridine, between petroleum ether and 80 %

aqueous methanol resulted in precipitation of the pigments in the aqueous phase; 92 % recovery. Standard acetylation and TLC (40 % APE) gave the acetates *1b*, *3b*, and *2b*; 59 % recovery. If partition was omitted acetylation of the crude pigment gave only 5 – 30 % recovery.

Batch 1, *ca.* 2 kg wet weight, provided 325 mg [E(1 %, 1 cm) = 1900] extracted pigment; 0.074 % of the dry extracted residue, purified by procedure B. Batch 2 gave 27 mg pigment, purified by procedure A.

Zeaxanthin (*1a*)

Characterization. 1a available 0.5 mg, purified by standard saponification and TLC; λ_{max} (acetone) 429, 451 and 474 nm, % III/II [30] = 29; *m/e* 568 (M), 550 (M – 18), 489 (M – 79), 476 (M – 92), 462 (M – 106), 422 (M – 146), 410 (M – 158), was inseparable from authentic *1a* and more strongly adsorbed than isozeaxanthin in direct comparison.

Zeaxanthin diacetate (1b). Standard acetylation of *1a* (0.2 mg) gave *1b*; *m/e* 652 (M), 592 (M – 60), 560 (M – 92) 546 (M – 106), 532 (M – 60 – 60), 500 (M – 60 – 92), 494 (M – 158), 486 (M – 60 – 106), inseparable from authentic *2a*.

Zeaxanthin dirhamnoside (*2a*)

Characterization. 2a (Batch 2), repeatedly crystallized from petroleum ether-methanol and pyridine-petroleum ether-acetone gave a semi-crystalline material, m.p. 216 – 218 °C; R_F = 0.16, TLC 35 % APE; λ_{max} (acetone) 428, 452, and 480 nm, % III/II = 27, λ_{max} (benzene-methanol 1:1) 436, 461 (ε = 75 700, compared with ε = 130 000 for authentic *1a* in acetone) and 488 nm; *m/e* (230 °C) 860 (M), 842 (M – 18), 824 (M – 18 – 18), 781 (M – 79), 768 (M – 92), 754 (M – 106), 734 (M – 126), 714 (M – 146), 702 (M – 158), 697 (M – 163), 696 (M – 164), 678 (M – 18 – 18 – 146), 622 (M – 92 – 146), 608 (M – 106 – 146), 604 (M – 18 – 92 – 146), 602 (M – 258), 568 (M – 146 – 146), 565, 550, 508, 472, 339, 264, 147; τ (deuteriopyridine) 8.85 (*gem.* Me), 8.73 (lipid imp.), 8.34 (*d*, J = 5.5 Hz, Me in rhamnose), 8.24 (18,18′-Me), 7.96 (in-chain Me).

For comparison L-rhamnose had C-5 methyl signals at τ 8.36 (β, d, J = 6 Hz) and 8.40 (α, d, J = 6 Hz). Authentic zeaxanthin dimethyl ether *1c* had τ 8.85 (*gem.* Me), 8.22 (18,18′-Me), 8.01 and 7.97 (in-chain Me) and 6.62 (OMe).

Glycoside hydrolysis. Methanolysis [11] of *2a* in 0.15 N dry HCl for 20 h, 22 °C, was not effected; pigment recovery 75 % and no reducing sugars formed after hydrolysis.[11,31] Methanolysis of *2a* (15 mg) in 0.2 N HCl for 5 h, 65 °C, followed by hydrolysis [11,31] with aqueous polystyrene sulfonic acid and ascending chromatography,[22] followed by development with aniline-phthalic acid, showed presence of rhamnose ($R_{glucose}$ = 1.44) only in co-chromatography tests with saccarose (0.45), mannose (0.65), galactose (0.81), glucose (1.00) rhamnose (1.44), and fucose (2.02).

An aliquot of the hydrolysate was acetylated and submitted to GLC (Perkin Elmer F11, silicon XE-60/ethyleneglycol succinate, 157 – 218 °C, 1 °/min) provided two peaks with retention time 20.4 min (75 %) and 22.9 min (25 %). Peracetylated α,β-L-rhamnose showed the same retention times and a ratio of 90:10.

Remaining tetraacetyl rhamnose was saponified and reduced with $NaBH_4$ in water. The rhamnitol, thus prepared, was acetylated and submitted to GLC as above and found identical (retention time 24.5 min) in direct comparison with authentic L-rhamnitol pentaacetate prepared from L-rhamnose.

Zeaxanthin dirhamnoside hexaacetate. (2b). Standard acetylation of *2a* (12 mg) gave *2b* (90 % yield), purified by TLC: λ_{max} as for *2a*; *m/e* 1112 (M), 1070 (M – 42), 1068 (M – 44), 1033 (M – 79), 1020 (M – 92), 1006 (M – 106), 992 (M – 60 – 60), 980 (M – 132), 976 (M – 44 – 92), 968 (M – 144), 956 (M – 156), 954 (M – 158), 868 (M – 244), 840 (M – 272), 824 (M – 288), 823 (M – 289), 822 (M – 290), 731 (M – 92 – 289), 717 (M – 106 – 289), 687 (M – 44 – 92 – 289), 568, 381, 351, 331.1025 ($C_{14}H_{19}O_9$), 273, 169, ν_{max} (KBr) 2925, 2855, 1752, 1445, 1370, 1225, 1180, 1150, 1135, 1050, 977, 910, 890, 840, 790 and 685 cm^{-1}; τ ($CDCl_3$), *cf. 2b*, Scheme 1, 8.91, 8.82 (*gem.* Me, *ca.* 12 H); 8.77 (*Me*–CH?); 8.28 (Me – 18,18′, *ca.* 6 H); 8.03 (in-chain Me, *ca.* 12 H); 8.01, 7.95, 7.85 (acetate Me, *ca.* 18 H); 7.38 (allylic CH_2); 4.0 – 6.0 (CH in rhamnose moiety); 3.0 – 4.0 (olefinic H), stepwise addition of Eu(dpm)$_3$ [32] until 3.1 mol/mol *2b* caused no shift of the aglycone methyl groups, 55 Hz downfield shift of allylic CH_2 and 30 – 19 Hz downfield shifts of CH_3 and $CH_3C{=}O$ of the rhamnose moiety; R_F = 0.65 in 10 % APE.

An acetylated compound more polar than *2b* by TLC and representing 4 % of *2b* was, from electronic spectrum (% III/II = 0), mass spectrum (as *2b*), and reversibility tests, shown to be a *cis* isomer of *2b*.

$LiAlH_4$-reduction of the hexaacetate 2b. Treatment of *2b* (0.5 mg) with $LiAlH_4$ in dry ether for 5 min – 24 h [11,12] gave no modified aglycone but only the free rhamnoside *2a*; pigment recovery 30 – 50 %.

Attempted allylic elimination of the hexaacetate 2b. 2b (0.3 mg) treated with 0.03 – 0.2 N dry HCl in ethanol-free chloroform for 2 – 15 min gave no new, coloured products.

Alkaline hydrolysis of the hexaacetate 2b. 2b (0.75 mg), in 2.5 N NaOEt in 96 % ethanol (10 ml) was kept for 3 h at 50 – 60 °C. The pigments were transferred to ether on dilution with water after acidification to pH 4; pigment recovery 42 %. Paper chromatography revealed the formation of zeaxanthin (*1a*). Acetylation of the products gave zeaxanthin diacetate (*1b*, 5 %, *m/e* 652 = M), zeaxanthin monorhamnoside tetraacetate (*3b*, 45 %, *m/e* 882 = M) and zeaxanthin dirhamnoside hexaacetate (*2b*, 50 %) with electronic spectra and R_F-values as authentic samples.

Treatment with potassium methoxytriethyleneglycolate [17] at room temperature was carried out with the hexaacetate (*2b*, 1.5 mg) in dioxane (1.4 ml, chromatographed on alumina and distilled under N_2) and potassium methoxytriethyleneglycolate (0.6 ml, *ca.* 0.5 N) added. The mixture was stirred mechanically for 1.5 h and worked up as above; pigment recovery 30 %. TLC gave two major zones, presumably comprising four products: *4a, b* (70 %, $R_F = 0.18$, TLC, 5 % APE), λ_{max} (acetone) 460 nm (round), *m/e* 548 (M_a, consistent with $C_{40}H_{52}O$), 546 (M_b, $C_{40}H_{50}O$?, 80 % of *m/e* 548), 456 ($M_a - 92$), 454 ($M_b - 92$), 442 ($M_a - 106$), 440 ($M_b - 106$) and *5a, b* (25 %, $R_F = 0.60$ in above system), λ_{max} (acetone) 442, 461, and (483) nm, *m/e* 534 (M_a, $C_{40}H_{54}$), 532 (M_b, $C_{40}H_{52}$, 50 % of *m/e* 534), 442 ($M_a - 92$,) 440 ($M_b - 92$), 428 ($M_a - 106$).

Zeaxanthin dirhamnoside hexamethyl ether (2c). Methylation of *2a* (9 mg) by the modified Kuhn procedure [33] gave *2c*; 40 % pigment recovery, quantitative conversion. Compound *2c* had λ_{max} as *2a*; *m/e* 944 (M), 942 (M − 2), 912 (M − 32), 883 (M − 61), 852 (M − 92), 850 (M − 2 − 92), 838 (M − 106), 830 (M − 114), 814 (M − 130), 812 (M − 132), 800 (M − 144), 786 (M − 158), 784 (M − 160), 770 (M − 174), 762 (M − 182), 756 (M − 188), 738 (M − 206), 694 (M − 92 − 158), 692 (M − 92 − 160), 664 (M − 92 − 188), 189, 157; $R_F = 0.90$, 10 % APE.

Alkali treatment with 2.5 % NaOEt as for *2b* caused no reaction.

Zeaxanthin monorhamnoside (*3a*)

Characterization. 3a, precipitated from methanol-benzene-petroleum ether, available 4 mg, m.p. 149 − 150 °C had μ_{max} (methanol-benzene 1:1) 436, 460 and 487 nm, % III/II = 23; *m/e* (220 °C) 714 (M), 696 (M − 18), 680 (M − 34), 672 (M − 42), 635 (M − 79), 622 (M − 92), 608 (M − 106), 602 (M − 112), 596 (M − 118), 588 (M − 34 − 92), 582 (M − 132), 568 (M − 146), 558 (M − 156), 556 (M − 158), 551 (M − 163), 550 (M − 164), 516 (M − 92 − 106), 508 (M − 206), 504 (M − 210), 476 (M − 92 − 146), 462 (M − 106 − 146), 339, 264, 163; τ (deuteriopyridine) 8.82 (*gem.* Me), 8.70 (lipid imp.), 8.34 (d, $J = 5.5$ Hz, Me in rhamnose), 8.24 (18 − Me), 8.17 (18′ − Me) and 7.95 (in-chain Me); $R_F = 0.50$, 35 % APE.

Zeaxanthin monorhamnoside tetraacetate (3b). 3a (0.4 mg) was quantitatively acetylated to *3b* λ_{max} as *3a*; *m/e* 882 (M), 840 (M − 42), 838 (M − 44), 822 (M − 60), 803 (M − 79), 790 (M − 92), 776 (M − 106), 748 (M − 42 − 92), 730 (M − 60 − 92), 726 (M − 156), 724 (M − 158), 716 (M − 60 − 106), 666 (M − 60 − 156), 664 (M − 60 − 158), 638 (M − 244), 610 (M − 272), 594 (M − 288), 593 (M − 289), 331 (15 % of *m/e* 273), 273, 153, (100 %); $R_F = 0.54$, 5 % APE.

Alkaline hydrolysis of tetraacetate 3b. 3b (0.5 mg) was treated with 2.5 % NaOEt as described above for *2b*; pigment recovery 40 %. Zeaxanthin (*1a*) was identified by paper chromatography. Acetylation of the product, followed by TLC gave zeaxanthin diacetate (*1b*, 13 %, inseparable from authentic *1b*) and *3b* (87 %, inseparable from the starting material *3b*).

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On the Structure of Rapidly Labeled Rat Liver RNA

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Rat liver total RNA, extracted by three phenol methods relying upon 4-aminosalicylate, naphthalene-1,5-disulfonate, and heat, respectively, gave different migration patterns, most pronounced for rapid label. The difference could not be attributed to differential extraction.

By the 4-aminosalicylate method, large amounts of rapidly labeled structures sedimenting up to 20 000 S were isolated. These structures, consisting of aggregates of heterogeneous RNA, were examined in the present report. They complied with most of the criteria used to classify giant heterogeneous nucleoplasmic RNA as a separate entity of covalent molecules, but they were not resistant to treatment with dimethyl sulfoxide or to heating in low-ionic-strength acetate buffers or in formaldehyde.

When such disaggregating treatment was performed on the RNAs extracted by the different methods, the rapidly labeled RNA >45 S disappeared, and several additional UV-absorbing constituents appeared in the cold-extracted RNAs. This caused a resemblance in the migration and sedimentation patterns of the different RNAs. Radioactive markers added to the extraction buffers prior to homogenization of the livers did not reveal any degradation during extraction or analysis. The results suggest that giant heterogeneous nucleoplasmic RNA from rat liver could essentially be an artifact of extraction.

The presence of giant heterogeneous nucleoplasmic RNA (giant hnRNA) (10 – 200 S) in eucaryotic cells has been established by many authors (review by Darnell [1]). It was identified in rat liver by Schütz *et al.*[2] A precursor relationship to cytoplasmic messenger RNA (mRNA) (6 – 30 S) [3,4] is widely accepted. However, conclusive evidence for specific cleavage has not been found. A remarkably reproducible base composition has been reported, with uridine content in excess of the corresponding DNA, and in excess of that of the cytoplasmic mRNA of the same cells.[3,5–8] Its biological role is thus still uncertain.

The existence of covalent molecules of corresponding size was questioned by Mayo and de Kloet [9] who showed that formaldehyde treatment converted rapidly sedimenting rapidly labeled RNA (rlRNA) to structures sedimenting as mRNA. Recent reports, using different disaggregating treatments, and different methods for analysis of the treated RNA, have been contradictory.[10–15] Even if such a conversion could be found to be accompanied by a corresponding change in the electrophoretic pattern, the possibility of giant size transcription with later conversion to smaller molecules could not be excluded.

In an examination of different methods for extraction of rat liver total RNA [16] large variations were found in the rapidly sedimenting rlRNA. These could not be attributed to differential extraction of the rapidly labeled molecules. Preliminary experiments showed that the amount and size of such molecules, when extracted by a 4-aminosalicylate method, were out of proportion to what could reasonably be expected to represent an *in vivo* state. The present study was undertaken to see whether these structures were consistent with the criteria of giant hnRNA. If so, such molecules could be mere artifacts of extraction, and not necessarily expression of giant size transcription. Besides, the criteria used to classify giant hnRNA as a separate entity of covalent molecules could be evaluated.

The results showed that the extracted aggregates met many of the criteria used for giant hnRNA, and that several of the disag-

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gregating treatments previously used to establish the covalency of this class of molecules in fact did not disaggregate them. However, they could be disaggregated by heating in low-ionic-strength acetate buffer at 50 °C, or by treatment with dimethyl sulfoxide (DMSO). When such treatment was applied to materials extracted by different methods, evidence was obtained that emphasized the tendency of newly synthesized RNA to aggregate during extraction. To exclude the possibility of RNA degradation, controls using both homogeneous and heterogeneous markers were included.

MATERIALS AND METHODS

Animals. Male 250 g CD*F inbred rats (Charles River Breeding Laboratories, Wilmington, Mass.) were used.

Cell fractionation procedures. Liver nuclei were prepared by homogenizing the liver in 13 ml 1.5 M sucrose containing 2 mg/ml bentonite (treated according to Fraenkel-Conrat *et al.*[17]). The homogenate was filtered through gauze and layered on a 35 ml linear 1.5 – 2.25 M sucrose gradient, using a 10 ml 2.3 M sucrose cushion. All sucrose solutions were made up in buffer (Tris 0.05 M, KCl 0.025 M, $CaCl_2$ 3.3 mM; pH 7.1) containing 0.1 mg/ml dextran sulfate. Centrifugation was performed in a Spinco SW 25.2 rotor for 45 min at 25 000 rpm and 5 °C. The pellet was collected.

Polysomes were prepared after homogenization of the liver in two volumes chilled 0.25 M sucrose in TKM-buffer (Tris 0.05 M, KCl 0.025 M, $MgCl_2$ 2.5 mM; pH 7.0), containing 2 mg/ml bentonite. The homogenate was then centrifuged at 15 000 *g* for 10 min at 2 °C, and the supernatant made 0.4 % w/v with respect to deoxycholate. 15 ml was applied on top of two layers of sucrose in TKM-buffer, 10 ml 0.5 M sucrose and 5 ml 1.0 M sucrose. Centrifugation was performed in a Spinco SW 25.1 rotor for 3 h at 25 000 rpm and 2 °C. The pellet was used as polysome source.

Extraction procedures. Method 1 was a cold-phenol method based on the 4-aminosalicylate method of Parish and Kirby.[18] Method 2 was modified from the cold-phenol naphthalene-1,5-disulfonate method of Attardi *et al.*[5] Method 3 was modified from the hot-phenol method of Warner *et al.*[19] Details have been given previously.[16]

Preparation of E. coli *16 S (10 S) rRNA marker. Escherichia coli* K12 cells were grown logarithmically for 2 h in 500 ml of a minimal salt medium containing 0.4 % w/v glucose and 200 μCi ^{3}H-uridine. Ribosomal RNA (rRNA) was extracted by a modified Method 3, extracting at 4 °C, and the 16 S rRNA purified by repeated ultracentrifugations. After DMSO-treatment, the peak was recovered as a 10 S peak.

Chemical analysis. Protein was determined by the method of Lowry *et al.*[20] DNA was determined by a diphenylamine reaction.[21]

Ultracentrifugation. Sucrose gradient analysis and sedimentation coefficient determinations have been described previously.[16] The very high *s* values (≥ 2000 S) were determined by examining pellet material after short runs at reduced speed (10 000 rpm), taking the sedimentation during the acceleration and deceleration of the rotor into consideration. The possibility that the pelleting could be due to irregular sedimentation, was excluded by running in a sector-formed partition cell in a Spinco model E analytical ultracentrifuge.

Before density equilibrium centrifugation, RNA was fixed at 4 °C in 0.05 M sodium phosphate – citrate pH 7.0, containing 5 – 8 % w/v formaldehyde. Cesium chloride gradients were run in a Spinco SW 65 rotor for 66 h at 25 °C. Mixed cesium chloride : cesium sulfate 4:1 v/v (equal densities) gradients were run in a Spinco 65 fixed angle rotor at 30 °C and 61 000 rpm. All solutions contained 1.5 % w/v formaldehyde and 0.05 M sodium phosphate-citrate pH 7.0.

Polyacrylamide gel electrophoresis was performed as described previously.[16]

^{32}P Base composition analysis. Sucrose gradient fractions were pooled according to the UV-pattern, precipitated after addition of carrier RNA, and hydrolyzed in 0.3 M potassium hydroxide at 37 °C for 17 h. The nucleotides were separated by high voltage electrophoresis, using Whatman No. 1 paper as support.

Treatment with disaggregating agents. Formaldehyde treatment was performed by heating RNA in 0.01 M sodium acetate pH 6.0, containing 7.5 % w/v formaldehyde, at 63 °C for 15 min before chilling. RNA samples to be treated with DMSO were dissolved in DMSO containing 0.01 M LiCl and 1 mM EDTA.[22]

RESULTS AND DISCUSSION

Aggregate characteristics

Physical state. $rlRNA_1$,* isolated 20 min after administration of radioactive orotic acid, sedimented heterogeneously with 3 % of the radioactivity at 2000 S or faster, and 1 % at 20 000 S or faster.

No DNA was found in material ≥ 140 S. The protein content was 5 – 30 μg per mg total RNA_1, all sedimenting at *s* values ≥ 140 S. A reduction in protein content upon re-extraction with phenol with no change in RNA-pattern,

* Subscript $_1$, $_2$, or $_3$: That which pertains to Extraction Method 1, 2, or 3.

as well as cesium salt density gradient centrifugations, indicated that this protein was merely co-sedimenting. The sedimentation patterns were also unaffected by treatment of the isolated RNA with the lipid solvents ethanol, ethyl-ether, or methanol-chloroform 1:2 v/v, or exposure to pronase, DNAase, or amylase. RNAase treatment caused both rapid label and optical density to be confined exclusively to the top fractions of the gradients. The rapidly sedimenting substances therefore appear to consist of RNA without other macromolecules. Their conversion to slower sedimenting, faster migrating structures on moderate heating in a low-ionic-strength acetate buffer or by DMSO-treatment (see below), indicates that they consist of hydrogen-bonded aggregates.

Origin. By separate extraction of RNA from nuclei and polysomes, rlRNA$_1$ sedimenting at *s* values comparable to those obtained by extracting whole liver tissue could be demonstrated only in the nuclear fraction. Polysomal rlRNA sedimented in the region 5 – 35 S.

Kinetics. All RNA sedimenting faster than 65 S had identical labeling kinetics. Except for the growing polynucleotide chains,[16] it was labeled faster than any other fraction. The absolute amount of radioactivity ≥ 140 S was maximal about 20 min after administration of radioactive orotic acid (Fig. 1a). Its relative specific activity

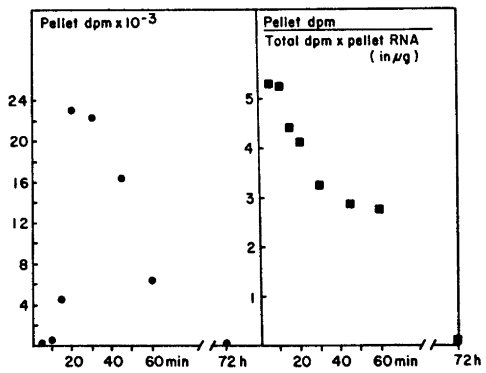

Fig. 1. Kinetics of labeling of apparent giant hnRNA$_1$. RNA$_1$ was isolated at different intervals after injection of 50 μCi ^{14}C-orotic acid. 1 mg RNA was subjected to ultracentrifugation (10 – 40 % w/w sucrose in 0.1 M sodium acetate pH 6.0, Spinco SW 25.1, 24 000 rpm, 5 °C, 14 h). The pellet (≥ 140 S) was eluted, and the amount of RNA and of radioactivity determined.

showed a steady decline with time (Fig. 1b), demonstrating the high turnover of the fast sedimenting RNA$_1$, and indicating that newly synthesized or even incomplete RNA strands are especially liable to aggregation.

Apparent base composition. Base composition analysis of RNA$_1$ isolated 1 h after intraperitoneal injection of ^{32}P$_i$ showed varying composition of the RNA in the heavy parts of the gradient. Even runs of the same material on parallel gradients showed considerable differences in base composition at the same gradient level. (Repeated analysis of material from the same hydrolysis tube showed identical results). However, the RNA in the regions > 65 S had adenine + uracil/guanine + cytosine ratios ≥ 1. With references to the polyadenylic sequences in nuclear and cytoplasmic mRNA, a relatively high adenine content, ranging from 25 to 31 mol per cent, was noteworthy. No substantial participation of rRNA/pre-rRNA could be detected.

Susceptibility to disaggregating conditions. Exposure to low ionic strength at room temperature [23] did not affect the rapidly sedimenting rlRNA$_1$, but retarded the 10 – 45 S rlRNA (Fig. 2a, b). This retardation was reversible (Fig. 2c), indicating a mere conformational change.

Heating at 50 °C for 3 min in the presence of 0.1 M sodium chloride left the rapid label pattern almost unaffected (Fig. 2d). When such heating was performed in 0.1 M sodium acetate, a substantial amount of rapidly sedimenting radioactivity was converted to material sedimenting in the 5 – 28 S region, and after heating in low-ionic-strength acetate buffer, nearly all radioactive material sedimented in the 5 – 35 S region (Fig. 2e). A parallel conversion was seen by gel electrophoresis (Fig. 5a, d). No specific release of material from the aggregates was found corresponding to rRNA or tRNA (Fig. 2f). Molecules without a firm secondary structure therefore seem to aggregate most readily. Local concentrations in the cell might also be of importance.

Five mM magnesium prevented any change during heating at 50 °C for 5 min. Concentrations of RNA above 1 mg/ml stabilized the original structures somewhat, increasing with greater concentrations. Treatment at room temperature with 1 % w/v SDS, 3 – 8 M urea or ethylurea and/or 1 – 10 mM EDTA did not

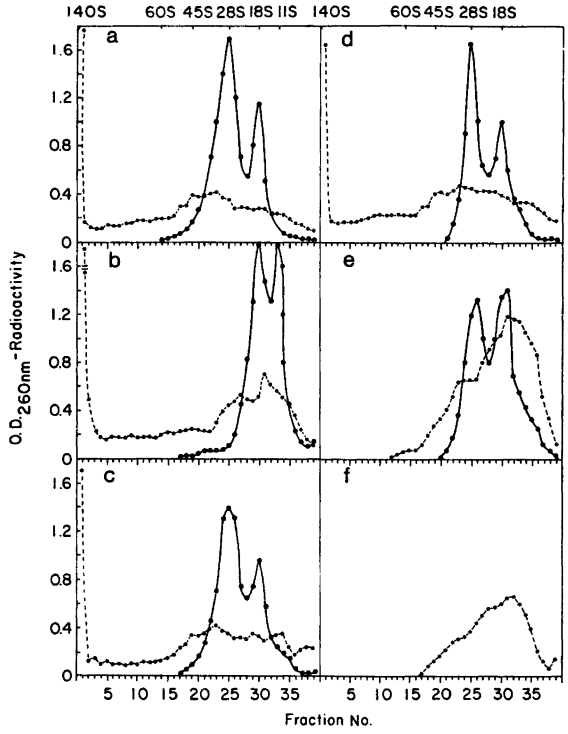

Fig. 2. The effects of low ionic strength and of elevated temperatures on the sedimentation pattern of RNA_1. (a–e) 1 mg RNA, extracted 10 min after administration of 300 μCi ^{3}H-orotic acid, was examined after incubation under the following conditions: (a) Control. No incubation. (b) In 5 mM EDTA-tris pH 7.0, at 25 °C for 1 h. (c) As under (b), with sodium acetate added to 0.1 M before chilling. (d) In 0.01 M sodium acetate containing 0.1 M sodium chloride and 1 mM EDTA at 50 °C for 3 min. (e) In 0.01 M sodium acetate pH 6.0 containing 1 mM EDTA, at 50 °C for 3 min before addition of acetate to 0.1 M. (f) rlRNA $\geq$140 S from an animal labeled for 30 min with 50 μCi ^{14}C-orotic acid was heated as under (e), and recentrifuged.

10–40 % w/w sucrose, Spinco SW 25.1, 24 000 rpm, 5 °C, 14 h, gradient buffer: (a,c–f) 0.1 M sodium acetate pH 6.0, (b) 5 mM EDTA-tris pH 7.0. ———, $OD_{260\ nm}$; (a–e) - - -, ^{3}H-dpm × 10^{-3}; (f) - - - ,^{14}C-dpm × 5 × 10^{-4}.

affect the gradient patterns, but abolished the adsorption of rlRNA to the centrifuge tubes.

After treatment with formaldehyde, the changes were more pronounced than after heating in low-ionic-strength buffer, but some fast-sedimenting, rapidly labeled structures persisted (Fig. 3a). The 28 S rRNA was then partly excluded from the gels, indicating a severe derangement of the molecular conformation, possibly also formation of intermolecular methylene bridges.[24] DMSO-treatment caused essentially the same changes as heating in buffer but some rlRNA, less than after formaldehyde-treatment, still sedimented at 2000 S or more (Fig. 3b).

Aggregate formation. When 20 min labeled RNA_1 >80 S was collected, dissociated by heating, and added to the aqueous phase before unlabeled rat liver total RNA was extracted, aggregation could directly be demonstrated, although to a less extent than observed after *in vivo* labeling (Fig. 4a, b). After re-dissociation, the radioactivity maximum sedimented as after the first dissociation (Fig. 4c).

Relationship to giant hnRNA. The rapidly sedimenting $rlRNA_1$ possesses several of the characteristics reported for giant hnRNA. It is the most rapidly labeled fraction found, except for the growing polynucleotide chains. It consists of pure RNA, and can be demonstrated

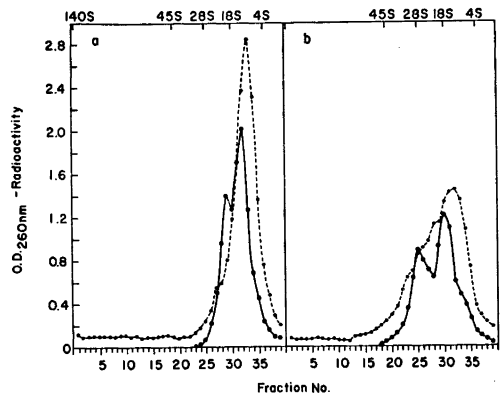

Fig. 3. Dissociation of rat liver RNA. RNA labeled for 10 min was examined by sucrose gradient centrifugation after treatment with (a) formaldehyde, (b) DMSO. The material had been stored for four years between the experiments reported in Fig. 2a–e and the experiments reported in this figure. Legend otherwise as in Fig. 2a.

only in the nuclear fraction. Its sedimentation pattern is fairly reproducible with 25–35 % of the total radioactivity sedimenting ≥140 S after a 20 min pulse. It resists treatment with 3–8 M urea or ethylurea, 1–2 % SDS, 1–10 mM EDTA, or low ionic strength at room temperature. It is also unaffected by heating at 50–60 °C in 0.1 M sodium chloride for 3–5 min. In spite of this, it is obviously an artifact of extraction, because it sediments at *s* values up to 20 000 S, and aggregation can directly be demonstrated.

To be designated as covalent, RNA molecules should be completely resistant to treatment with DMSO or to heating in low-ionic-strength acetate buffer containing 1 mM EDTA for removal of divalent ions. The difference between sodium chloride and sodium acetate is not understood, but Tal [25] noticed that potassium acetate depressed the melting temperature of rRNA. Parallel examination by ultracentrifugation and gel electrophoresis should be performed to determine whether changes in charge or in conformation *versus* aggregation/disaggregation have taken place. Because formaldehyde-treated RNA is partly excluded from acrylamide gels, the use of this denaturing agent is not advocated.

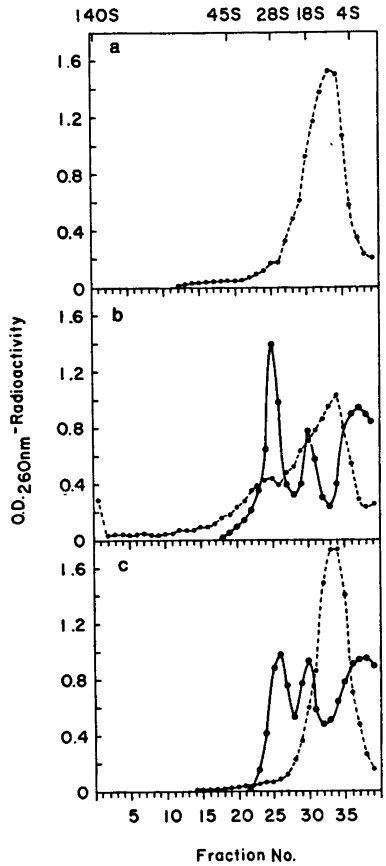

Fig. 4. Aggregation/disaggregation of rlRNA$_1$. (a) 20 min labeled RNA, originally sedimenting ≥80 S, was collected and dissociated by heating at 57 °C for 7 min in 0.01 M sodium acetate pH 6.0. The dissociated material was added to aqueous solution$_1$ before extraction of unlabeled rat liver total RNA. The extracted material was then dissociated by heating. (a) Dissociated rlRNA before addition to aqueous solution$_1$. (b) Total RNA after extraction. (c) Total RNA after dissociation.

10–40 % w/w sucrose in 0.1 M sodium acetate pH 6.0, Spinco SW 25.1, 24 000 rpm, 5 °C, 14 h. ———, OD $_{265\ nm}$; ----, ^{14}C-dpm × 10^{-2}.

Although DNA-like with respect to a high adenine + uracil content, our apparent giant hnRNA$_1$ did not have the high uracil content reported for giant hnRNAs. A specific base composition would suggest a separate molecular species, but determinations made by phosphorus-32 nearest neighbor technique must be regarded with caution.

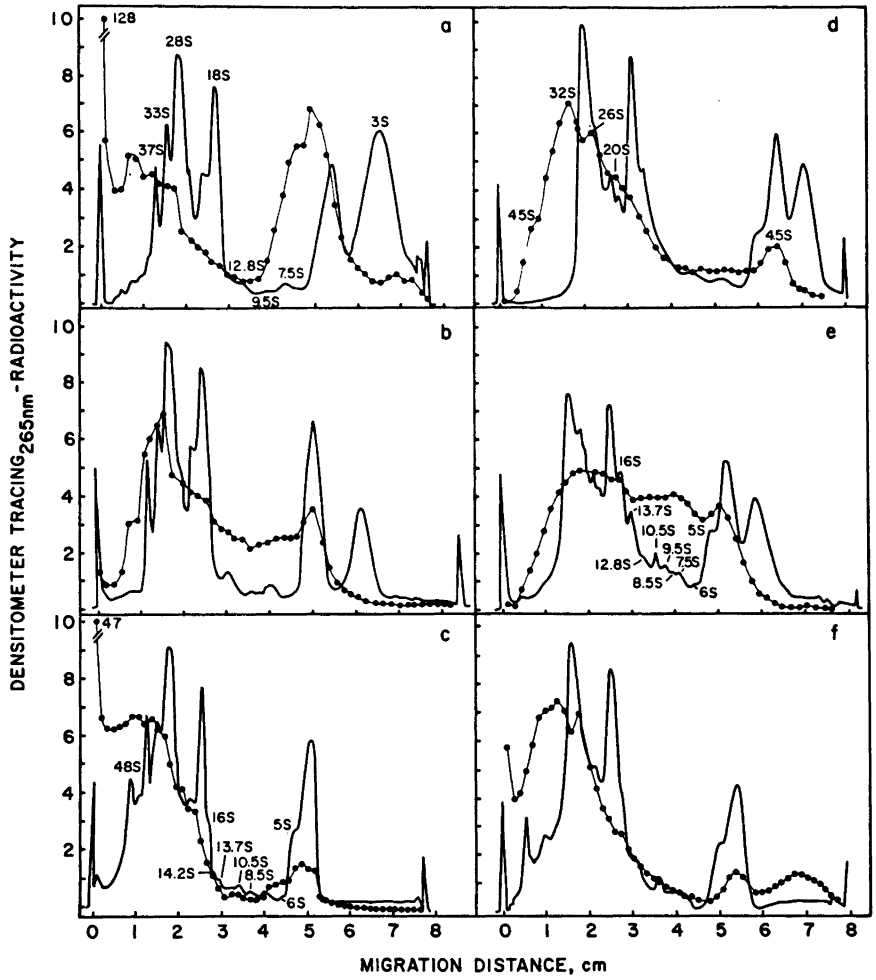

Fig. 5. Disaggregation of rat liver total RNA extracted according to three methods. RNA, extracted 20 min after administration of 50 μCi ^{14}C-orotic acid, heated in 0.01 M sodium acetate pH 6.0 at 50 °C for 3 min prior to electrophoresis. (a) RNA_1, (b) RNA_2, and (c) RNA_3: Unheated, run in magnesium-buffer. (d) RNA_1, (e) RNA_2, and (f) RNA_3: After heating, run in EDTA-buffer. 2.6 % w/v acrylamide, 12 mA/tube, 20 °C, 2 3/4 h. ———, densitometer tracing$_{265\ nm}$; ●, ^{14}C-dpm/mg gel.

Disaggregation patterns

When materials extracted by the 3 different methods were examined after heating in low-ionic-strength acetate buffer, it was found that the aggregates of $rRNA_3$ at 48, 37, and 33 S were harder to dissociate than those obtained by the other methods (Fig. 5a–f). The 28 S peak of RNA_1 and RNA_2 became reduced, while the 18 S peak remained unaffected. Additional peaks between the ribosomal peaks and in the 4–18 S region made the UV-patterns of RNA_1 and RNA_2 approach that of RNA_3, demonstrating which components of RNA_3 that could be attributed to heating during extraction. A comparison of the UV-patterns of unheated RNA with those obtained after heating (Fig. 5) indicates that the 25 S, 21.5 S, and 20 S peaks as well as the 12.8 S, 9.5 S, and 7.5 S peaks may represent *in vivo* existing separate molecules.

Heating at 50 °C for 3 min in 0.01 M sodium acetate usually made all $rlRNA_1$ enter the gels, with only traces of radioactivity migrating slower than the 45 S component, and with a main peak at 32 S (Fig. 5d). The amount of $rlRNA_3$ on top of the gels was reduced, but some radioactivity usually persisted (Fig. 5f), unless all aggregates of rRNA were dissociated. Such heating made these radioactivity patterns approach that of unheated $rlRNA_2$. However, parallel heating of $rlRNA_2$ also altered the migration patterns of this rlRNA species, with a decrease in the amount of radioactivity in the region > 18 S, and an increase in the 4 – 18 S region. Some label still migrated as a shoulder at 45 S (Fig. 5e). Heating of $rlRNA_1$ and $rlRNA_3$ at higher temperatures (> 60 °C) caused a further conversion in their patterns towards that of heated $rlRNA_2$.

After heating at 50 °C, similarity in the patterns of the different rlRNAs was in general found when materials with labeling times ≤ 10 min were investigated, and the UV-absorbing rRNA aggregates were dissociated. In these materials the amount of radioactivity in pre-rRNA/rRNA was negligible. Increase in the incubation times from 3 – 5 min to 1 h did not affect the patterns significantly. A further conversion was seen on increased heating, occurring in a parallel fashion for the different materials.

The conversion of the isolated giant hnRNAs to slower sedimenting, faster migrating structures demonstrates their aggregate nature, and supports the findings of Mayo and de Kloet.[9] All phenol methods examined caused aggregation of RNA. The preferential aggregation of newly synthesized RNA probably reflects a less firm secondary and tertiary structure of these molecules. The tendency towards aggregate formation varies according to the extraction method,[16] as does the dissociation temperature of the formed aggregates. One cannot exclude a transcription giving giant molecules which are immediately cleaved without dissociation of the fragments. However, hard evidence for the *in vivo* existence of rlRNA molecules larger than corresponding to 45 – 50 S cannot be found.

The rapid label in the 45 S and 36 S regions became reduced on heating, and the radioactivity accumulated as a 32 S peak. Convincing evidence has been obtained by electron microscopy (review by Bush and Smetana [26]) and by disaggregating experiments [9,27] to demonstrate the transcription of 45 S pre-rRNA as a single polynucleotide chain. The possibility exists, however, that the molecules do not immediately dissociate after scission. Possible co-migration of other rlRNA species in our experiments necessitates the use of subcellular fractionation to settle the question.

After heat treatment, rlRNA peaks were observed at 28 – 32 S, 26 S, 20 S and 4.5 S. Long label (24 – 48 h) followed the UV-pattern closely.

The possibility of RNA degradation

The size of the rat liver rlRNA molecules seemed smaller than could be expected from most previous reports. It was therefore imperative to exclude the possibility of degradation of the molecules during their extraction, storage or analysis.

RNAase activity during the extraction seemed unlikely: The ice-cold homogenized livers were mixed with phenol within 1 min after decapitation of the animals, and RNAase inhibitors were used throughout. Removal of DNA and glycogen by chemical [18] instead of enzymatic methods did not influence the dissociation. The sedimentation pattern of dissociated heterogeneous rlRNA seemed unaffected by passage of the rlRNA through the extraction procedure (Fig. 4a, c). Also unaffected was the migration pattern of a homogeneous marker, 16 S (10 S) rRNA from *E. coli* (Fig. 6a – c). As these molecules were partly unfolded by the DMSO-treatment during their purification, their sensitivity towards degradation should be increased. These results, therefore, strongly argue against any degradation during the extraction.

Degradation during storage could be excluded, as the dissociation patterns were unchanged, even after 4 years of storage.

The changes found on dissociation by heating at 50 °C could not be attributed to degradation during the incubation: Parallel changes were observed after treatment with DMSO at room temperature, which obviates both RNAase activity and thermal scissions,[28] and no further changes were found when the incubation was increased from 5 min to 1 h. At temperatures ≥ 60 °C, the patterns were not as stable,

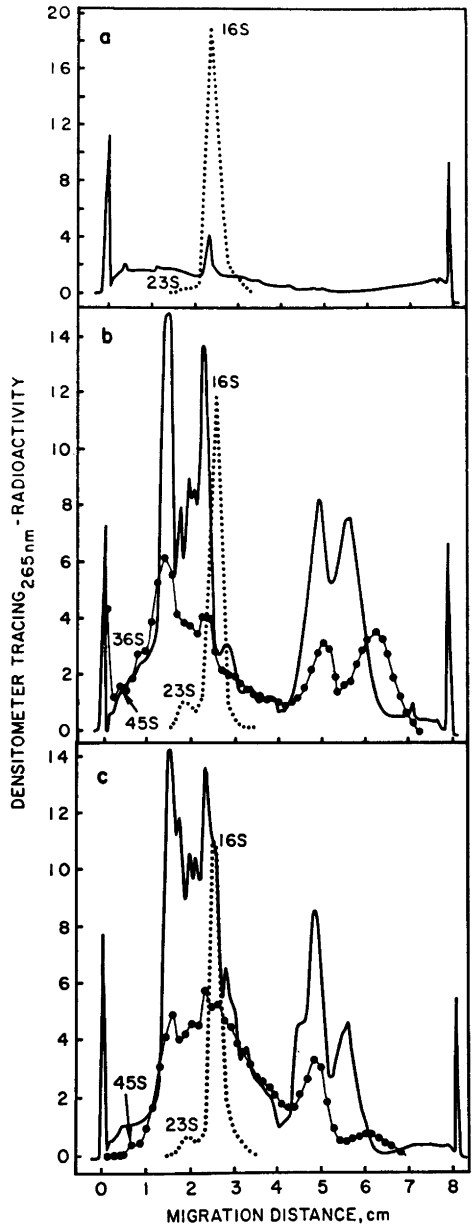

Fig. 6. Disaggregation of rat liver total RNA_2, extracted with *E. coli* 16 S (10 S) rRNA marker. The marker was prepared as described under Materials and Methods. Traces of 23 S rRNA were present. The purified ^3H-labeled marker was added to aqueous solution$_2$, before the chopped liver from a rat labeled for 45 min with 50 μCi ^{14}C-orotic acid was added, homogenized, and extracted. Disaggregation was performed by heating at 50 °C in 0.01 M sodium acetate pH 6.0 for 3 min. (a) Purified disaggregated marker material (25 μg). (b) Extracted total RNA (300 μg). (c) Extracted total RNA after disaggregation.

2.6 % w/v acrylamide, EDTA-buffer, 12 mA/tube, 20 °C, 3 h. ———, densitometer tracing$_{265\ nm}$; · · · ·, ^3H-dpm × 10^{-2}/mg gel; ●, ^{14}C-dpm/mg gel.

indicating a possible contribution from hydrolytic/thermal scissions.

As degradation could not be detected during extraction or analysis of the RNA, only trace amounts – if any – of the rat liver RNA molecules can be larger than corresponding to about 45 S. The strong tendency to aggregation of the rapidly labeled molecules should be considered whenever their structure, or factors affecting their turnover, are discussed.

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Thermodynamics of Vinyl Ethers. IX.* The Relative Stabilities of 4-Methylene-1,3-dioxane and 4-Methyl-1,3-dioxene-(4)

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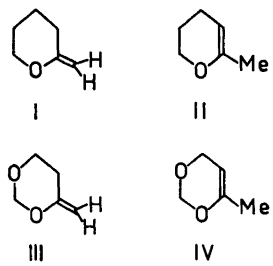
Equilibrium concentrations of the title compounds in cyclohexane solution at various temperatures have been determined. The following results were obtained for the isomerization of 4-methylene-1,3-dioxane to 4-methyl-1,3-dioxene-(4) at 298.15 K: $\Delta G° = -19.9 \pm 0.2$ kJ mol^{-1}, $\Delta H° = -16.1 \pm 0.8$ kJ mol^{-1} and $\Delta S° = 12.8 \pm 2.3$ J K^{-1} mol^{-1}. In the gas phase at 298.15 K, the values of $\Delta H°$ and $\Delta S°$ were obtained as -18.3 ± 0.9 kJ mol^{-1} and 10.2 ± 2.4 J K^{-1} mol^{-1}, respectively.

In a recent work by this author,[1] thermodynamics of the *exo*→*endo* isomerization of a number of five- and six-membered unsaturated heterocyclic ethers, such as 2-methylenetetrahydropyran (I) and 6-methyl-3,4-dihydro-2*H*-pyran (II), were investigated. In diethyl ether



Scheme 1.

solution at 298.15 K, the standard Gibbs free energy change for I→II was obtained to be -21.8 kJ mol^{-1}. The corresponding value for the isomerization of 4-methylene-1,3-dioxane (III) to 4-methyl-1,3-dioxene-(4) (IV) has been reported to be about -12.2 kJ mol^{-1},[2] which appeared amazing, since it is difficult to see any factor which could give rise to such a large difference between the values of $\Delta G°$ for the two similar isomerization reactions. Hence it was considered necessary to make a reinvestigation of the relative stabilities of III and IV.

EXPERIMENTAL

4-Chloromethyl-1,3-dioxane was prepared from paraformaldehyde (200 g), allyl chloride (320 cm^3) and concentrated sulfuric acid (120 cm^3) as described by Price and Krishnamutri.[3] The yield was 90 g (17 %), boiling temperature 352 to 356 K at 2.1 kPa. The product was distilled from an excess of potassium hydroxide to give a mixture of water and III, which was collected over potassium carbonate. The organic layer was separated, dried (K_2CO_3), and distilled. The yield of III, boiling temperature 337 to 338 K at 15.6 kPa, was 55 %. The product was converted into IV (boiling temperature 381 K at 101.3 kPa) by slow distillation at atmospheric pressure.

The *NMR spectra* were taken in CCl_4 solution with TMS as internal standard. III: τ 5.05 (O$-$CH$_2-$O), 6.13 (O$-$CH$_2-$C), 7.77 (C$-$CH$_2-$C), 5.60 (H *trans* to O), 5.42 (H *cis* to O); IV: τ 5.05 (O$-$CH$_2-$O), 5.87 (O$-$CH$_2-$C), 5.87 (C=C$-$H), 8.26 (CH$_3$).

Determination of normal boiling temperatures.[4] Reference curve (compound, normal b.t., relative retention time): isobutylidene ethyl ether, 366.7 K, 0.312; 1-methoxycyclopentene, 387.0 K, 0.511; 1-methoxycyclohexene, 417.1 K, 1.000. The relative retention times of III and IV were 0.597 and 0.443, respectively, corresponding to normal boiling temperatures of about 395.0 (III) and 380.9 K (IV).

Procedure. The equilibrations were carried out in cyclohexane with iodine as catalyst. Substrate concentration was about 4 mol dm^{-3} and catalyst concentration 0.01 mol dm^{-3}. Two initial mixtures were available for the equilibra-

* Part VIII: Taskinen, E. *Acta Chem. Scand. B 28* (1974) 357.

tions: "pure" III and "pure" IV; although the concentration of III in the latter was less than 1 %, it exceeded the equilibrium concentration of III, and hence the position of equilibrium was always approached from the side of the *exo* isomer (III). To be sure that the true equilibrium had been achieved, the progress of isomerization was followed over sufficiently extended periods of time (several times the time necessary for the isomer ratio to become constant). The column used in the gaschromatographic analyses was an 8 m column containing 10 % Carbowax 20 M on Chromosorb G. The compounds were eluted in the order IV, III. After the first isomer had been eluted through the column, the attenuation of the detector was decreased by a nominal factor of 250 to get a peak of detectable size for the less stable isomer (III). The real change of attenuation was found to be 218 ± 2 by a procedure described previously.[1] The relative peak areas were determined by the cut-and-weigh method, and the area ratio, A_{IV}/A_{III}, was multiplied by 218 to get the true mol ratio at equilibrium. The practical performance of the equilibrations has been described in more detail previously.[4]

RESULTS

Table 1 shows the values of the mean equilibrium constant K and its standard error at various temperatures. The values of the thermodynamic functions of isomerization at 298.15 K were obtained by a linear least-squares treatment of ΔG° against temperature T. The results are: $\Delta G^\circ = -19.9 \pm 0.2$ kJ mol^{-1}, $\Delta H^\circ = -16.1 \pm 0.8$ kJ mol^{-1} and $\Delta S^\circ = 12.8 \pm 2.3$ J K^{-1} mol^{-1} for the isomerization of III to IV in cyclohexane solution at 298.15 K (the errors are twice the standard errors). The values of ΔH°(g) and ΔS°(g) were evaluated to be -18.3 ± 0.9 kJ mol^{-1} and 10.2 ± 2.4 J K^{-1} mol^{-1}, respectively, from the liquid phase data and the normal boiling temperatures.[4]

Table 1. Values of the mean equilibrium constant K and its standard error for the reaction 4-methylene-1,3-dioxolane ⇌ 4-methyl-1,3-dioxene-(4) in cyclohexane solution at various temperatures; n denotes the number of independent determinations.

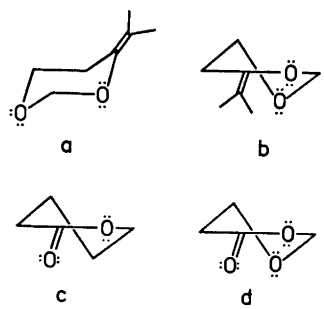
T/K	n	K (*endo*/*exo*)
282.2	2	4160 ± 260
298.2	5	3060 ± 130
316.2	3	2130 ± 90
326.2	2	1860 ± 100
336.2	2	1540 ± 70
358.2	3	932 ± 52
375.2	3	777 ± 27
391.2	3	680 ± 23
399.2	2	578 ± 20
413.2	2	507 ± 7

DISCUSSION

According to the results of this study, the difference (-19.9 kJ mol^{-1}) in the standard Gibbs free energy between IV and III is close to that (-21.8 kJ mol^{-1}, Ref. 1) between II and I. Interestingly, the corresponding difference between 1-methylcyclohexene (VI) and methylenecyclohexane (V) is -13.2 kJ mol^{-1}.[5] Herling *et al.*[5] have shown that the value of ΔS°(g) in V→VI is about 13 J K^{-1} mol^{-1}, which, within experimental error, is equal to that in III→IV, 10.2 ± 2.4 J K^{-1} mol^{-1}. The value of ΔS°(l) in diethyl ether for I = II has been obtained to be 28 ± 13 J K^{-1} mol^{-1} (the error quoted here is twice the standard error),[1] but it is evident that the high value of ΔS° in the latter case is due to solvation, since the values of ΔS° for other similar reactions studied in the same work [1] were considerably less positive if hexane was used as solvent in the equilibrations (for instance, ΔS°(l) for (*Z*)-2-ethylidenetetrahydropyran → 6-ethyl-3,4-dihydro-2*H*-pyran was obtained to be about 17 J K^{-1} mol^{-1} in diethyl ether but only about 9 J K^{-1} mol^{-1} in hexane). Thus it is likely that the entropy changes in the reactions I→II, III→IV, and V→VI are quite similar, and the differences in the values of ΔG° are due to enthalpy differences. It is remarkable that the difference in the standard Gibbs free energy between V and VI is *smaller* than that between I and II (or between III and IV). In the case of the five-membered analogues of V and VI, methylenecyclopentane and 1-methylcyclopentene, respectively, the value of ΔG°(l, 298.15 K) in *exo*→*endo* is -17.3 kJ mol^{-1},[5] while that for the reaction between the five-membered analogues of I and II, 2-methylenetetrahydrofuran and 5-methyl-2,3-dihydrofuran, respectively, is -7.1 kJ mol^{-1}.[1] The latter result is "natural" in the sense that on going from *exo* to *endo*, the number of alkyl groups attached to the double bond increases by one, and since alkyl groups do not stabilize the double bond of a

vinyl ether as effectively as they do an "ordinary" olefinic double bond,[1,6] the value of $\Delta G°$ (and that of $\Delta H°$, too, since entropy changes are be similar in both reactions) ought to be less negative in the latter reaction, in accord with experiment. Clearly, the situation is more complicated in the six-membered ring compounds. In a previous study,[1] the low thermodynamic stability of the *exo*-cyclic member of the *exo-endo* pair, I and II, was assumed to be due to an unfavorable orientation of the *p*-orbitals of the lone-pair electrons of the oxygen atom for an effective conjugation with the π-orbital of the double bond in the *exo*-cyclic isomer (I), which was assumed to have a chair conformation. Support for decreased conjugation in the *exo* compounds I and III is obtained from their NMR spectra: in most acyclic alkyl vinyl ethers, the chemical shifts of the terminal hydrogen atoms of the vinyl group are located about 1 ppm upfield from the corresponding shifts in simple olefins, or at about τ 6.2. On the other hand, the hydrogen atoms of the *exo*-cyclic methylene groups of I and III absorb at about τ 5.9 (Ref. 1) and τ 5.5, respectively. For comparison, the corresponding signals of 2-methylenetetrahydrofuran and 4-methylene-1,3-dioxolane are found at τ 6.1 (Ref. 1) and τ 5.9,[7] respectively. Hence the lower τ-values of the signals due to the protons of the *exo*-cyclic methylene group in the six-membered heterocycles indicate increased double-bond character of the ethylenic linkage resulting from the reduced resonance interaction between the lone-pair electrons of the oxygen atom and the π-electrons of the double bond.[8–10]

The chair conformation (a) is not the only imaginable conformation for III (and for I), since it is possible that an energetically more favorable spatial structure might be the half-chair conformation (b), where effective conjugation between the lone-pair electrons and π-electrons seems more likely. This aspect is in accordance with the probable conformations of unsubstituted δ-lactones (c) and 4-oxo-1,3-dioxanes (d),[11–13] but as stated above, the NMR shift data suggest that conjugation is weak in the *exo* forms I and III, which is best interpretable in terms of the chair structure.



Scheme 2.

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Short Communications

Absolute Configuration of 2,3-Dihydroxy-2-isopentylbutanedioic Acid, a Component of the Alkaloid Isoharringtonine

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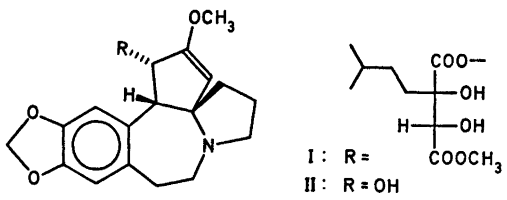
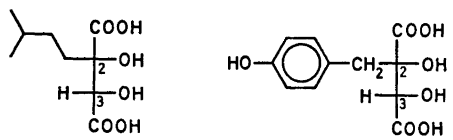
Isoharringtonine (I) is one of the alkaloids isolated from *Cephalotaxus harringtonia* which show antileukemia activity.[1] The alkaloid I is an ester of an amino alcohol, cephalotaxine (II) with a monomethyl ester of an optically active



diacid, 2,3-dihydroxy-2-isopentylbutanedioic acid (III). The absolute configuration of cephalotaxine has been determined in an X-ray investigation.[2] The relative configuration of the natural diacid III has been shown to be *erythro*,[3] but its absolute configuration has not been determined.

We here report that the absolute configuration of III is 2*R*, 3*S*, as found by comparison between the CD spectra of molybdate com-



III IV

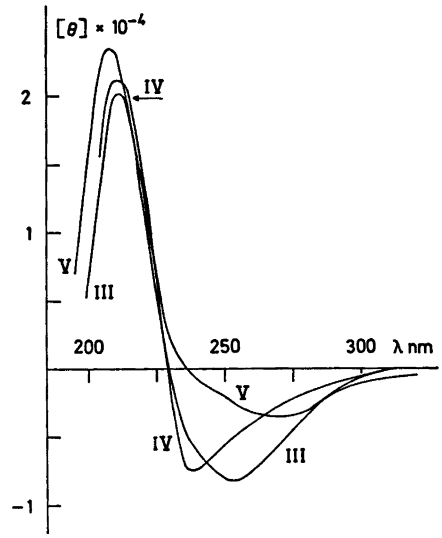


Fig. 1. CD spectra of molybdate complexes of the acid from isoharringtonine (III), piscidic acid (IV), and the hexahydro derivative of piscidic acid (V). The solutions had pH 2.9, 2.9, and 3.1, respectively.

plexes[4] of III and piscidic acid (IV). Acid IV is of natural origin and has a known absolute configuration (2*R*, 3*S*).[5] (In this paper[5] the reversed numbering is used.) The similarity between the CD spectra of the molybdate complexes (Fig. 1), taken together with the known *erythro* configuration[3] of III gave its absolute stereochemistry as 2*R*, 3*S*, thereby completing the knowledge about the configuration of isoharringtonine.

Other alkyltartaric acids of natural origin than the two ones mentioned above are known. The methyl homologue has been detected in wine,[6] and the isobutyl homologue has been found as a component of a glucoside from *Orchis militaris*.[7] Fukiic acid, the 3,4-dihydroxyphenyl analogue of piscidic acid, is also of natural origin.[8]

Experimental. The dimethyl esters of III and IV were hydrolysed with 4 M hydrochloric acid (reflux, 4 days), and excess hydrochloric acid was evaporated under diminished pressure. The crude acids so obtained were used directly in

preparation of CD solutions, which were 3.0 mM with respect to hydroxy acid and 2.7 mM with respect to sodium molybdate. Hydrochloric acid and sodium hydroxide solution were added until pH 2.9 – 3.1 was reached. Measurements of the CD spectra were carried out in a 0.5 mm cell using a Cary 60 spectropolarimeter (a Jasco J-40 instrument was used for the measurement on the hexahydro derivative of IV) five days after the solutions had been prepared.

The hexahydro derivative of IV was prepared by hydrogenation (1 atm, 23 °C, 24 h) of the dimethyl ester of IV (3.5 mg) in methanol (3 ml) using 5 % rhodium on alumina as catalyst (16 mg) followed by hydrolysis. The starting material was contaminated by approximately 5 % of an unknown compound, and after hydrogenation one further compound, probably dimethyl (cyclohexylmethyl)tartrate (GLC-MS), contaminated the desired hydrogenation product to the extent of about 5 %. Hydrolysis of the ester and preparation of the molybdate complex were performed as described above.

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Strained Heterocyclic Compounds. 7. Preparation of α-Phthalimido-β-lactams from α-Halo-β-lactams

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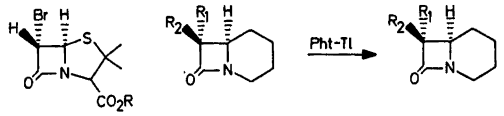
For some time we have been trying to develop a general synthesis of penicillins. In particular we are interested in synthesizing penicillin analogues containing a modified nucleus. In one approach carbene insertion has been used to produce halo-β-lactams of the types *1* and *2*.[1]



1

2a R_1=Br, R_2=H
2b R_1=H, R_2=Br
2c R_1=Cl, R_2=H

3a R_1=H, R_2=Pht
3b R_1=Pht, R_2=H
(Pht=Phthalimido)

As expected, these cyclizations yield predominantly the more stable *trans*-halo-β-lactams, *e.g. 1*, *2a*, and *2c*. Nucleophilic displacement of the halogen with an amine function should therefore yield amino-β-lactams with the *cis*-configuration characteristic for the penicillins and cephalosporins. Simple amines were found to destroy the β-lactams *2a* – *2c*, which were used as model compounds. Likewise, the use of metal amides were of no success.[2] Nor was it possible to use sodium azide which has been used for an unfused halo-β-lactam.[3] Therefore, we turned our attention to phthalimide salts. Potassium phthalimide reacted with the halo-β-lactams *2a* and *2c* to give a very low yield of phthalimido-β-lactam *3a*. The major part of the starting material was decomposed. On the other hand mercury(II) and silver(I) phthalimides were completely unreactive towards α-bromo-β-lactams. (In fact, silver phthalimide reacts reluctantly even with dilute HCl.)

In the search for compounds of intermediate reactivity we have now found that thallium(I) phthalimide reacts fairly readily in dimethyl sulfoxide at 150 °C with the bromo-β-lactams *2a* and *2b* to give phthalimido-β-lactams. The *trans*-compound *2a* gave the *cis*-7-phthalimido-8-oxo-1-azabicyclo[4.2.0]octane *3a* (55 % yield, 90 % stereoselectivity as determined by NMR and TLC), while the *cis*-compound *2b* gave *trans*-7-phthalimido-8-oxo-1-azabicyclo[4.2.0]-octane *3b* (24 % yield) with nearly complete stereospecificity. Since the halo-β-lactams isomerize slowly at 150 °C, the 10 % *trans*-phthalimido-β-lactam obtained from *trans*-bromo-β-

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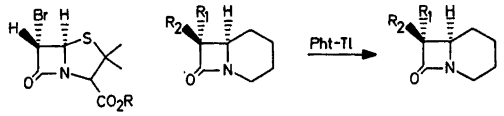

1

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lactam probably arises from isomerization rather than from lack of stereospecificity in the displacement reaction. Also the chloro-β-lactam *2c* reacts with thallium phthalimide, but the reaction is sluggish and only low yields of the product *3a* have so far been realized (5 %).

Acknowledgement. We thank the Swedish Board for Technical Development for financial support.

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Tobacco Chemistry. 30. The Absolute Configuration of 11-Nor-8-hydroxy-9-drimanone, a Constituent of Greek *Nicotiana tabacum* L.

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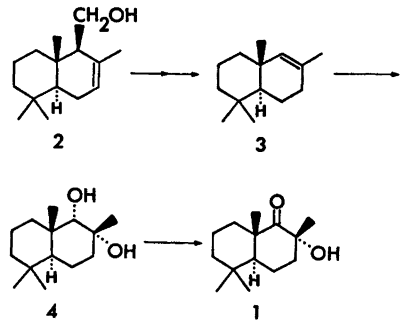
Research Department, Swedish Tobacco Co., Box 17 007, S-104 62 Stockholm 17, Sweden

Schumacher and Vestal [1] have recently reported 11-nor-8-hydroxy-9-drimanone** (*1*) as a new constituent of Turkish tobacco leaves but without elaborating on the chirality of its three asymmetric centres. The gross structure of this ketol indicates a genetic relationship with tobacco terpenoids of the drimane/labdane group or, although less likely, of the carotenoid group.[2] Since all tobacco terpenoids of the labdane/drimane group possess the "normal" stereochemistry at the A/B-ring junction (5α-H, 10β-CH_3) and this would not be expected in the case of carotenoid derived tobacco nor-isoprenoids, elucidation of the absolute configuration of the ketol (*1*), now isolated from Greek tobacco, would indicate the nature of its precursor(s).

** Nomenclature and stereochemistry as defined in Ref. 3.



The absolute configuration of drimenol (*2*) has been determined through correlation with oleanolic and abietic acid,[3] and it was envisaged that a stereospecific conversion of drimenol to 11-nor-8-hydroxy-9-drimanone (*1*) would unravel the chirality of the three asymmetric centres of the latter compound. This was accomplished using as starting material the olefin *3*, which was prepared from drimenol (*2*) as previously described [4] by catalytic hydrogenation followed by oxidation to the corresponding saturated acid and subsequent decarboxylation using lead tetraacetate. Preferential attack by osmium tetroxide from the less hindered α-side of the olefin (*3*) furnished the *cis*-diol (*4*) in good yield. Subsequent mild oxidation employing chromic acid in a two-phase system [5] gave the desired product, 11-nor-8-hydroxy-9-drimanone (*1*), which possesses a cedar-like, woody fragrance.[1] The synthetic and natural materials were found indistinguishable when comparing NMR, IR, MS and retention times on a capillary column (co-injection), and they exhibited rotatory powers of the same sign and magnitude. Since several syntheses of drimenol have been accomplished,[6–10] its conversion, portrayed above, to 11-nor-8-hydroxy-9-drimanone (*1*) formally represents a total synthesis of the latter.

Possessing the same absolute configuration as the previously detected tobacco terpenoids of the drimane/labdane group,[2] it seems highly probable that the ketol (*1*) is derived from representatives of this group, *e.g.* from 8,11-drimandiol [11] by a route similar to the present synthetic one or by direct oxidation of 9,11-didehydronorambreinolide.[1]

Experimental. NMR, IR, UV, and mass spectra were recorded on Varian XL-100, Digilab FTS-14, Beckman DB-2A and LKB 9000 instruments, respectively. Rotations were measured on a Perkin-Elmer 141 instrument.

11-Nor-8-hydroxy-9-drimanone (*1*, 4 mg) was isolated from a volatile, neutral fraction (B 5) of an extract obtained from 295 kg sun-cured Greek *Nicotiana tabacum* L.[12] The separation of this fraction will be described elsewhere.[13] $[\alpha]^{20}$ +39.5° (589 mn), +41° (578), +50.5° (546), +124° (436), +337° (365) (*c* 0.29, $CHCl_3$);

lactam probably arises from isomerization rather than from lack of stereospecificity in the displacement reaction. Also the chloro-β-lactam *2c* reacts with thallium phthalimide, but the reaction is sluggish and only low yields of the product *3a* have so far been realized (5 %).

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Tobacco Chemistry. 30. The Absolute Configuration of 11-Nor-8-hydroxy-9-drimanone, a Constituent of Greek *Nicotiana tabacum* L.

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Schumacher and Vestal [1] have recently reported 11-nor-8-hydroxy-9-drimanone** (*1*) as a new constituent of Turkish tobacco leaves but without elaborating on the chirality of its three asymmetric centres. The gross structure of this ketol indicates a genetic relationship with tobacco terpenoids of the drimane/labdane group or, although less likely, of the carotenoid group.[2] Since all tobacco terpenoids of the labdane/drimane group possess the "normal" stereochemistry at the A/B-ring junction (5α-H, 10β-CH_3) and this would not be expected in the case of carotenoid derived tobacco nor-isoprenoids, elucidation of the absolute configuration of the ketol (*1*), now isolated from Greek tobacco, would indicate the nature of its precursor(s).

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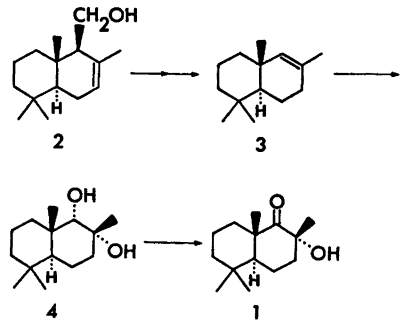

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NMR, IR, and mass spectra: see under synthetic 11-nor-8-hydroxy-9-drimanone.

11-Nor-8,9α-drimandiol (4). 11-Nor-8-drimene (*3*, 100 mg) [4] dissolved in dry pyridine (10 ml) was added to a cooled (−30 °C) solution of osmium tetroxide (150 mg) in pyridine (10 ml) and the mixture stirred for 3 h while the disappearance of the hydrocarbon was monitored by TLC. A solution of $NaHSO_3$ (1.8 g) in water (30 ml)/pyridine (20 ml) was added and the solution stirred at room temperature for 20 min after which the mixture was diluted with water and extracted with CH_2Cl_2. The extract was washed with H_2SO_4 (5 %), water, brine, dried, and evaporated leaving a slightly coloured oil. Chromatography on silica gel furnished 11-nor-8,9α-drimandiol (*4*, 87 mg) which crystallized on standing at 5 °C; m.p. 58−59 °C; MS: *m/e* 226 ($M^{+\cdot}$ 12), 43 (100), 95 (86), 41 (84), 69 (81), 123 (73), 81 (72), 138 (65), 82 (64), 71 (60), 137 (50), 55 (49), 109 (39), 67 (33), 96 (28), 84 (28), 83 (23), 57 (20), 196 (20), 177 (19); ν_{max}(KBr): 3478 (shoulder), 3400 (broad), 2949 (s), 2874 (s), 1456 (m), 1377 (m), 1360 (w), 1124 (w), 1092 (w), 1072 (w), 1056 (m), 1026 (w), 1012 (w), 994 (w), 978 (w), 960 (w), 955 (w), 942 (w), 895 (w), 876 (w), 828 (w), 728 (w); $[\alpha]^{20}$ −11.6° (589 nm), −18° (578), −19.5° (546), −27.4° (436), −46° (365) (*c* 0.38, $CHCl_3$); $\delta(CDCl_3)$: 0.81 (3 H, s), 0.89 (3 H, s), 0.97 (3 H, s), 1.37 (3 H, s), 2.30 (OH, broad s), 2.74 (OH, d, *J* 2.5 Hz), 2.94 (1 H, d, *J ca.* 2.5 Hz), the signals at δ 2.30 and 2.74 disappeared and the resonance at δ 2.94 collapsed to a singlet on addition of D_2O.

11-Nor-8-hydroxy-9-drimanone (1). To a cooled (ice-bath) solution of the diol *4* (43 mg) in ether (10 ml) was added ten drops of the CrO_3-solution described by Brown *et al.*[5] After stirring for 30 min water was added and the mixture extracted with ether which was washed with $NaHCO_3$, water, brine, dried, and evaporated leaving an oil (38 mg). Chromatography on silica gel yielded pure 11-nor-8-hydroxy-9-drimanone (*1*, 17 mg) which was indistinguishable from the natural ketol *1* (NMR, IR, MS, retention time, when co-injected on a capillary GC-column). MS: *m/e* 224 ($M^{+\cdot}$ 22) 43 (100), 69 (86), 123 (72), 41 (64), 55 (50), 95 (50), 82 (49), 109 (48), 67 (46), 81 (44), 71 (43), 83 (42), 163 (42), 125 (40), 68 (37), 196 (25); minor intensity-differences were observed when compared with the spectrum recorded by Dr. Schumacher;[14] ν_{max}(film): 3485 (broad), 2932(s), 2870 (s), 1696 (s), 1466 (m), 1393 (m), 1380 (m), 1371 (m), 1343 (w), 1245 (m), 1175 (m), 1163 (m), 1142 (m), 1114 (w), 1100 (w), 1064 (w), 1026 (w), 1012 (w), 992 (s), 976 (m), 949 (m), 918 (w), 843 (w), 842, (w), 693 (w); lit.[1] ν_{max}(film): 3475, 1690, 1242 1160, 990; $[\alpha]^{20}$ +46° (589 nm), +49.3° (578), +59° (546), +142° (436), +395° (365) (*c* 0.3, $CHCl_3$); $\delta(CDCl_3)$: 0.93 (6 H, s), 1.18 (3 H, s), 1.41 (3 H, s), 2.00−2.35 (1 H, m); lit.[1] $\delta(CDCl_3)$: 0.93 (6 H), 1.16 (3 H), 1.40 (3 H).

Note added in proof. Dr. J. N. Schumacher (private communication) has confirmed our results by degrading 9,11-didehydronorambreinolide to 11-nor-8,9-drimandiol which exhibited the same optical activity as the corresponding diol derived from natural 11-nor-8-hydroxy-9-drimanone (*1*) by $LiAlH_4$ reduction.

Acknowledgements. The authors are indebted to Miss Ann-Marie Eklund for skilful technical assistance, Professor H. H. Appel, Universidad Tecnica Federico Santa Maria, Valparaiso, for a generous gift of drimenol, and Dr. J. N. Schumacher, R. J. Reynolds Tobacco Co., Winston-Salem, for a copy of the mass spectrum of the title compound.

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The Direct Observation of Intermediates during the Oxidative Cyclization of Phenol Ethers

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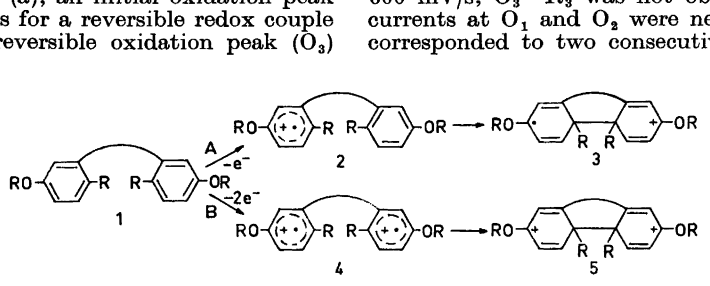
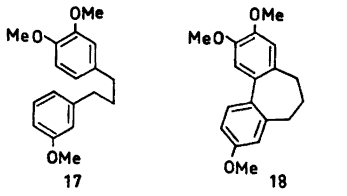
The oxidative cyclization of phenyl ethers is a topic of great current interest to organic chemists due to the importance of such coupling reactions in natural systems.[1-5] Various mechanisms, such as attack of a cation radical moiety upon an unoxidized aromatic ring (path A)[4] or initial oxidation to a dication diradical followed by coupling (path B)[2] have been proposed. The only mechanistic evidence that has been presented is voltammetric evidence for path B.[2] We now present conclusive evidence for the occurrence of path A during the oxidative cyclization of 1-(3,4-dimethoxyphenyl)-3-(4-methoxyphenyl)-propane (*6*).

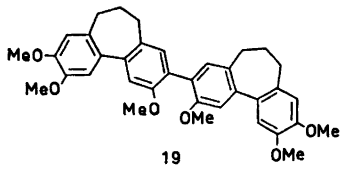
Anodic oxidation of *6* in dichloromethane-trifluoroacetic acid (TFA) (3:1) resulted in the formation of *11* which was isolated in 71 % yield. (Found: C 75.9; H 7.1. Calc. for $C_{18}H_{20}O_3$: C 76.1; H 7.0). The NMR assignment of the aromatic protons are as follows; H_A 7.14 (d, $J_{AB}=8.1$ Hz), H_B 6.79 (d,d, $J_{AB}=8.1$ Hz, $J_{BC}=2.8$ Hz), H_C 6.93 (d, $J_{BC}=2.8$ Hz), H_D 6.93 (s), and H_E 6.76 (s). Shifts are in δ values and the subscripts are those used in structure *11*. The remaining protons gave signals at δ: 3.85 (3 H, methoxy), 3.91 (3 H, methoxy), 3.93 (3 H, methoxy), 2.41 (t, 4 H, aliphatic) and 2.12 (q, 2 H, aliphatic). *M/e* 284 (M^+). An isomer of *11* was prepared by the oxidation of *17*. The isomer (*18*) showed a different NMR spectrum and did not form a dimer while *11* with an unsubstituted position *para* to the biphenyl linkage gave *19* upon further oxidation (V. D. Parker and A. Ronlán, unpublished results).

Cyclic voltammograms of *6* in the same solvent are shown in Fig. 1. At a voltage sweep rate of 31 mV/s (a), an initial oxidation peak (O_1), small peaks for a reversible redox couple (O_2-R_2), an irreversible oxidation peak (O_3) and a reduction peak (R_4) were observed on a complete cycle. The peaks O_3 and R_4 matched those observed during cyclic voltammetry of the product, *11*. The height of O_3 indicated that substrate was being oxidized nearly quantitatively to *11* at that voltage sweep rate. The effect of increasing the sweep rate is illustrated in (b) and (c).

The redox couple (O_2-R_2) became more pronounced with increasing sweep rate while the relative current at O_3 was diminished. A reduction peak (R_3) corresponding to O_3 was also observed. At 310 mV/s O_3-R_3 was very small (c). At voltage sweep rates greater than 600 mV/s, O_3-R_3 was not observed and the currents at O_1 and O_2 were nearly equal and corresponded to two consecutive one electron







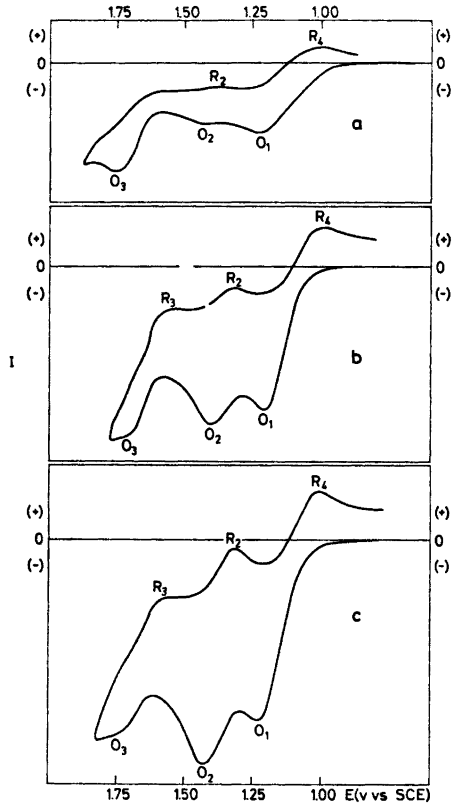


Fig. 1. Cyclic voltammograms for the oxidative cyclization of *6* in dichloromethane-TFA (3:1) containing Bu_4NBF_4 (0.2 M). Voltage sweep rate: (a) 31 mV/s, (b) 151 mV/s, (c) 310 mV/s.

transfers. Thus, it is clear that O_2-R_2 is a reversible redox couple of a short lived intermediate formed during oxidation of *6* to *11* which has a lifetime of the order of 1 s in dichloromethane-TFA (3:1) at room temperature. The reduction peak (R_4) was observed even at high sweep rates which indicates that under those conditions it corresponds to a further reduction of the species formed at R_2. Voltammetry in acetonitrile gave similar results with the height of O_2 showing a marked sweep rate dependence. On the other hand, R_2 was not observed under any conditions in acetonitrile indicating that the intermediate formed at O_2 was consumed in a rapid chemical reaction.

The sweep rate dependence for the observation of the reversible couple (O_2-R_2) suggested that the intermediate oxidized at O_2 undergoes a relatively slow chemical reaction which is outrun as the voltage sweep rate is increased. The latter suggested that it might be possible to observe different products if the chemical step was precluded by carrying out the oxidation at a potential greater than O_2. In fact, in acetonitrile *11* was formed in nearly quantitative yield when the reaction was carried out at a potential of + 1.25 V and the unrearranged dienone (*14*) was the exlusive product when the reaction was conducted at potentials greater than + 1.60 V.*

The intermediate undergoing oxidation at O_2 is a one electron oxidation product of (*6*) which reacts slow enough to be detected by slow sweep cyclic voltammetry. Mechanism B requires that the intermediate be the cation radical of *6* which is further oxidized to the dication diradical at O_2 (+ 1.42 V *vs.* SCE). We can rule out the latter mechanism on the basis that the dication diradical would not form at such a low potential** and would be a very reactive species. Mechanism A suggests that the intermediate could be a cation radical in which the cationic center and the unpaired electron are in different rings separated by saturated carbon (*8*) or the radical (*15*). On the basis of the data, we cannot definitively choose between *8* and *15* as the intermediate (Scheme). However, if *15* were the intermediate, O_2-R_2 would correspond to the couple, *15*⇌*13*. We have found that *14* oxidizes about 100 mV more easily than O_2 and this must involve oxidation of the ring containing two methoxy groups. It seems almost inconceivable that *15* should be more difficult to oxidize than *14*. Furthermore, the only product formed at potentials below O_2 is *11* which forms *via* a dienone phenol rearrangement of the intermediate and it seems much

* Compound (*14*) was isolated in 91 % yield and identified by comparison of the NMR, mass and IR spectra with that of the authentic compound prepared by either chemical [6] or electrochemical [7] oxidation of the corresponding phenol.

** The ring containing only one methoxy group in *6* would not be expected to oxidize more easily than anisole, + 1.7 V.

more likely that the cation radical (*8*) is the rearranging species rather than *15*. Two additional points favor *8* over *15* for the intermediate: At high sweep rates the cyclic voltammogram of *6* shows R_4 which must be due to reduction of the intermediate. The radical *15* would reduce at negative potentials. Also R_2 is not observed in acetonitrile, most likely due to rapid deprotonation of *12*. Thus, we favor the reaction pathway involving the cation radical (*8*) for the reactions producing both *11* and *14*.

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Non-enzymic Oxidation of Lower Aliphatic Alcohols by Ascorbic Acid in Tissue Extracts

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It has been shown that ethanol is oxidized to acetaldehyde by ascorbic acid,[1] and since it is not oxidized further, the aldehyde accumulates. The ascorbic acid content of different tissues can vary considerably,[2] so that it can be assumed that the reaction proceeds at different speeds in different tissue extracts. As far as is known, no reports have been published on the non enzymic oxidation of other alcohols than ethanol.

Only small amounts of formaldehyde were formed from methanol when 20 mM alcohol was incubated in a citrate buffer, pH 4.0, containing 2.0 mM ascorbic acid (Table 1). The other normal and iso alcohols tested all formed more than 100 nmol aldehyde in a reaction mixture of 1 ml. With the exception of methanol the oxidation rate decreased with increasing chain length.

Table 1. Oxidation of some aliphatic alcohols by ascorbic acid. Alcohols (20 mM) in 0.1 M citrate buffer, pH 4.0, containing 2.0 mM ascorbic acid was incubated for 60 min at 65 °C. The aldehydes formed except formaldehyde were estimated gas chromatographically.[10] Formaldehyde was estimated spectrophotometrically by the Eegriwes' method.[11] The values given are the means of two experiments.

Alcohol added	Aldehyde formed	(μM)
Methanol	Formaldehyde	< 100
Ethanol	Acetaldehyde	530
Propanol	Propionaldehyde	310
Isobutanol	Isobutyraldehyde	230
Butanol	Butyraldehyde	220
Isopentanol	Isovaleraldehyde	120
Pentanol	Valeraldehyde	110

Extracts contained only a part of the ascorbic acid found in the intact tissue because of the dilution with perchloric acid (Table 2). Bovine liver has been reported to contain 1.7 mmol ascorbic acid/kg, kidney 0.7 mmol/kg, heart 0.3 mmol/kg and blood 0.1 mmol/kg.[2] Of the four tissue extracts examined the liver contained

more likely that the cation radical (*8*) is the rearranging species rather than *15*. Two additional points favor *8* over *15* for the intermediate: At high sweep rates the cyclic voltammogram of *6* shows R_4 which must be due to reduction of the intermediate. The radical *15* would reduce at negative potentials. Also R_2 is not observed in acetonitrile, most likely due to rapid deprotonation of *12*. Thus, we favor the reaction pathway involving the cation radical (*8*) for the reactions producing both *11* and *14*.

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It has been shown that ethanol is oxidized to acetaldehyde by ascorbic acid,[1] and since it is not oxidized further, the aldehyde accumulates. The ascorbic acid content of different tissues can vary considerably,[2] so that it can be assumed that the reaction proceeds at different speeds in different tissue extracts. As far as is known, no reports have been published on the non enzymic oxidation of other alcohols than ethanol.

Only small amounts of formaldehyde were formed from methanol when 20 mM alcohol was incubated in a citrate buffer, pH 4.0, containing 2.0 mM ascorbic acid (Table 1). The other normal and iso alcohols tested all formed more than 100 nmol aldehyde in a reaction mixture of 1 ml. With the exception of methanol the oxidation rate decreased with increasing chain length.

Table 1. Oxidation of some aliphatic alcohols by ascorbic acid. Alcohols (20 mM) in 0.1 M citrate buffer, pH 4.0, containing 2.0 mM ascorbic acid was incubated for 60 min at 65 °C. The aldehydes formed except formaldehyde were estimated gas chromatographically.[10] Formaldehyde was estimated spectrophotometrically by the Eegriwes' method.[11] The values given are the means of two experiments.

Alcohol added	Aldehyde formed	(μM)
Methanol	Formaldehyde	< 100
Ethanol	Acetaldehyde	530
Propanol	Propionaldehyde	310
Isobutanol	Isobutyraldehyde	230
Butanol	Butyraldehyde	220
Isopentanol	Isovaleraldehyde	120
Pentanol	Valeraldehyde	110

Extracts contained only a part of the ascorbic acid found in the intact tissue because of the dilution with perchloric acid (Table 2). Bovine liver has been reported to contain 1.7 mmol ascorbic acid/kg, kidney 0.7 mmol/kg, heart 0.3 mmol/kg and blood 0.1 mmol/kg.[2] Of the four tissue extracts examined the liver contained

Table 2. Formation of acetaldehyde from ethanol in tissue extracts. Ethanol 20 mM in PCA-precipitated tissue extracts, pH 4.0, was incubated for 60 min at 45 °C both without and with addition of ascorbic acid. The acetaldehyde formed was estimated gas chromatographically.[10] The values given are the means of two experiments.

Tissue extract	Without ascorbic acid addition		Ascorbic acid added	
	Ascorbic acid (mM)	Acetaldehyde produced (μM)	Ascorbic acid (mM)	Acetaldehyde produced (μM)
Liver	0.34	41.0	1.0	75.6
Kidney	0.15	65.9	0.91	97.9
Heart	0.03	9.0	0.94	108.4
Blood	0	3.4	0.89	66.4

the highest amount of ascorbic acid (Table 2). The kidney extract contained less than half the ascorbic acid present in the liver extract, but nevertheless formed the greatest amount of acetaldehyde from ethanol. Heart and blood extracts contained little ascorbic acid, and the non-enzymic aldehyde formation was low. The ascorbic acid content in the blood extract was so low that it could not be measured. The addition of a similar quantity of ascorbic acid to each of the extracts increased the formation of acetaldehyde in each case, but by an amount dependent on the tissue type (Table 2). This could be explained by the presence of varying amounts of substances in the extracts which can modify the rate of the oxidation. It has been assumed [1] that ethanol is oxidized by a semidehydroascorbate peroxy radical produced in the incubation solution when ascorbic acid is oxidized. Thiols and disulfides strongly inhibit this reaction by acting as free radical acceptors.[3,4] Liver is the tissue richest in thiols and disulfides, while their content in heart muscle and blood is rather low.[5,6]

The non-enzymic oxidation rate of other aliphatic alcohols in tissue extracts containing

Table 3. Formation of aldehydes from some aliphatic alcohols in tissue extracts. Aliphatic alcohols (20mM) were incubated in PCA-precipitated tissue extracts, pH 4.0, for 60 min at 45 °C after addition of ascorbic acid. The ascorbic acid content in the incubation solution after addition was 0.93 ± 0.04 mM. The aldehydes formed were estimated gas chromatographically.[10] The values given are the means of two experiments.

Alcohol added	Aldehyde produced (μM)			
	Liver	Kidney	Heart	Blood
Propanol	50.6	30.4	73.6	47.2
Butanol	31.2	38.6	46.7	24.3
Isobutanol	51.6	31.3	54.2	53.3
Pentanol	22.7	8.6	17.0	22.3
Isopentanol	11.2	10.9	21.8	25.7

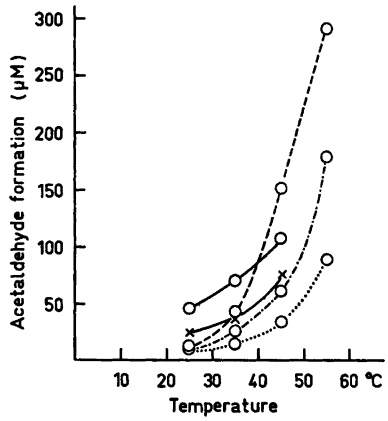

Fig. 1. Formation of acetaldehyde from ethanol at different temperatures and with different amounts of ascorbic acid. Ethanol (20 mM) in 0.1 M citrate buffer, pH 4.0, containing different amounts of ascorbic acid, and in liver and heart extracts (pH 4.0) to which ascorbic acid had been added, was incubated for 60 min at different temperatures. The acetaldehyde formed was estimated gas chromatographically.[10] Every point represents the mean value obtained from two experiments. - - - 2.0 mM, — · — 1.0 mM and ··· 0.5 mM ascorbic acid in citrate buffer; × liver extract containing 1.0 mM ascorbic acid, — heart extract containing 1.0 mM ascorbic acid.

0.9 mM ascorbic acid was lower than that of ethanol (Table 3). Propanol and isobutanol were oxidized at the same rate, while pentanol and isopentanol were oxidized slowly.

The acetaldehyde formed from a given amount of ethanol increased with increasing amounts of ascorbic acid (Fig. 1). With 20 mM ethanol and 1 – 2 mM ascorbic acid in citrate buffer, pH 4.0, rather much acetaldehyde was formed at 55 °C. At 37 °C, however, the aldehyde formation was low, especially at low ascorbic acid concentrations.

As shown in Fig. 1, more acetaldehyde was formed in tissue extract incubated at 25 °C than in ascorbic acid solution. An explanation for this may be that tissue extracts usually contain activators like Cu^{2+} and Fe^{3+} which catalyse the autoxidation of ascorbic acid, especially at low temperatures.[7] At physiological pH, however, the formation of acetaldehyde is much less since the reaction becomes much slower as the pH rises over 5.[1] In addition, ascorbic acid-rich tissues also contain a relatively high amount of SH groups,[8,6,5] which inhibit the non-enzymic oxidation *in vitro*.[1] It can be assumed, therefore, that aliphatic alcohols cannot be oxidized to aldehydes by ascorbic acid *in vivo* at such a speed that the products would produce harmful effects.

Experimental. Bovine liver, kidney, heart and blood were separately homogenized in 0.6 N perchloric acid (PCA) (35 g tissue/100 ml) and the homogenates filtered. Before use, the pH of the acidic filtrate was adjusted to 4.0 by adding 1.25 ml of 5 M KOH and 1 M citrate buffer (pH 4.0) to 10 ml of the filtrate. The samples were incubated for 60 min in a thermostated water bath in the presence of different aliphatic alcohols. After the incubation thiourea was added to prevent alcohol oxidation during the subsequent analysis of the aldehydes. Thiourea was added to the control samples before incubation. In two experiments 0.1 M citrate buffer, pH 4.0, containing different amounts of ascorbic acid was used instead of tissue extract. The ascorbic acid content was estimated by the method described by Roe.[9] The alcohols and aldehydes except formaldehyde were estimated on a Perkin-Elmer F 40 head-space gas liquid chromatograph, as reported previously.[10] Formaldehyde was estimated spectrophotometrically by the violet color which develops on heating with chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid) in the presence of strong sulfuric acid. The absorbance was measured at 570 nm.[11]

The analytical procedure was standardized with samples of diluted formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde, valeraldehyde, isovaleraldehyde, methanol, ethanol, propanol, butanol, isobutanol, pentanol, and isopentanol of analytical grade. Standard and unknown samples were determined by identical procedures.

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Correction of the Amino Acid Sequence of Soybean Leghemoglobin *a*

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A comparison of the sequence of soybean leghemoglobin *a* (Lb*a*) [1,2] with the sequence of soybean leghemoglobin *c* (*to be published*) and kidney bean leghemoglobin *a* [3] revealed a genetically inexplicable difference at the site 50 – 54 in the leghemoglobin *a* chain. The order of these amino acids in both soybean leghemoglobin *c* and kidney bean leghemoglobin *a* is *Gly-Val-Asp-Pro-Thr*, whereas *Pro-Thr-Asp-Gly-Val* was proposed for leghemoglobin *a* from soybean. This

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The analytical procedure was standardized with samples of diluted formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde, valeraldehyde, isovaleraldehyde, methanol, ethanol, propanol, butanol, isobutanol, pentanol, and isopentanol of analytical grade. Standard and unknown samples were determined by identical procedures.

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Table 1. Amino acid compositions of substilopeptidase A peptides from the tryptic peptide *a*T14.

Amino acid	S1	S2	S3	S4	S5
Aspartic acid	1.13	1.02		2.06	0.89
Threonine				0.97	
Serine			0.94		
Proline				1.90	
Glycine				0.53	
Alanine		1.13			1.11
Valine				1.07	
Leucine	0.94	0.86			
Phenylalanine	0.92		1.06		
Lysine				1.02	
E_{asp}	0.52	neutr.	neutr.	neutr.	neutr.

prompted us to re-investigate the sequence of the tryptic peptide *a*T14.

Materials and methods. The methods used were mainly those described previously.[2] Peptide *a*T14 was isolated from the tryptic hydrolysate of apoLb*a* using high voltage electrophoresis at pH 6.5, pH 1.9, and paper chromatography. Peptide *a*T14 was digested with subtilopeptidase A (Sigma Chemical Company, St. Louis, USA), and the peptides were isolated as described previously. The amino acid composition of the peptides was determined on a modified Beckman/Spinco 120 amino acid analyzer. The sequence of the partial peptides was determined by the dansyl-Edman method.[4] The dansyl amino acids were identified by high voltage electrophoresis on silica gel plates.[5] or by miniature thin layer chromatography on polyamide sheets (3 × 3 cm).[6]

Results and discussion The amino acid contents of the subtilopeptidase A peptides (denoted by S) are presented in Table 1. The low glycine content of peptide S4 indicates that it is a mixture of two peptides. The only difference between the peptides is at the amino end, at which the *N*-terminal glycine is missing from the smaller one (Fig. 1). This minor difference makes it impossible to separate the peptides by conventional means.

In the previous determination of the sequence of *a*T14, the subtilopeptidase A peptides were obtained in very low yield and, apparently contaminated by impurities, which led to an incorrect deduction of the sequence. The chymotryptic [7] and thermolytic [8] hydrolysates of apoLb*a* also yield peptides covering this portion of the Lb*a* chain. However, these peptides, *a*C18 and *a*Th10, respectively, were investigated only to elucidate the bridges between the tryptic peptides and were therefore not completely sequenced. Consequently the misinterpretation of the data was not observed. The correct sequence of peptide *a*T14 is given in Fig. 1.

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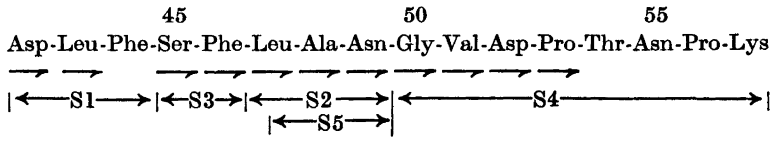

Fig. 1. The amino acid sequence of the tryptic peptide *a*T14 (residues 42 – 57 in the Lb*a* chain). The arrows show the amino acids identified by the dansyl-Edman method. The subtilopeptidase A peptides are shown below the sequence.

Animal Carotenoids. 9*. Trikentriorhodin

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In his thesis Smith [1] described the quantitative distribution pattern of carotenoids in the marine sponge *Trikentrion helium* Dickinson (family Cyamonidae) and a partial characterization of the individual carotenoids. The structure of a major carotenoid P474, now called trikentriorhodin, occurring both in esterified and unesterified form [1] has been further studied in our laboratory.

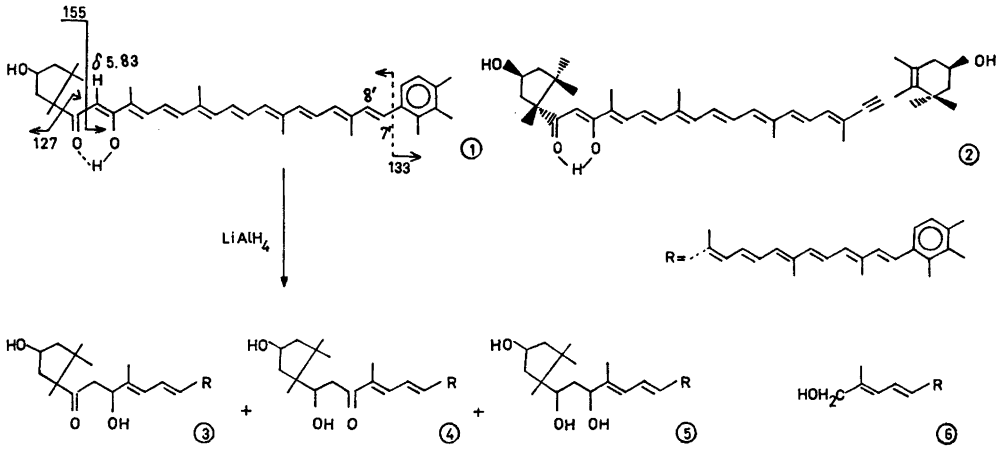
The work was complicated by the presence of much lipid contaminants. Esterified trikentriorhodin (26 % of total carotenoids) on saponification gave free trikentriorhodin (*1*), identical with naturally occurring trikentriorhodin (comprising 21 % of total carotenoids). The structural assignment is based on the following evidence; the position of the hydroxy group in the cyclopentane end group being tentative. Formally trikentriorhodin (*1*) is 3-hydroxy-$\varkappa,\chi$-caroten-6,8-dione. Trikentriorhodin (*1*) exhibited a round-shaped absorption spectrum in all solvents with λ_{max} 518 mn in CS_2; 497 nm in pyridine; (468), 488 and (520) nm in petroleum ether, 478 nm in acetone and 475 nm in diethyl ether, bathochromically displaced relative to astaxanthin [2] and hypsochromically shifted relative to rhodoxanthin,[3] caused by a so far unknown chromophore.

The molecular formula $C_{40}H_{52}O_3$ (*m/e* 580.3908, calc. 580.3917) was established by high precision mass spectrometry.

Fragment ions at M − 92 and M − 106 [4] confirm this assignment. The occurrence of trikentriorhodin as an ester and a prominent M − 18 ion in the mass spectrum of the hydrolyzed pigment indicated the presence of hydroxyl. Trikentriorhodin (*1*) was destroyed under standard conditions for silylation [5] or acetylation,[5] although a monoacetate *1b* (*m/e* 622 = M, M − 60) with unchanged electronic spectrum was obtained in low yield. Attempted methylation of allylic hydroxyl with HCl-MeOH [6] gave 60 % recovery of *1* and no methyl ether, under conditions where lutein gave the monomethyl ether [7] in high yield. Treatment with NaH/MeI [8,9] caused complete decomposition and gave no methyl ether. Attempted allylic oxidation with I_2/O_2 [10] or DDQ [11] again caused decomposition and provided no oxidation products. Trikentriorhodin (*1*) thus appeared to contain a non-allylic hydroxy group, the localization of which was further indicated from the mass spectrum with diagnostically useful fragment ions at M − 127 and *m/e* 127 (*a*, 22 % of base peak) and base peak *m/e* 109.1013 (calc. 109.1017 for $C_8H_{13} = b$, consistent with $b = a - 18$), compatible with a hydroxylated $\varkappa$ [12] end group,[13] Scheme 1.

Careful $LiAlH_4$-reduction gave more polar products with hypsochromically displaced electronic spectra, see below, demonstrating the presence of conjugated carbonyl. IR-absorption at 1610, 1568 cm^{-1} of trikentriorhodin compatible with enolized α- or β-diketone,[14] suggested an end group similar to that recently established by Khare *et al.* in mytiloxanthin (*2*).[15] Support was derived from an M − 155 peak [15] in the mass spectrum of *1*, attributed to α-cleavage, and ^1H NMR signals ($CDCl_3$) at δ 5.83 s (1 H) [15] and methyl singlets at δ 0.85, 1.20 and 1.34 [15,16] compatible with the $\varkappa$ end

* No. 8 *Acta Chem. Scand.* *27* (1973) 1401.



Scheme 1.

group. Weak acidic properties of the enolized β-diketone was consistent with irreversible adsorption of trikentriorhodin (*1*) on alumina, although less evident from partition tests without and in the presence of alkali.

Having now accounted for all oxygen functions, consideration of the chromophore and the remaining double bond equivalences, require that the second end group should be a trimethylphenyl end group. Confirmation was obtained from a broad, unresolved ^{1}H NMR-signal at δ 1.8–2.3 compatible with in-chain methyl and aromatic methyl.[4] Absence of M – 133 and *m/e* ions in the mass spectrum of *1* is ascribed to other preferred cleavages. Preference for 1,2,3-trimethyl substitution follows from the electronic spectrum of trikentriorhodin (*1*) and derivatives to be discussed next.

Micro scale $LiAlH_4$-reduction of *1* gave products with spectral properties compatible with the conjugated ketone *4* [λ_{max} in ether 456, (482) nm], the unconjugated ketone *3* (*m/e* 582 = M, M – 18); λ_{max} in ether 335, 351, 441, 468 nm), and the allylic alcohol *5* (λ_{max} in ether 335, 350, 441 and 465 nm; *m/e* 584 = M, M – 18, M – 18 – 18, M – 106 – 18, M – 158).

Direct comparison of the electronic spectra in diethyl ether of the triol *5* (λ_{max} 441, 468 nm) with that of renierol (*6*, λ_{max} 439, 465 nm), prepared by $LiAlH_4$-reduction of renieral, require 1,2,3- rather than 1,2,5-trimethyl substitution of the aryl ring; *cf.* Ref. 18. Consideration of the absorption maximum of mytiloxanthin (*2*) [15] at lower wavelength than that of trikentriorhodin (*1*) leads to the same conclusion. Evidence obtained by Smith [1] suggests the presence of β-isorenieratene with a 1,2,3-trimethylphenyl end group in *T. helium*.

Experimental. Methods were as commonly employed in this laboratory.[19,5] The isolation involved extraction with acetone, removal of colourless contaminants by deep-freezing, followed by filtration of the concentrated acetone extract, repeated TLC (silica gel G, 1 mm, 5 % acetone in petroleum ether), standard saponification of individual fractions, again followed by repeated TLC of saponified pigments. The carotenoid composition of *T. helium* studied here corresponded to that found by Smith;[1] available *ca.* 7 mg, purified *1*.

R_F-values on Schleicher and Schüll No. 287 kieselguhr paper in acetone-petroleum ether (APE) were: β-carotene 1.00 (0 % APE), *1* 0.29 (5 % APE), *1*-esters 0.27, 0.31 (0 % APE). Relative polarity on silica gel G plates (20 % APE) were *7* < *1* < *3* < *4* < *5*.

Acknowledgement. We thank Dr. V. E. Smith for passing this problem over to us and him and Professor D. L. Fox, Scripps Institution of Oceanography, La Jolla, California, for arranging collection of the biological material used in the present study. M. A.-M. was supported by a NORAD fellowship.

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