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Thermodynamics of Vinyl Ethers. XIV.* Effect of Aromatic and Heteroaromatic α-Substituents on the Relative Stabilities of Geometric Isomers

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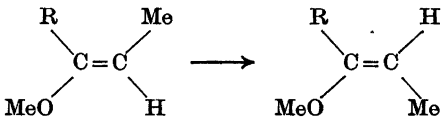
The thermodynamics of *cis* – *trans* isomerization in vinyl ethers of the type RC(OR′)=CHMe, where R is an aromatic or a heteroaromatic substituent and R′=Me or Et_2CH, have been studied by chemical equilibration. For R=Ph and R′=Me, $\Delta G^\ominus = -1.60 \pm 0.06$ kJ mol^{-1}, $\Delta H^\ominus = 1.8 \pm 0.8$ kJ mol^{-1} and $\Delta S^\ominus = 11.5 \pm 2.5$ J K^{-1} mol^{-1} for the $E \rightarrow Z$ isomerization in cyclohexane solution at 298.15 K. Substituents in the phenyl group can significantly alter the relative stability of the *E*,*Z* pair only if they are in the *ortho*-position, the stability of the *E* isomer being favored by *ortho*-substituents. 2-Furyl and 2-thienyl groups are practically equivalent to a phenyl group as the α-substituent. Bulky alkoxy groups favor the relative stability of the *Z* isomer.

The present paper describes the results of a study of the effect of aromatic and heteroaromatic α-substituents (R) on the thermodynamics of reaction (1). In addition, the effect of the size of the alkoxy group was clarified by the reaction (2).



(1)

I: R=C_6H_5 (phenyl); II: R=*p*-Me−C_6H_4; III: R=*p*-MeO−C_6H_4; IV: R=*p*-Cl−C_6H_4; V: R=*p*-F−C_6H_4; VI: R=*m*-Me−C_6H_4; VII: R=*o*-Me−C_6H_4; VIII: R=*o*-MeO−C_6H_4; IX: R=*o*-Cl−C_6H_4; X: R=C_4H_3O (2-furyl); XI: R=C_4H_3S (2-thienyl).

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(*E*)-PhC(OCHEt_2)=CHMe
XII*E*

(*Z*)-PhC(OCHEt_2)=CHMe (2)
XII*Z*

EXPERIMENTAL

Materials. The vinyl ethers were prepared from the appropriate ketones *via* the route $R^1R^2CO \rightarrow R^1R^2C(OMe)_2 \rightarrow$ a vinyl ether (*e.g.* propiophenone → propiophenone dimethyl acetal → 1-methoxy-1-phenylpropene) as illustrated in the previous parts of this series. The ketones, unless commercially available, were prepared by conventional methods. The vinyl ethers were isolated as mixtures of the two geometric isomers with an *E*:*Z* ratio of about 0.5 for I−VI, X, XI, 2 for VII−IX, and 0.3 for XII. The following boiling temperatures were observed during final distillation: I 478 to 479 K at 100.0 kPa; II 496 to 501 K at 101.3 kPa; III 410 to 417 K at 2.5 kPa; IV 400 to 401 K at 2.1 kPa; V 355 to 356 K at 1.2 kPa; VI 366 to 367 K at 1.1 kPa; VII 355 K at 1.2 kPa; VIII 380 to 381 K at 1.3 kPa; IX 369 to 372 K at 1.2 kPa; X 331 to 340 K at 1.3 kPa; XI 360 to 363 K at 1.5 kPa; XII about 393 K at 1.3 kPa (the compound was prepared from I and 3-pentanol by transetherification, for examples see Ref. 1). If necessary, the synthetic products were purified by preparative GLC, but isolation of the pure isomers in preparative scale was too laborious due to their similar retention times (exception: I). *NMR spectra.* The δ values (CCl_4, TMS) of some characteristic peaks are given in Table 1.

Equilibrations. The equilibrations were carried out at about five temperatures from about 300 to 405 K (298 to 373 K for XII) with cyclohexane as solvent and iodine as catalyst (see previous papers of this series). The total number of independent determinations varied

Table 1. NMR shift data (δ values, CCl_4, TMS) for some characteristic peaks of the compounds studied in this work.

Compound	MeO		β-Me		Olefinic proton	
	E	*Z*	*E*	*Z*	*E*	*Z*
I	3.52	3.43	1.64	1.73	4.65	5.18
II	3.51	3.42	1.64	1.71	4.63	5.15
III	3.51	3.42	1.65	1.71	4.59	5.08
IV	3.54	3.44	1.64	1.73	4.68	5.23
V	3.54	3.44	1.64	1.74	4.67	5.18
VI	3.52	3.44	1.64	1.73	4.66	5.21
VII	3.50	3.22	1.40	1.70	4.69	4.64
VIII	3.51	3.31	1.40	1.71	4.68	4.91
IX	3.54	3.31	1.41	1.74	4.74	4.80
X	3.54	3.59	1.87	1.73	4.74	5.41
XI	3.56	3.56	1.82	1.70	4.74	5.25
XII	...	...	?[a]	1.71	4.83	5.06

[a] Obscured by other signals.

from 20 to 40. The equilibrated samples were analyzed by GLC. This method, however, was not applicable to XII, since the isomers had practically identical retention times. Thus the samples were analyzed by NMR from the relative integrated intensities of the signals at δ 4.83 (*E*) and 5.06 (*Z*).

CONFIGURATIONAL ASSIGNMENT

Configurational assignment of I*E* and I*Z* from NMR data has been accomplished by Schmid and Heinola,[2] and the NMR shift data and positive entropy change in the direction taken to represent the $E \rightarrow Z$ isomerization (see Discussion) lead us to the same conclusion.

RESULTS

In most cases ln K proved to be a nonlinear function of T^{-1} (*i.e.* $\Delta C^{\ominus}_p \neq 0$). Then the experimental values of K were fitted to the equation [3]

$$\ln K = A + BT^{-1} + C \ln T \qquad (3)$$

from which the values of $\Delta C^{\ominus}_p$ (independent of T) could be obtained. The values of the thermodynamic parameters, derived from the van't Hoff equation ($\Delta C^{\ominus}_p = 0$) or eqn. (3), are shown in Table 2. In the synthetic products of the *para*-substituted compounds, II–VI, the *E*:*Z* ratio was about 0.5 suggesting that for all these compounds the values of the thermodynamic parameters of the $E \rightarrow Z$ isomerization are similar. Hence only one of them (V) was equilibrated.

DISCUSSION

It is likely that conjugation between the olefinic double bond and the (substituted) phenyl group (compounds I–IX and XII) is more enhanced in the *Z* isomer, since there are fewer resonance-inhibiting steric interactions between the aromatic ring and the rest of the molecule in this configuration (*cf.* styrene appears [4] to be a planar molecule, but the interplanar angle between the planes of the aromatic ring and the double bond in *cis*-β-methylstyrene is [4,5] about 30–35°). Thus it might be expected that the inductive and resonance effects of the substituents could

Table 2. Thermodynamic data at 298.15 K for the $E \rightarrow Z$ isomerization reactions studied in this work. Solvent: cyclohexane. The errors are twice the standard errors.

Compound	Eqn.[a]	$\Delta G^{\ominus}$/ kJ mol^{-1}	$\Delta H^{\ominus}$/ kJ mol^{-1}	$\Delta S^{\ominus}$/ J K^{-1} mol^{-1}	$\Delta C^{\ominus}_p$/ J K^{-1} mol^{-1}
I	C	−1.60(6)	1.8(8)	11.5(25)	−22(14)
V	C	−1.08(3)	2.6(5)	12.5(17)	−18(11)
VI	L	−1.52(5)	0.1(3)	5.5(9)	...
VII	C	2.24(4)	5.3(5)	10.1(17)	−25(9)
VIII	C	1.07(4)	3.8(7)	9.2(23)	−20(14)
IX	L	2.94(10)	5.4(6)	8.1(18)	...
X	C	−1.70(5)	1.2(8)	9.6(25)	−12(15)
XI	L	−0.08(1)	2.2(1)	7.7(3)	...
XII	L	−5.73(7)	−12.3(5)	−22.0(16)	...

[a] The equation used in the calculations: L = van't Hoff eqn. (linear), C = eqn. (3) (curvilinear).

affect the extent of conjugation in the two isomers in unequal amounts. However, the data shown in Table 2 reveal that only *ortho*-substituents can significantly alter the relative stability of the *E*,*Z* pair. Hence the relative stability is mainly affected by the steric effect of the particular substituent, and this effect is negligible for substituents in the *meta*- or *para*-position. *ortho*-Substituents raise the relative enthalpy of the *Z* isomer suggesting that the decrease in conjugation caused by *ortho*-substitution is more severe in the *Z* isomer. The steric requirements of *ortho*-substituents are also reflected in the standard enthalpies of formation of some isomeric derivatives of benzoic acid: the *o*-Me, *o*-F, *o*-Cl, and *o*-I derivatives have less negative $\Delta H^{\ominus}_{f}(c)$-values than the corresponding *m*- and *p*-derivatives.[6] Similarly, steric effects affect the carbonyl frequency, νCO, in *ortho*-substituted acetophenones, benzoic acids, *etc.*[7,8]

2-Furyl and 2-thienyl groups are practically equivalent to a phenyl group as to their effect on the enthalpy and entropy difference between the geometric isomers.

The NMR shift data (Table 1) are interesting. In the *E* form of compounds I–XI, the signal of the MeO group is constantly found at δ 3.53 ± 0.03 and that of the olefinic proton at about δ 4.7 (max. 4.74, min. 4.59). The inability of R to affect the position of the signal of the MeO group is understandable if this group exists in the planar s-*cis* configuration (the most stable configuration of methyl vinyl ether, see Ref. 9 and the references cited therein), in which the distance R···Me (in MeO) has the maximum value. Similarly, the signal of the β methyl group of the *Z* isomer is found at δ 1.72 ± 0.02. However, *ortho*-substituents move (i) the signal of the β methyl group in the *E* isomer about 0.25 ppm to a higher field, and (ii) the signals of the MeO group and the olefinic proton in the *Z* isomer 0.1–0.2 and 0.3–0.6 ppm, respectively, to a higher field. This is reasonable, since *ortho*-substituents inevitably increase the interplanar angle between the aromatic ring and the double bond in both isomers. Thus the "face" of the phenyl group is forced to turn towards the β *cis* substituent (Me or H), and the *shielding* diamagnetic anisotropy effect [10] of the aromatic ring (for protons above or below the plane of the ring) leads to the upfield shifts observed. In the *Z* isomer, the MeO group probably assumes the *gauche* or s-*trans* configuration (see previous papers of this series), and the shielding effect of the aromatic ring is also directed towards this group. It is remarkable that in the *E* isomers of X and XI the signal of the β methyl group is found at about δ 1.85, *i.e.* 0.2 ppm to a lower field than the corresponding signal in I–IX. This might imply that the interplanar angle in X*E* and XI*E* is very small whereby the *deshielding* diamagnetic anisotropy effect of the furan and thiophene rings [10] causes the downfield shifts observed. If this interpretation is correct, the coplanarity must be ascribed to the smaller steric requirements of the five-membered rings in question.

For R = Me in reaction (1), $\Delta H^{\ominus}_{1} = 10.3 \pm 0.3$ kJ mol^{-1} and $\Delta S^{\ominus}_{1} = 13.9 \pm 0.8$ J K^{-1} mol^{-1}.[11] Since the entropy change is positive, it might be assumed that this should be the case for I*E* → I*Z*, too, and our configurational assignment for these isomers, based on the NMR shift data, is in agreement with this expectation. The difference (about 8 kJ mol^{-1}) between the values of $\Delta H^{\ominus}_{1}$ for the two reactions in question exceeds the difference (about 3 kJ mol^{-1}, Ref. 12) between Me···Me and Me···Ph *cis* interaction energies, which affect the stability of the *E* isomers. Thus it appears that I*Z* has some unexpected stabilization.

Replacement of the MeO group in I by an Et_2CHO group (XII) favors the stability of the *Z* isomer, in agreement with some previous results.[13] On going from I to XII, the increments in the values of the thermodynamic functions of isomerization are the following: $\Delta(\Delta G^{\ominus}_{1}) = -4.1$ kJ mol^{-1}, $\Delta(\Delta H^{\ominus}_{1}) = -14.1$ kJ mol^{-1}, and $\Delta(\Delta S^{\ominus}_{1}) = -34$ J K^{-1} mol^{-1}. For R = Me in reaction (1) the corresponding increments are [13] as follows: $\Delta(\Delta G^{\ominus}_{1}) = -4.3$ kJ mol^{-1}, $\Delta(\Delta H^{\ominus}_{1}) = -8.3$ kJ mol^{-1}, and $\Delta(\Delta S^{\ominus}_{1}) = -13$ J K^{-1} mol^{-1}.

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Electroörganic Preparations. XXXVII. Electrosynthesis of Cinnolines by Reductive Ring Closure of Dinitrocompounds

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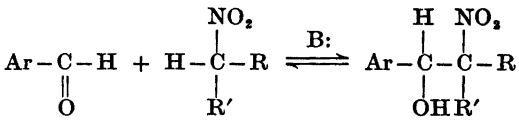
Cinnoline and its 3-methyl and 3-phenyl derivatives have been prepared in good yields by controlled potential reduction of suitable dinitroalcohols; the reduction yields the corresponding dihydroxylaminoalcohols which in presence of oxygen condense in a base catalysed reaction to the cyclic compounds. The 1-*N*-oxide was also formed in appreciable amounts in the preparation of 3-phenylcinnoline besides some 2,1-benzisoxazole (anthranil). The yields of cinnolines were found to depend mainly on the pH and the temperature during the electrolysis.

Among the benzodiazines the cinnolines are some of the least accessible.[1] Most synthetic routes involve several steps which besides being tedious often result in low over-all yields. For example, the preparation of 3-phenylcinnoline *via* *N*-benzylideneamino isatin involves five consecutive steps starting with benzaldehyde phenylhydrazone.[2] Controlled potential electrolysis is a valuable tool to induce ring closure reactions[3,4] by bringing the reaction centres in suitable oxidation states, and below is reported an application of this method for a convenient synthesis of 3-substituted cinnolines.

RESULTS AND DISCUSSION

Dinitroalcohols. As starting materials serve the dinitroalcohols I (R=H) which are easily available by the Henry addition between *o*-nitrobenzaldehyde and primary or secondary nitroalkanes.



Ar=2-nitrophenyl
Ia, R=R′=H
Ib, R=H, R′=Me
Ic, R=R′=Me
Id, R=H, R′=Ph

I

The presence of the *o*-nitro group made the dinitroalcohols (I) stable in solution and they showed no tendency to eliminate water with the formation of *β*-nitrostyrenes contrarily to the corresponding unsubstituted nitroalcohols derived from benzaldehyde.[5,6] Compounds Ib and Id were obtained as 1:1 and 1:2 mixtures of the (±)-*erythro* and (±)-*threo* stereoisomers, respectively, by using triethylamine as the basic catalyst for the addition. The stereoisomeric dinitroalcohols were separated by column chromatography on silica. Acetic acid was added to the stationary phase and to the developing solvent; otherwise, the dinitroalcohols were partly reconverted to *o*-nitrobenzaldehyde and the parent nitroalkane.

The NMR spectra of the investigated dinitroalcohols in deuteriochloroform solution are collected in Table 1. The stereochemical assignments of the *erythro*- and *threo*-isomers rest upon a comparison between the data in Table 1 and the NMR spectrum (see Experimental) of (±)-*threo*-α-(1-nitrobenzyl)benzyl alcohol, the stereochemistry of which is well established.[7,8] Although there are considerable differences in the *δ*-values of the H^a protons in the stereoisomeric dinitroalcohols (due to the *o*-nitro

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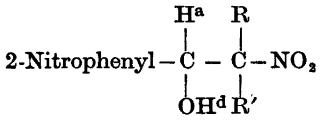
Table 1. NMR parameters of *o*-nitro-α-(1-nitroalkyl/aralkyl)benzyl alcohols, I. ($CDCl_3$; chemical shift values in δ (ppm); coupling constants J in Hz; TMS as internal standard).



Compound	R	R′	H^a	H^b	H^c	H^d	Arom. protons	J_{ab}	J_{ac}	J_{ad}	J_{bc}
Ia	H^b	H^c	5.99	4.83	4.56	3.49	7.3–8.1	2.4	9.2	–	–13.7
erythro-Ib	H^b	$CH_3{}^c$	6.08	4.97	1.51	3.32	7.3–8.2	3.0	–	4.5	7
threo-Ib	H^b	$CH_3{}^c$	5.68	4.97	1.44	3.55	7.3–8.2	7.0	–	5.5	7
Ic	$CH_3{}^b$	$CH_3{}^c$	6.28	1.38	1.52	3.39	7.3–8.0	–	–	–	–
erythro-Id	H^b	C_6H_5	6.32	5.93		3.45	7.1–8.2	4.2	–	–	–
threo-Id	H^b	C_6H_5	6.38	5.87		4.08	7.1–8.2	9.5	–	–	–

group) and the reference compound, it seems characteristic that the compound which was assigned the *threo*-Id structure had a coupling constant J_{ab}, identical with the value (9.5 Hz) found for the (±)-*threo*-α-(1-nitrobenzyl)benzyl alcohol. The coupling constants between H^a and H^b in benzene-d_6 of both *threo*-Id and the reference compound were practically unaltered from the values found in deuteriochloroform. In dimethyl sulfoxide-d_6, the H^a and H^b protons of *threo*-Id by chance had the same δ-value which excluded the determination of the coupling constant. For both the *erythro*-Id and the reference compound, however, only a small variation in the value of J_{ab} was observed in this solvent.

The assignment of the *erythro*- and *threo*-isomers of Ib was more uncertain, but was based on a comparison of the coupling constants and also on the chromatographic behaviour of *erythro*- and *threo*-Id. The J_{ab} coupling constants seem to indicate preference for all the stereoisomeric dinitroalcohols of conformations which enable a hydrogen bonding between the hydrogen of the alcohol function and the aliphatically bound nitro group.

Polarographic investigation. The dinitroalcohols show two well-defined polarographic waves at pH < 7; in Fig. 1 are depicted the half-wave potentials of (±)-*erythro*-*o*-nitro-α-(1-nitroethyl)-benzyl alcohol (*erythro*-Ib) in dependence of pH in acidic to neutral solution. At higher pH a third pH-independent wave with $E_{\frac{1}{2}} = -0.56$ V (*vs.* SCE) grows up which probably is due to the reduction of the *o*-nitro group of *o*-nitrobenzaldehyde generated by a reversal of the Henry addition. Both waves were shown by controlled potential electrolysis to be four-electron waves. The half-wave potential of the first wave which has a slope of the $E_{\frac{1}{2}}$-pH curve of 0.086 V/pH corresponds to a reduction of the "aromatic" *o*-nitro group to a hydroxylamino function,[9] while the second wave corresponds to a reduction of the "aliphatic" nitro group to the hydroxylamino stage (Scheme 1).

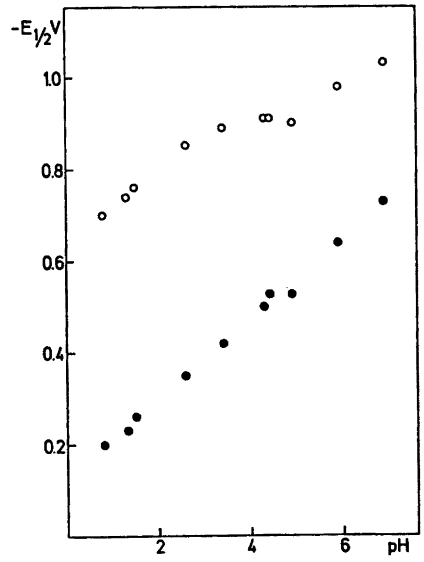
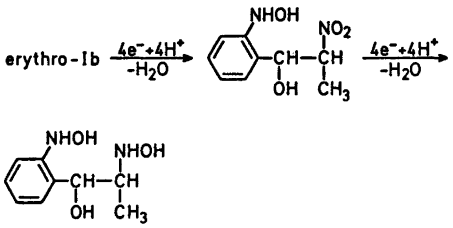


Fig. 1. Half-wave potentials (V *vs.* SCE) of (±)-*erythro*-*o*-nitro-α-(1-nitroethyl)benzyl alcohol in 40 % aqueous ethanol. Concentration 80 mg/l. ● ○, Data of first and second wave, respectively.



Scheme 1.

Although the dihydroxylamino compound thus produced apparently was quite stable in solution when kept under nitrogen, no attempt was made to isolate this strongly hydrophilic product neither in analytical nor in preparative scale.

Table 2 shows the half-wave potentials of the investigated dinitroalcohols I in an acetate buffer at pH 5.

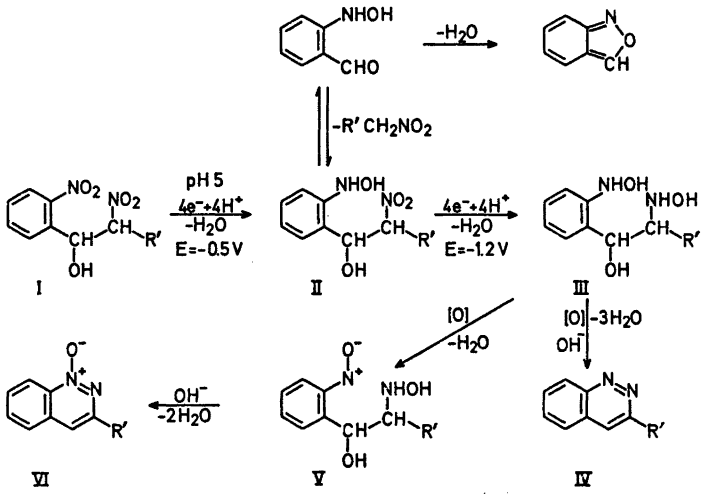
The successive introduction of methyl groups α to the aliphatic nitro group causes a cathodic shift of 70 mV of the half-wave potential of this group; the presence of an α-phenyl group causes an anodic shift of $E_{\frac{1}{2}}$ of the aliphatic nitro group of 25 mV. The half-wave potentials of the *o*-nitro group of *erythro*-Id and *threo*-Id show a significant difference (50 mV); in the case of *erythro*-Ib and *threo*-Ib the difference is only about 10 mV. This finding probably reflects the preferred conformations of the stereoisomeric dinitroalcohols, but a detailed conformation analysis is needed before a definite conclusion can be drawn concerning the origin of this stereochemical effect.

Table 2. Half-wave potentials of *o*-nitro-α-(1-nitroalkyl/aralkyl)benzyl alcohols I in 40 % ethanolic acetate buffer at pH 5.

Compound	$-E_{\frac{1}{2}}$ (1.)	$-E_{\frac{1}{2}}$ (2.)
Ia	0.53	0.85
erythro-Ib	0.54	0.92
threo-Ib	0.55	0.91
Ic	0.57	0.98
erythro-Id	0.47	0.83
threo-Id	0.52	0.82

Preparation of cinnolines. Controlled potential reduction at 0 °C of *erythro*-Ib in an acetate buffer containing 25 % ethanol at a mercury electrode at the potential of the second wave (−1.20 V *vs.* SCE) consumed 8 F/mol; after the reduction the solution was made alkaline and was allowed to stand over night in contact with air before further work-up.

Besides the main product, 3-methylcinnoline, which was isolated after chromatography in 59 % yield, some anthranil could be detected by NMR-spectroscopy of the crude reaction mixture. From the reduction of *threo*-Id at −0.95 V *vs.* SCE was isolated, after a similar work-up, 3-phenylcinnoline and some 3-phenylcinnoline-1-*N*-oxide; anthranil was also here detected in the crude reaction mixture in a yield of 5 − 10 %. These results are rationalized in Scheme 2.



Scheme 2.

Table 3. Polarographically determined yields of cinnolines (IV) and cinnoline 1-*N*-oxides (VI) from the reduction of *o*-nitro-α-(1-nitroalkyl/aralkyl)benzyl alcohols I in 25 % ethanolic acetate buffer at pH 5.

Compound	$-E$(V *vs.* SCE)	Yield % IV	VI
Ia	1.20	51	0
erythro-Ib	1.20	56 – 63	0
threo-Ib	1.20	67	0
erythro-Id	0.95	49	20
threo-Id	0.95	47	16

In Table 3 are given polarographically determined yields of the cinnolines and the *N*-oxides from a series of runs at pH 5 and 0 – 5 °C, followed by the standard work-up.

Anthranil is a reduction product of *o*-nitrobenzaldehyde. The tendency of a reversal of the Henry addition is low for I, but probably higher for II, as the anthranil formation would remove the *o*-hydroxylaminobenzaldehyde from the equilibrium and thereby favour the cleavage.

This is in accordance with the fact that higher temperatures cause higher yield of anthranil and lower yield of cinnolines. Furthermore, reduction at the potential of the first wave followed by a reduction at the potential of the second wave similarily gives a higher yield of anthranil and a lower one of cinnoline. This substantiates the polarographic results that the first wave is caused by the reduction of the "aromatic" nitro group.

The reduction of the aromatic nitro group may also facilitate the loss of water from II with the formation of an *o*-hydroxylaminonitrostyrene. A formation of such a compound, followed by its reduction and further reactions, may help to explain why the yield of cinnoline never exceeds 65 %. The dehydration of II may also explain why an attempt to suppress the reversal of the Henry addition by conducting the reduction in N sulfuric acid was not successful (yield of 3-methylcinnoline 21 %).

The formation of the cinnoline-1-*N*-oxides is readily explained by an air-oxidation of a hydroxylamino group to a nitroso group followed by a condensation with the other hydroxylamino group to an azoxy compound; aromatization occurs then by loss of the hydroxyl group as water.

Whereas the reaction mechanisms leading to the by-products, anthranil and the cinnoline-*N*-oxides, seem well established, this is not so for the main reaction, Formally, loss of 3 mol of water from III would lead to a cinnoline; any reaction mechanism must explain the following findings.

(a) The ring closure does not proceed at a measurable rate in the absence of oxygen. III is stable in solution under nitrogen, even at high pH; this is important for the synthesis, otherwise the cinnoline formed during the electrolysis would be reduced in preference to the starting material.

(b) In the presence of oxygen the condensation takes place; it goes faster at high pH, but the yield of cinnoline is the same whether the condensation takes place at pH 1 or 12.

(c) The oxidation state of III and IV is the same; any initial oxidation by oxygen in the beginning of the reaction sequence must be followed by a corresponding reduction later in the reaction.

(d) Additions of oxidation agents, such as cupric ions, hexacyanoferrate, or dinitrogen tetroxide, which are recognized as one-electron oxidation reagents, did not increase the yield of cyclized products, although it increased the formation of *N*-oxide at the expense of the cinnoline.

One could assume that the initial step was a one-electron oxidation of III, followed by formation of a nitrogen-nitrogen bond and successive loss of 3 mol of water. The energy gained by the aromatization may make it possible for the radical to oxidize (abstract an electron from) another molecule of III. It has, however, not been possible to obtain experimental verification of this or any other hypothesis.

CONCLUSION

Despite the fact that yields are not quantitative, the ease of preparing the starting materials and conducting the reduction without isolation of intermediates, makes the cinnolines a more accessible class of compounds than hitherto. From a synthetic point of view, it is a further advantage that the tedious separation of the stereoisomeric dinitroalcohols I is superfluous as judged from Table 3.

A better understanding of the critical cycliza-

tion step might possibly result in even better yields than we have been able to obtain. On the other hand, if the loss is exclusively caused by a cleavage or loss of water from II, the chance of increasing the yields of cinnolines seems not very promising; possibly reduction at low temperatures may be an advantage.

EXPERIMENTAL

The equipment used for polarography and electrolysis at controlled potential was a recording polarograph P04 (Radiometer, Copenhagen) and a fully transistorized potentiostat (Tage Juul Electronics, Copenhagen) respectively. NMR spectra were obtained with a Varian A-60 spectrometer. Mass spectra were taken on a CEC/MS-21-104 mass spectrometer. M.p's were determined with a Mettler FP-instrument.

General procedure for the preparation of the o-nitro-α-(1-nitroalkyl/aralkyl)benzyl alcohols (I). To a stirred solution of *o*-nitrobenzaldehyde (7.55 g, 0.05 mol) and the nitroalkane (0.1 mol) in benzene (25 ml) was added triethylamine (300 μl, 2 mmol). The reaction mixture was kept below 25 °C with external cooling and was then allowed to stand at room temperature over-night. The reaction mixture was diluted with further 100 ml benzene and was extracted with portions of saturated sodium hydrogensulfite solutions (3 × 50 ml) to remove the base catalyst, unreacted aldehyde and coloured impurities. The benzene layer was dried over calcium sulfate and the benzene removed *in vacuo* together with excess nitroalkane.

Deviation from the general procedure. Erythro- and *threo*-Id were for practical reasons prepared from *o*-nitrobenzaldehyde and phenylnitromethane in the molar ratio 2:1. The excess of aldehyde was again removed by extractions with saturated sodium hydrogensulfite solutions (3 × 100 ml) as above.

Separation of the stereoisomeric dinitroalcohols. erythro- and *threo*-Ib and Id, respectively, were separated by partition chromatography on Merck Silica 40. The eluent was prepared by shaking chloroform (1.5 l) with a mixture of methanol (50 ml), 80 % acetic acid (4 ml) and water (150 ml). The silica was deactivated with a small amount of the water phase (4 % by weight). In the case of Id unreacted phenylnitromethane was eluted before the stereoisomeric dinitroalcohols. Typically, crude Id (1.48 g) by partition chromatography as described above (column 16 × 3 cm) yields phenylnitromethane (261 mg), *erythro*-Id (278 mg) and *threo*-Id (741 mg) by elution with 750 ml of the developing solvent.

Characterisation of the dinitroalcohols (I): o-*Nitro-α-(nitromethyl)benzyl alcohol (Ia)* was prepared according to the general procedure in 85 % yield. Recrystallization from benzene/cyclohexane gave m.p. 57.1 °C (lit.[12] 59.5–60 °C).

(±)-erythro-o-*Nitro-α-(1-nitroethyl)benzyl alcohol* (erythro-*Ib*) was obtained in 40 % yield by the general procedure as a 1:1 mixture (overall yield 80 %) with the (±)-*threo*-isomer, from which it was separated either by repeated crystallization from benzene or by column chromatography. M.p. 94.5 °C (lit.[12] 92–93 °C).

(±)-threo-o-*Nitro-α-(1-nitroethyl)benzyl alcohol* (threo-*Ib*) was separated from the *erythro*-Ib isomer by column chromatography. Light orange oil. (Found: C 47.65; H 4.7; N 12.32. Calc. for $C_9H_{10}N_2O_5$ (226.19): C 47.79; H 4.5; N 12.39).

o-*Nitro-α-(1-nitroisopropyl)benzyl alcohol (Ic)* was prepared according to the general procedure in 68 % yield as a slowly crystallizing orange oil. Recrystallized from lukewarm benzene/cyclohexane. M.p. 54.2–56.3 °C. (Found: C 49.92; H 5.2; N 11.59. Calc. for $C_{10}H_{12}N_2O_5$ (240.21): C 50.00; H 5.1; N 11.66).

(±)-erythro-o-*Nitro-α-(1-nitrobenzyl)benzyl alcohol* (erythro-*Id*) was isolated in 16 % yield from the *threo*-isomer and unreacted phenylnitromethane by column chromatography. Recrystallized from benzene. M.p. 115–116 °C. (Found: C 58.44; H 4.3; N 9.72. Calc. for $C_{14}H_{12}N_2O_5$ (288.25): C 58.33; H 4.2; N 9.72). ^{1}H NMR (C_6H_6):* δ 2.38 (1 H, s, O*H*), 5.81 (1 H, d, H^b, J_{ab} 4.5 Hz), 6.09 (1 H, d H^a), 6.5–7.7 (9 H, m, arom. H). (DMSO-d_6): δ 6.13 (1 H, d, H^b, J_{ab} 5.5 Hz), 6.31 (1 H, d, H^a), 6.60 (1 H, s, O*H*), 7.2–8.2 (9 H, m, arom. H).

(±)-threo-o-*Nitro-α-(1-nitrobenzyl)benzyl alcohol* (threo-*Id*) was obtained in 32 % yield as a light orange oil, which crystallized after standing at room temperature for three months. The crystals were washed with benzene/cyclohexane 2:1. M.p. 81–83 °C. (Found: C 58.36; H 4.5; N 9.37. Calc. for $C_{14}H_{12}N_2O_5$ (288.25): C 58.33; H 4.5; N 9.72). ^{1}H NMR (C_5D_5): δ 3.07 (1 H, s, O*H*), 5.77 (1 H, d, H^b, J_{ab} 9.0 Hz), 6.34 (1 H, d, H^a), 6.5–7.4 (9 H, m, arom. H). (DMSO-d_6): δ 6.16 (2 H, s, H^a+H^b), 6.84 (1 H, s, O*H*), 7.2–8.0 (9 H, m, arom. H).

(±)-threo-α-*(1-Nitrobenzyl)benzyl alcohol* was prepared according to the procedure of Bordwell and Garbisch [7] and recrystallized from benzene/cyclohexane. M.p. 103–104 °C (lit.[7] 103.5–104 °C). ^{1}H NMR ($CDCl_3$): δ 3.05 (1 H, s, O*H*), 5.51 (1 H, d, H^a, J_{ab} 9.5 Hz), 5.63 (1 H, d, H^b), 7.1–7.4 (10 H, arom. H). (C_6D_6): δ 2.51 (1 H, d, O*H*, J_{aOH} 3.5 Hz), 5.39 (1 H, dd, H^a, J_{ab} 10 Hz), 5.57 (1 H, d, H^b), 6.8–7.3 (10 H, arom. H). (DMSO-d_6): δ 5.59 (1 H, dd, H^a, J_{ab} 10.5 Hz, J_{aOH} 4 Hz), 5.94 (1 H, d, H^b), 6.36 (1 H, d, O*H*), 7.0–7.7 (10 H, arom. H).

Analytical determination of yields. The dinitroalcohols (I) (0.5 mmol) were reduced at the selected potential in 25 % aqueous ethanol (150 ml) adjusted to the appropriate pH and kept

* Proton indices, see Table 1.

under nitrogen at 0 – 5 °C. After complete reduction of the nitro groups (the reaction was followed by polarography directly in the cell) the solution was separated from the mercury. The mercury was further washed with 50 ml ethanol and the combined solution was made slightly alkaline (pH 10 – 12) with solid potassium carbonate and allowed to stand overnight in contact with air. The solution was diluted to 250 ml with water and portions (25 ml) were adjusted with 4 N hydrogen chloride to pH 1 and diluted to 50 ml. Yields were calculated from polarograms of these solutions by comparison with standard curves of the corresponding cinnolines (*cf.* Ref. 10).

Preparative reductions

Reduction of (±)-erythro-o-*nitro*-α-*(1-nitroethyl)benzyl alcohol (*erythro-*Ib). erythro*-Ib (1.00 g) was reduced at 0 °C at – 1.2 V (SCE, sec. wave) in a 25 % ethanolic acetate buffer adjusted to pH 5. The reduction consumed eight electrons per molecule. The solution was then made alkaline with solid potassium carbonate and was left over-night in the presence of air. The polarographically determined yield of 3-methylcinnoline in this solution was 63 %. The solution was then extracted with portions of methylene chloride (3 × 150 ml). The combined extracts were dried over solid potassium carbonate and the solvent evaporated *in vacuo*. The crude product (515 mg) was purified by column chromatography (2.5 × 19 cm) on alumina (Merck, activity I) by elution with ether-light petroleum (b.p. 60 – 80 °C) 1:1 to yield 3-methylcinnoline (375 mg, 59 %; $R_F = 0.18$). The compound was identified from its NMR-spectrum ($CDCl_3$): δ 2.90 (3 H, s), 7.57 – 7.93 (4 H, m), 8.32 – 8.60 (1 H, m) and its quantitative conversion to the hydrochloride, m.p. 120 – 122 °C (dec.), which was identical with that of an authentic sample.[13]

TLC-evidence and the NMR-spectrum of the crude product revealed a small admixture (5 – 10 %) of anthranil. Furthermore, the spectrum had broad unresolved signals at δ 6.5 – 7.4 probably due to polymeric materials. By a similar reduction of *erythro*-Ib (2.26 g) conducted at room temperature, there was isolated 115 mg (10 %) anthranil (purified by chromatography on silica gel by elution with the earlier described chloroform-methanol-acetic acid-water system; $R_F = 0.40$).

Reduction of o-*nitrobenzaldehyde.* o-Nitrobenzaldehyde (2.00 g) was reduced at – 0.55 V (SCE, first wave) in acetate buffer containing 25 % ethanol with an electron consumption of 4F per mol. The reduced solution was extracted with methylene chloride, which was dried over calcium sulfate and evaporated *in vacuo* at 10 °C; the yield was 1 g of crude product. As TLC revealed a small admixture of starting material, the product was dissolved in benzene (50 ml) and shaken with a saturated aqueous solution of sodium hydrogensulfite. The benzene layer was dried over calcium sulfate and evaporated *in vacuo* to yield anthranil (803 mg, 51 %). ^{1}H NMR ($CDCl_3$): δ 6.77 – 7.80 (4 H, m), 9.13 (1 H, d, 1.1 Hz). MS (70 eV) *m/e* (% I): 119 (M^+, 88), 93 (7), 92 (100), 91 (38), 90 (8), 65 (22), 64 (84), 63 (60) (*cf.* Ref. 1).

Reduction of (±)-threo-o-*nitro*-α-*(1-nitrobenzyl)benzyl alcohol (*threo-*Id). threo*-Id (0.50 g) was reduced at – 0.95 V (SCE, sec. wave). The reduction consumed approximately eight electrons per molecule. The solution was made alkaline with potassium carbonate and left overnight in contact with air. A white precipitate was filtered off and the solution was further extracted with methylene chloride (3 × 150 ml). After drying over potassium carbonate the solvent was removed and the solid residue combined with the precipitate and subjected to column chromatography on alumina (activity I) by elution with ether-light petroleum (b.p. 60 – 80 % °C) 1:1. By this procedure were isolated 3-phenylcinnoline (168 mg, 47 %; $R_F = 0.39$), m.p. 118 – 119 °C (lit.[2] 119 °C) and 3-phenylcinnoline 1-*N*-oxide (85 mg, 22 %; $R_F = 0.22$), m.p. 134 – 136 °C after washing with a little cold methanol (lit.[14] 138 – 139 °C). MS (70 eV) *m/e* (% I): 223 (15), 222 ($M^{+\cdot}$, 100), 206 (17), 178 (28), 176 (12), 165 (10), 119 (13), 97 (10), 92 (60), 91 (37), 89 (26), 83 (13), 77 (22), 76 (20), 71 (12), 69 (22), 64 (20), 63 (24). Before 3-phenylcinnoline 98 mg was eluted, which according to the NMR-spectrum contained anthranil and probably o-hydroxylaminobenzaldehyde.

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Syntheses of Some Furanosidic D-Fructose Derivatives

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The syntheses of α- and β-penta-*O*-acetyl-D-fructofuranose and of α- and β-1,3,4,6-tetra-*O*-acetyl-D-fructofuranosyl fluoride are reported. The substances were characterized by their ^{1}H and ^{13}C NMR spectra.

The chemistry of D-fructose and its derivatives has been summarized.[1,2] When glycosidically linked in natural products, D-fructose always occurs in the β-D-furanose form, therefore the synthesis of furanosidic D-fructose derivatives is of some interest. The recent finding that dextran may be synthesized by the action of dextransucrase on α-D-glucopyranosyl fluoride [3] raises the possibility of an analogous synthesis of levan from β-D-fructofuranosyl fluoride. We now report the synthesis of the latter substance, as the acetate, and of some other D-fructofuranose derivatives.

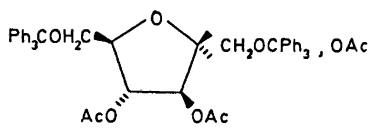
On acetolysis of 2,3,4-tri-*O*-acetyl-1,6-di-*O*-trityl-D-fructofuranose (*1*), Bredereck and coworkers [4] obtained a mixture of fully acetylated D-fructofuranoses. These have now been separated by chromatography on silica gel into the amorphous α-form (*2*, $[\alpha]_{578}^{22}$ +58°) and the β-form (*3*, m.p. 57–58 °C, $[\alpha]_{578}^{22}$ −9°). The starting material also is an anomeric mixture, as evident from its ^{13}C NMR spectrum (Table 1).

Treatment of the mixture of D-fructofuranose acetates with liquid hydrogen fluoride yielded the tetra-*O*-acetyl-α- and β-D-fructofuranosyl fluorides, which were fractionated by chromatography on silica gel. Neither of the products crystallized. The optical rotations were remarkably similar, $[\alpha]_{578}^{22}$ +45° for the α-form (*4*) and $[\alpha]_{578}^{22}$ +36° for the β-form (*5*), indicating different conformation for the two anomers.

The structures as well as the anomeric assignments of the penta-*O*-acetyl-D-fructofuranosyl fluorides follow from considerations of chemical shifts and coupling constants in the ^{1}H and ^{13}C NMR spectra (Table 1. For comparison, the chemical shifts of penta-*O*-acetyl-β-D-fructopyranose are also given). In agreement with previous findings,[5–8] the ^{13}C chemical shifts of the furanoses were 3–7 ppm higher than those for the same carbon atoms of the corresponding fructopyranoses. In their studies

Table 1. The ^{13}C NMR shifts in δ(ppm from TMS) of α- and β-D-fructofuranose pentaacetate (*2* and *3*); α- and β-D-fructofuranosyl fluoride tetraacetate (*4* and *5*); β-D-fructopyranose pentaacetate (*6*); the anomeric mixture of 1,6-dityityl-D-fructofuranose triacetate (*1*) and β-D-fructofuranose derivatives (Ref. 6).

	1	*2*	*3*	*4*	*5*	*6*	Ref. 6
C–2	109.4 105.4	107.9	104.9	117.5	113.9	102.3	~104.6
C–3	76.5–86.8	78.4	75.7	78.2	75.0	62.9–68.2	~77.6
C–4		80.6	79.5	83.5	80.2		~82.6
C–5		76.1	75.6	76.7	74.7		~75.1
C–1	62.3–64.6	61.8	63.7	61.2	61.7		~63.3
C–6		62.8	63.9	62.7	63.6		~62.6



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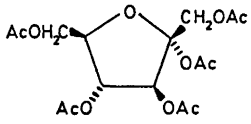

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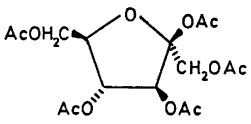

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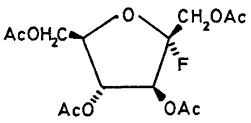

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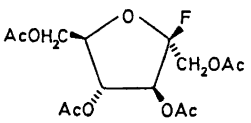

5

of the ^{13}C NMR spectra of various furanosidic carbohydrates, Perlin and coworkers have shown that in hexulofuranoses with the *arabino*- and *ribo*-configurations (fructose and psicose), a *cis*-disposition of the hydroxyl group at C-3 and the methoxy group at C-2 gives rise to stronger shielding at C-2 than does the corresponding *trans*-disposition (methyl α-D-fructofuranoside 105.2 ppm and methyl β-D-fructofuranoside 102.4 ppm).[5] The anomeric C-2 therefore comes into resonance at lower field for the α-anomers than for the corresponding β-anomers. Application of this finding to the *2* and *3* furanosides gives the assignments shown (Table 1). Thus, the fructofuranose pentaacetate with δ 107.9 for the anomeric carbon atom is assigned the α-configuration (*2*), while that with the corresponding δ 104.9 is assigned the β-configuration (*3*). Similarly, in the tetra-*O*-acetyl-fructofuranosyl fluorides, the anomer with δ 117.5 for C-2 is assigned the α-configuration (*4*) and that with δ 113.9 for C-2 the β-configuration (*5*). The assignments for the pentaacetates are corroborated by the observed optical rotations. No such confirmation has been obtained for the tetra-*O*-acetyl-furanosyl fluorides (*4* and *5*). The reason for this is discussed below. However, these latter configurational assignments are confirmed by the observed ^{19}F-2, ^{1}H-3 coupling constants. Hall and coworkers [9] have reported $^{3}J_{H,F}$ 20.6 Hz for 2,3,5-tri-*O*-benzoyl-α-D-ribofuranosyl fluoride (H-2 and F-1 *trans*-disposed), in agreement with the generally observed angular dependence of $^{3}J_{H,F}$ coupling constants.[10] We therefore assign the $^{3}J_{H,F}$ coupling constant of 7 Hz to tetra-*O*-acetyl-α-D-fructofuranosyl fluoride and that of about 16 Hz to the corresponding β-anomer.

The similarly of the optical rotations of the anomeric fructofuranosyl fluorides most probably reflects different conformational preferences. A preference for a quasi-axial orientation of the fluorine atom in each of the two anomers (anomeric effect) would lead to two different ring conformations. Conformations E_2 or $^{3}T_2$ [11] for the α-anomer appear compatible with the observed NMR coupling constants and ^{2}E or $^{2}T_0$ correspondingly for the β-anomer. (The indexes refer to the numbering in D-fructose.)

EXPERIMENTAL

General methods. Concentrations were performed under reduced pressure. Precoated plates with Silica Gel F_{254} (Merck) were used for TLC, and Silica Gel 60 (230–400 mesh, Merck) was used for column chromatography. Light petroleum refers to a fraction with b.p. 60–71 °C. NMR spectra were recorded with Varian A 60-A and Varian XL-100 instruments ($CDCl_3$ with TMS as internal reference). ^{1}H spectra were recorded at 60 and 100.1 MHz and ^{13}C spectra at 25.2 MHz. Optical rotations were determined with a Perkin-Elmer 141 polarimeter.

2,3,4-Tri-O-acetyl-1,6-di-O-trityl-D-fructo-furanose (1). 1,6-Di-*O*-trityl-D-fructose was prepared according to Bredereck *et al.*,[12] except that crystallization from pyridine was omitted. This product (16 g) was acetylated, worked up [12] and purified by chromatography on a silica gel column (80 × 8 cm), using light petroleum–ethyl acetate (3:1) as irrigant. The main product (8 g) was identified as a mixture of anomeric furanosides by ^{13}C NMR (Table 1).

Penta-O-acetyl-α- and β-D-fructofuranose (2 and 3). The trityl derivative *1* (5.8 g) was subjected to acetolysis and worked up as described by Bredereck.[4] TLC using light petroleum–ethyl acetate, 1:1, revealed two components in the derived syrup, the minor one having low mobility. The product was fractionated on a silica gel column (40 × 4 cm) using

light petroleum – ethyl acetate, 1:1, as irrigant. The minor component (0.3 g) was probably a tetraacetate as acetylation yielded a product with the same mobility as the pentaacetate. TLC demonstrated that the major component (2.4 g) was a mixture of two substances with slightly different mobilities. Separation of this material (1.4 g) on a silica gel column (30 × 3 cm), using the same irrigant, yielded the pure anomers, identified by their NMR spectra.

Penta-*O*-acetyl-α-D-fructofuranose (*2*, 1.05 g), $[\alpha]_{576}^{22}$ +58° (*c* 1.0, $CHCl_3$), was the faster component.

Penta-*O*-acetyl-β-D-fructofuranose (*3*, 330 mg), $[\alpha]_{578}^{22}$ −9° (*c* 1.0, $CHCl_3$). Crystals (ethanol), m.p. 57 – 58 °C. (Found: C 48.9; H 5.59. $C_{16}H_{22}O_{11}$ requires: C 49.2; H 5.68.)

*1,3,4,6-Tetra-*O*-acetyl-α- and β-D-fructofuranosyl fluoride (4 and 5).* The anomeric mixture of fructofuranose pentaacetates (*2* and *3*, 2.2 g) in liquid hydrogen fluoride (10 ml) was kept for 15 min at −15 °C and then for 15 min at 20 °C. The solution was poured into a stirred mixture of ice-water (100 ml) and chloroform (100 ml). The chloroform layer was separated and the aqueous phase extracted with chloroform (50 ml). The combined chloroform phases were washed with cold 1 M sodium hydrogen carbonate (2 × 50 ml), cold water (5 × 50 ml), dried ($MgSO_4$) and concentrated to a syrup. This was fractionated on a column of silica gel (40 × 4 cm) using light petroleum – ethyl acetate (1:1) as irrigant.

1,3,4,6-Tetra-*O*-acetyl-α-D-fructofuranosyl fluoride (*4*, 690 mg), $[\alpha]_{578}^{22}$ +45°, was eluted first.

1,3,4,6-Tetra-*O*-acetyl-β-D-fructofuranosyl fluoride (*5*, 430 mg), showed $[\alpha]_{578}^{22}$ +36°.

^1H NMR shifts and coupling constants. α-D-fructofuranose pentaacetate (*2*): δ 5.9 (H-3), 5.2 (H-4), 4.1 – 4.8 (H-1, H-5, H-6); $J_{3,4}$ 4 Hz, $J_{4,5}$ 6 Hz.

β-D-fructofuranose pentaacetate (*3*): δ ~5.7 (H-3, H-4), 4.5 – 4.7 (H-1, H-5, H-6); $J_{3,4}$ and $J_{4,5}$ probably small.

α-D-fructofuranosyl fluoride tetraacetate (*4*): δ 5.55 (H-3), 5.0 (H-4), 4.2 – 4.5 (H-1, H-5, H-6); $J_{F,3}$ 7; $J_{3,4}$ 1; $J_{4,5}$ 5.

β-D-fructofuranosyl fluoride tetraacetate (*5*): δ 5.4 – 5.7 (H-3, H-4), 4.2 – 4.5 (H-1, H-5, H-6); $J_{F,3}$ = 16; $J_{3,4}$ 7; $J_{4,5}$ small.

$^{13}C - ^{19}F$ coupling constants (Hz).

α-D-fructofuranosyl fluoride tetraacetate (*4*): $J_{F,1}$ 28.8; $J_{F,6}$ 0.5; $J_{F,3}$, $J_{F,4}$, $J_{F,5}$ 46.0, 1.5, 0.2; $J_{F,2}$ 226.8.

β-D-fructofuranosyl fluoride tetraacetate (*5*): $J_{F,1}$ 43.7; $J_{F,3}$, $J_{F,4}$, $J_{F,5}$ 20.9, 2.8, 0.4; $J_{F,2}$ 232.8.

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Kinetics of the Reaction of 1-Halo-2,4-dinitrobenzenes with *N*-(3-Dimethylaminopropyl)-*p*-anisidine and *N*-Isohexyl-*p*-anisidine in Benzene

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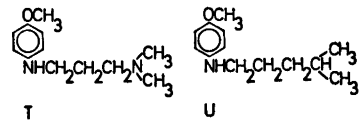
The reactions of *N*-(3-dimethylaminopropyl)-*p*-anisidine and *N*-isohexyl-*p*-anisidine with 1-X-2,4-dinitrobenzene, X = F, Cl and Br, have been studied in benzene with and without 1,4-diazabicyclo[2.2.2]octane as a catalyst. With the first of these amines, the fluorine compound was found to react much faster than the two other halogeno compounds. With the second amine, however, fluoride ion as the leaving group gives the slowest reaction, indicating a rate-limiting second step. Intramolecular base catalysis is supposed to be operating with the diamine as nucleophile.

In an earlier paper,[1] the reaction of 2,6-dinitroanisole with a series of α,ω-diaminoalkanes, $H_2N(CH_2)_nNH_2$, $n = 2-5$, was kinetically studied. From the results, no definite conclusions could be drawn whether intramolecular base catalysis was operating. In dry dioxane, the reaction is entirely second-order with respect to diamine. In aqueous dioxane or methanol, the rate can be expressed as a sum of a first- and a second-order term with respect to diamine. The first-order term would indicate intramolecular base catalysis if the second step of the reaction were rate-limiting and the solvent did not act as a base. Only small rate changes were found upon varying the chain length of the diamines. It was supposed that proton transfer *via* solvent molecules takes place in the collapse of the intermediate to products.

A different system has now been studied, namely the reaction of 1-halo-2,4-dinitrobenzenes in which the halogen is fluorine, chlorine or bromine, with the amines *N*-(3-dimethylaminopropyl)-*p*-anisidine and *N*-isohexyl-*p*-anisidine, designated for brevity, T and U. The reactions were studied in benzene at 25 °C, with and without 1,4-diazabicyclo[2.2.2]octane (DABCO) as a catalyst.



T U

The choice of substituted *p*-anisidines is based on work by Bernasconi and Zollinger,[2] who demonstrated base catalysis in the reaction of 1-halo-2,4-dinitrobenzenes with *p*-anisidine in benzene. The diamine T is a substituted *p*-anisidine, carrying an aliphatic chain with a terminal tertiary amino group. The terminal amino group might serve as a built-in catalyst. The compound U was designed to be sterically closely similar to T.

EXPERIMENTAL

General. For spectral identification, a Varian Model A 60 and a Bruker WH 270 NMR spectrometer were used. The UV spectra were recorded on a Cary Model 15 spectrophotometer. A Perkin-Elmer Model 900 instrument was used for gas chromatography. The purity of the chemicals was also checked by TLC. Melting points were determined on a Kofler Hot Stage microscope.

Synthesis. N-*(3-Dimethylaminopropyl)*-p-*anisidine* was prepared from 3-dimethylaminopropyl chloride hydrochloride, *p*-anisidine and anhydrous sodium carbonate in toluene, fol-

lowing the directions by Wright and co-workers.[3] The oily residue was distilled and the diamine was obtained in a 54 % yield, b.p. 128 °C/26.7 Pa. The product was found to contain a minor amount of *p*-anisidine, which was removed in the following way. The impure product (10 g) was dissolved in 500 ml of an aqueous phosphate buffer of pH 6.5. Addition of a few drops of phosphoric acid was necessary in order to restore the pH value to 6.5. Extractions with 15 ml portions of benzene were repeated until no more *p*-anisidine could be detected in the extracts (GLC). The aqueous phase was made strongly alkaline with potassium hydroxide solution and the diamine extracted with ether. Drying over potassium carbonate and evaporation of the solvent gave an oily residue, which could be recrystallized from hexane at −20 °C. Colourless crystals (5 g) of m.p. 23 − 24 °C were obtained.

4-Methylpentanoyl chloride was prepared from 100 g (0.86 mol) of 4-methylpentanoic acid and 154.7 g (1.3 mol) of thionyl chloride. After a 3 h reflux period, the reaction mixture was distilled, yielding 105 g of product (91 % yield), b.p. 143 − 144 °C, lit.[4] 143.8 − 144.5 °C.

N-(4'-Methoxyphenyl)-4-methylpentanamide. A solution of 56.7 g (0.46 mol) of *p*-anisidine in 500 ml of dry benzene was chilled in an ice bath, and 31 g (0.23 mol) of the above acid chloride was slowly added. After a stirring period of 1.5 h, the reaction mixture was washed with dilute hydrochloric acid, water and 5 % sodium hydrogen carbonate solution. The organic layer was dried and the solvent removed *in vacuo*. Recrystallization from ligroin gave 38.9 g of the product (76 % yield), m.p. 80.5 − 81.5 °C.

N-Isohexyl-p-*anisidine* was prepared from 22.1 g (0.10 mol) of the above amide by the method of Mićović and Mihailović.[5] Lithium tetrahydridoaluminate (5.7 g, 0.15 mol) was used. The amide was only slightly soluble in ether and was therefore initially placed in an extractor thimble through which the refluxing ether percolated. The reaction required 24 h for completion. Excess hydride was destroyed with a 1:1 mixture of ethanol and ether, and dilute sodium hydroxide solution was then added. The ethereal phase was separated off, dried and the solvent evaporated. The remainder was distilled on a 60 cm spinning-band column (Normag, Teflon band) and gave 19 g (92 %) of an almost colourless oil, b.p. 114 °C/40 Pa.

N-(3-Dimethylaminopropyl)-2,4-dinitro-4'-methoxydiphenylamine was prepared for spectral comparison by mixing 1-chloro-2,4-dinitrobenzene and *N*-(3-dimethylaminopropyl)-*p*-anisidine in the molar proportions 1:2 in dry benzene. After three days at room temperature, the mixture was washed with water. The benzene solution was dried and the solvent removed *in vacuo*. Recrystallization of the residue from ethanol gave red crystals in 61 % yield, m.p. 75.5 − 76.5 °C.

N-Isohexyl-2,4-dinitro-4'-methoxydiphenylamine was analogously prepared from 1-chloro-2,4-dinitrobenzene and *N*-isohexyl-*p*-anisidine. Recrystallization from 2-propanol gave orange-red crystals in 60 % yield, m.p. 83.5 − 84.5 °C.

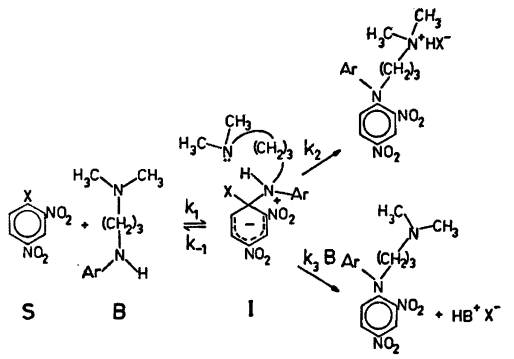
Other chemicals used in this work were of commercial origin or available from previous work in the laboratory. They were recrystallized before use, and their purity was verified by NMR, GLC and/or TLC. The benzene used as solvent in the kinetic runs was dried over Molecular Sieves 4 A and distilled through an effective column.

Kinetic procedure. For the rate measurements, the apparatus described in previous work,[1] a Beckmann Model DU spectrophotometer, equipped with a thermostated water bath in the optical path, was first used. In the course of the present work, the instrument was modified. The original null detection bridge was replaced with a digital transmission readout device. The photomultiplier current was converted into voltage in a FET operational amplifier. The voltage was read off a digital voltmeter. Furthermore, the tungsten lamp and photomultiplier were fed by suitably regulated power supplies. The long-time stability of the system was found to be superior to that of the original one.

The optical measurements were performed at a temperature of 25.00 ± 0.05 °C of the thermostated water bath at the wavelength 420 nm. Care was taken to exclude oxygen and carbon dioxide from the solutions by flushing with argon.

In order to facilitate the treatment of the data, first-order conditions were chosen. The 1-halo-2,4-dinitrobenzenes had initial concentrations 1.0×10^{-3} M or, for the fluorine compound in its reaction with T, 1.0×10^{-4} M, whereas the amine concentration range was 0.010 to 0.233 M. The DABCO concentration was 0.022 M. For each kinetic run about 20 points were taken, and all runs were made in duplicate or triplicate. The absorbance infinity values, A_∞, were obtained by preparing solutions of the appropriate concentration from the independently synthesized reaction products. These solutions had the same content of amine and DABCO as those in the runs. The molar absorptivities are, for the terminal dimethylaminopropyl compound, 10470 and for the isohexyl compound, 9670 at 420 nm. The entire UV spectra of the synthesized products and those obtained in the kinetic runs agreed, thus identifying the reactions under study. Precipitation of amine hydrochloride was not observed during the time the reaction was followed.

A possibility that must be considered is that, for the diamine T, the terminal dimethylamino group may initially attack the 1-halo-2,4-dinitrobenzene, forming a quaternary ammonium compound, which may then rearrange to the final product. No evidence was found for this,



Scheme 1. Scheme for the reaction of 1-X-2,4-dinitrobenzene with *N*-(3-dimethylaminopropyl)-*p*-anisidine.

however, neither by following the reaction with UV nor with NMR.

The tertiary amine DABCO, used as a catalyst in the present study might also react with the 1-halo-2,4-dinitrobenzene. However, solutions with these two compounds present in the same concentrations as in the kinetic runs, showed no spectral changes even after standing for several days at room temperature.

Treatment of data. From the kinetic runs, absorbance values A or, after modification of the spectrophotometer, transmittance values T were obtained. The function ln $(A_\infty - A)$ or ln log (T/T_∞) was plotted against time and the slope was calculated by the least-squares method. All calculations were made on an Olivetti Programma 101 electronic desk-top computer or on a Compucorp 425 Scientist computer. The standard deviation in each separate run, typically 1 – 2 %, equals the deviations between the different runs.

RESULTS AND DISCUSSION

A kinetic scheme for activated nucleophilic aromatic substitution with a diamine is shown in Scheme 1. Two routes have been drawn for the conversion of the intermediate to product. One involves intramolecular proton transfer from the "inner" to the "outer" nitrogen atom, whereas in the other, intermolecular proton transfer to another base takes place. Whether departure of the leaving group is concerted with this proton transfer or occurs in a separate step is hard to tell. A careful kinetic analysis by Ross [6] shows that other possibilities lead to the same dependence of the observed rate upon the concentrations of nucleophile and base. For example, in one mechanism, a rapid equilibrium between two intermediates involving proton transfer is established, followed by a slow, acid-catalyzed loss of the leaving group. Another possibility is a rate-limiting proton transfer from the first intermediate I to the base, forming a second, anionic intermediate, followed by a rapid loss of the leaving group.[7]

From Scheme 1 we obtain, using the steady state approximation, the following expression

$$k_A = \frac{k_1(k_2 + k_3[B])}{k_{-1} + k_2 + k_3[B]} \quad (1)$$

Tables 1 – 3. First- and second-order rate constants in the reactions of 1-X-2,4-dinitrobenzene, X = F, Cl and Br, with *N*-(3-dimethylaminopropyl)-*p*-anisidine (T). Substrate concentration 1.0×10^{-4} M for X = F and 1.0×10^{-3} M for X = Cl and Br.

Table 1.

[Amine]/M	$k_{obs}/10^{-4}$ s^{-1}	$k_A/10^{-2}$ dm^3 mol^{-1} s^{-1}
X = F, [DABCO] = 0 M		
0.010	5.44	5.44
0.020	11.28	5.64
0.025	14.30	5.72
0.035	21.18	6.05
X = F, [DABCO] = 0.022 M		
0.010	5.35	5.35
0.020	11.01	5.50
0.025	14.01	5.60
0.035	19.83	5.67

Table 2.

[Amine]/M	$k_{obs}/10^{-3}$ s^{-1}	$k_A/10^{-5}$ dm^3 mol^{-1} s^{-1}
X = Cl, [DABCO] = 0 M		
0.050	3.23	6.47
0.075	4.99	6.66
0.100	6.84	6.84
0.125	8.60	6.88
0.150	10.43	6.95
X = Cl, [DABCO] = 0.022 M		
0.050	3.21	6.42
0.075	4.98	6.65
0.100	6.74	6.74
0.125	8.56	6.85
0.150	10.34	6.89

Table 3.

[Amine]/M	$k_{obs}/10^{-6}$ s^{-1}	$k_A/10^{-5}$ dm^3 mol^{-1} s^{-1}
X = Br, [DABCO] = 0 M		
0.050	7.50	15.01
0.075	11.33	15.11
0.100	15.47	15.47
0.150	23.75	15.83
0.200	32.01	16.01
X = Br, [DABCO] = 0.022 M		
0.050	7.61	15.23
0.075	11.56	15.42
0.100	15.44	15.44
0.150	23.86	15.91
0.200	32.29	16.14

where k_A, the effective second-order rate constant, is related to the experimentally obtained first-order rate constants, k_{obs}, through $k_A = k_{obs}/[B]$. If bases different from B are kinetically effective in step 3, the $k_3[B]$ term in eqn. (1) should be replaced by sum a of the type $\sum_i k_{3,i}[B_i]$.

If $k_{-1} \gg k_2 + k_3[B]$, then eqn. (1) reduces to

$$k_A = \frac{k_1 k_2}{k_{-1}} + \frac{k_1 k_3}{k_{-1}}[B] \qquad (2)$$

and the reaction could be sensitive to base catalysis provided that $k_3[B]$ is not too small compared to k_2.

If $k_{-1} \ll k_2 + k_3$ [B], we obtain $k_A = k_1$, and the reaction is of first order with respect to the base concentration.

If $k_{-1} \approx k_2 + k_3$ [B], a curvilinear dependence of k_A on the base concentration is observed.[8]

An important feature of the rate constants obtained in this work (Tables 1 – 6), is that those for the fluorine compound are somewhat lower than those for the chlorine and bromine compounds in the reaction with U, whereas in the reaction with T, the order of reactivity is F ≫ Br > Cl.

It is known from many kinetic studies that the choice of fluoride ion instead of a heavier halogen as the leaving group produces dramatic rate differences. An analysis of the factors causing these differences has been given in a book by Miller.[9] In reactions with amines, one generally finds the reactivity order F ≫ Cl ≈ Br,

Tables 4 – 6. First- and second-order rate constants in the reactions of 1-X-2,4-dinitrobenzene, X = F,Cl and Br, with *N*-isohexyl-*p*-anisidine (U). Substrate concentration 1.0×10^{-3} M.

Table 4.

[Amine]/M	[DABCO]/M	$k_{obs}/10^{-7}$ s^{-1}	$k_A/10^{-6}$ dm^3 mol^{-1} s^{-1}
X = F			
0.200	0	0.80	0.4
0.200	0.022	5.17	2.6

Table 5.

[Amine]/M	$k_{obs}/10^{-7}$ s^{-1}	$k_A/10^{-6}$ dm^3 mol^{-1} s^{-1}
X = Cl, [DABCO] = 0 M		
0.100	1.13	1.13
0.150	1.69	1.13
0.175	2.00	1.14
0.200	2.33	1.17
0.225	2.70	1.20
X = Cl, [DABCO] = 0.022 M		
0.100	1.12	1.12
0.150	1.75	1.16
0.175	2.03	1.16
0.200	2.40	1.20
0.225	2.79	1.24

Table 6.

[Amine]/M	$k_{obs}/10^{-7}$ s^{-1}	$k_A/10^{-6}$ dm^3 mol^{-1} s^{-1}
X = Br, [DABCO] = 0 M		
0.100	4.19	4.19
0.117	4.99	4.27
0.150	6.58	4.39
0.200	9.01	4.51
0.225	10.37	4.61
X = Br, [DABCO] = 0.022 M		
0.100	5.58	5.58
0.117	6.45	5.51
0.150	8.28	5.52
0.200	11.05	5.53
0.233	13.02	5.59

indicating a rate-limiting first step. There are, however, also cases in which F < Cl < Br, for example, in the reactions of 1-halo-2,4-dinitrobenzenes with *N*-methylaniline in nitrobenzene solution.[10] When the *N*-methylaniline reaction was performed in ethanol,[11] the F/Cl ratio was found to be 1. With potassium acetate also present, the F/Cl ratio increased. Base catalysis is thus more important for the fluorine compound. The effect of the base is to increase the k_3[B] term in the kinetic expression of eqn. (1). Bernasconi and Zollinger [2] studied the reactions of 1-fluoro- and 1-chloro-2,4-dinitrobenzene with *p*-anisidine in benzene solution. The ratio of the rate constants for the F and the Cl reaction, at the same *p*-anisidine concentration, 0.2 M, in the absence of the catalyst DABCO is close to 100.

Rossi and Rossi [12] have measured the rates for the reactions of the same two dinitro compounds with aniline in acetone at 50 °C. They found an F/Cl ratio of 9.7 at 0.1 M base concentration.

Both in the Bernasconi and Zollinger [2] and the Rossi and Rossi paper,[12] a strong base catalysis by DABCO is observed with fluorine, whereas with chlorine moderate rate accelerations are observed.[2]

We introduce the convenient notation $k_{F,U}$ for the observed, first-order rate constant of the reaction of the fluorine compound with U, *etc.* When ratios like $k_{Cl,U}/k_{F,U}$ are calculated, the U concentration in the different kinetic runs is the same, otherwise the difference is taken into account.

First fluoride and chloride ion as leaving groups will be compared. Inspection of the data obtained in this work for the F,U- and the Cl,U-reactions shows that the $k_{F,U}/k_{Cl,U}$ ratio is 0.3, at 0.2 M amine concentration in the absence of DABCO. The rate of the Cl,U-reaction is not influenced by DABCO. Application of eqn. (1) to the Cl,U-reaction leaves us with two possibilities. Either, $k_{-1,Cl,U} \ll k_{2,Cl,U} + \sum_i k_{3,i,Cl,U}[B_i]$, *i.e.* the first step is rate-limiting, or

$k_{-1,Cl,U} \gtrsim k_{2,Cl,U} \gg \sum_i k_{3,i,Cl,U}[B_i]$ which leads to

$$k_{A,Cl,U} = \frac{k_{1,Cl,U}k_{2,Cl,U}}{k_{-1,Cl,U} + k_{2,Cl,U}}$$

We assume that the first step is rate-limiting since chloride ion is a good leaving group.

On the contrary, the F,U-reaction is catalyzed by DABCO. Due to the very slow reaction, the the F,U-reaction was measured at only one concentration of the nucleophile. From the data we can conclude that the decomposition of the intermediate I is involved in the rate-limiting step.

It is found from Tables 1 – 6, that $k_{Cl,T}/k_{Cl,U} = 61$ and that $k_{F,T}/k_{F,U}$ is approximately 250 000 (by linear extrapolation). Catalysis by DABCO is absent for the Cl,T-reaction. For the Cl,T-reaction, an analysis similar to the one just performed, gives the same two possibilities, *i.e.*, the first step is rate-limiting or $k_{-1,Cl,T} \gtrsim k_{2,Cl,T} \gg \sum_i k_{3,i,Cl,T}[B_i]$. The same assumption as above, that of a rate-limiting first step, will be made. Assuming that k_1 is solely rate-limiting, the factor $k_{Cl,T}/k_{Cl,U}$ reflects the increased nucleophilicity of T compared to U. Let us assume that a comparable factor, for example 100, can be applied to the k_1's for the F,T- and the F,U-reaction. This gives

$$250\,000 = 100\left(\frac{k_2 + \sum_i k_{3,i}[B_i]}{k_{-1} + k_2 + \sum_i k_{3,i}[B_i]}\right)_{F,T} \Bigg/ \left(\frac{k_2 + \sum_i k_{3,i}[B_i]}{k_{-1} + k_2 + \sum_i k_{3,i}[B_i]}\right)_{F,U}$$

We suggest that in the F,T-reaction the first step is completely rate-limiting, in other words, $k_{-1,F,T} \ll k_{2,F,T} + \sum_i k_{3,i,F,T}[B_i]$, *e.g.*, the F,T-parenthesis equals 1. This leads to

$$\left(\frac{k_{-1} + k_2 + \sum_i k_{3,i}[B_i]}{k_2 + \sum_i k_{3,i}[B_i]}\right)_{F,U} \approx 2500$$

or $k_{-1,F,U} \approx 2500(k_{2,F,U} + \sum_i k_{3,i,F,U}[B_i])$, in other words, the second step of the reaction is rate-limiting. This reaction is also catalyzed by DABCO.

As regards the difference between chloride and bromide ion as the leaving group, our data show that the bromine compound consistently reacts faster than the chlorine compound by a factor of 2 – 4. In activated nucleophilic aromatic substitution, the rate constants for the chlorine and bromine compounds are often remarkably similar, and the present results

provide no exception. It is apparent, that the Br,U-reaction is weakly catalyzed by DABCO, whereas the Cl,U-reaction is not catalyzed. We can offer no explanation for this interesting difference.

To summarize, we suggest that the dramatic increase of the rate constant in going from the F,U- to the F,T-system, can be explained by intramolecular base catalysis operating with the nucleophile T.

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Kinetics of the Reaction of 2,4-Dinitroanisole with 1,3-Diaminopropane and 1,3-Diamino-2,2-dimethylpropane in Benzene

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The kinetics of the reaction between 2,4-dinitroanisole and the two diamines 1,3-diaminopropane and 1,3-diamino-2,2-dimethylpropane in benzene has been studied.

The choice of the dimethyl-substituted diamine was expected to favour intramolecular base catalysis of the second step of the reaction. This was not observed, however.

In an earlier publication by the present authors,[1] the kinetics of the reaction between 2,6-dinitroanisole and a series of α,ω-diaminoalkanes $H_2N(CH_2)_nNH_2$, $n = 2-5$, was studied in methanol, dioxane, and 60 % dioxane – 40 % water. The purpose of that investigation was to demonstrate intramolecular base catalysis for the system concerned. One amino group should act as a nucleophile and the other amino group within the same molecule as a base. The relative importance of this intramolecular effect should depend on the number of methylene groups joining the two amino nitrogens. From the results, however, no definite conclusions could be drawn.

In an NMR study [2] of some nitro-substituted *N*-alkylanilines, hydrogen exchange in the compound *N*-(2,4-dinitrophenyl)-1,3-diamino-2,2-dimethylpropane was investigated. Evidence was found for intramolecular hydrogen exchange between the two different nitrogen atoms.

In a branched bifunctional compound such as 1,3-diamino-2,2-dimethylpropane, the two geminal methyl groups should favour a spatial arrangement in which the two amino groups come close to each other. This so-called "*gem*-dimethyl effect" has been utilized in various fields of physical organic chemistry.[3]

A comparison of the reaction rates of 1,3-diaminopropane and 1,3-diamino-2,2-dimethylpropane with a suitable aromatic compound, *e.g.*, 2,4-dinitroanisole, has therefore now been undertaken. The substrate 2,4-dinitroanisole was chosen instead of the 2,6-dinitro isomer used in a previous study,[1] since the reaction product *N*-(2,4-dinitrophenyl)-1,3-diamino-2,2-dimethylpropane and the similar *N*-(2,4-dinitrophenyl)-1,2-diaminoethane have been used earlier for NMR studies.[2,4] Methoxy was chosen as the leaving group since it is supposed to be a poor one in nucleophilic aromatic substitution [5] and should favour a rate-limiting decomposition of the intermediate I in the Scheme. It is in this step that the base catalysis might operate.

The same dimethyl-substituted diamine has also been studied by Pratt and Lawlor,[6] who found that 1,3-diamino-2,2-dimethylpropane aminolyzed phenyl acetate at about the same rate as butylamine.

The reactions in the present work were studied in benzene at 25 °C by the direct spectrophotometric method described earlier.[7]

EXPERIMENTAL

General. The NMR spectra were recorded on a Varian Model A 60 or on a Bruker WH 270 spectrometer and the UV spectra on a Cary Model 15 spectrophotometer. A Perkin-Elmer Model 900 instrument was used for gas chromatography. Melting points were determined on a Kofler Hot Stage microscope. The purity of the chemicals was also checked by TLC.

Synthesis. *1,3-Dinitro-2,2-dimethylpropane* was prepared from acetone and nitromethane,

using diethylamine as a catalyst.[2,8] An almost colourless product was obtained in a 31 % yield. B.p. 142–144 °C/2.4 kPa, lit.[8] 130–132 °C/1.3 kPa.

1,3-Diamino-2,2-dimethylpropane was obtained by catalytic hydrogenation over Raney Nickel of the above nitro compound according to the method of Lamm and Nordfält.[2] The crude product was distilled on a spinning band column (Normag, Teflon band, 60 cm). A colourless product with b.p. 153–154 °C, lit.[2] 154 °C and lit.[8] 154–156 °C, was obtained in a 29 % yield.

N-(2,4-Dinitrophenyl)-1,3-diaminopropane was prepared for spectral comparison by mixing 2,4-dinitroanisole and 1,3-diaminopropane in the molar proportions 1:2 in dry benzene. The reaction mixture was allowed to stand for three days at room temperature. After evaporation of the solvent and recrystallization from isopropyl alcohol, yellow crystals were obtained in a 96 % yield. M.p. 87–88 °C, lit.[9] 86–87 °C.

N-(2,4-Dinitrophenyl)-1,3-diamino-2,2-dimethylpropane was analogously prepared from 2,4-dinitroanisole and 1,3-diamino-2,2-dimethylpropane. Recrystallization from isopropyl alcohol gave yellow crystals in an 88 % yield. M.p. 114–115 °C, lit.[2] 108–110 °C.

Benzene was dried with Molecular Sieves 4 Å, and then distilled on an effective column.

1,3-Diaminopropane was purified as described earlier.[1]

2,4-Dinitroanisole was available from previous work and was recrystallized from methanol. M.p. 87–88 °C, lit.[10] 87–88 °C (unst.) and 94.5–95.5 °C (st.).

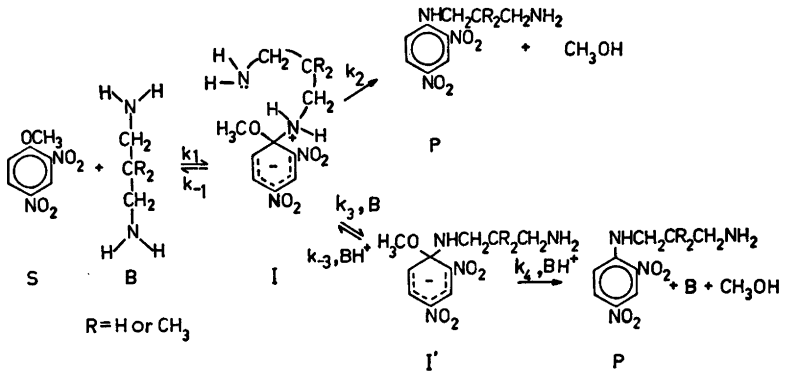
Kinetic procedure. The same apparatus as described earlier[7] was used for the kinetic measurements. The reactions were studied at 25.00 ± 0.05 °C in benzene at the wavelength 420 nm. First-order conditions were chosen, with the diamine in large excess over the substrate 2,4-dinitroanisole. The initial substrate concentration in all runs was 1.0×10^{-4} M. The amine concentration was 0.052 to 0.281 M. For each kinetic run, about 20 points were taken and all runs were made in duplicate or triplicate. To obtain the absorbance infinity value, A_∞, a mock infinity solution was prepared,[7] of the same composition as expected at infinite time in the kinetic runs. The molar absorptivity for the product in the reaction with 1,3-diaminopropane is 4850 and, for the dimethyl-substituted diamine, 5120 at 420 nm.

Bunnett and Garst have reported[11] that in the nucleophilic attack of piperidine on the 1-carbon of 2,4-dinitroanisole in methanol at 67.9 °C a side reaction may take place to form *N*-methylpiperidine and 2,4-dinitrophenol *via* S_N2 displacement on the methyl carbon.

In the present work, the possibility of the same side reaction, *i.e.*, *N*-methylation of the

Table 1. First- and second-order rate constants in the reactions of 1,3-diaminopropane and 1,3-diamino-2,2-dimethylpropane with 2,4-dinitroanisole at 25 °C in benzene. Substrate concentration 1.0×10^{-4} M.

[Amine]/ M	k_{obs}/ 10^{-5} s^{-1}	(k_{obs}/[B])/ 10^{-4} dm^3 mol^{-1} s^{-1}
1,3-Diaminopropane		
0.052	2.13	4.10
0.104	4.58	4.40
0.156	7.57	4.85
0.208	10.84	5.21
0.260	14.88	5.71
1,3-Diamino-2,2-dimethylpropane		
0.056	1.47	2.62
0.112	3.33	2.98
0.169	5.39	3.20
0.225	7.50	3.34
0.281	10.21	3.63



Scheme 1. Scheme for the reaction between a diamine and 2,4-dinitroanisole.

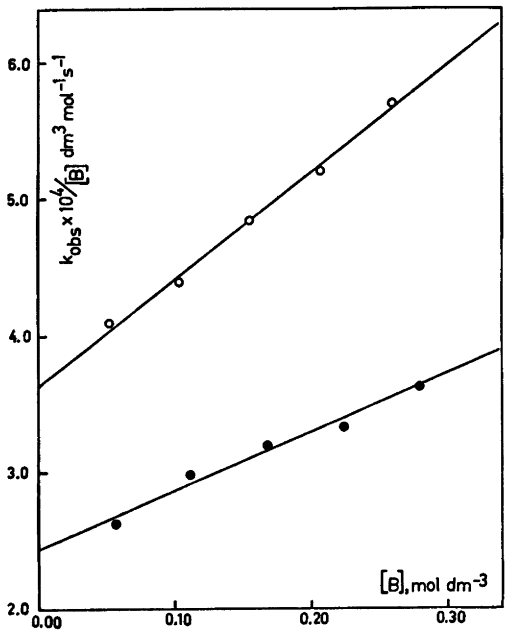


Fig. 1. Plot of the apparent second-order rate constant *versus* base concentration in the reactions of 1,3-diaminopropane (open circles) and 1,3-diamino-2,2-dimethylpropane (filled circles) with 2,4-dinitroanisole in benzene.

diamines by 2,4-dinitroanisole, was regarded. The entire UV spectrum was therefore recorded in a few runs during the reaction and after completion. The UV spectra of the mock infinity solutions were identical with those obtained from the kinetic runs. The conclusion was therefore drawn that this side reaction is unimportant in the present work, in which the rates were determined at 25 °C.

Treatment of data. The same treatment of data as described earlier [7] was used. The standard deviation in each separate run, typically less than 1 %, equals the deviations between the different runs.

RESULTS AND DISCUSSION

If intramolecular base catalysis is to be kinetically observable, it requires that the second step of the reaction, *i.e.*, the decomposition of the intermediate I, is rate-limiting. Using the symbols given in Scheme 1 and the same deduction as in a previous paper,[1] we can then express the observed first-order rate constant as a sum of two terms

$$k_{obs} = k_2'[B] + k_3'[B]^2 \quad (1)$$

The second- and third-order rate constants k_2' and k_3' can be obtained from the values in Table 1 as the intercept and the slope, respectively, from a plot of $k_{obs}/[B]$ *versus* [B] as in Fig. 1.

For the reaction with 1,3-diaminopropane, k_2' and k_3' are found to be $(3.64 \pm 0.06) \times 10^{-4}$ dm^3 mol^{-1} s^{-1} and $(7.8 \pm 0.4) \times 10^{-4}$ (dm^3)^2 mol^{-2} s^{-1}, respectively, and in the reaction with 1,3-diamino-2,2-dimethylpropane, $(2.44 \pm 0.07) \times 10^{-4}$ dm^3 mol^{-1} s^{-1} and $(4.2 \pm 0.4) \times 10^{-4}$ (dm^3)^2 mol^{-2} s^{-1}.

The ratio k_2'/k_3' is, for the former diamine, 0.47 mol dm^{-3} and, for the latter, 0.58 mol dm^{-3}. Our expectation was that this ratio should be larger for the dimethyl-substituted diamine, where the "*gem*-dimethyl effect" might favour intramolecular base catalysis. The ratio is in fact somewhat larger for the branched compound, but the difference is too small to allow any conclusions to be drawn whether intramolecular base catalysis is operating or not. The constant k_2' accounts for all kinds or catalysis except for that by B and even for the possibility that the proton is removed from nitrogen in a rapid third step.

We must also consider the possibility that the first step in the reaction is rate-limiting and that the observed dependence of k_{obs} on [B] is merely a medium effect. At any given amine concentration [B], the rate constants for the unsubstituted and the branched diamine differ by a factor of about 1.6 (see Fig. 1). This would mean that the medium effect on the rate of the addition step is unaffected by the dimethyl-substitution in the diamine.

Acknowledgement. We wish to thank Professor Lars Melander for constructive criticism and helpful discussions.

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Pyrylium Salts. Part VII.* A Derivative of *anti*-1,5:6,10-Bisepithiocyclodecene

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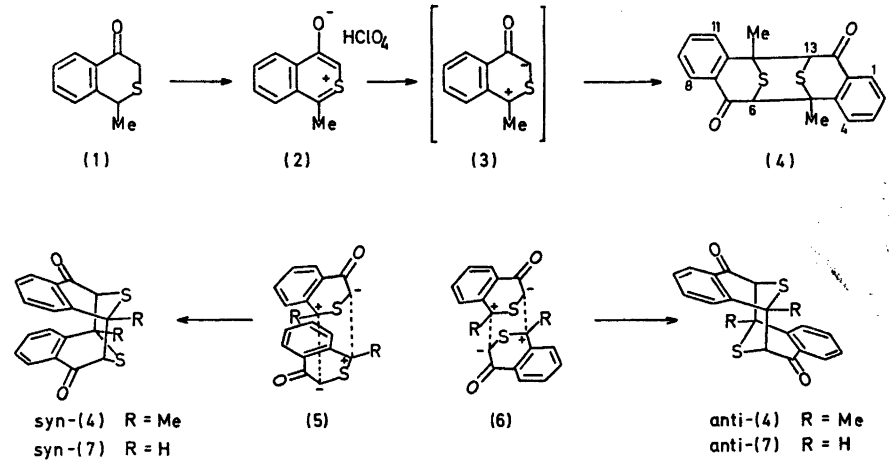
The perchloric acid salt of 1-methyl-2-benzothiopyrylium-4-olate has been obtained from 1-methylisothiochroman-4-one. The pyrylium salt is rapidly dimerised in the presence of a base. From spectroscopic and single crystal X-ray data the product has been identified as *anti*-5,12-dimethyl-5,6,12,13-tetrahydro-5,13:6,12-bisepithiodibenzo[*a*,*f*]cyclodecene-7,14-dione.

1,3-Dipolar character has been demonstrated for pyridinium-3-olates [2] and *N*-methylisoquinolium-4-olate [3] by cycloaddition reactions under forcing conditions. The aromatically less stabilised isoelectronic 2-benzothiopyrylium-4-olate, however, undergoes preferential 1,3-dimerisation to the bisepithiocyclodecene (*7*),[4] while the dimerisation of the parent, thiopyrylium-3-olate and its 5-methyl derivative takes another course.[1] 2-Benzopyrylium-4-olate which is the least aromatically stabilised member of the present hetero-analogue series, has yielded some 1,3-dimerised product besides polymeric material.[5] Stabilisation of the pyrylium system by phenyl group substitution such as in 1,3-diphenyl-2-benzopyrylium-4-olate, which is formed by valence isomerisation of 2,3-epoxy-2,3-diphenylindan-1-one, results in derivatives which will behave as 1,3-dipolar reactants.[6] Photolysis of 2,3-epoxy-2-methyl-3-phenylindan-1-one, however, has yielded a minor dimeric by-product for which the substituted dioxa-analogue of (*7*) was suggested without specification of the stereoisomer involved.[7] The *syn*:*anti*-isomer ratio for (*7*) was about 7:1 in the formation from 2-benzothiopyrylium-4-olate;[4] the *syn*-isomer was also the major product in the dimerisation of thiopyrylium-3-olate and its 5-methyl analogue.[1] The *anti*-isomer, however, is probably thermodynamically more stable because of less non-bonded interaction. X-Ray data of *syn*-(*7*) show short interplanar distances and stretching of the C5—C6 and C12—C13 bonds implying repulsion between the periplanar faces.[8]

The directional face-to-face dimerisation can be visualised through the electron density distribution as indicated by the charges in structures (*5*) and (*6*). The incipient positive charge on C1 is stabilised by the fused benzo group. In this work we have studied the effect of an electron donating 1-substituent on the reactivity. For this purpose 1-methylbenzo[*c*]-thiopyrylium-4-olate (*2*) was synthesised and its dimerisation studied. The 1-methyl derivative (*2*) was prepared by hydride abstraction from 1-methylisothiochroman-4-one (*1*) by means of triphenylmethyl perchlorate as previously reported in the syntheses of some of its analogues.[1,4] (*1*) can be prepared by cyclisation of (1-phenylethylthio)acetic acid *via* its acid chloride under Friedel-Crafts conditions [9] or from the acid by means of phosphorus pentoxide as reported [10] for isothiochroman-4-one. The yields, however, are low because of the easy cleavage of the benzylic sulfide bond. The recently reported [11] cyclisation of [1-(2-carboxyphenyl)ethylthio]acetic acid under alkaline conditions circumvents the ready acid-catalysed benzyl thioether cleavage.

The structure of the thiopyrylium betaine (*2*) isolated as the perchloric acid salt is evident from spectroscopic data. The NMR spectrum (CD_3CN) has a methyl proton singlet at δ 3.4

* For Part VI, see Ref. 1.



Scheme 1.

and otherwise only aromatic protons; The UV spectrum (MeCN) has several absorption maxima with the highest wavelength band at 392 nm.

Treatment of the perchloric acid salt (*2*) dissolved in THF with triethylamine smoothly gave the dimeric product (*4*) at room temperature. UV absorptions recorded for a reaction run at −60 °C showed the disappearance of the longwave 392 nm band of the perchloric acid salt (*2*) with the transient appearance of a longwave band at *ca.* 450 nm; the latter is attributed to the intermediate thiopyrylium betaine. The progress of the reaction was also evident by the fading of the greenish-yellow colour of the solution. It is apparent that the methyl substituent has decreased the rate of dimerisation in comparison with the unsubstituted betaine. The reaction gave essentially one product. The molecular ion at *m/e* 352 confirms dimerisation. The NMR spectrum (DMSO-d_6) has the six protons for Me-5 and Me-12 as a singlet at δ 1.5 and H-6 and H-13 as a singlet at δ 4.0. In the unsubstituted dimer (*7*) the methine proton signals are split by 2 Hz ascribed to coupling between the methine protons on either side of the sulfur bridge.[4] Two of the aromatic protons resonate in the region δ 7.9 − 8.2 while the remaining aromatic proton signals are in the region δ 7.5 − 7.7. The lower field signals are ascribed to the aromatic protons in the *ortho*-position to the carbonyl groups. In the parent analogue (*7*) the corresponding protons in the *anti*-isomer resonate at lower fields than the other aromatic protons which was ascribed to the carbonyl anisotropy effect; these protons in the *syn*-isomer are in the aromatic shielding zone and therefore resonate at higher fields.[4] The NMR data are thus in accordance with formation of the *anti*-isomer which was confirmed by single crystal X-ray analysis. The oscillation diagram showed no symmetry. Zero-level Weissenberg diagram showed plane group *pgg* and the space group is therefore uniquely determined to be $P2_1/c$. Measurements on the films gave $a = 7.46$ Å, $b = 10.60$ Å and c sin $\beta = 10.33$ Å. The corresponding unit cell volume is $V = 817$ Å^3. The calculated density with two molecules in the cell is 1.44 g/cm^3 ($\varrho_{obs} \sim 1.45$ g/cm^3). For an *ordered* structure in space group $P2_1/c$ two molecules per unit cell are possible if and only if the molecules themselves possess a centre of symmetry which shows that the dimer must be the *anti*-isomer.

The base peak in the mass spectrum was at *m/e* 177 ($C_{10}H_9OS$); the relative intensities for the molecular ion and the ions at half of its mass number (*m/e* 176) were 22 and 31 %, respectively. The spectrum of the perchloric acid salt of the thiopyrylium betaine shows substantial pyrolytic dimerisation. The relative intensities vary somewhat in accordance with pyrolytic reactions. In a representative spectrum the base peak has the mass number of the betaine (*m/e* 176) while *m/e* 177 is 39 %

and the dimeric molecular ion (*m/e* 352) intensity 5 %. The data are consistent with competition between direct evaporation of a monomeric species and dimerisation before evaporation.

The dimeric structures can be regarded as 1,4-dithiane derivatives fixed rigidly in the boat or the chair conformation. The dihedral angle in the *syn*-isomer (boat conformation) is small. In the *anti*-isomer (chair conformation), however, the dihedral angle is probably not far from 60° and this isomer therefore has the less vicinal steric repulsion. The effect of the 1-methyl group is therefore to stabilise the incipient positive charge on C1, which is expected to increase the selectivity in the reaction of this species, as well as to increase the relative activation energy for the formation of the *syn*-isomer due to larger non-bonded interaction than in the *anti*-isomer. It is suggested that further substitution will favour *anti*-isomer formation when dimerisation occurs. The vicinal interaction in the dimer may be an important reason why 1,3-diphenyl derivatives do not dimerise in this way, but undergo 1,3-dipolar cycloadditions.

EXPERIMENTAL

The NMR spectra were recorded on a Varian A-60A or a Varian A-100 instrument, the UV spectra on a Cary 14 spectrophotometer and the mass spectra on an AEI-902 mass spectrometer.

4-Hydroxy-1-methyl-2-benzothiopyrylium perchlorate (2). 1-Methylisothiochroman-4-one (1.78 g, 0.01 mol) was dissolved in anhydrous acetonitrile (10 ml) and triphenylmethyl perchlorate (3.42 g, 0.01 mol) added. The reaction medium was heated at 60 °C for 10 min, left to cool and poured into anhydrous ether (200 ml) The precipitated greenish perchlorate was recrystallised from acetic acid; yield 1.90 g (70 %), m.p. 160–161 °C (decomp.) (Found: C 43.45; H 3.13. Calc. for $C_{10}H_8OS.HClO_4$: C 43.40; H 3.28); $\delta(CD_3CN)$ 3.4 (Me), 8.0–8.3 (H-arom); λ_{max} (MeCN) 228 (log ε 4.12), 258 (4.26), 293 (3.34), 312 (3.33), 324 (3.10), and 392 nm (3.81).

anti-*5,12-Dimethyl-5,6,12,13-tetrahydro-5,13:-6,12-bisepithiodibenzo*[a,f]*cyclodecene-7,14-dione (4).* 4-Hydroxy-1-methyl-2-benzothiopyrylium perchlorate (2.76 g, 0.01 mol) was dissolved in anhydrous THF (200 ml) and a solution of triethylamine (1.01 g, 0.01 mol) in anhydrous THF (50 ml) added dropwise over 1 h with stirring at room temperature. The solution was coloured deeper yellowish-green during the triethylamine addition and the colour disappeared when the reaction was completed. The solution was then washed with water, dried and evaporated. The residual material was dissolved in methylene chloride and the solution chromatographed on silica gel (0.2–0.5 mm). The material eluted with methylene chloride was recrystallized from chloroform; yield 1.23 g (70 %), m.p. 276–277 °C. (Found: C 67.99; H 4.37. Calc. for $C_{20}H_{16}O_2S_2$: C 68.17; H 4.55); δ(DMSO-d_6) 1.5 (s, Me-5, Me-12) 4.0 (s, H-6, H-13), 7.5–7.7 (6*H*-Ph), and 7.9–8.3 (2H-Ph *peri* to CO); λ_{max} (MeCN) 230 (log ε 4.30), 249 (4.32), 301 (3.55), 360 (2.37), 378 (2.82), and 395 nm (2.72); λ_{max} (KBr) 1650 cm^{-1} (CO); *m/e* (m.s.) 352 (22 %, M), 319 (19), 208 (18), 177 (100), 176 (31), 148 (20), and 147 (24).

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Formation of 1,4-Anhydro-3-deoxypentitol-2-carboxylic Acids by Alkaline Degradation of Cellulose

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An anhydrosaccharinic acid obtained in large amounts by end-wise degradation of cellulose in alkaline media and in small amounts in hydrolysates of cellulose has been identified as a 1,4-anhydro-3-deoxypentitol-2-carboxylic acid (3-hydroxy-5-(hydroxymethyl)oxolane-3-carboxylic acid). Structural evidence was obtained by GC-MS studies of the compounds (as Me_3Si derivatives) obtained on degradation of the acid to 1,4-anhydro-3-deoxypentitol by reduction of the methyl ester followed by periodate oxidation and borohydride reduction.

In aqueous alkali, the acid is likely to be formed from a dicarbonyl precursor by benzilic acid rearrangement.

In aqueous alkali, cellulose is degraded from the reducing end. A main reaction pathway is isomerization of the glucose end-groups followed by β-alkoxyelimination and the formation of a new glucose end-group. The dicarbonyl intermediate formed is converted to the two diastereomeric 3-deoxy-2-*C*-(hydroxymethyl)-pentonic (isosaccharinic) acids by a benzilic acid rearrangement. Several carboxylic acids formed in competing fragmentation reactions have been isolated. Under certain working conditions [1] the third most abundant acid (on weight basis) in the solution is an anhydro acid, previously denoted anhydroisosaccharinic acid. In the present paper this acid is shown to be a 1,4-anhydro-3-deoxypentitol-2-carboxylic acid. The acid is formed in appreciable amounts also during alkali treatment in the presence of oxygen [2] or polysulfide.[3] Trace amounts of the diastereomer were isolated by anion exchange chromatography from the solution obtained on oxygen-alkali treatment of cellulose.[2]

Interestingly, the major diastereomer was isolated from hydrolysates obtained by acid hydrolysis of cellulose with hydrochloric acid. Model experiments with glucose under conditions simulating those used to determine carboxylic acid groups in cellulose showed that a small amount of the acid was formed from glucose.[4] The procedure used included treatment at pH 8 at room temperature to hydrolyze ester linkages. Experiments carried out more recently in which the hydrolysate was separated directly on the acetate form of an anion exchange resin showed that the anhydro acid was formed. The amount obtained from cellulose decreased on lowering the temperature and acid concentration during hydrolysis with hydrochloric acid and was negligible after mild hydrolysis with trifluoroacetic acid.

STRUCTURE DETERMINATION

In the previous study,[1] the composition of the anhydro acid was shown to be $C_5H_7O(OH)_2COOH$. Periodate oxidation showed that no vicinal hydroxyl groups were present. Reduction of the carboxylic acid group resulted in a polyalcohol which on oxidation with periodate gave rise to formaldehyde. This demonstrates that one of the hydroxyl groups was present in the α-position. Ring opening gave rise to the two diastereomeric 3-deoxy-2-*C*-(hydroxymethyl)pentonic acids.

From these observations, it is concluded that the acid has the furanoid structure *1* (Fig. 1) or the corresponding pyranoid structure. To confirm this conclusion and to distinguish between the two ring forms, the acid was converted

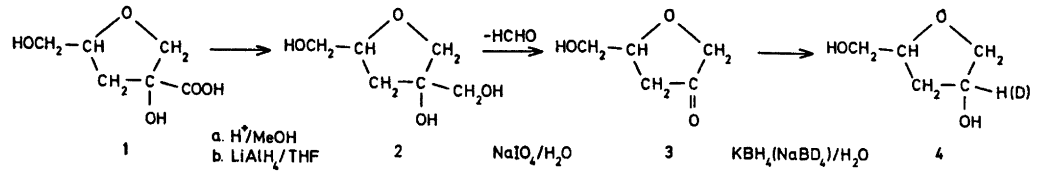

Fig. 1. Reactions of 1,4-anhydro-3-deoxypentitol-2-carboxylic acids for structure determination.

to its methyl ester and reduced with lithium aluminium hydride. The polyalcohol *2* was oxidized with periodate and the resulting ketose *3* reduced with borohydride to obtain the alcohol(s) *4*. A reaction scheme valid for the furanoid form is given in Fig. 1. All species were analysed by GC-MS after preparation of the trimethylsilyl (Me_3Si) derivatives under conditions previously used.[5] The GC results indicated almost quantitative yield in each of the reaction steps. The mass spectra of *1* and *2* (Fig. 2) are in full agreement with the structural features mentioned above but could not be used to unequivocally distinguish between the furanoid and the pyranoid structures. In contrast to the pyranoid derivative of *4*, the furanoid derivative can be predicted to give an abundant *m/e* 159 ion by loss (α-cleavage) of the

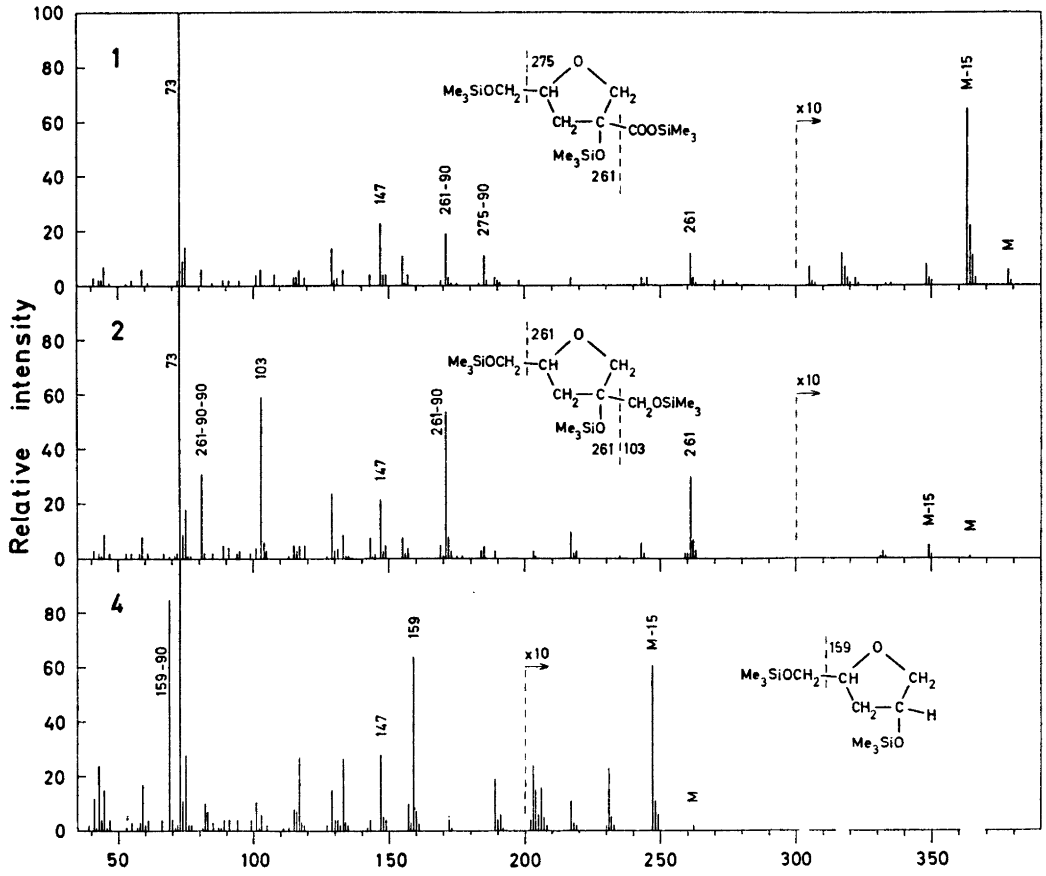

Fig. 2. Mass spectra at 70 eV of the Me_3Si derivatives of 1,4-anhydro-3-deoxypentitol-2-carboxylic acid (*1*), 1,4-anhydro-3-deoxy-2-*C*-(hydroxymethyl)pentitol (*2*) and 1,4-anhydro-3-deoxypentitol (*4*).

CH_2OSiMe_3 branch. Charge delocalization over the ring oxygen should promote such fragmentation. A corresponding prominent peak present in the spectrum (Fig. 2) demonstrates a furanoid structure. This structure was confirmed by an almost complete shift to *m/e* 160 when the last reduction step was made with borodeuteride. For the pyranoid derivative, an *m/e* 159 ion might be formed by a rearrangement involving the loss of a CH_2OSiMe_3 group, although this fragmentation reaction appears unlikely. The pyranoid derivative would, however, contain two symmetrically located $CHOSiMe_3$ groups, and in the deuterated derivative only one of these would contain deuterium. Hence, approximately 50 % of the *m/e* 159 ions would not be shifted to *m/e* 160 for the deuterated form. A spectrum of the pyranoid derivative would also be expected to differ from the observed spectrum in several other respects. The main fragmentation features are indicated in Fig. 2 and include the formation of the M − 15, *m/e* 73 and *m/e* 147 ions which are characteristic of Me_3Si derivatives. The remaining ions, marked by their mass, are the specific α-cleavage ions and the ions produced by further elimination of Me_3SiOH (90 mass units). The mass spectra of the Me_3Si derivatives of *3* and the *syn* and *anti* isomers of its oxime were also recorded and were compatible with the anticipated structures.

The configuration at the two chiral carbon atoms were not determined. Lactone formation might be possible for the diastereomer with the carboxyl and the primary hydroxyl groups on the same side of the ring. Efforts to prepare a lactone by evaporation with 1 M acetic acid or 6 N hydrochloric acid at 50 °C failed and resulted in the production of intermolecular esters. Previous indications for the formation of a lactone [1] should, therefore, be disregarded.

DISCUSSION

Anhydroisosaccharinic acid, now denoted 1,4-anhydro-3-deoxypentitol-2-carboxylic acid, is obtained in high yield from hydrocellulose at a temperature so low that attack along the cellulose chain is excluded. This demonstrates that the acid is formed by rearrangement and elimination of the terminal reducing glucose moiety. A new reducing glucose end-group is formed in this reaction. The observation that the anhydro acid like 3-deoxy-2-*C*-(hydroxymethyl)pentonic acids contains a methylene group at C-3 which in these species corresponds to C-4 in the reducing glucose moiety strongly suggests that the reaction which gives rise to the methylene group is a β-alkoxyelimination. Accordingly, the acid was formed also by alkali treatment of cellobiose but not from laminarin. Separate experiments have shown that no detectable amount of the anhydro acid was formed when the 3-deoxy-2-*C*-(hydroxymethyl)pentonic acids were heated in alkaline solution at 100 °C for several hours.

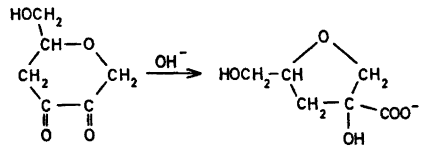

Fig. 3. Formation of 1,4-anhydro-3-deoxypentitol-2-carboxylic acids from 1,5-anhydro-4-deoxy-2,3-hexodiulose.

Since dicarbonyl compounds formed as intermediates after β-alkoxyelimination undergo benzilic acid rearrangement on treatment with alkali, it can be concluded that 1,5-anhydro-4-deoxy-2,3-hexodiulose is a precursor of the 1,4-anhydro-3-deoxypentitol-2-carboxylic acids (Fig. 3). Formation of these acids from cellobial by this route has been postulated by Corbett and Kidd.[6] Paper chromatographic evidence was presented for the formation of one of the acids. Benzilic acid rearrangements of other pyranoid dicarbonyl compounds have been demonstrated.[7] In the present case the predominance of one of the diastereomers demonstrates a high stereospecificity for the rearrangement.

EXPERIMENTAL

Degradation of 1,4-anhydro-3-deoxypentitol-2-carboxylic acid. Each of the reaction steps shown in Fig. 1 was first carried out on an analytical scale starting with approximately 10 mg of the substances. The products were studied by GC and the reaction conditions modified to maximize the yield. Subsequently, the compounds were prepared in the 100 mg range by scaling up the experiments.

The methyl ester was prepared by treating the acid (15 mg/ml) with methanolic hydrochloric acid (1.5 M) for 1 h at ambient temperature. The ester (10 mg/ml) was reduced in

tetrahydrofuran with 2 mg $LiAlH_4$/mg ester. The solvent was prepurified by passing it through a column of aluminium oxide. The hydride was added carefully and the reaction mixture kept at 40 °C overnight with ultrasonic treatment. Excess hydride was destroyed and cations removed by the addition of wet cation exchanger (H^+) until the solution became clear. The product was isolated by transfer of the slurry to a column, rinsing with water and evaporation to dryness.

The periodate oxidation was achieved by mixing equal volumes of aqueous solutions of the polyalcohol and sodium periodate in a dark flask. The concentrations were chosen to give a two-fold molar excess and a 0.05 M final concentration of periodate. The reaction mixture was kept overnight at 30 °C. The solution was then passed through columns packed with an anion exchanger (Ac^-) and a cation exchanger (H^+). The product was isolated from the effluent by evaporation.

For the final reduction, aqueous solutions of KBH_4 or $NaBD_4$ (5 mg/ml) and of the product from the previous step (10 mg/ml) were prepared, cooled to 0 °C and mixed to give an approximately two-fold molar excess of the hydrides. The mixture was kept in a refrigerator overnight, after which a cation exchanger (H^+) was added in excess of the amount needed for hydrogen formation to cease. The resin was washed with water and boric acid was removed by twice evaporating with methanol to give the pure 1,4-anhydro-3-deoxypentitol(s).

Gas chromatographic data. Relative retentions of the Me_3Si derivatives, directly comparable with previously published data for related compounds,[5] were determined with the derivative of glucitol as reference. The same equipment and instrumental settings as in the previous study were used. The relative retention of the 1,4-anhydro-3-deoxypentitol-2-carboxylic acid (*1*) was 0.206 on OV-1 (160 °C), 0.436 on OV-17 (160 °C), 0.456 on QF-1 (120 °C) and 0.530 on XE-60 (120 °C). The value for the methyl ester on QF-1 (120 °C) was 0.361. The corresponding data for the other compounds were 0.298 for the 1,4-anhydro-3-deoxy-2-*C*-(hydroxymethyl)pentitol (*2*), 0.099 for the 1,4-anhydro-3-deoxy-2-pentulose (*3*), 0.088 for the 1,4-anhydro-3-deoxypentitol(s) (*4*) and 0.101 and 0.117 for the two isomers of the 1,4-anhydro-3-deoxy-2-pentulose oxime.

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The Mass Spectrometric Fragmentation of Some 5,5-Dimethyl-2-oxo-1,3,2-dioxaphosphorinanes

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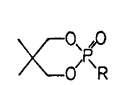
The mass spectra of some 5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinanes are examined. Study of deuterated analogues and high resolution measurements allow the observed fragmentation to be rationalised. The system is typified by multiple hydrogen transfers during fragmentation. The site of charge location and the measured ionisation potentials are briefly considered.

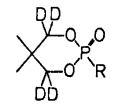
A number of reports in the literature discuss the mass spectra of organic esters of the phosphorus oxyacids.[1–12] 2-Oxo-1,3,2-dioxaphosphorinane (*1*) is a cyclic ester of phosphonic acid and the majority of the compounds encompassed by the present work may be regarded in this way also. The compounds here examined and whose structures (*2* – *14*) are given in Scheme 1 were prepared by established procedures [13–18] or by simple modification of these to allow of deuteration The mass spectra are presented in Fig. 1.

1

The fragmentation of the compounds *2* – *14* may be readily compartmentalised according to whether it is common to the system as a whole or depends on the presence of a particular substituent. In all cases hydrogen migration from the cyclic alkyl residue is a feature of the first type of fragmentation, while the most typical charged fragments of the second class are the result of simple bond fission.



2, R = Me; *3*, R = CH_2Ph; *4*, R = CMe_3; *5*, R = Ph; *6*, R = OPh; *7*, R = OMe; *8*, R = SMe; *9*, R = NMe_2; *10*, R = CPh_3; *11*, R = Cl; *12*, R = CCl_3



13, R = Me; *14*, R = CH_2Ph

Scheme 1. Structures of the compounds (*2* – *14*) studied in the present investigation.

CHARACTERISTIC FRAGMENTATION

Although the term characteristic fragmentation may be used to describe the discussion which follows, it must be borne in mind that the observation of such fragmentation depends upon the initial site of charge location and on the stability of the particular fragment under consideration. Thus, drastic changes in the electronic properties of substituents must inevitably give rise to considerable changes in the type of spectrum observed.

The identity of the various fragments in the discussion which follows in this section is based on the spectra of the deuterated compounds (*13* and *14*) and on high resolution studies carried out on the compounds *2* and *3*. The measured masses for the various fragments are given in the experimental part.

Characteristic fragments occur at M – 15, M – 28, M – 30, M – 55, M – 67 and M – 85 mass units in most of the accompanying spectra of the compounds *2* – *9*, while these fragments are largely absent from the spectra of *10* – *12*. This difference may be ascribed to a variation

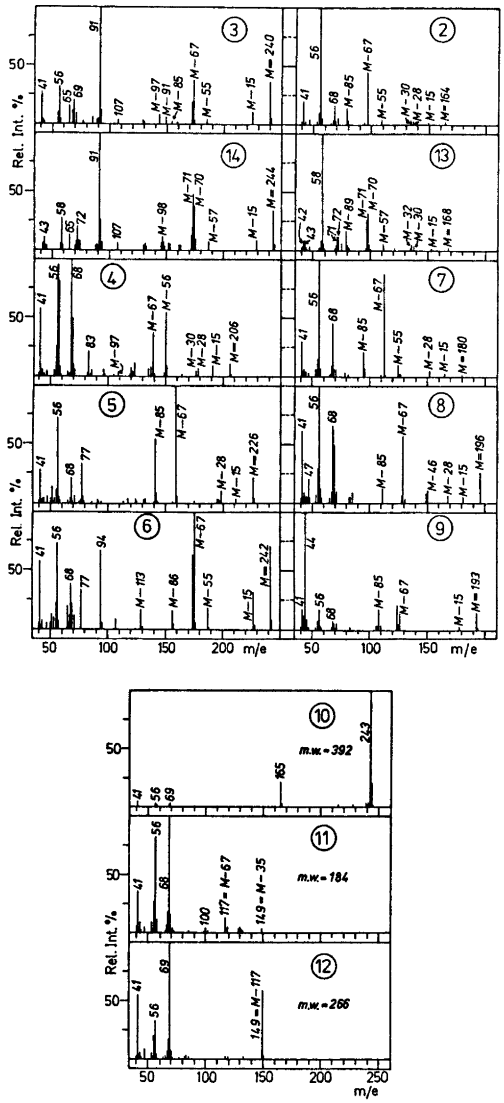
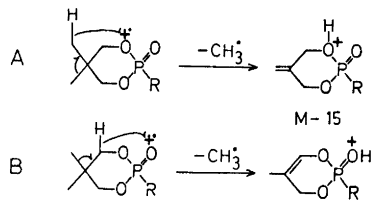


Fig. 1. Mass spectra of the compounds *2* – *14*.

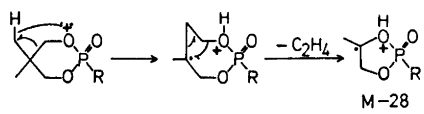
in the site of initial charge location and/or stability factors. The three characteristic low mass ions at *m/e* 41, 56 and 68 (and/or 69) are present in all cases.

The M – 15 ion is due to the loss of a methyl radical and as can be seen from the spectra of the deuterated compounds *13* and *14* the fragment eliminated comes entirely from the *gem*-methyl groups at position 5. Two possible fragmentation paths may be considered according to whether the charge is placed initially on the ester oxygen atoms or on the phosphoryl oxygen atom. Both paths involve hydrogen transfer and it is not possible to distinguish between them on the basis of the present evidence (Scheme 2).



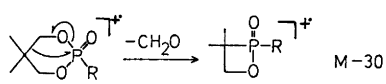
Scheme 2.

Deuteration shows that the M – 28 ion is shifted quantitatively by two mass units and thus its formation must involve loss of the carbon atoms 4 or 6. The other carbon atom lost is most likely derived from the *gem*-methyl groups and a tentative mechanism (Scheme 3), satifying these requirements, may be conceived based on the same hydrogen shift as in Scheme 2A.



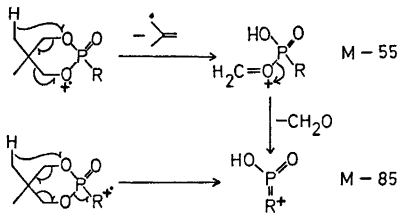
Scheme 3.

The M – 30 ion is generally of low intensity, but like that above is shifted by two mass units on deuteration. This fact and the high resolution data accord with its origin through elimination of formaldehyde from the ring (Scheme 4).



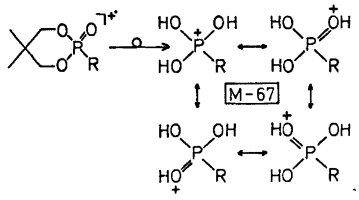
Scheme 4.

The M – 55 and M – 85 mass unit ions retain, respectively, two and none of the deuterium atoms from the molecule in the case of the compounds *13* and *14*, and their origin may thus be formulated as in Scheme 5. The M – 85 ion may also be formed by a concerted process where suitable charge location is available (Scheme 5).



Scheme 5.

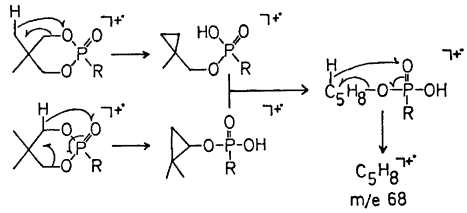
The ion at M – 67 is due to the loss of the hydrocarbon residue of the phosphorinane ring with prior transfer of three hydrogen atoms to the charged phophorus-containing fragment eliminated. The occurrence of this ion in the esters of phosphonic acid has previously been commented on by McLafferty [1] and Occolowitz and White,[2] and its formation related to the ability of the resultant even electron ion to stabilise the charge through resonance. That this is probably the case is supported by the fact that this ion provides the base peak in the case of the phenyl derivative *5* and the phenoxy compound *6* where additional resonance structures may be written. Only the general resonance forms are shown in Scheme 6. The methoxy, thiomethoxy and dimethylamino compounds (*7* – *9*) show a somewhat lower intensity for this ion, but the differences between these cases may be explained in terms of variation in charge pattern in the molecular ion, and this point will not be further pursued.



Scheme 6.

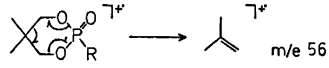
The origin of the hydrogen atoms transferred to the charged fragment in the formation of the M – 67 ion is somewhat surprising, in that the hydrogens involved, as evidenced by the deuterated compounds, are largely derived from the *gem*-methyl groups. This provides some circumstantial evidence for the siting of the charge in the molecular ion at the ester oxygen atoms since the required hydrogen shifts seem more probable under these conditions. While a detailed mechanism cannot be suggested at the present time, the comments made below on the formation of the *m/e* 68 ion also apply to this ion in a general way.

The ion at *m/e* 68 is shifted in the deuterated analogues to masses 71 and 72, thus showing that hydrogens of different origin may be transferred. Scheme 7 suggests possible origins for this ion.



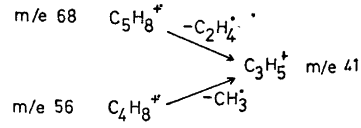
Scheme 7.

The *m/e* 56 ion is shifted quantitatively to *m/e* 58 in the compounds *13* and *14*, and may be readily rationalised as resulting from bond rearrangement in the phosphorinane ring (Scheme 8). That this ion is normally the strongest in the lower end of the mass spectrum fits well with such a mechanism which involves a simpler process than that described immediately above or that giving rise to the *m/e* 41 ion below.



Scheme 8.

The ion at *m/e* 41 (C_3H_5) can be formed only as a result of considerable prior rearrangement and seems likely to go *via* either the *m/e* 68 or *m/e* 56 species. Such an explanation requires the loss of an ethenyl or a methyl radical, respectively, as the final stage of the process, Scheme 9.

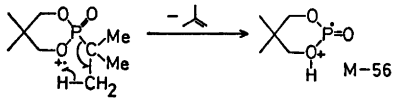


Scheme 9.

SPECIFIC FRAGMENTATION ATTRIBUTABLE TO SUBSTITUENTS AT PHOSPHORUS

While the presence of substituents alters, sometimes drastically, the intensities of the characteristic ions described above, a number of specific fragments attributable to particular substituents normally occur. That the former normally dominate in the spectrum is largely explicable in terms of the highly directive effects of the dioxaphosphorinane ring system. That this is the case is seen in the fact that specific fragmentation is most marked in the presence of those substituents expected to have the largest effects on the electronic distribution.

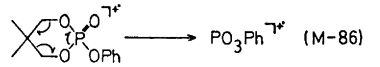
The compound *2* having a *P*-methyl group provides no fragment ion specific for the presence of the methyl group. However, when the bulkier *tert*-butyl group is present, compound *4*, a change occurs in the characteristic fragmentation in that the M − 55 ion is apparently largely replaced by an M − 56 ion. This may be rationalised in terms of the loss of isobutene (Scheme 10), a process involving H-transfer to one of the ring oxygen atoms and hence in direct competition with that envisaged by Scheme 2A.



Scheme 10.

As expected, the presence of the *P*-benzyl and *P*-phenyl groups in the compounds *3* and *5* leads to the observation of *m/e* 91 and 77 ions, respectively, with charge retention on the aromatic moiety. A relatively low intensity M − 91 ion with charge retention on the phosphorinane ring is also observed in the former case.

The ability of aromatic moieties to stabilise the charge is further seen in the spectrum of the compound *6* which has a phenoxy substituent. Ions at *m/e* 77 and 94 are readily explicable as phenyl and phenol species, respectively. However, the M − 86 ion is apparently specifically favoured in this case over the normal M − 85 ion, and represents the most unique feature of the spectrum (see Scheme 11).



Scheme 11.

While the methoxy function in compound *7* is not revealed by specific fragmentation, ions of low intensity at *m/e* 47 and M − 46 mass units in compound *8* can be readily associated with the presence of the *S*-methyl group. These observations are in contrast with the finding in the spectrum of the dimethylamino compound (*9*) that the low mass *m/e* 44 ion is by far the strongest ion in the whole mass range. This probably reflects initial charge siting at nitrogen in agreement with the known data for the relative abundances of similar ions in amino-alcohols.

None of the remaining compounds (*10* − *12*) provide molecular ions and the high mass part of the characteristic fragmentation of the system is inhibited. It should also be noted that in both *10* and *12* the *m/e* 68 ion is replaced by an *m/e* 69 ion, presumably of similar origin but reflecting the drastic changes in electron distribution and charge stabilisation caused by the substituents here present. In the case of the trityl compound (*10*) it is apparent that the charge is preferentially located on the trityl moiety as evidenced by the intense trityl and diphenylmethenyl ions which completely dominate the spectrum. The chloro substituent in compound *11* and the trichloromethyl group in compound *12* each make available an energetically favourable radical loss to yield M − 35 and M − 117 ions, respectively.

GENERAL CONSIDERATIONS

The compounds studied here are unusual in having present so many hetero-atoms in such proximity. This, and the wide variation expected in the electronic distribution within the molecule as highly divergent substituents are attached to the phosphorus atom make intuitive consideration of charge siting in the molecular ion more difficult than in most organic molecules.

Previous work by Bafus *et al.*[3] has shown that in the case of trimethyl and triethyl phosphates (*15* and *16*) initial charge siting in the molecular ion was at one of the ester oxygen atoms Consideration of the mass spectrometric ionisation potentials of the sulfur analogues, *17*–*19*, of *15*, where one of the oxygen atoms had been replaced by a sulfur atom led Santoro [19] to the conclusion that in the latter cases the first electron removed was a sulfur non-bonding electron.

Molecular orbital calculations (CNDO/2) by one of the present authors showed [13] that the atom making the greatest electronic contribution to the highest bonding molecular orbital, *i.e.* having the largest coefficient in the HOMO, varies according to the substituent present on phosphorus. However, in all cases where such calculations were carried out the highest electronic contribution to the HOMO was made by one of the cyclic ester oxygen atoms or by the substituent at the phosphorus atom. These calculations were in general accord with the experimentally measured ionisation potentials (for the latter see Table 1).

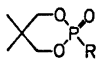
Among the compounds whose ionisation potentials were measured (*2*–*6*, *8* and *9*) the lowest values were obtained for *3*, *5* and *6* where the substituent was, respectively, benzyl, phenyl and phenoxy. These I.P.'s (8.25–8.81 eV) we feel able to associate with the removal of an electron from the benzene nucleus: CNDO/2 calculations show that in *5* and *6* the highest electronic contribution to the HOMO is made by the atoms of the aromatic nucleus.[13]

The I.P. values for the thiomethoxy and dimethylamino compounds, *8* and *9*, lie close together at 9.21 and 9.07 eV, respectively, and compare well with the values found for the compounds *17*–*19* (9.5, 9.2 and 9.0 eV) by Santoro.[19] Further, the MO calculations [13] indicate that the highest HOMO contribution is made by the sulfur atom in *8* and by the nitrogen in *9*. It thus seems likely that in *8* and *9*, as in *17*–*19*,[14] the electron removed on formation of the molecular ion originates as a non-bonding electron on the hetero-substituent.

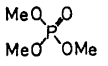
The somewhat higher I.P. values found for the compounds *2* and *4* (10.55 and 9.74 eV) which have *P*-alkyl substituents compare well with those reported for triethyl phosphate (10.1 eV) and trimethyl phosphate (10.7 eV).[3,12] The conclusion [3,12] that in the latter compounds the electron removed was from an ester oxygen atom may thus be extended to the present case. This is further supported by the MO calculations which show that the highest electronic contribution to the HOMO is made by an ester oxygen atom in each case.

While extension of the above results to 70 eV spectra is fraught with difficulties,[20] the general conclusion that the charge in the molecular ion is carried by either an ester oxygen atom or the substituent at phosphorus seems valid. Examination of the characteristic fragmentation modes shows that when they give rise to high mass ions they are usually ex-

Table 1. Ionisation potentials (I.P., eV) in electron volts as determined for the compounds *2*–*9* in the present investigation and from the literature [3,14] for the compounds *15*–*19*. The atom(s) whose electrons make the greatest contribution(s) to the highest occupied molecular orbital are indicated (XS) for the compounds *2*–*9*.[13]



2–*9*



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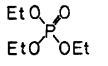

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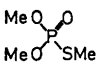

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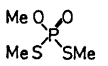

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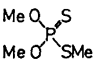

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Compound No.	R	IP eV	XS
2	Me	10.55	ring O
3	CH_2Ph	8.25	Ph
4	CMe_3	9.74	ring O
5	Ph	8.81	Ph
6	OPh	8.43	Ph
7	OMe	–	ring O
8	SMe	9.21	S
9	NMe_2	9.07	N
15[a]		10.77	
15[b]		10.7	
16[a]		10.06	
17[b]		9.5	
18[b]		9.2	
19[b]		9.0	

[a] Ref. 3. [b] Ref. 14.

plicable in terms of charge location on an ester oxygen atom.

Turning to the specific fragmentation, it is apparent that here too the results just discussed can be usefully applied. The compounds with *P*-alkyl substituents (*2* and *4*), where we would expect the initial charge site to be on an ester oxygen atom, show no characteristic fragmentation. However, while this is true of the methoxy compound (*7*) with a similar charge siting, the *S*-methyl compound (*8*) where some charge siting on the sulfur atom might be expected does indeed show an ion, albeit of only moderate intensity, at *m/e* 47.

The expected charge siting in the dimethylamino compound (*9*) is reflected in the spectrum by the dominance of the *m/e* 44 ion: no other ion has a relative intensity of more than 20 %. Further, in all compounds containing phenyl groups (*3*, *5*, *6*, and *10*), that at least part of the charge siting is on these groups is seen in the observation of appropriate ions.

It is interesting to compare the spectra of the compounds *10* and *12* since both triphenylmethyl and trichloromethyl radicals are known to be energetically favourable species. The difference between these spectra must thus be the result of variation in initial charge siting and an extension of the theoretical calculations to these compounds seems desirable.

In conclusion, it seems that the system here examined provides a case where mass spectral data and theoretical calculations may be usefully compared and yield mutual support.

EXPERIMENTAL

The syntheses of the compounds used in this study have been described elsewhere [13] and follow general literature procedures.[14–18] The mass spectra were obtained on two AEI MS9 instruments with the kind cooperation of the Universities of Oslo and Trondheim. The spectra reproduced were recorded at 70 eV and at the lowest temperature of the ion source allowing of volatilisation. Accurate mass measurements and ionisation potentials were determined using suitable standards.

2,5,5-Trimethyl-2-oxo-1,3,2-dioxaphosphorinane (*2*). M, measured *m/e* 164.061, calc. 164.060 for $C_6H_{13}O_3P$. M − 15, measured *m/e* 149.037, calc. 149.037 for $C_5H_{10}O_3P$ (M − CH_3). M − 28, measured *m/e* 136.031, calc. 136.029 for $C_4H_9O_3P$ (M − C_2H_4). M − 30, measured *m/e* 134.052, calc. 134.050 for $C_5H_{11}O_2P$ (M − CH_2O). M − 55, measured *m/e* 109.006, calc. 109.005 for $C_2H_6O_3P$ (M − C_4H_7). M − 67, measures *m/e* 97.005, calc. 97.006 for CH_6O_3P (M − C_5H_7). *m/e* 68, measured *m/e* 68.063, calc. 68.063 for C_5H_8. *m/e* 56, measured *m/e* 56.066, calc. 56.063 for C_4H_8.

2-Benzyl-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinane (*3*). M, measured *m/e* 240.086, calc. 240.087 for $C_{12}H_{17}O_3P$. M − 15, measured *m/e* 225.066, calc. 225.068 for $C_{11}H_{14}O_3P$ (M − CH_3). M − 28, measured *m/e* 212.060, calc. 212.060 for $C_{10}H_{13}O_3P$ (M − C_2H_4). M − 30, measured *m/e* 210.081, calc. 210.081 for $C_{11}H_{15}O_2P$ (M − CH_2O). M − 55, measured *m/e* 185.037, calc.185.037 for $C_9H_{10}O_3P$ (M − C_4H_7). M − 67, measured *m/e* 173.034, calc. 173.037 for $C_7H_{10}O_3P$ (M − C_5H_8). *m/e* 68, measured *m/e* 68.062, calc. 68.063 for C_5H_8. *m/e* 56, measured *m/e* 56.065, calc. 56.063 for C_4H_8.

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Proton Magnetic Resonance Studies of 1,2-Bis(2,4,6-trineopentylphenyl)ethane and the Diastereomers of 2,3-Bis(2,4,6-trineopentylphenyl)butane

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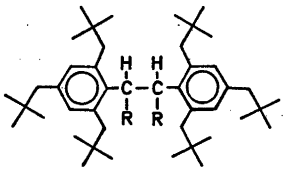
1,2-Bis(2,4,6-trineopentylphenyl)ethane, (±)-2,3-bis(2,4,6-trineopentylphenyl)butane and *meso*-2,3-bis(2,4,6-trineopentylphenyl)butane have been synthesized and studied by ^{1}H NMR to obtain information about free energies of internal rotation at the temperature of coalescence and about the conformations of the molecules.

The present work deals with the determination of barriers to internal rotation and conformations of 1,2-bis(2,4,6-trineopentylphenyl)ethane (*1*), *meso*-2,3-bis(2,4,6-trineopentylphenyl)butane (*2*) and (±)-2,3-bis(2,4,6-trineopentylphenyl)butane (*3*) by NMR studies, and also with the syntheses of these compounds by organolithium reactions.

RESULTS and DISCUSSION

Compounds *2* and *3* were synthesized by treating 2-(1-chloroethyl)-1,3,5-trineopentylbenzene [1] with methyllithium.[2] They were separated by means of liquid chromatography, and further purified by recrystallization. Compound 1 was synthesized by treating 2-chloromethyl-1,3,5-trineopentylbenzene [3] with methyllithium.



1 : R = H

2, 3 : R = CH_3

Compound 1. The NMR spectrum of 1,2-bis-(2,4,6-trineopentylphenyl)ethane showed only singlets at ambient probe temperature (Table 1). By comparison with the NMR spectrum of 2-ethyl-1,3,5-trineopentylbenzene [1] it could be concluded that the four methylene protons that appeared at δ 2.34 belonged to the 4-neopentyls while the four methylene protons at δ 2.73 belonged to the ethane moiety. The other signals could be assigned by means of the chemical shifts and integrals. On cooling the sample in carbon disulfide to −70 °C the singlet at δ 2.41 (2- and 6-methylenes) disappeared and instead an AB pattern with $\Delta\nu_{AB} = 38$ Hz and $J_{AB} = 13$ Hz was formed (determined from 60 MHz spectra). On raising the temperature the AB pattern gradually broadened and coalesced at −46 °C, which corresponds to a barrier to internal rotation ($\Delta G^{\ddagger}$) of 46.1 kJ/mol.*

In 1,3,5-trineopentylbenzene all signals appear as singlets at −70 °C. The nonequivalence within the 2- and 6-methylene groups of *1* at low temperature can only be explained by restricted rotation about the aryl-methylene bonds of the ethane moiety. Below the tempera-

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* A complete bandshape analysis of this compound will be published separately.

Table 1. NMR data of compounds *1*, *2* and *3* determined from 100 MHz spectra (s = singlet, d = doublet and q = quartet).

	1	*3* (±)	*2* (*meso*)
tert-Butyl; 2,6	0.83s	0.62s	0.87s
		0.98s	0.99s
tert-Butyl; 4	0.88s	0.77s	0.89s
Methyl; 2,3		1.48[a]	0.93[b]
Methylene; 2,6	2.41s	1.51q	2.81q
J_{AB} (Hz)		14.1	13.7
$\Delta\nu_{AB}$ (Hz)		34.1	85.0
		2.75q	2.93
J_{AB} (Hz)		14.0	14.0
$\Delta\nu_{BA}$ (Hz)		43.7	68.2
Methylene; 4	2.34s	2.21s	2.38s
Methylene; 1,2	2.73s		
Methine; 2,3		3.31[c]	3.61[d]
Aryl H; 3,5	6.59s	6.22d	6.70d
		6.82d	6.92d
J (Hz)		1.9	1.8

[a] X_3X_3' part of $X_3AA'X_3'$ spectrum: $J_{AX} = +7.80(5)$ Hz, $J_{AX'} = -0.35(5)$ Hz (see text). [b] X_3X_3' part of $X_3AA'X_3'$ spectrum: $J_{AX} = 7.25(10)$ Hz, $J_{AX'} = -0.36(5)$ Hz (see text). [c] AA' part of $X_3AA'X_3'$ spectrum: $J_{AA'} = 8.8(1)$ Hz. [d] AA' part of $X_3AA'X_3'$ spectrum: $J_{AA'} = 12.0(2)$ Hz.

ture of coalescence *1* will prefer to exist in a conformation in which the plane defined by the ethane bond and the bond between one of the aryl and methylene groups of the ethane moiety is approximately perpendicular to the plane of the mentioned aryl group. The 2- and 6-methylene groups are then enantiotopic due to the real or averaged symmetry of the molecule, the rotation around the ethane CC-bond still being rapid, but the hydrogens of the 2- and 6-methylene groups are diastereotopic. The hydrogens A and A' (see Fig. 1) will then be equivalent and different from the equivalent hydrogens B and B', and an AB pattern will be formed. Due to the symmetry

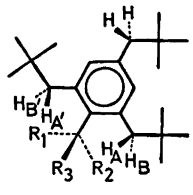

Fig. 1. 1: $R_1 = R_2 = H$, $R_3 = -CH_2-R$. *2*, *3*: $R_1 = CH_3$, $R_2 = H$, $R_3 = -CH(CH_3)-R$. R = 2,4,6-trineopentylphenyl.

properties of *1*, the nonequivalence between the two methylene protons of the 2- and 6-neopentyl groups, respectively, will remain even if these groups are free to rotate. According to Reuvers *et al.*[4] the spectrum of 1,2-bis(2,6-dimethylphenyl)ethane did not change on cooling to −60 °C. Even if the interannular bridge is locked in one position in this compound, the methyl groups will still give rise to a singlet, because the methyl hydrogens will not be diastereotopic.

1,2-Bis(4-methylphenyl)ethane has been found[5] by X-ray crystallography to have an *anti* conformation with respect to the aryl groups. IR and Raman studies [6] of *p,p'*-disubstituted 1,2-diphenylethanes showed that these compounds exist in *anti* conformations in the solid state, but they also showed that in solution there was an equilibrium between *anti* and *gauche* conformers. The NMR data for *1* do not allow conclusions to be drawn concerning the preferred conformation of the compound at ambient temperature in solution.

*Compounds 2 (*meso*) and 3(±).* In several cases [4,7] a higher melting point has been reported for a *meso* compound than for a corresponding *racemic* mixture. This indicates that *2* (m.p. 232–233 °C) is a *meso* compound and that *3* (m.p. 155–156 °C) is a *racemic* mixture. In the following text, we will first discuss the case in which the above assignements are correct. Later in the text we will also discuss the case in which the high-melting compound is assumed to be the *racemic* mixture and the low-melting the *meso* compound.

At ambient probe temperature the aromatic protons of *2* and *3* in carbon disulfide solution both appeared as two *meta*-coupled doublets. This shows that the protons on one and the same aryl group are nonequivalent, but that the aryl groups are equivalent. The 2- and 6-methylene groups of both *2* and *3* formed two AB patterns, due to restricted rotation about the aryl-methine bonds and the chirality at carbons 2 and 3 of the butane moiety.

Compounds *2* and *3* each showed three *tert*-butyl resonances. On heating the sample of *3* in deuteriobromoform, two *tert*-butyl peaks (appearing at δ 0.62 and 0.98 in carbon disulfide) coalesced ($t_C = 185$ °C) to a singlet, which shows that they should be assigned to the 2- and 6-neopentyl groups. The aromatic

protons of *3* also coalesced to a singlet at 185 °C. This corresponds to a barrier to internal rotation ($\Delta G^{\ddagger}$) of 96.3 kJ/mol for both measured coalescences. The two AB patterns of *3* broadened but did not coalesce completely even at 190 °C. The NMR spectrum of *2* was unchanged up to 190 °C ($\Delta G^{\ddagger} > 97$ kJ/mol).

The protons of the methine and methyl groups of the butane moieties of *2* and *3* gave rise to $X_3AA'X_3'$ patterns, which were analysed by a general method described by Anet [8] to obtain input data for the iterative computer program UEAITR.[9] Unfortunately, insufficient spectral detail was resolved to allow automatic iteration, and thus the search for the "best fit" of theoretical to experimental bandshape was performed manually. Analysis of the AA' part of the 100 MHz spectrum of *3* at ambient temperature in carbon disulfide solution gave, after considerable work with trial and error, $J_{AA'} = 8.8 \pm 0.1$ Hz. Analysis of the AA' part of the spectrum of *2* at ambient temperature in the same solvent gave $J_{AA'} = 12.0 \pm 0.2$ Hz The values of J_{AX} and $J_{AX'}$ were 7.80 ± 0.05 Hz and -0.35 ± 0.05 Hz for *3*, and 7.25 ± 0.10 Hz and -0.36 ± 0.05 Hz for *2*. Opposite signs for J_{AX} and $J_{AX'}$ were also found in most of the 2,3-disubstituted butanes studied by Anet [8] and Bothner-By and Naar-Colin.[10] $J_{XX'}$ was set equal to zero in all iterations.

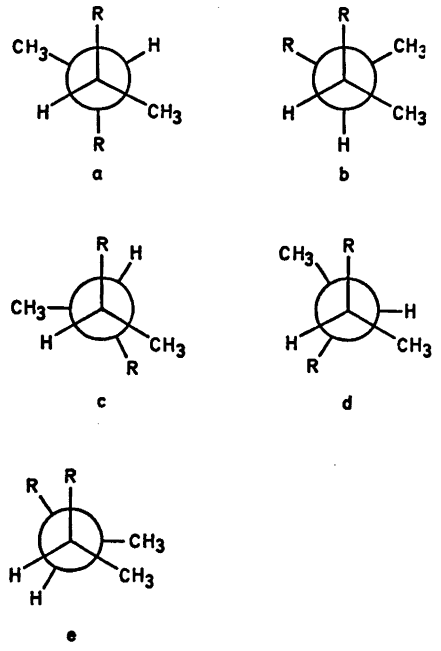

Fig. 2. Conformations of *meso*-2,3-bis(2,4,6-trineopentylphenyl)butane. R = 2,4,6-trineopentylphenyl.

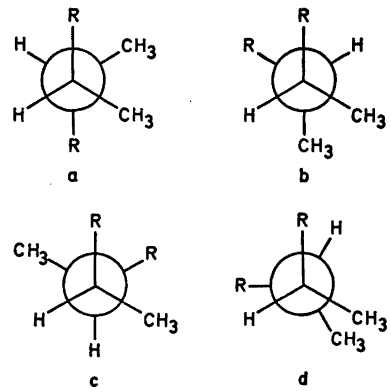

Fig. 3. Conformations of (+)- or (−)-2,3-bis-(2,4,6-trineopentylphenyl)butane. R = 2,4,6-trineopentylphenyl.

In 2,3-diphenylbutane the coupling constants $J_{AA'}$ were found to be 7.0 and 9.9 Hz for the *racemic* mixture and the *meso* compound, respectively, in carbon disulfide solution.[10] In the corresponding isomers of 2,3-bis(2,6-dimethylphenyl)butane, $J_{AA'}$ was found [4] to be 9.5 and 12 Hz, and in 3,4-bis(2,6-dimethylphenyl)2,2,5,5-tetramethylhexane about 5 and 12 Hz, for the *racemic* mixture and the *meso* isomer, respectively, in carbon tetrachloride solution. The latter values were estimated from ^{13}C satellites.[4] A comparison of the chemical shift values of the isomers of 2,3-bis(2,6-dimethylphenyl)butane at ordinary and elevated temperatures led to the suggestion [4] that each of these isomers exists in only one conformation at ordinary temperatures. The *meso* isomer was proposed to exist in the conformation shown in Fig. 2a (with R = 2,6-dimethylphenyl) and the *racemic* mixture in a conformation resembling Fig. 3a (with R = 2,6-dimethylphenyl), with a distortion of 30° from the "ideal" H−H dihedral angle of 60° in this conformation. Studies of molecular models [4] indicated that the dihedral angle between the methyl groups was smaller than 60°. On the basis of the chemical shifts and coupling constants, it was proposed [4] that in the *meso* isomer of 3,4-bis(2,6-dimethylphenyl)2,2,5,5-

tetramethylhexane, the two *tert*-butyl groups, the two aryl groups and the two methine protons were pairwise *anti* to each other, and in the racemic isomer the *tert*-butyl groups were *anti*, while the methine protons were *gauche* to each other.

Due to the great steric hindrance and to the symmetry of the *meso* isomers of 2,3-bis(2,6-dimethylphenyl)butane and 3,4-bis(2,6-dimethylphenyl)2,2,5,5-tetramethylhexane, when the aryl groups are *anti* to each other, it seems reasonable to assume that a coupling constant of 12 Hz between the methine protons is very close to that for an "ideal" *anti* position of these protons. This conclusion is further strengthened by the fact that $J_{AA'}$ is 12.0 Hz in compound *2*.

Using the Karplus equation, $J_{AA'} = K \cos^2 \theta$,[11] with $K = 12$ Hz, the coupling constant can be calculated to be 3 Hz, if the dihedral angle between the methine protons is 60° as in a *gauche* conformation.

If the population of the *anti* conformation is denoted X_{anti}, and rapid interconversion is assumed, the following relation can easily be obtained

$$X_{anti} = (J_{AA'} - J_g)/(J_a - J_g)$$

where J_g and J_a are the expected coupling constants 3 and 12 Hz for the *gauche* and *anti* positions of the methine protons, respectively).

On the basis of the iterated value of $J_{AA'}$ for *2* (12.0 Hz), this compound is found to exist essentially in the *anti* conformation shown in Fig. 2a. The chemical shift differences between the 2- and 6- methylene groups (0.12 ppm), the 2- and 6-*tert*-butyl groups (0.12 ppm) and the aromatic protons (0.22 ppm) on each aryl group of *2*, can be explained by their location near centers of chirality.

For *3*, an *anti*:*gauche* ratio of 64:36 may be obtained on the basis of the iterated value of $J_{AA'}$ (8.8 Hz) if it is assumed that an equilibrium exists between the conformations. On the other hand, a single intermediate conformation may also be assumed. By means of the Karplus equation,[11] with $K = 12$ Hz, the dihedral angle between the methine protons is found to be either 30 or 150° for *3*. In conformations 3a and 3c (with an H–H dihedral angle of 30°), the 2- and 3-methyl groups will be located in the shielding zone of the neighboring aryl group, which would result in an upfield shift of these protons, contrary to the observed behavior (δ 1.48 for these protons, while the corresponding protons of *2* appear at δ 0.93). A dihedral angle of 150° is thus more reasonable. This is nearly consistent with conformation 3b. The fact that the methine protons of *3* are shifted 0.30 ppm upfield with respect to those of *2* indicates that the former protons are in the shielding zone of the neighboring aryl group, and that the aryl groups of *3* are thus forced apart from each other. With an H–H dihedral angle of 150° the dihedral angle between the aryl groups will, in this case, be 90° (see Fig. 3d), and the large upfield shift of one of the 2- or 6-methylene groups, one of the 2- or 6-*tert-butyl*-groups and one of the aromatic protons on each aryl group (1.24, 0.36 and 0.60 ppm with respect to the downfield shifted groups, respectively) can be explained by their location in the shielding zone of the other aryl group, while the downfield shifts of the 2-and 3-methyl groups (0.55 ppm with respect to those of *2*) can be explained by their location in deshielding zones of the aryl groups. The racemic mixture thus seems to prefer to exist in conformation 3d in carbon disulfide solution at ambient temperature.

A comparison between the chemical shifts of *2* and *3* and those of 1,3,5-trineopentylbenzene, which shows *tert*-butyl, methylene and aromatic resonances at δ 0.86, 2.37 and 6.61, respectively, in carbon disulfide solution, indicates that the above assignments are correct, and we prefer the single conformation 3d to an *anti*/*gauche* mixture as the explanation of the iterated value of $J_{AA'}$.

Contrary to the above assignment, we can assume that the low-melting isomer *3* is the *meso* compound. An H–H dihedral angle of 150° is in accord with conformations 2c and 2d. In these conformations the methyl groups will be expected to have different chemical shifts. Furthermore, the chemical shift difference between the 2- and 6-methylene groups, the 2- and 6-*tert*-butyl groups and the aromatic protons, respectively, on each aryl group will be expected to be small, which is not the case (see Table 1). An H–H dihedral angle of 30° is in accord with conformation 2e. Perhaps the small chemical shift difference, in this conformation, between the methyl groups is not resolved due to instrumental limitations.

In that case, all the chemical shift values can be explained by means of the same arguments that were used above when *3* was assumed to be the *racemic* mixture. However, if *3* is the meso compound, the high-melting compound *2* must be the *racemic* mixture. In *2* there is an H−H dihedral angle of 0 or 180°. Assuming that *2* is the *racemic* mixture the value 180° is consistent with conformation 3b. In this conformation the methyl groups would be expected to be shifted downfield to about the same extent as those of *3* (compare 2e and 3b), which is not the case. A larger chemical shift difference than is found between the 2- and 6-methylene groups, the 2- and 6-*tert*-butyl groups and the aromatic protons, respectively, on each aryl group would also be expected. In a conformation with an H−H dihedral angle of 0°, both the aryl groups eclipse a methyl group. Although this conformation cannot be rejected on the basis of the chemical shift values, it seems unreasonable that it should be the lowest energy conformation of the compound.

The *meso* configuration can thus be assigned to the high-melting compound *2*, while the low-melting *3* is the *racemic* mixture.

EXPERIMENTAL

Gas chromatographic (GLC) analyses were carried out on a Perkin-Elmer 900 gas chromatograph fitted with a flame ionization detector (column: 2 m × 2 mm 3 % SE-30 silicon gum on Gaschrom 100−200 mesh).

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 mass spectra were determined (at the Department of Medical Biochemistry, University of Göteborg) on an AEI MS 902 mass spectrometer with the following conditions: electron energy 70 eV, accelerating voltage 8 kV and emission 100 μA. The intensities of the peaks are reported in parentheses 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.

The NMR spectra were recorded on Varian A 60, XL-100, and/or JEOL JNM-MH-100 spectrometers. About 10 % by weight solutions in carbon disulfide were used for identification of the compounds at a probe temperature of 38 °C. The chemical shifts are reported in ppm downfield from tetramethylsilane as internal standard. For the variable temperature studies, the same concentrations as mentioned above were used, but *2* and *3* were dissolved in deuteriobromoform. The temperatures were measured by methanol or 1,2-ethanediol shifts with separate NMR tubes. The accuracy of the temperature is estimated to be within ±5 °C. The $\Delta G^{\ddagger}$ values were calculated as described by Mannschreck *et al.*[12]

(±)-2,3-Bis(2,4,6-trineopentylphenyl)butane (3) and meso*-2,3-bis(2,4,6-trineopentylphenyl)-butane (2).* A solution of 250 mg (0.71 mmol) of 2-(1-chloroethyl)1,3,5-trineopentylbenzene [1] in dry ether was added to a solution of 25 mmol of methyllithium [2] in 20 ml of diethyl ether under nitrogen. The reaction mixture was then stirred overnight at room temperature, after which the excess of methyllithium was destroyed with water, and the mixture was neutralized with 6 M hydrochloric acid. The aqueous layer was then extracted several times with diethyl ether, and the combined ethereal layers were dried over magnesium sulfate. Compounds *2* and *3* were separated on a column of silica gel (< 0.08 mm) by elution with hexane, and further purified by repeated recrystallization from ethanol. Compounds *2* and *3* were identified as *meso*-2,3-bis(2,4,6-trineopentylphenyl)butane and (±)-2,3-bis(2,4,-6-trineopentylphenyl)butane from their spectral characteristics (see the above discussion). The yields of *2* and *3* were 90 mg (40 %) and 69 mg (31 %), respectively.

Data for *3* were: M.p., 155−156 °C. IR, 2950vs, 290w, 2865 m, 1608m, 1568w, 1475vs, 1390s, 1378w, 1362vs, 1238s, 1200 m, 1135 w, 1048m, 878m, 750w, 604w. MS, 29(4),41(10), 43(18), 57(27), 69(24), 71(27), 315(100), 316(27), 630(0.127), 631(0.058).

Data for *2* were: M.p. 232−233 °C. IR, 2960vs, 2910w, 2865m, 1610m, 1569m, 1480vs, 1392s, 1378w, 1365vs, 1338w, 1240s, 1200m, 1168w, 1060m, 1000m, 883s, 793m, 751w, 633m. MS, 29(5), 41(10), 43(21), 57(33), 69(32), 71(33), 145(6), 146(6), 315(100), 316(19), 630(0.091), 631(0.049).

1,2-Bis(2,4,6-trineopentylphenyl)ethane (1). To a solution of 15 mmol of methyllithium [2] in 12 ml of diethyl ether, under nitrogen, 500 mg (1.5 mmol) of 2-chloromethyl-1,3,5-trineopentylbenzene [3] was added. The reaction mixture was stirred at room temperature, and at regular time intervals small aliquots were withdrawn and analyzed by GLC. After 20 h a main product had been formed in 80 % yield. The work-up procedure described above for *2* and *3* was then applied, and the main product was purified by repeated recrystallization from ethanol. Its physical properties were identical with those previously reported [1] for 1,2-bis(2,4,6-trineopentylphenyl)ethane and the yield was 334 mg (74 %).

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Purification of Alkaline Phosphatase of the Halotolerant Yeast *Debaryomyces hansenii*

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Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) of the halotolerant yeast *Debaryomyces hansenii* was purified by a procedure involving cell disruption, DNAase treatment, ethanol precipitation, gel filtration, chromatography on DEAE-Sephadex, and preparative polyacrylamide gel electrophoresis. The specific activity was increased 1250-fold as compared to the activity of cell free extract. The total recovery was 30 %. Various modifications of the growth conditions had slight or no effect on the yield of enzyme.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activity is widely distributed in nature. Homogenous preparations of the microbial enzyme have been obtained from *Escherichia coli*,[1,2] *Serratia marcescens*,[3] *Bacillus subtilis*,[4] *Bacillus licheniformis*,[5] *Micrococcus sodonensis*,[6] *Neurospora crassa*,[7,8] and *Aspergillus nidulans*.[9] Attias *et al.*[10] have described a partial purification of the enzyme from *Saccharomyces cerevisiae*. The present report describes the methods used to purify alkaline phosphatase of *Debaryomyces hansenii*, a strongly halotolerant yeast,[11] and the effect on the enzyme yield by various growth conditions. This work was undertaken to permit a comparative study of the chemical and enzymatic properties of the enzyme prepared from cells cultured at different salinity along with comparisons with alkaline phosphatase from less halotolerant sources.

MATERIAL AND METHODS

Cultures and growth. Debaryomyces hansenii (Zopf) van Rij strain 26, previously described,[11] was maintained on nutrient agar WA.[12] Cells were cultured in a glucose-salt medium with the required growth supplements[13] (medium S) or in a basal complex medium (BCM) with 1.0 g urea and 5.0 g Bacto yeast extract per litre of distilled water. BCM was supplemented with various major carbon sources. After autoclaving, the pH of BCM was close to 6.5. Cultures of 500 ml were routinely grown in 2.5 l Fernbach-flasks at 25 °C on a rotatory shaker. Studies concering the phosphatase yield from cells grown in BCM were done with 500 ml cultures supplemented with one of the following additions: 50 mM glucose; 50 mM galactose; 50 mM arabinose; 50 mM maltose; 25 mM sucrose; 25 mM sucrose plus 20 mM Tris buffer, pH 8.0; 100 mM glycerol; 100 mM lactic acid, pH 6.5, or 150 mM ethanol. The flasks were inoculated with 8×10^6 cells/ml from an appropriate broth culture in the exponential growth phase. All cultures were harvested at a cell density of 6 mg dry wt cells/ml except the lactate culture which was halted at 1.2 mg dry wt cells/ml. To follow the production of phosphatase during phosphate starvation, phosphate-free medium S was inoculated with 0.2 mg dry wt/ml of washed cells precultured in medium S containing 50 μM phosphate. For large scale preparation of alkaline phosphatase the organism was grown in BCM supplemented with 25 mM sucrose. A 20 l Pyrex carboy containing 15 l medium was inoculated with 500 ml of a 40-hours culture. One ml of antifoam emulsion FG (Midland Silicone, England) was added. After 20 h incubation at 25 °C under vigorous aeration the growth was halted at approximately 7 mg dry wt cells/ml, corresponding to 9×10^8 cells/ml. The organisms were collected at 4 °C by a Sorvall RC 2-B centrifuge with a KSB continous flow system. The SS-1 rotor used was run at 20 000 *g* with a flow rate of 125 ml/min.

Growth determination and preparation of extract. Growth was followed at 610 nm in a Beckman spectrophotometer, model DBG.

Absorbance was related to dry wt by use of standard curves. Harvested cells were washed three times in cold 20 mM Tris buffer (pH 7.2) containing 10 mM $MgCl_2$ and 75 mM NaCl (starting buffer). $MgCl_2$ was necessary for full enzymatic activity during the purification steps. For large scale preparation of cell extract, 40 g wet wt cells were suspended per 100 ml starting buffer and the suspension was disrupted by treatment with glass beads (ϕ 0.45 – 0.50 mm, 70 g/100 ml) for 3 min in a Braun cell homogenizer, model MSK (Melsungen Apparatebau, Germany). Cell extract from 500 ml cultures was prepared in an MSE ultrasonic disintegrator, Model 60 W (MSE, Ltd., England). Aliquots of 40 ml were washed and suspended in 0.1 volume 20 mM Tris-buffer (pH 7.2) containing 10 mM $MgCl_2$ before sonic treatment for 30 min.

Enzyme assay. Alkaline phosphatase activity was assayed with *p*-nitrophenyl phosphate (Sigma 104, Sigma, USA) as substrate. The enzymatic liberation of *p*-nitrophenol was followed at 410 nm in a recording Beckman Acta II spectrophotometer. Sample cuvettes contained 0.1 M Tris buffer (pH 8.5), 10 mM $MgCl_2$ and 1 mM *p*-nitrophenyl phosphate in a final volume of 3 ml. All solutions used were equilibrated in a water bath at 25 °C. Linear reaction rates were observed when the change in absorbance was ≤ 1.0, corresponding to a decrease of ≤ 0.057 mM in substrate concentration. To determine the activity of intact cells, assay mixture was incubated with constant agitation at 25 °C for 60 min. Enzyme units are expressed as μmol of *p*-nitrophenol released per min at 25 °C. Specific activities refer to the hydrolysis of 1 μmol of substrate per min per mg of protein. Protein was determined by the method of Lowry *et al.*[14] with tyrosine as the standard.

Enzyme purification

Sephadex gels were hydrated, equilibrated and packed as recommended by the supplier (Pharmacia Fine Chemicals, Sweden). Columns (CK 14 and CK 32) were obtained from the Department of Biochemistry, Uppsala, Sweden. Flow rates were regulated by means of peristaltic pumps (Vario Perpex 2000, LKB, Sweden). A Radiometer conductivity meter CDM 3 with a CDC 304 electrode (Radiometer, Denmark) was used for determinations of conductivity. Protein solutions were concentrated in an Amicon ultrafiltration cell model 52 using PM 30 membranes (Amicon, Holland) or by dialysis against solid polyethyleneglycol 20 000 (Union Carbide, USA). Gel electrophoresis was carried out in a Gradipore 3 cell electrophoresis chamber (Universal Scientific Ltd., England). All operations were carried out in a cold room (2 – 5 °C).

Step 1. Cell extract. Cell homogenates prepared from a 45 l cell culture were centrifuged at 20 000 *g* for 10 min to remove cell debris and glass beads. The pellet material was resuspended in 1.5 volumes of starting buffer and reprecipitated by centrifugation as above. The combined supernatants contained approximately 90 % of the total alkaline phosphatase activity.

Step 2. Treatment with DNAase (EC 3.1.4.5). Material from the preceding step (1000 ml) was exposed to 10 μg DNAase per ml for 2 h at 35 °C and was dialysed overnight at 2 – 5 °C against starting buffer. After dialysis the cloudy precipitate was removed by centrifugation (20 000 *g* for 10 min).

Step 3. Precipitation with ethanol. The supernatant from *Step 2*, 1000 ml, containing 6.5 mg of protein/ml, was mixed with equal volumes of cold ethanol (– 15 °C) at the rate of 25 ml/min with stirring. The mixture was placed in an ice-NaCl bath for 1 h. The precipitate formed was recovered by centrifugation (5000 *g* for 10 min) and pellet material was gently suspended in cold starting buffer and dialysed against the same agent overnight. The supernatant (250 ml) remaining after centrifugation (10 000 *g* for 10 min) was concentrated 7-fold. The recovery obtained in this step was more than 100 %, possibly due to the removal of inhibitors of the enzyme.

Step 4. Sephadex G-200 gel filtration I. The concentrated enzyme solution from the preceding step (35 ml) was subjected to gel filtration on Sephadex G-200. The enzyme appeared in a broad peak behind a large amount of ultraviolet absorbing material, eluted in the void volume (Fig. 1).

Step 5. Sephadex G-200 gel filtration II. Active fractions from *Step 4* were collected, concentrated as above and recycled under the conditions used for the first gel filtration.

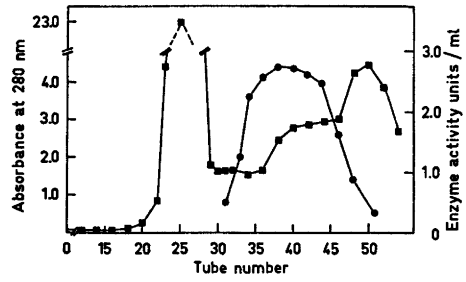

Fig. 1. Step 4, purification of *Debaryomyces hansenii* alkaline phosphatase on Sephadex G-200. 35 ml enzyme solution from *Step 3* was filtered through a Sephadex G-200 column (3.2 × 90 cm) in starting buffer at a flow rate of 15 ml/h and 11 ml fractions were collected. Fraction Nos. 31 to 48 were pooled. ■, absorbance at 280 nm; ●, enzyme activity.

Step 6. DEAE-Sephadex A-50 chromatography I. The pooled fractions of phosphatase activity from *Step 5* were concentrated 10-fold. The material was centrifuged (10 000 *g* for 10 min) and then applied to a DEAE-Sephadex column (1.4 × 10 cm) equilibrated with starting buffer. After adsorption of the enzyme, the column was washed with three column volumes of starting buffer and then developed with a linear salt gradient. The gradient system was composed of two equal volumes (60 ml) of 10 mM $MgCl_2$ in 20 mM Tris buffer (pH 7.2) containing 75 and 250 mM NaCl, respectively. Fractions of 3 ml were collected. A major peak of inactive ultraviolet absorbing material emerged from the column ahead of a minor peak of absorbance containing 80 – 85 % of the applied phosphatase activity.

Step 7. DEAE-Sephadex A-50 chromatography II. The pooled active material from the gradient elution (25 ml) was dialysed against starting buffer and reapplied to a DEAE-Sephadex column pre-equilibrated with the same agent. Column dimensions and the conditions used for elution are given in the caption to Fig. 2. As can be seen in the figure a single peak of absorbance eluted at 0.13 – 0.15 M NaCl. The phosphatase activity coincided roughly with the ultraviolet peak. The A_{280}/A_{260} ratio, averaging 1.6 in the most active fractions, indicates that the enzyme was essentially free of contaminating nucleic acids.[15]

Step 8. Preparative polyacrylamide electrophoresis. Electrophoresis was performed in a gel column (1.6 × 8 cm) composed of 5 % acrylamide, 0.17 % methylenebisacrylamide, 0.033 % tetramethylethylenediamine, and 0.06 % ammonium persulfate. Polymerisation was performed in a buffer (pH 8.1) containing 0.2 g Tris and 2.8 g glycine per 1000 ml. The same buffer adjusted to pH 7.9 with HCl was used as electrode buffer. The most active material from *Step 7* was concentrated to 1.5 ml and dialysed against electrode buffer, diluted 1:5.[16] The sample was layered on the gel after centrifugation (20 000 *g* for 10 min) and the addition of sucrose to give a concentration of 10 %. The electrophoresis was started at 100 V for 15 min followed by 250 V, 10 mA for 2.5 h. The extruded gel was cut into 3.5 mm wide sections. Each segment was crushed and eluted for 24 h at 4 °C with 3 ml starting buffer. The elution profile is shown in Fig. 3. All of the recoverable activity, about 50 % of the initial activity, was eluted from a 2 cm wide band located 4.2 cm from the origin.

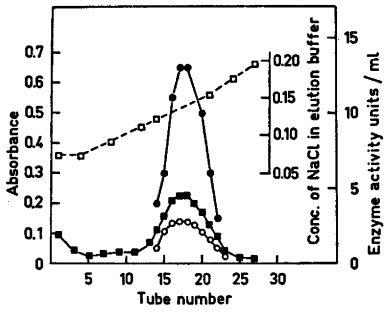

Fig. 2. Step 7, linear gradient elution profile of partially purified alkaline phosphatase from a DEAE-Sephadex column. 25 ml of enzyme solution from *Step 6*, was applied to the column (1.4 × 4 cm). Elution was conducted with 100 ml of a linear salt gradient from 75 to 200 mM NaCl in 20 mM Tris buffer (pH 7.2) containing 10 mM $MgCl_2$ at a rate of 12 ml/h. Fractions of 3 ml were collected. ●, enzyme activity; ○, absorbance at 260 nm; ■ absorbance at 280 nm; □, concentration of NaCl in elution buffer (M).

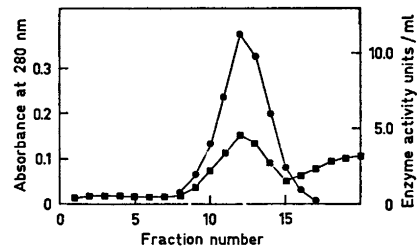

Fig. 3. Step 8, preparative polyacrylamide electrophoresis of pooled alkaline phosphatase from *Step 7*. Approximately 2 mg of protein was subjected to electrophoresis at pH 7.9 in a 5 % acrylamide gel (1.6 × 8 cm). After electrophoresis the gel was fractioned in segments of 3.5 mm. Each segment was eluted with 3 ml starting buffer. ■, absorbance at 280 nm; ●, enzyme activity.

Analytical gel electrophoresis. Protein samples were subjected to polyacrylamide electrophoresis following the general method of Davies[17] except that spacer and sample gels were not used. Tank buffers were those described by Reisfeld and Small[18] with urea omitted. 10 – 100 μg of protein in 100 μl buffer containing 10 % sucrose (or glycerol) was applied to each gel. Extruded gels were stained with Coomassie Brilliant Blue R according to Gabriel.[19] Alkaline phosphatase activity was visualized by incubating gels for 1 h at 25 °C in 15 mM $CaCl_2$, 10 mM sodium β-glycerophosphate, and 30 mM Tris buffer (pH 8.9).[20]

Electrophoresis in 0.1 % sodium dodecyl sulfate (SDS) was performed at room temperature by the method of Weber and Osborn.[21]

RESULTS

Growth conditions and alkaline phosphatase formation. Typical curves for growth and enzyme production in *D. hansenii* cultures

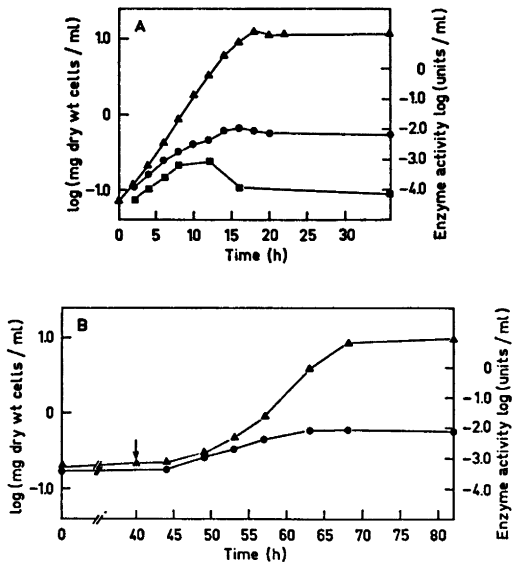

Fig. 4. Growth and formation of alkaline phosphatase by *Debaryomyces hansenii*. Cells were grown in (A) BCM plus 25 mM sucrose and (B) phosphate-free medium S. Samples of 50 ml were withdrawn at intervals for determination of cell growth and alkaline phosphatase activity. ▲, log mg dry wt cells/ml; ■, log enzyme activity of intact cells; ●, log enzyme activity of crude extract. The arrow indicates the addition of 10 μmol KH_2PO_4/ml.

supplied with exponentially growing inoculum, are illustrated in Fig. 4 A. The exponential growth rate was 0.5 generations per h and the culture entered the maximum stationary phase at a cell density of 12 mg dry wt cells/ml ($1-2 \times 10^9$ cells/ml). Alkaline phosphatase activity as monitored in crude extract increased to a maximum value at the end of the exponential phase of the growth curve followed by a slow decrease. The enzyme activity exhibited by intact cells during growth showed a deviation from the above pattern. A sharp rise and a subsequent decline in activity occurred while the culture was still in the exponential growth phase. Intact cells showed considerably less alkaline phosphatase activity than disrupted cells which provides evidence for a location of the enzyme behind a permeability barrier.

There are many reports of derepression of alkaline phosphatase in microorganisms cultured under conditions of limiting phosphate.[4-6,9,22-24] Fig. 4B presents growth and formation of alkaline phosphatase in medium S cultures of *D. hansenii* during phosphorus starvation and after addition of excess (10 μmol/ml) phosphate. Following the growth curve the alkaline phosphatase production was halted in the phosphate deprived culture. After the introduction of phosphate, growth and formation of phosphatase was restored. Thus, the differential rate of alkaline phosphatase synthesis was not increased by growth-limiting concentration of inorganic phosphate. It may, however, be noted that the activity of acid phosphatase (measured as described by Torriani [24]) was markedly derepressed in the phosphate deprived culture (at maximum by a factor of 18, data not included in Fig. 4B) indicating that the biosynthetic functions of the organism were not completely interrupted during the phosphorus starvation.

Various fermentable and nonfermentable carbon sources used as supplement in BCM (for details, see Material and Methods) did not markedly change the production of alkaline phosphatase by *D. hansenii*. The enzyme activity ($10^3 \times$ units per mg cell dry wt) in cell homogenates ranged between 0.9 to 1.1.

Table 1. Summary of purification of *Debaryomyces hansenii* alkaline phosphatase.

Preparation	Total volume (ml)	Protein (mg/ml)	Total units	Specific activity	Recovery (%)
Crude	1000	6.5	360	0.06	100
Ethanol	250	5.2	420	0.32	117
Sephadex G-100 I	190	2.8	400	0.8	112
Sephadex G-200 II	110	0.6	320	4.8	89
DEAE Sephadex I	25	0.35	280	32	78
DEAE Sephadex II	25	0.16	220	55	61
Polyacrylamide electrophoresis	16	0.1	120	75	30

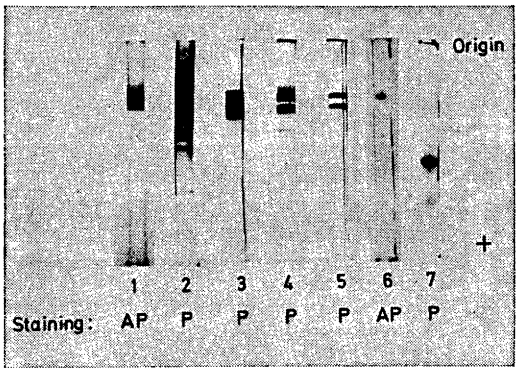

Fig. 5. Polyacrylamide gel electrophoresis of material from different steps in the purification of *Debaryomyces hansenii* alkaline phosphatase. Samples were subjected to analytical polyacrylamide gel electrophoresis with (gel 7), and without (gels 1 to 6), SDS. Gels were stained for alkaline phosphatase activity (AP) or for protein (P). Contents of the gels were: gels 1 and 2, crude extract (*Step 1*); gel 3, peak fraction of Sephadex G-200 gel filtration (*Step 4*); gel 4, peak fraction of DEAE-Sephadex recromatography (*Step 7*); gels 5, 6 and 7, peak tube of preparative electrophoresis (*Step 8*).

This is in contrast to results reported for *Bacillus subtilis*.[25]

Summary of enzyme purification. Table 1 summarizes the purification of alkaline phosphatase from a 45 l culture. The specific activity of alkaline phosphatase was increased about 1250-fold with an overall yield of 30 %. Activation of the enzyme after the ethanol precipitation (*Step 3*) was observed while a substantial reduction of the total activity occurred during the last purification step. Assuming a molecular weight of 110 000 daltons,[26] the specific activity of purified enzyme (75 units/mg) corresponds to a turnover number of 8.3×10^3 min^{-1}. This value is within the same order of magnitude as the turnover number reported for the *E. coli* phosphatase.[2]

Homogeneity of the enzyme preparations. Fig. 5 shows the results of analytical polyacryalmide gel electrophoresis of enzyme from various purification steps. The purification scheme resulted in a preparation that produced two bands of protein of approximately equal intensity. Using glycerophosphate as a substrate, alkaline phosphatase activity coincided with the band of lower mobility. When activity was detected with *p*-nitrophenyl phosphate, the same pattern was seen but the band was less distinct, probably due to diffusion of the developed colour. Electrophoresis of purified enzyme on SDS polyacrylamide gel showed a diffuse minor band migrating in advance of a major narrow band.

DISCUSSION

The significant difference between the alkaline phosphatase activity of intact and disintegrated cells (Fig. 4A), suggests a location of the enzyme within the cell. This observation appears to agree well with the reported distribution of alkaline phosphatase in baker's yeast [27] and *Saccharomyces carlsbergensis*.[28] The relation between the activity exhibited by disintegrated cells and that of intact cells varies considerably during growth (Fig. 4A). This may be a reflection of changes in the location of alkaline phosphatase in aging cultures. Such changes in distribution patterns have been observed in *Saccharomyces carlsbergensis*.[28] Alternatively, it may be an effect of altered permeability for the substrate along with growth.

Many of the characterized alkaline phosphatases of microbial origin are orthophosphate repressible.[4-6,9,22-24] Wilkins [29] has demonstrated, however, that the synthesis of the *E. coli* enzyme is regulated by the internal concentration of one or more nucleotide species rather than the orthophosphate concentration itself. Observations by Kuo and Blumenthal [30] indicate that the regulation of the formation of alkaline phosphatase by the concentration of inorganic phosphate in the growth medium is not a common phenomenon among microorganisms. The production of alkaline phosphatase by *D. hansenii* appeared unaffected by the extracellular phosphate level (Fig. 4B) as well as other environmental and nutritional factors known to influence phosphatase production.

Crude cell-free extract revealed after electrophoresis a diffuse zone of alkaline phosphatase activity (Fig. 5) indicating heterogeneity of the enzyme or, more likely, the presence of other non-specific phosphatase activities in the unfractioned extract. Microheterogeneity is, however, reported for alkaline phosphatase

from several sources.[31-32] The apparent heterogeneity of the *D. hansenii* phosphatase activity was reduced throughout the purification procedure. Fractions of purified enzyme displayed only one narrow band of activity after analytical polyacrylamide gel electrophoresis (Fig. 5). Therefore, it would appear that material from the last purification step contains a single enzyme species possessing activity. Although the specific activity of the *D. hansenii* alkaline phosphatase was increased 1250-fold by the purification procedure, the final enzyme preparation displayed, after analytical polyacrylamide gel electrophoresis, two bands of protein of which one was active (Fig. 5). The marked difference in distribution of protein material between the two bands seen on electrophoresis with and without SDS suggests a dissociable subunit structure of the enzyme.

Further purification of the enzyme has been restricted by the low yield of the alkaline phosphatase. With a total recovery of 30 % in the purification steps, 1 – 2 mg of enzyme were obtained from a 45 l culture. This reflects a low level of phosphatase biosynthesis and further progress in the purification process seems to be dependent on isolation of mutants or more extensive studies of the growth conditions in order to improve the yield of the enzyme.

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Photochemical Dechlorination of 1,2,4-Trichlorobenzene

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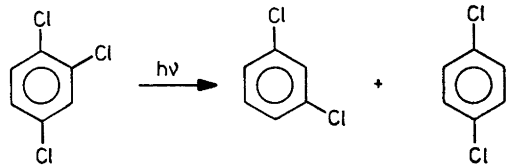
The photochemical dechlorination of 1,2,4-trichlorobenzene in cyclohexane and 2-propanol has been investigated. The primary products formed are 1,3- and 1,4-dichlorobenzene. The product ratio is significantly different on direct irradiation and on acetone sensitization indicating that two different reactive species give rise to dechlorination. The quantum yield for the dechlorination is 0.46 in 2-propanol under aerated conditions.

Man-spread chlorinated hydrocarbons have created a difficult environmental problem due to their persistence under natural conditions. Since these hydrocarbons are susceptible to photochemical degradation, a number of groups, including ours, have recently been studying photochemical dehalogenation of aromatic hydrocarbons. These studies show that, *e.g.*, PCB analogues may indeed be dehalogenated by photolysis, but many of these investigations have dealt with complex mixtures, making a determination of quantum efficiency and mechanism difficult.[1–4] In some recent publications a more detailed treatment has been given.[5–7] This prompts us to report our results on the dechlorination of 1,2,4-trichlorobenzene (*1*). These results support some of the conclusions of previous studies, but also add some new aspects.

RESULTS AND DISCUSSION

Light in the 300 nm range was used to simulate natural conditions.* Dechlorination



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takes place both on direct irradiation and on sensitization with a high energy sensitizer, *e.g.*, acetone and leads mainly to a mixture of 1,3- and 1,4-dichlorobenzene but the product distribution varies substantially depending on the reaction conditions. Only traces of 1,2-dichlorobenzene were detected. The 1,3:1,4-product ratio ranges from 0.15 on direct irradiation of aerated solutions in 2-propanol or cyclohexane, to 4.8 on acetone sensitization in oxygen-free cyclohexane (Table 1).

The fate of the chlorine lost and the hydrogen donor is of mechanistic and practical interest since the formation of chlorinated secondary

Table 1. Relative yields of dichlorobenzenes in the photochemical monodehalogenation of compound *1* on direct irradiation and acetone sensitization.

Conditions	1,3:1,4-Dichlorobenzene ratio	
	Direct	Acetone sensitized
Oxygen-free 2-propanol	0.65	3.1
Aerated 2-propanol	0.15	
Oxygen-free cyclohexane	1.51	4.8
Aerated cyclohexane	0.15	

* Irradiations at 254 nm showed that the same products were formed at shorter wavelengths.

products in nature is undesirable. Irradiation in 2-propanol gave in addition to dichlorobenzenes, hydrochloric acid and acetone as the only products. Under rigorously oxygen-free conditions, a small amount of pinacol was also formed. In aerated cyclohexane solution, the major products were hydrochloric acid, cyclohexanol and cyclohexanone. No chlorocyclohexane but traces of bicyclohexyl could be detected. Under oxygen-free conditions hydrochloric acid, substantial amounts of bicyclohexyl and some chlorocyclohexane were formed. Cyclohexene is probably also formed by disproportionation of cyclohexyl radicals.

Acetone sensitization in the absence of oxygen led to increased pinacol formation in 2-propanol and increased formation of bicyclohexyl in cyclohexane. In cyclohexane some 2-cyclohexyl-2-propanol was formed. The quantitative evaluation of these results in relation to the sensitized photolysis of 1,2,4-trichlorobenzene is difficult, since the same products are formed on irradiation of acetone itself in the absence of trichlorobenzene.

The acetone-sensitized reaction should go *via* the triplet.* If the dechlorination were of triplet origin only as has been suggested for chlorobiphenyls,[7,8] direct irradiation and sensitization should yield identical product patterns, which is not the case. Furthermore, the reaction rate but not the product pattern should be influenced by oxygen, which is a very efficient triplet quencher. The fact that a non-quenchable reaction persists on direct irradiation of continuously aerated solutions and that the product ratio is significantly different from that observed in the triplet case, implies that both the singlet and triplet-excited states of 1,2,4-trichlorobenzene lead to dechlorination in a hydrogen-donating solvent. The low 1,3:1,4 ratio (0.15) obtained in aerated solution is evidently characteristic of the singlet reaction while the high ratio (4.8) obtained on sensitization is characteristic of the triplet reaction. On direct irradiation of air-free solutions in cyclohexane and 2-propanol, intermediate ratios of 1.5 and 0.65 were obtained (Table 1), indicating that dehalogenation from the two states occur at comparable rates. The presence of a hydrogen donor is essential as indicated by the fact that little photochemical degradation of 1,2,4-trichlorobenzene takes place in 1,1,2-trichlorotrifluoroethane solution.*

The existence of concurrent intermolecular triplet and singlet reactions of comparable efficiencies is fairly unusual in the liquid state. (For a discussion of alkanone singlet and triplet hydrogen abstraction, see Ref. 9). The relative rates of singlet and triplet reactions are slightly dependent on the solvent as shown by the lower 1,3:1,4 ratio obtained in 2-propanol (0.65) than in cyclohexane (1.5).

An interesting question is whether π-chlorine derivatives are formed in the photolysis of 1,2,4-trichlorobenzene as suggested by Lemal for chlorobenzene.[5] The main argument for π-complex formation in the latter case is that photolysis in cyclohexane solution yields benzene and chlorocyclohexane and no phenylcyclohexane in spite of the fact that free chlorine atoms should be more efficient hydrogen abstractors than phenyl radicals by about four powers of ten.[5] Chlorocyclohexane was formed even in the presence of oxygen, which further supports the existence of π-chlorobenzene species. In the photolysis of 1,2,4-trichlorobenzene under similar conditions, the formation of chlorocyclohexane is completely inhibited by oxygen. However, under oxygen-free conditions, chlorocyclohexane is formed from the trichlorobenzene, indicating that a complex is formed also in this case. This complex must be weaker than π-chlorobenzene since the formation of chlorocyclohexane is quenched by oxygen.

In order to get an estimate of efficiency of the photodehalogenation of 1,2,4-trichlorobenzene, the quantum yield was determined for the non-quenchable part of the reaction by direct irradiation in 2-propanol in the presence of oxygen. The quantum yields were 0.46 for the consumption of 1,2,4-trichlorobenzene, 0.38 for

* At high acceptor concentrations singlet energy transfer can occur from acetone on account of relatively slow intersystem crossing. To ensure that no singlet sensitization was taking place, the concentration of 1,2,4-trichlorobenzene was varied over two powers of ten (0.15 – 0.0015 M) which did not change product ratio appreciably.

* 1,2,4-Trichlorobenzene was quantitatively recovered after irradiation in 1,1,2-trichlorotrifluoroethane solution under conditions which led to approximately 30 % dehalogenation when 2-propanol was used as solvent.

formation of 1,4-dichlorobenzene and 0.06 for formation of 1,3-dichlorobenzene. These values are considerably higher than those observed for tetrachlorobiphenyls [7] but of the same order as that determined for chlorobenzene by Lemal and co-workers.[5]

The identification of a highly efficient singlet path for dehalogenation of polychlorinated aromatic compounds is of interest also in relation to their destruction in nature. The decomposition of trichlorobenzene *via* the triplet is inhibited even by acetophenone and it is reasonable to assume that a number of efficient quenchers, including oxygen, are available in nature. Furthermore, the increased efficiency of the singlet pathway when a good hydrogen donor is present, can perhaps be utilized to increase destruction of halogenated aromatics even under natural conditions.

EXPERIMENTAL

Photochemical procedure. All irradiations were made in Pyrex glass tubing with a wall thickness of 1 mm (cut-off at 285 nm) in a Rayonette photochemical reactor fitted with a total of 16 RPR 3000 Å and RPR 3100 Å fluorescent lamps. Deaeration was effected by purging the solution with nitrogen purified by passage through a 3.5 × 50 cm tower of BASF R3-11 copper catalyst maintained at 90 °C followed by a 2 × 25 cm tower of 4 Å molecular sieves > 30 min prior to and during irradiation. The nitrogen was introduced at the bottom of the reaction tube by Intramedic PE 60 polyethylene tubing *via* a rubber septum equipped with a narrow syringe needle as a gas outlet. Alternatively deaeration was carried out by three freeze-thaw cycles, namely evacuation of the solution at −80 °C, followed by equilibration at atmospheric pressure under purified nitrogen. The samples were then irradiated in a stoppered tube. Aeration was accomplished with the aid of an aquarium pump. Standard samples were prepared by diluting 50 µl (0.072 g, 0.397 mmol) of commercial, redistilled 1,2,4-trichlorobenzene with solvent to 25 ml in a volumetric flask, giving a 0.0158 M solution, which was transferred to a 1 × 50 cm Pyrex tube, in which irradiation was performed. In sensitized reactions 24 ml of solvent and 1 ml of acetone were used which allowed the sensitizer to capture > 99 % of the light at 300 nm.

Analytical procedure. A PYE-104 gas chromatograph with a flame ionization detector was used. Reaction mixtures in 2-propanol were analyzed at 118 °C on a 6 mm × 1.5 m glass column packed with 10 % PEG 20 M on Chromosorb W 80 − 100 mesh. (1,4-Dichlorobenzene and dicyclohexyl did not separate on this column.) Reaction mixtures in cyclohexane were analysed at 105 °C on a 6 mm × 2.7 m glass column packed with a 1:9 mixture of 10 % SF and 10 % PEG 4000 on Chromosorb W 100 − 120 mesh. The nitrogen carrier gas flow-rate was 50 ml/min in both cases.

Peak areas were determined by the method of peak height × peak width at 0.5 of the peak height. The peak height was measured with a ruler graduated in mm and the peak width with a magnifying glass and scale with 0.1 mm divisions. Naphthalene was used as an internal standard. Solutions of the internal standard and the reaction mixture were measured out with a 100 µl SGE syringe and mixed prior to analysis. Individual response factors were determined.

Product identification. Compounds were identified by coinjection of authentic samples and by combined GC-MS analyses using an LKB 9000 mass spectrometer connected to a Hewlett-Packard model 5750 gas chromatograph fitted with 3 mm stainless steel columns. The columns employed were a 1.5 m 10 % PEG on Chromosorb W 100 − 120 mesh and a 7 m 1 % SF-96 on Chromosorb W 100 − 120 mesh. Commercial samples were used except for bicyclohexyl which was made from cyclohexylmagnesium chloride and silver bromide [10] and 2-cyclohexyl-2-propanol which was made by the addition of acetone to cyclohexylmagnesium chloride.[11]

Irradiation of 1 *in 2-propanol under nitrogen.* A solution of 0.072 g (0.397 mmol) of *1* in 25 ml of 2-propanol was deaerated by the freeze-thaw method and irradiated for 30 min. GC analysis on 10 % PEG gave the following results: 1,3-dichlorobenzene, 0.019 mmol, 4.7 % (4.8 min)*; 1,4-dichlorobenzene, 0.029 mmol, 7.3 % (5.5 min); pinacol, trace (6.3 min); 1,2,4-trichlorobenzene, 0.356 mmol, 89.4 % (13.4 min).

Irradiation of 1 *in 2-propanol with aeration.* A solution of *1* (0.072 g) in 25 ml of 2-propanol was aerated during irradiation and irradiated for 70 min. GC analysis gave the following results: 1,3-dichlorobenzene, 0.008 mmol, 2.0 %; 1,4-dichlorobenzene, 0.061 mmol, 15.4 %; 1,2,4-trichlorobenzene, 0.322 mmol, 81.1 %.

Irradiation of 1 *in 2-propanol with acetone as sensitizer.* A mixture of *1* (0.072 g) and 1 ml of acetone was diluted to 25 ml with 2-propanol deaerated with a stream of nitrogen and was irradiated for 34 min. GC analysis gave the following results: 1,3-dichlorobenzene, 0.080 mmol, 20.4 %; 1,4-dichlorobenzene, 0.025 mmol, 6.2 %; pinacol, 0.034 mmol; 1,2,4-trichlorobenzene, 0.292 mmol, 74.1 %.

Irradiation of 1 *in cyclohexane under nitrogen.* A solution of *1* (0.072 g) in 25 ml of cyclohexane was deaerated by the freeze-thaw method and irradiated for 33 min. GC analysis on the 1:9 SF, PEG 4000 column gave the following

* Percentage based on initial starting material.

results: chlorocyclohexane, 0.004 mmol; 1,3-dichlorobenzene, 0.025 mmol, 6.4 %; 1,4-dichlorobenzene, 0.017 mmol, 4.3 %; bicyclohexyl, 0.013 mmol; 1,2,4-trichlorobenzene, 0.353 mmol, 89.8 %.

Irradiation of 1 *in cyclohexane with aeration.* A solution of *1* (0.076 mg, 0.418 mmol) in 25 ml of cyclohexane was aerated with a stream of air-bubbles from the bottom of the tube during irradiation for 60 min. Analysis on the 1:9 SF, PEG 4000 column gave the following results: cyclohexanone, 0.028 mmol (9.2 min); cyclohexanol, 0.036 mmol (13.6 min); 1,3-dichlorobenzene, 0.004 mmol, 0.8 % (15.6 min); 1,4-dichlorobenzene, 0.025 mmol, 5.7 % (17.5 min); 1,2,4-trichlorobenzene,0.409 mmol, 93.3 % (44.6 min).

Irradiation of 1 *in cyclohexane with acetone as sensitizer.* A mixture of *1* (0.072 g) and 1 ml of acetone was diluted to 25 ml with cyclohexane, deaerated with a stream of nitrogen and irradiated for 30 min. GC analysis gave the following results: chlorocyclohexane, 0.008 mmol; 1,3-dichlorobenzene, 0.063 mmol, 15.9 %, 1,4-dichlorobenzene, 0.013 mmol, 3.8 %, 2-cyclohexyl-2-propanol, 0.022 mmol; bicyclohexyl, 0.040 mmol; 1,2,4-trichlorobenzene, 0.310 mmol, 78.2 %.

Quantum yield apparatus. The quantum yield determination was carried out using a "Black Box" apparatus as described by Zimmermann.[12] As a slight modification the reflector was machined into the shape of a nose-cone and a hole was drilled from the side to fit a PEK SEB side-mounted cooling assembly. A PEK AH-6-1-B lamp was used as the light source. The light output and the light absorbed by the photolysis mixture were determined in the following way as described by Zimmermann:[12] A quartz plate beam splitter was placed at 45° angle between the filter cell and the double compartment photolysis cell to reflect a fraction of the light into a small side cell. The splitting ratio for the specific filter combination used was determined by filling potassium ferrioxalate in the side cell and in both compartments of the photolysis cell. The amount of light absorbed by each cell was determined by the standard procedure of Hatchard and Parker.[13] During irradiation of the reaction mixture, the side cell and the back cell of the photolysis cell were filled with potassium ferrioxalate. The amount of light absorbed by the reaction mixture was then determined by substraction of the light transmitted to the back cell from the total amount as calculated from the splitting ratio and the amount absorbed in the side cell. The use of a double compartment cell is important, since it may otherwise be difficult to quantitatively determine the light transmitted by the photolysis mixture, resulting in calculated quantum yields which are too low.

Filter solutions. A triple compartment filter cell was used. Cell 1 was filled with 2 M nickel(II) sulfate in 5 % sulfuric acid, cell 2 with 1 M cobolt(II) sulfate in 5 % sulfuric acid, and cell 3 with 0.0012 M bismuth(III) chloride in 14 % hydrochloric acid. This combination was opaque below 260 nm and above 300 nm and showed a maximum transmittance at 280 nm.

Quantum yield irradiations. 2-Propanol was distilled through a 30 cm Widmer column and checked for UV transparency in the region used for irradiation. A solution of 1 g of 1,2,4-trichlorobenzene in 750 ml of 2-propanol was mechanically stirred and 125 ml/min of air was pumped through the solution during irradiation. Photolyses were run to 4–5 % conversion and less than 4 % of the light was transmitted to the back cell.

Run 1. Compound *1* (1.05 g, 5.80 mmol) captured 0.66 mE of light. GC analysis gave: compound *1* consumed, 0.31 mmol, $\Phi = 0.47$; 1,3-dichlorobenzene formed, 0.4 mmol, $\Phi = 0.06$; 1,4-dichlorobenzene formed, 0.24 mmol, $\Phi = 0.36$.

Run 2. Compound *1* (1.06 g, 5.86 mmol) captured 0.57 mE of light. GC analysis gave: compound *1* consumed, 0.25 mmol, $\Phi = 0.44$; 1,3-dichlorobenzene formed, 0.03 mmol, $\Phi = 0.06$; 1,4 dichlorobenzene formed, 0.23 mmol, $\Phi = 0.40$.

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α-*N*-Benzoylarginine-2-naphthylamide Hydrolase (Cathepsin B1?) from Rat Skin. III. Substrate Specificity, Modifier Characteristics, and Transformation of the Enzyme at Acidic pH

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Some properties of rat skin benzoylarginine-2-naphthylamide hydrolase types I (preparations I and AI) and II (preparations II and NII) were studied. Both types were activated by dithiothreitol and EDTA, but responded differently to 1 mM KCN, when benzoylarginine-2-naphthylamide (BANA) was used as a substrate: type I was inhibited, while type II was activated. When leucine-2-naphthylamide was used as a substrate, both types were activated by KCN. Thiol proteinase inhibiting substances, like heavy metals, iodoacetic acid, 4-chloromercuribenzoic acid, and tosyllysine chloromethylketone, inhibited the enzymes. Diisopropylfluorophosphate, phenylmethylsulfonylfluoride, 4-aminobenzamidine, and high-molecular-weight trypsin inhibitors were without effect. The substrate specificity of rat skin BANA hydrolase resembled that of an amino acid naphthylamidase, naphthylamides of methionine, lysine, arginine, and alanine being hydrolyzed most rapidly. The rate of hydrolysis of BANA was only 11 % of that of methionine naphthylamide. Amino acid esters with a free α-amino group were also good substrates. The transformation of type II to type I at acidic pH was studied. During the transformation amino acids or peptides were formed and probably some inhibitor present in type II was destroyed proteolytically.

In a previous report the purification of the rat skin α-*N*-benzoylarginine-2-naphthylamide hydrolyzing enzyme was presented.[1] Two enzyme types, preparations I and II, were separated by DEAE cellulose chromatography. The specific activity of preparation II increased markedly, when its concentration in the reaction mixture was lowered by dilution, while dilution had no effect on the specific activity of preparation I. Treatment of preparation II at pH 4 and 55 °C increased the activity of the undiluted enzyme 4.5 times and the effect of dilution on the specific activity of the enzyme was lost. The isoelectric point and chromatographic behavior of acid treated preparation II were similar to those of preparation I suggesting that a transformation of enzyme type II to type I had occurred as a consequence of the acid treatment. In this report some characteristics of the transformation of type II to type I as well as substrate specificity and modifier characteristics of the enzyme are presented.

MATERIALS AND METHODS

Enzyme preparations. Rat skin BANA hydrolase preparations (I, AI, II, and NII) were purified as described previously.[1]

Substrates. α-*N*-Benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA) and α-*N*-benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPA) were obtained from Fluka A.G.; α-*N*-benzoyl-DL-arginine amide hydrochloride hydrate (BAA) was from Merck A.G.; L-alanine-2-naphthylamide hydrobromide (Ala-NA), L-arginine-2-naphthylamide (Arg-NA), and L-lysine-2-naphthylamide carbonate (Lys-NA) were from Nutritional Biochemicals Corp.: 1-L-glutamic acid-2-naphthylamide (Glu-NA), L-methionine-2-naphthylamide (Met-NA), and α-*N*-tosyl-L-arginine-2-naphthylamide (Tos-Arg-NA) were from Schwarz/Mann, and L-arginine methylester (Arg-ME), *N*-acetyl-L-tyrosine ethylester (ATEE), α-*N*-benzoyl-L-arginine methylester (BAME), bovine hemoglobin (Hb), and L-leucine-2-naphthylamide (Leu-NA) from Sigma Chem. Co.

Modifiers. Bovine lung trypsin-kallikrein inhibitor (Tracylol^R, 10.000 kallikrein inhibiting

equivalents (KIE)/ml) was obtained from Bayer A.G.; dithiothreitol (DTT) from Calbiochem; diisopropylfluorophosphate (DFP) and 4-chloromercuribenzoic acid were from Fluka A.G.; benzethonium chloride and phenylmethylsulfonylfluoride (PMSF) from Schwarz/Mann; 4-aminobenzamidine, *N*-ethylmaleimide, iodoacetic acid, iodoacetamide, puromycin, α-*N*-*p*-tosyl-L-lysine chloromethylketone hydrochloride (TLCK), ovomucoid trypsin inhibitor (type II-O), and soybean trypsin inhibitor (SBTI, type II-S) were from Sigma Chem. Co.

Activity determinations. The hydrolysis rates of naphthylamides, amides, 4-nitroanilides, esters, and proteins were determined as described in previous reports.[1,2] In modifier experiments with metal ions the Britton-Robinson buffer[2] was replaced with a Tris-maleate buffer,[3] pH 5.8, containing 0.6 mM DTT and 3 mM KCN. All modifiers were dissolved in the assay buffers and preincubated for 15 min at room temperature with the enzyme solution. Undiluted enzyme preparations were used unless otherwise stated.

Isolation of peptides formed during acid activation of preparation II. Preparation II (20 ml) was dialyzed against 5 mM ammonium formate buffer, pH 4.0, at +4 °C. The solution was ultrafiltrated with a Diaflo [R] 8-MC apparatus using a UM-10 membrane (Amicon N. V., Holland) and 10 ml of the filtrate was collected as a control. The volume of the enzyme solution was adjusted to 20 ml with the ammonium formate buffer, and the enzyme solution incubated for 20 min at 55 °C. The enzyme solution was again ultrafiltrated and 10 ml of the filtrate collected. The filtrates were then lyophilized (the ammonium formate buffer being evaporated during the lyophilization). The dry residues were dissolved in 1 ml of 50 % (*v*/*v*) methanol and the amino acids in the solutions were determined with ninhydrin, using L-leucine (Sigma Chem. Co.) as a standard.[4] Samples of filtrates were pipetted onto silica gel thin layer plates (Kieselgel G, Merck A. G.) and ascending chromatography was performed with a 1-buthanol-acetic acid-water solvent system (60:20:20 *v*:*v*:*v*), and stained with ninhydrin.[5]

RESULTS

Activation of BANA hydrolase with thiol activators. The effects of DTT, EDTA, and KCN were tested on purified enzyme preparations I, AI, and NII using BANA and Leu-NA as substrates (Fig. 1). DTT activated the hydrolysis of both substrates by all enzyme preparations, maximally at concentrations over 0.1 mM. EDTA activated the enzyme preparations only slightly, which indicated that heavy metals were not present in the enzyme solutions. Enzyme types I and II responded oppositely

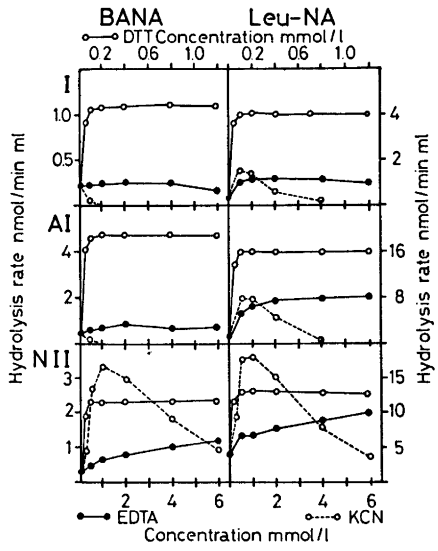

Fig. 1. Effects of DTT, EDTA, and KCN on the Leu-NA and BANA hydrolyzing activities of enzyme preparations I, AI, and NII.

on KCN, when BANA was used as a substrate; type I was inhibited and type II activated by 1 mM KCN. On the other hand, both types were activated by KCN, when Leu-NA was used as a substrate. KCN caused similar

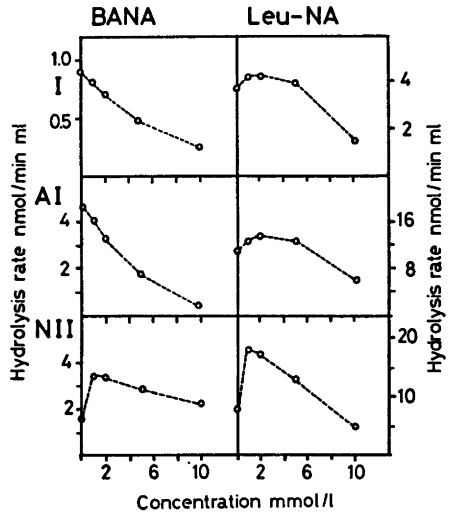

Fig. 2. Effect of KCN on the Leu-NA and BANA hydrolyzing activities of purified preparations I, AI, and NII, when the reaction mixtures contained DTT (0.2 mM) and EDTA (1 mM).

Table 1. Effects of some inhibitors on the activity of BANA hydrolase. TM = Tris-maleate buffer, BR = Britton-Robinson buffer, KIE = kallikrein inhibiting equivalent (see "Materials and Methods").

Inhibitor	Conc. mM	Buffer	Relative activities of preparations I	AI	NII
Control		BR, TM	100	100	100
Pb-acetate	1	TM	0	0	0
$CuCl_2$	1	TM	0	0	0
$HgCl_2$	1	TM	0	0	0
$CoCl_2$	1	TM	0	0	0
$FeCl_2$	1	TM	10	10	20
$CdCl_2$	1	TM	0	0	0
$CaCl_2$	1	TM	80	80	90
$ZnCl_2$	1	TM	0	0	0
$MnCl_2$	1	TM	50	50	60
$MgCl_2$	1	TM	90	90	90
Iodoacetic acid	1	BR	0	0	5
Iodoacetamide	1	BR	10	10	5
4-Chloromercuribenzoic acid	0.1	BR	20	20	20
N-Ethylmaleimide	1	BR	10	10	30
TLCK	0.1	BR	0	0	0
4-Aminobenzamidine	1	BR	100	100	100
Benzethonium chloride	1	BR	150	150	160
Puromycin	1	BR	70	70	70
DFP	1	BR	100	100	100
PMSF	1	BR	100	100	100
SBTI	0.1[a]	BR	104	100	100
Tracylol[R]	16.6[b]	BR	110	110	110
Ovomucoid	0.1[a]	BR	100	100	100

[a] mg/ml. [b] KIE/ml.

effects, when the reaction mixture contained optimal concentrations of DTT and EDTA (0.2 and 1 mM, Fig. 2), but higher concentrations of KCN (> 10 mM) were needed for total inhibition of the BANA hydrolyzing activity of type I.

Inhibitors. In Table 1 the effects of some inhibitors on the hydrolysis of BANA by preparations I, AI, and NII are presented. Heavy metal ions Pb^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+}, and Zn^{2+} were found to be strong inhibitors, while Mg^{2+}, Mn^{2+}, and Ca^{2+} were only slightly inhibitory. Tosyllysine chloromethylketone, iodoacetic acid, iodoacetamide, 4-chloromercuribenzoic acid, and *N*-ethylmaleimide strongly inhibited all BANA hydrolase preparations. The high-molecular-weight trypsin inhibitors from soybean, ovomucoid, and bovine lung (Tracylol [R]) were without effect on the activity, while puromycin exerted a slight inhibition. Benzethonium chloride caused a slight activation at 1 – 5 mM concentrations, but inhibited BANA hydrolase preparations at higher concentrations (over 10 mM). NaCl (0.01 – 0.20 M) and methanol (1 – 10 %) had no effects on the enzyme activities.

Substrate specificity. In Table 2 relative hydrolysis rates of various substrates in comparison to the hydrolysis rate of BANA are presented. The BANA hydrolase preparations hydrolyzed naphthylamides, nitroanilides, amides, and esters of various amino acids. The most readily hydrolyzed substrates were Met-NA, Arg-ME, Lys-NA, Arg-NA, and Ala-NA, in decreasing order. Substrates with a free α-amino group were hydrolyzed faster than corresponding substrates with an acylated α-amino group: Arg-NA 4.7 times faster than BANA and Arg-ME 8.7 times faster than BAME. Nitroanilides were hydrolyzed slower than corresponding naphthylamides: BAPA was not hydrolyzed at all and the hydrolysis rate of Leu-PA was 50 – 65 % of that of Leu-NA, at pH 5.8. Tos-Arg-NA was not hydrolyzed,

Table 2. Substrate specificity of rat skin BANA hydrolase. Relative hydrolysis rates, in comparison to that of BANA, are given. The values are expressed as the means of the activities of the enzyme preparations from three separate purification procedures. The specific activities of the preparations were (nmol min^{-1} mg^{-1}): preparation I, 49.3, 77.1, and 71.3; preparation AI, 394, 495, and 460; and preparation NII, 68.6, 99.3, and 95.2.

Substrate	Concentration Substr. mM	Methanol % (v/v)	pH	Relative activities of preparations I	AI	NII
BANA	1.66	6.66	5.8	100	100	100
Met-NA	1.66	6.66	5.8	790	740	920
Lys-NA	1.66	6.66	5.8	540	590	590
Arg-NA	1.66	6.66	5.8	470	470	490
Ala-NA	1.66	6.66	5.8	540	550	500
Leu-NA	1.66	6.66	5.8	240	260	450
Leu-NA	1.66	6.66	7.0	390	340	510
Val-NA	1.66	6.66	5.8	210	250	280
Glu-NA	1.66	6.66	5.8	160	150	180
Tos-Arg-NA	1.66	6.66	5.8	0	0	0
Leu-PA	0.33	6.66	5.8	160	130	210
BAPA	0.33	6.66	5.8	0	0	0
BAA	20	0	5.8	30	40	10
Arg-ME	3.33	0	5.8	760	780	670
TEE	3.33	0	5.8	360	400	230
BAME	3.33	0	5.8	90	90	90
ATEE	3.33	0	5.8	10	10	5
Hemoglobin	0.66[a]	0	4.3	0	0	80

[a] %.

and the rate of hydrolysis of BAA by preparations I and AI was 30 – 40 %, and by preparation NII 10 % of that of BANA. The relative activity of BAA/BANA always increased when preparation II was pretreated at pH 4 and 55 °C. Hemoglobin was hydrolyzed by preparation NII, but not by preparations I and AI. The hemoglobin hydrolyzing activity was not inhibited by iodoacetic acid or TLCK, suggesting that a cathepsin D contaminant was present in preparation NII.

The substrate specificities of preparations I and AI (type I) were similar, while some differences in their specificities in comparison to that of preparation NII (type II) was noticed. The relative activity (as compared to the hydrolysis rate of BANA) of type II was higher than that of type I, when amino acid naphthylamides were used as substrates, while the relative activity of type I was higher than that of type II, when BAA or amino acid esters were used as substrates.

The Michaelis constants of preparations I, AI, and NII were determined for BANA by the Lineweaver-Burk method. A Michaelis constant of 1.4 mM was obtained for preparations I and AI, and 5.0 mM for preparation NII. BANA inhibited the hydrolysis of Leu-NA by preparation NII, at pH 5.8. The specific activity of the undiluted enzyme was 9.4 nmol min^{-1} mg^{-1}, when BANA was used as a substrate, and 40.9 nmol min^{-1} mg^{-1}, when Leu-NA was used as a substrate. If BANA and Leu-NA were simultaneously in the reaction mixture, liberation of naphtylamine was only 30.1 nmol min^{-1} mg^{-1}.

Transformation of enzyme type II to type I. When enzyme type II (preparation II) was preincubated at pH 4 and 55 °C, the activity of the undiluted enzyme was increased and in a subsequent DEAE cellulose chromatography the enzyme was eluted as type I (preparation AI).[1] In Fig. 3 the dependence of this activation on pH and temperature is presented, using a fixed incubation time (20 min). At pH 7.5 the enzyme was labile at 45 °C. At pH 4.5, the activity increased by increasing temperature up to 60 °C, and at pH 4.0 the maximal activity was reached at 55 – 60 °C. At more acidic pH's (3.5 – 3.0) the maximal activity was

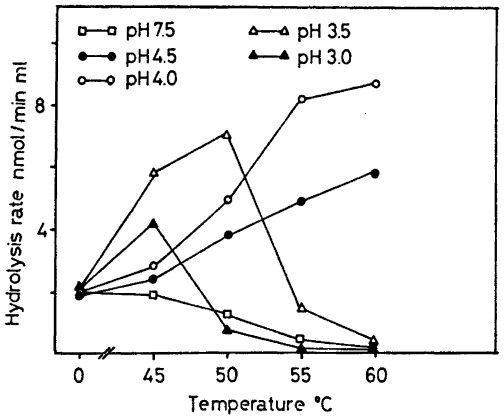

Fig. 3. Effect of preincubation of preparation II at various pH's and temperatures, for 20 min, on the BANA hydrolase activity of the undiluted enzyme.

obtained at lower temperatures (50 – 45 °C), and higher temperatures destroyed the enzyme. In Fig. 4 the activation effects of preincubation times at a fixed temperature (55 °C) and different pH's are presented. At pH 7.5 the activity of the enzyme was destroyed within 30 min. At acidic pH's (5.0 or below) activation of BANA hydrolase was noticed. The initial rate of the activation reaction increased with increasing acidity of the solution, but at pH's below 4.0 the activated enzyme was labile at 55 °C. At pH 4.0 the activity reached a plateau within 10 min and remained unchanged during the next 20 min. The most reproducible activation of preparation II was obtained by using an incubation time of 20 min, at pH 4.0

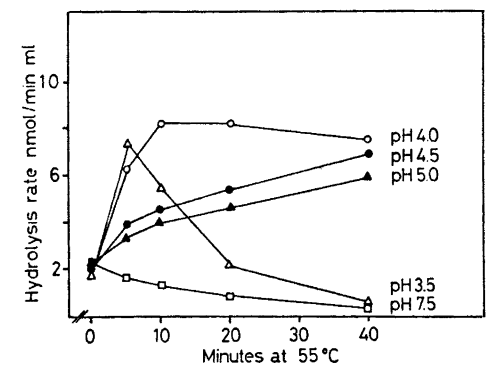

Fig. 4. Effect of preincubation time at various pH's, at 55 °C on the BANA hydrolyzing activity of preparation II.

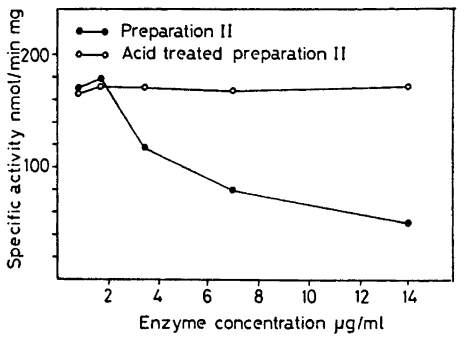

Fig. 5. Effect of enzyme concentration on the specific activity of preparation II and acid treated preparation II.

and 55 °C. After acid activation, the specific activity of preparation II did not increase by diluting the enzyme solution. In Fig. 5 the specific activities of preparation II and acid activated preparation II as a function of enzyme concentration are presented. It is interesting to notice that the specific activities of the acid treated and nontreated enzymes approach each other at low enzyme concentrations.

Since the activation of various proteolytic enzymes is a proteolytic reaction itself an investigation was made on the protease activity of preparation II, at pH 4.0 and 55 °C. When BANA or Leu-NA were added to the activation mixture at a final concentration of 1.66 mM, no formation of naphthylamine was observed. However, when hemoglobin, at a final concentration of 0.66 %, was incubated with preparation II at pH 4.0 and 55 °C, a marked hydrolysis occurred.

The role of an autolytic protease in the activation of preparation II at pH 4, at 55 °C, was studied by isolating possible peptides formed during the reaction, by an ultrafiltration technique (details are given in "Materials and Methods"). During the incubation of preparation II (20 ml) at pH 4, 55 °C, the total activity of the undiluted enzyme increased from 52 nmol/min to 218 nmol/min, with a concomitant release of 40.1 nmol of amino acids. A thin-layer chromatography of isolated peptides or amino acids is presented in Fig. 6.

Distinct spots of amino acids or peptides were found in the ultrafiltrate of the acid treated preparation II, the most prominent spot having an R_F value similar to that of glycine

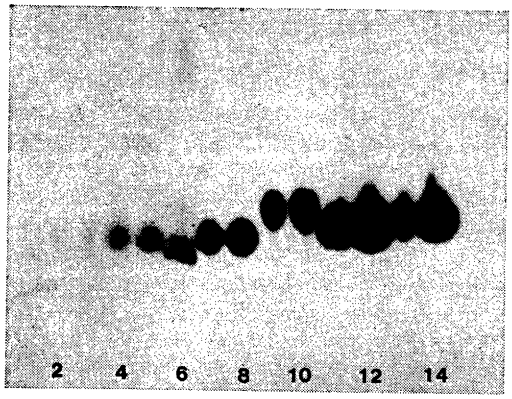

Fig. 6. A thin layer chromatography of ultrafiltrates of preparation II and acid-treated preparation II. 1 – 3, ultrafiltrates of preparation II; 4 – 6, ultrafiltrates of acid treated preparation II (10, 20, and 40 μl); 7 – 8, glycine; 9 – 10, glutamic acid; 11 – 12, serine; 13 – 14, threonine (5 and 10 μg).

(0.40), while the ultrafiltrate of the untreated preparation II contained only very little ninhydrin positive material. The experiments suggest that transformation of type II to type I is a proteolytic reaction.

DISCUSSION

The rat skin BANA hydrolase preparations are, like cathepsin B1 isolated from various tissues, activated by thiols (DTT) and chelating reagent (EDTA). Activation by EDTA was not very marked while DTT strongly activated the enzyme. BANA hydrolase types I and II responded oppositely to 1 mM KCN, when BANA was used as a substrate; type I was inhibited and type II activated. When Leu-NA was used as a substrate, both types were activated by KCN. The actual reason for different responses of enzyme types I and II to KCN remained unsolved, as did the differences in the effects of KCN on the hydrolysis of Leu-NA and BANA, by enzyme type I. In the literature, the reported effect of KCN on the activity of cathepsin B1 varies.[6–8] It seems that various cathepsin B1 like enzymes respond differently to KCN, as do rat skin BANA hydrolase types I and II.

In inhibitor experiments, the rat skin BANA hydrolase preparations behaved as expected for thiol-activated proteinases. Serine proteases acylating inhibitors and high-molecular-weight trypsin inhibitors were not inhibitory. 4-Aminobenzamidine, a potent competitive inhibitor of trypsin [9] did not inhibit, and puromycin, which inhibits a pituitary thiol-activated arylamidase,[10] inhibited BANA hydrolase preparations only slightly. Benzethonium chloride, which inhibits some salivary arylamidases [11] and cathepsin B1 of beef spleen,[12] activated rat skin BANA hydrolase preparations at low concentrations (up to 5 mM), but caused inhibition at higher concentrations.

The substrate specificity of rat skin BANA hydrolases differed markedly from the specificities of cathepsin B1 preparations of various tissues. Substrates with a free α-amino group were hydrolyzed much faster than substrates with a blocked α-amino group. This was evident with both naphthylamide and ester substrates, and is in contrast with the specificity of most known cathepsin B1 preparations, which do not hydrolyze substrates with a free α-amino group.[7,8,12–16] However, a highly purified cathepsin B1 preparation from bovine spleen hydrolyzes Leu-NA.[17] The following facts suggest that rat skin BANA hydrolase also hydrolyzes Leu-NA:

1. The BANA and Leu-NA hydrolyzing activities were not separated by salt precipitation, gel chromatography, ion exchange chromatography, isoelectric focusing, or polyacrylamide gel electrophoresis.[1]

2. Acid treatment of type II enzyme at pH 4 and 55 °C caused similar changes in the properties of BANA and Leu-NA hydrolyzing activities (with the exception of the activation by KCN).

3. BANA inhibited the hydrolysis of Leu-NA by preparation NII.

Besides BANA, the rat skin enzyme preparations also hydrolyzed BAA, but BAPA was not hydrolyzed at all. The deficiency in the hydrolysis of BAPA by cathepsin B1 preparations has also been noticed in rat liver [16] and sheep thyroid gland.[15] Preparations II and NII hydrolyzed hemoglobin. This activity was not inhibited by iodoacetate or TLCK, which suggested that a hemoglobin hydrolyzing cathepsin D contaminate was present in preparations II and NII.[18] Cathepsin B1 is known to be a powerfull proteinase [8,12,14,19–21] and this proteolytic activi-

ty distinguishes it from rat skin BANA hydrolase.

BANA hydrolase of the rat skin behaved like an aminopeptidase-like arylamidase. Thiol-activated amino acid naphthylamidases have been found in rat skin,[22,23] testis,[24] kidney,[25,26] and liver.[26] The naphthylamidase from rat kidney and liver lysosomes [25,26] has properties very similar to those of skin BANA hydrolase. The lysosomal naphthylamidase is active optimally at pH 7.0, and is activated by dithiothreitol.[26] The enzyme preferentially hydrolyzes the naphthylamides of arginine, lysine, phenylalanine, and leucine, and blocking of the α-amino group of Arg-NA with a carbobenzoxy group lowers the hydrolysis rate by 76 %. It is, however, known that the lysosomal amino acid naphthylamidase differs from the BANA hydrolase purified from rat liver lysosomes.[16]

Treatment of BANA hydrolase type II at pH 4 and 55 °C for 20 min resulted in formation of type I with concomitant changes in isoelectric point, chromatographic behavior,[1] and effect of KCN on BANA hydrolyzing activity of the enzyme. During the acid treatment amino acids or peptides were formed and any added hemoglobin was hydrolyzed, while BANA and Leu-NA remained unchanged. These facts suggest that the activation at acidic pH is a proteolytic reaction probably catalyzed by an acidic protease, cathepsic D, present in type II enzyme preparations.[18] The molecular modification may take place in the BANA hydrolase molecule itself, or through some inhibitor associated with it. The fact that the specific activity of type II increased by dilution, while the specific activity of type I was uneffected suggests the latter mechanism. However, an inhibitor destroyed proteolytically must be a comparatively small molecule, since no difference in molecular sizes of types I and II was noticed by gel filtration.[1] Such a small inhibitor has been isolated from mouse haptoglobin.[27] The rat skin inhibitors of BANA hydrolase demonstrated in the previous report [1] have molecular weights of about 74 000 and 13 000, and the dissociation of these inhibitors from BANA hydrolase should cause a positive change in the molecular weight of the enzyme.

Slight activation of cathepsin B1 isolated from various tissue extracts has been noticed after the extracts were autolyzed at pH 3 – 4.[8,20,28] The reported activation is comparatively small, 20 – 30 %, while rat skin BANA hydrolase was activated 400 %. Barret [8] also reports a two-fold activation of purified human liver cathepsin B1, after storage of the enzyme at pH 5.0, in the presence of EDTA, at +4 °C. The isoelectric point of the human liver enzyme was not changed during storage, suggesting that the mechanism of the activation of the human liver enzyme differs from that of the rat skin BANA hydrolase. No activation of the rat liver lysosomal BANA hydrolase has been noticed, after the enzyme had been incubated at pH's below 5.0.[16] The rat skin BANA hydrolase is, like cathepsin B1 preparations of various tissues, readily inactivated at alkaline pH and elevated temperatures.[8,12,16]

α-*N*-Benzoylarginine-2-naphthylamide has widely been used as a substrate of cathepsin B1. However, Distelmaier *et al.*[29] have shown that testing of the BANA hydrolyzing activity of a tissue extract may lead to erroneous conclusions on the presence of cathepsin B1 in the tissue. Rat skin BANA hydrolase also has many properties that cannot be attributed to cathepsin B1 and the BANA hydrolyzing activity of the skin seems to be associated with a thiol-activated arylamidase.

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Oxidation—Degradation of Methyl 4-*O*-(2,3-Di-*O*-methyl-α-D-glucopyranosyl)-2,3,6-tri-*O*-methyl-β-D-glucoside

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Methyl 4-*O*-(2,3-di-*O*-methyl-α-D-glucopyranosyl)-2,3,6-tri-*O*-methyl-β-D-glucoside has been synthesized. The product obtained after oxidation was subjected to degradation by base and/or mild acid treatment. The use of these degradation procedures for specific degradation of polysaccharides is discussed.

In previous publications [1–4] a specific degradation procedure, using model compounds, is reported. Methylated polysaccharides or glycoconjugates having a limited number of free hydroxyl groups at specific positions [5] are suitable for sequential degradation. The degradation method involves oxidation of the free hydroxyl groups to carbonyl functions, β-elimination with formation of α,β-unsaturated ketones or aldehydes and further degradation of these derivatives by mild acid hydrolysis. Employing this method the glycosidic linkage of a methylated sugar residue with a free hydroxyl group in the 2-, 3-, 4-, or 6-position can be specifically cleaved. The degradation method has been applied in structural studies of polysaccharides.[6–8]

Several bacterial polysaccharides contain hexopyranoside residues substituted with pyruvic acid, ketalically bound to O-4 and O-6. Formaldehyde acetalically bound to the same positions has also been observed.[9] It should be possible to use the oxidation-degradation procedure after selective removal of such groups from a methylated polysaccharide. The removal of the ketal group, when preceded by carboxyl reduction, may be performed by acid hydrolysis under mild conditions without simultaneous hydrolysis of glycopyranosidic linkages. The possibility of degrading polysaccharides by this route has now been investigated using the disaccharide methyl 4-*O*-(2,3-di-*O*-methyl-α-D-glucopyranosyl)-2,3,6-tri-*O*-methyl-β-D-glucoside as a model substance.

RESULTS AND DISCUSSION

Methyl β-maltoside was treated with benzylidene bromide in pyridine [10] to yield methyl 4-*O*-(4,6-*O*-benzylidene-α-D-glucosyl)-β-D-glucoside (I). Methylation of I with methyl iodide/sodium hydride in dioxane gave crystalline methyl 4-*O*-(4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-glucosyl)-2,3,6-tri-*O*-methyl-β-D-glucoside (II), which after hydrogenation afforded the desired model compound methyl 4-*O*-(2,3-di-*O*-methyl-α-D-glucopyranosyl)-2,3,6-tri-*O*-methyl-β-D-glucoside (III). The structure of III was confirmed by NMR spectroscopy and sugar analysis of its hydrolysate.

The model compound (III) was oxidised with a chlorine–dimethyl sulfoxide complex,[11] but part of the reaction mixture was withdrawn before the addition of triethylamine. Sugar analysis of the latter product showed no oxidation of the hydroxyl groups.

The oxidised product was treated with base and acid under different conditions. The cleavage of the glycosidic linkage was evaluated by GLC from the amount of liberated methyl 2,3,6-tri-*O*-methyl-β-D-glucoside. Methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside was used as an internal standard. These analyses showed that

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about 25 % of the glucosidic bond was cleaved during the treatment with triethylamine, the work-up procedure and/or by thermal degradation in the gas chromatograph.

On treatment of oxidised III with sodium ethoxide in dichloromethane-ethanol followed by hydrolysis under mild conditions (50 % aqueous acetic acid, 100 °C, 3 h), a quantitative cleavage of the glycosidic linkage of the oxidised residue was obtained. It was, however, also possible to generate glycosidic cleavage by either base or mild acid hydrolysis alone. Selective degradation is therefore feasible in the presence of groups that are acid labile.

In a separate experiment the product from the oxidation of III was reduced with sodium borodeuteride, hydrolysed and the resulting mixture of sugars analysed, as alditol acetates, by GLC-MS.[12] The ratio of 2,3-di-*O*-methyl-D-glucose to 2,3,6-tri-*O*-methyl-D-glucose was 1:7, indicating that most of the oxidised residues were degraded during the alkaline conditions of the borohydride reduction. The 2,3-di-*O*-methyl-D-glucose was deuterated at C-6, showing that the small percentage of oxidised residues that not degraded had been oxidised at C-6 but not at C-4.

The results thus demonstrate that the degradation procedure can be applied to methylated polysaccharides with free hydroxyls at C-4 and C-6 of hexopyranoside residues. The glycosidic linkages of the oxidised residues may be cleaved either by treatment with base or with acid under mild conditions. The consecutive treatment with base and acid is, however, recommended, as complete degradation of only partially oxidised residues may otherwise not be obtained.

EXPERIMENTAL

Concentrations were performed at reduced pressure at bath temperatures not exceeding 40 °C. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. NMR spectra were recorded with a Varian A60 A spectrometer, using tetramethylsilane as internal reference. NMR spectra were recorded for all substances and were in agreement with the postulated structures. GLC separations were performed on a Perkin-Elmer model 900 instrument using a glass capillary column (25 m × 0.25 mm) wall-coated with SP-1000 (LKB-Products, Sweden). Peak areas were measured with a Hewlett-Packard 3370B electronic integrator. For GLC-MS a Perkin-Elmer 270 gas chromatograph – mass spectrometer fitted with an OV-225 S.C.O.T. column was used. Mass spectra were recorded at an ionisation potential of 70 eV and with an ion source temperature of 120 °C. Hydrolyses were performed with 90 % formic acid for 1 h at 100 °C followed by treatment with 0.25 M sulfuric acid for 16 h at 100 °C.

Methyl 4-O-(4,6-O-benzylidene-α-D-glucosyl)-β-D-glucoside (I). Methyl β-maltoside[13] (1.1 g) and benzylidene bromide (1.0 g) in pyridine (18 ml) were refluxed for 2 h. The solution was then allowed to cool and concentrated to dryness. The reaction mixture was separated on a Silica gel column (3 × 50 cm) using ethyl acetate as irrigant. The separation was monitored using TLC and the fractions containing I were concentrated to dryness yielding chromatographically pure I (1.0 g) as a syrup, $[\alpha]_D^{25}$ + 35° (*c* 0.4, ethanol).

Methyl 4-O-(4,6-O-benzylidene-2,3-di-O-methyl-α-D-glucosyl)-2,3,6-tri-O-methyl-β-D-glucoside (II). Methyl iodide (10 ml) was added to a mixture of I (1.0 g) and sodium hydride (1.0 g) in dioxane (20 ml). The mixture was refluxed for 4 h, cooled, and excess sodium hydride was destroyed by the addition of ethanol (10 ml). The solution was concentrated to dryness and partitioned between chloroform and water. The chloroform phase was collected and evaporated, yielding crystalline II which was recrystallised from ethanol – hexane (1:3, v/v). The yield of II was 1.1 g, m.p. 161 – 162 °C, $[\alpha]_D^{25}$ + 52° (*c* 1.0, chloroform). (Found: C 58.78; H 7.38. $C_{25}H_{38}O_{11}$ requires: C 58.35; H 7.44).

Methyl 4-O-(2,3-di-O-methyl-α-D-glucopyranosyl)-2,3,6-tri-O-methyl-β-D-glucoside (III). A solution of II (1.1 g) in ethanol (50 ml) was hydrogenated over palladium on carbon (10 %, 0.1 g) at room temperature and atmospheric pressure. When the hydrogen consumption had ceased, the catalyst was filtered off and the solution concentrated to dryness, yielding III as a chromatographically (TLC) pure syrup (0.9 g), $[\alpha]_D^{25}$ + 74° (*c* 1.4, chloroform). Sugar analysis of the hydrolysate gave equimolar amounts of 2,3,6-tri-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-glucose, analysed by GLC-MS as their alditol acetates.

Oxidation of III. The oxidation reagent was prepared by adding dimethyl sulfoxide (0.3 ml) to a 1 M solution of chlorine in anhydrous dichloromethane (1 ml) under vigorous stirring at − 45 °C. A white precipitate appeared during the addition. Compound III (24 mg) and methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside (13 mg) as an internal standard (relative molar proportion 1.08:1) in dichloromethane (2 ml) were added with the aid of a syringe, and the reaction mixture was stirred for 4 h at − 45 °C.

To show that III was quantitatively regenerated from the oxidation complex, part of the reaction mixture (1/10) was withdrawn, hydrolysed and the resulting sugars were analysed

as their alditol acetates. Triethylamine (0.25 ml) was added to the remaining part and the mixture was kept at −45 °C for another 10 minutes, and then allowed to rise to room temperature. TLC showed that no starting material was left. Part of the reaction mixture (1/10) was hydrolysed and the sugars analysed as their alditol acetates by GLC-MS. The ratio of 2,3-di-*O*-methyl-D-glucose to 2,3,6-tri-*O*-methyl-D-glucose was 0.03:1, demonstrating that most of the residues with free hydroxyl groups had been oxidised. Dimethyl sulfoxide and triethylamine hydrochloride were removed by chromatography on a Silica gel column (3 × 10 cm) using acetone−ethylacetate (1:1, v/v) as eluent and the eluate was concentrated to dryness.

Degradation of oxidised III. A. Part of the product (10 %) was analysed directly on GLC. The relative molar proportion of methyl 2,3,6-tri-*O*-methyl-β-D-glucoside and methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside (the internal standard) was 0.25:1.

B. Part of the product (30 %) was treated with 1 M sodium ethoxide in ethanol (0.2 ml) and dichloromethane (1.0 ml) for 1 h at room temperature, neutralized with glacial acetic acid and concentrated to dryness. The product was treated with 50 % aqueous acetic acid (1 ml) at 100 °C. Samples were withdrawn at intervals and analysed on GLC. The relative molar proportion of methyl 2,3,6-tri-*O*-methyl-β-D-glucopyranoside to the internal standard was 1.05:1 after 3 h.

C. Part of the product (30 %) was treated with 1 M sodium ethoxide in ethanol (0.2 ml) and dichloromethane (1 ml) at room temperature. Samples were withdrawn at intervals, neutralised with Dowex 50 (H^+) and analysed on GLC. The relative molar proportion of methyl 2,3,6-tri-*O*-methyl-β-D-glucopyranoside to the internal standard was 1.05:1 after 2 h.

D. Part of the product (30 %) was treated with 50 % aqueous acetic acid (1 ml) at 100 °C. Samples were withdrawn at intervals and analysed on GLC. The relative molar proportion of methyl 2,3,6-tri-*O*-methyl-β-D-glucopyranoside to the internal standard was 1.05:1 after 4 h.

Reduction of oxidised III. Compound III (2 mg) was oxidised as above and the organic solvents removed upon evaporation. Sodium borodeuteride (20 mg) was added to oxidised III in ethanol−water (1:1, 2 ml). The reaction mixture was kept for 14 h at room temperature, neutralised with Dowex 50 (H^+), filtered, hydrolysed, and the sugars analysed by GLC-MS of their alditol acetates. 2,3,6-Tri-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-glucose were obtained in the proportion 1:0.14. MS demonstrated that the latter substance was monodeuterated at C-6 but not at C-4.

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Periodate Oxidation of Phenols. XIX.* Nondimerizing *o*-Quinols, *o*-Quinol Ethers, and *o*-Quinone Ketals

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Several 6-hydroxy-6-methyl-2,4-cyclohexadienones (*o*-quinols) and their 6-ethoxy analogues carrying a CH_3 or OCH_3 substituent in the 5-position were obtained by oxidation of the appropriate phenols with sodium periodate in aqueous ethanol. The 6-methoxy analogues were prepared similarly, using periodic acid in methanol. These 2,4-cyclohexadienones showed no tendency to dimerize, in contrast to the corresponding dienones lacking the 5-substituent which undergo rapid Diels-Alder dimerization.

The *o*-quinol formed on oxidation of 2,3-dimethylphenol, although resistant to dimerization, could not be isolated since it gave Diels-Alder adducts with 2,3-dimethyl-*p*-quinone and 3,4-dimethyl-*o*-quinone being formed simultaneously. Part of the *o*-quinol suffered oxidative ring cleavage.

Treatment of 2,3-dimethoxyphenol with HIO_4/CH_3OH produced a nondimerizing *o*-quinone dimethyl ketal, 5,6,6-trimethoxy-2,4-cyclohexadienone.

A characteristic reaction of 2,4-cyclohexadienones [1] is their Diels-Alder dimerization. *o*-Quinols *1a*—*1e* and spiro-epoxydienones of type *2*, either unsubstituted or carrying small substituents such as CH_3 or OCH_3 in positions 2 to 4, dimerize so rapidly that they cannot be obtained as monomers.[2] The same is true for most of the *o*-quinone dimethyl ketals (*1*, $R = R^6 = OCH_3$) recently investigated.[3] Only the 3-methoxy derivative could be isolated as monomer, but it dimerized within two days at room temperature and thus behaved like *o*-quinol methyl ethers (*1*, $R = OCH_3$, $R^6 = CH_3$)[4] and 6,6-dialkyl-2,4-cyclohexadienones (*1*, $R = R^6 =$ alkyl).[1,5]

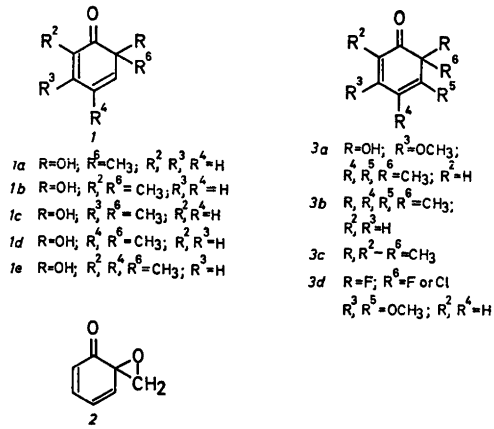

Chart 1.

The acetates derived from *o*-quinols *1a* and *1b*, although stable at room temperature, dimerize when heated at 120 °C,[6-8] whereas those of *1d* and *1e* proved to be stable even at 160 °C.[9] The dimerization of 2,4-cyclohexadienones is inhibited by bulky substituents such as aryl groups in the 2-, 4-, and 6-positions,[10] *t*-butyl [11] or iodine [8,11] in the 4-position, or a 2-cyclohexyl group.[11]

A few monomeric 2,4-cyclohexadienones lacking bulky substituents have been reported. Wessely *et al.*[12,13] obtained small amounts of *o*-quinols *4a* and *5a* (Chart 2) on thermolysis of the dimers of *1b* and *1e*, respectively. The retro Diels-Alder reaction of these dimers was followed by an acyloin rearrangement of the initially formed monomers, *1b* and *1e*.[7] *o*-Quinol *3a*,[14] the 6,6-dimethylcyclohexadienones

* Part XVIII, see Ref. 3.

3b [15] and *3c*,[16] and the 6,6-dihalo compounds *3d* [17] have also been described only as monomers.

In his review article (Ref. 1, p. 226), Waring assumed that substituents in positions 3 and 5 of a 2,4-cyclohexadienone hinder dimerization by steric interaction with the 6,6-groups of the other partner. This assumption seemed plausible as far as it concerned the 5-substituent, which is a common feature of compounds *3a-d*. It was incompatible, however, with the earlier reported spontaneous dimerization of *o*-quinol *1c*, which carries a 3-substituent [7,18] (*cf.* also Ref. 2a). The recently described dimerizations of a 3-methyl-substituted *o*-quinol ether [4] and of a similarly substituted spiro-epoxy-2,4-cyclohexadienone,[2a] as well as the dimerization of the above-mentioned 3-methoxy derivative of *o*-quinone dimethyl ketal,[3] also prove that a small 3-substituent is not critical in dimerization.

In the present work, 5-substituted *o*-quinols and *o*-quinol ethers, as well as a 5-substituted *o*-quinone dimethyl ketal, were prepared by the oxidation of phenols *4*–*8*, which carry a 3-methyl or a 3-methoxyl group, using either sodium periodate in water/ethanol (8:1) or periodic acid in methanol (Chart 2). No dimer of these 2,4-cyclohexadienones was detected in the reaction mixtures, nor could the isolated monomers be dimerized by heat treatment. These results firmly establish the dimerization-preventing effect of a small 5-substituent.

Oxidation with periodate in water/ethanol (Charts 2 and 3). Phenols *4*–*7* provided *o*-quinols (*5a* and *6a*) and *o*-quinol ethyl ethers (*4b*, *5b*, *7b*). Secondary reactions of these *ortho* oxidation products, as well as competing oxidation in the other *o*-position or in the *p*-position, gave rise to the additional products shown in Chart 3.

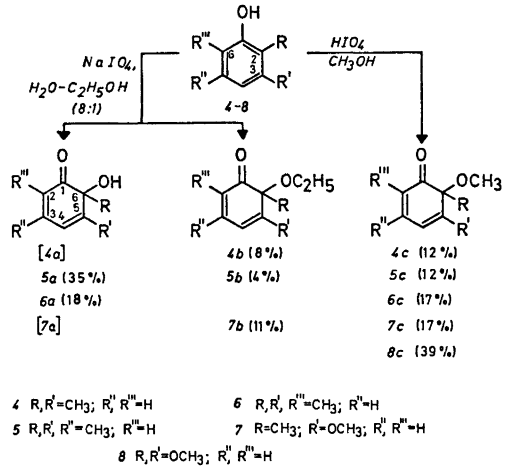

Chart 2.

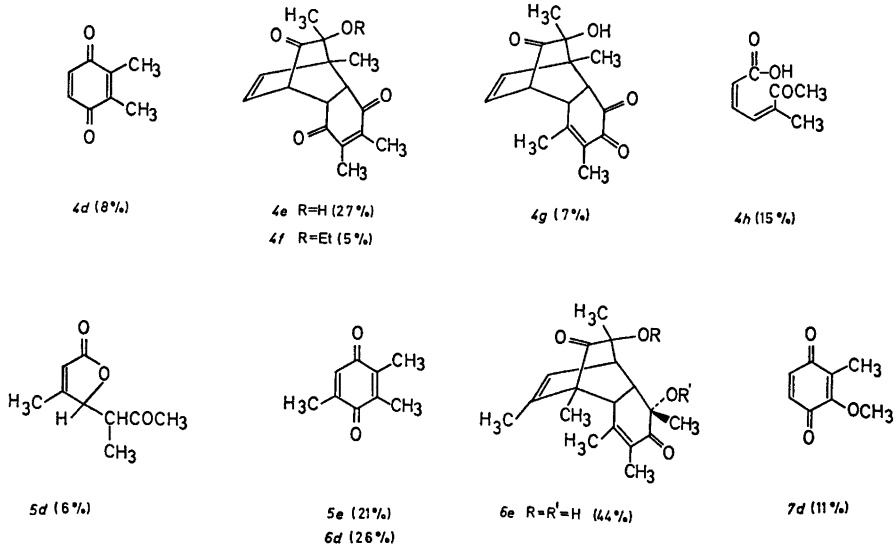

Chart 3. Products obtained, in addition to the 2,4-cyclohexadienones *a* and *b* (Chart 2), on oxidation of phenols *4*–*7* with $NaIO_4$ in H_2O/C_2H_5OH (8:1).

2,3-Dimethylphenol (*4*) was consumed very slowly, 33 % of the phenol being recovered after a reaction time of 21 h. (The yields of products derived from *4* are based on consumed phenol. The other phenols investigated were completely consumed within the reaction times used; see Experimental.) The expected *o*-quinol *4a* was not found in the reaction mixture. It had reacted as diene with simultaneously formed *p*-quinone *4d* and 3,4-dimethyl-*o*-benzoquinone to give the Diels-Alder adducts *4e* and *4g*, and had been also oxidized by periodate to the oxodienoic acid *4h*. A portion of the *o*-quinol ethyl ether also appeared as a *p*-quinone adduct (*4f*).

2,3,5-Trimethylphenol (*5*) gave, in addition to *5a* and *5b*, a minor amount of lactone *5d*, formed by comparatively slow periodate cleavage of *5a* followed by cyclization of the resulting oxodienoic acid. Furthermore, *para* oxidation produced the *p*-quinone *5e*.

In all the oxidations investigated (Chart 2), except for the oxidation of 2,3,6-trimethylphenol (*6*), the reaction mixtures acquired a red colour indicating the formation of the corresponding *o*-benzoquinones which, however, were removed during the work-up procedures (see Experimental). The presence of methyl groups in both *o*-positions of phenol *6* prevented *o*-quinone formation. Instead, oxidation at the 6-position of this phenol gave 2,3,6-trimethyl-*o*-quinol, which is isomeric with *6a*. Having no 5-substituent, it dimerized to give product *6e*. Competing *para* oxidation resulted in the formation of *6d*.

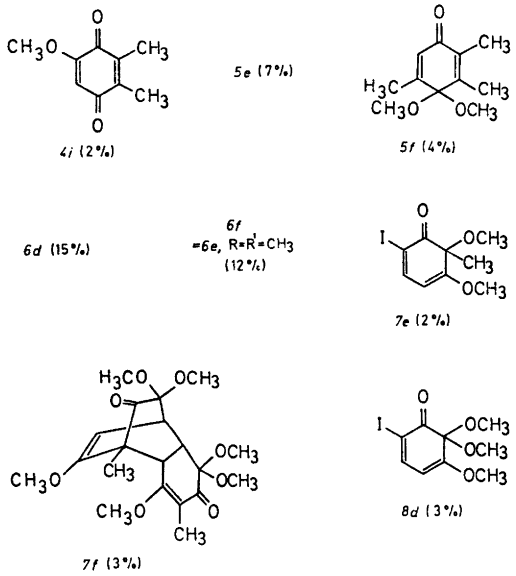

Chart 4. Products obtained, in addition to *4c – 8c* (Chart 2), on oxidation of phenols *4 – 8* with HIO_4 in CH_3OH.

Similar *para* oxidation of 3-methoxy-2-methylphenol (*7*) gave the *p*-quinone *7d* in addition to the *ortho* oxidation product *7b*. *o*-Quinol *7a* could not be detected, probably because it had suffered ketol cleavage.

Oxidation with periodic acid in methanol (Charts 2 and 4). Treatment of phenols *4 – 7* with HIO_4 in CH_3OH provided the *o*-quinol methyl ethers *4c – 7c*, and similar treatment of 2,3-dimethoxyphenol (*8*) gave the 5-methoxy-substituted 2,4-cyclohexadienone *8c*, an *o*-quinone dimethyl ketal (Chart 2).

Oxidative methoxylation at the free *o*-position of phenol *4*, followed by *para* oxidation, produced a small amount of *4i*. In the case of phenol *5*, two consecutive *para* methoxylations gave the *p*-quinone ketal *5f*, the *p*-quinone *5e* arising possibly by hydrolysis of *5f*. The formation of the dimeric *o*-quinone ketal *7f* can be understood as being due to a similar twofold *ortho*-oxidative methoxylation of phenol *7* with subsequent dimerization of the resulting 3,6,6-trimethoxy-2-methyl-2,4-cyclohexadienone.

The formation of the Diels-Alder dimer *6f* is similar to that of *6e* discussed above. As expected, both dimers have the same stereochemistry and regiochemistry, as shown by the methylation of *6e* to give *6f*. The orientations presented for *6e* and *6f* are analogous to those established earlier for other *o*-quinol dimers [2] and their dimethyl ethers.[4]

Initial iodination of the starting phenols, as observed previously in the system HIO_4/CH_3OH,[4] explains the formation of the 2-iodocyclohexadienones *7e* and *8d*.

In a following paper, mixed Diels-Alder reactions of nondimerizing 2,4-cyclohexadienones will be discussed.

EXPERIMENTAL

UV spectra were run in ethanol on a Beckman DK-2A, and IR spectra were recorded in KBr on a Beckman 9A instrument. ^1H NMR spectra were taken in $CDCl_3$ on a Varian A-60 spectrometer, unless otherwise stated. UV data

are noted as λ_{max} values in nm, with log ε values indicated in parentheses, IR data as ν_{max} values in cm^{-1}. Thin layer chromatography was performed on silica gel with benzene/ethyl acetate (4:1) as mobile phase.

Oxidation procedures. (A). For the oxidation in *aqueous* medium, a solution of the starting phenol (about 30 mmol) in a 7:1 mixture of water and ethanol (1600 ml) was mixed with an aqueous solution (200 ml) of $NaIO_4$ (about 90 mmol). After the reaction time (*t*) given below, the mixture was extracted with dichloromethane (6 × 100 ml), and the extract was dried over anhydrous $CaSO_4$ and evaporated. A solution of the resulting oil in ethyl acetate was passed through a column of aluminium oxide ("neutral", Woelm, 4 × 15 cm) which then was washed with the same solvent, dark-brown material remaining adsorbed in the upper part of the column. The combined filtrates, on evaporation, gave a yellow, oily or partly crystalline product which was chromatographed on a column (3 × 110 cm) of silica gel (Silicic Acid, Mallinckrodt, 100 mesh), benzene/ethyl acetate (4:1) being used as eluent.

Deviations from this general procedure are noted below.

(B) The oxidations in *methanolic* solution were carried out by mixing a solution of the phenol (about 30 mmol) in absolute methanol (50 ml) with that of an equimolar amount of anhydrous periodic acid (HIO_4)[19] in the same solvent (400 ml). (This actually implies an excess of oxidant, since the iodic acid formed also oxidizes the phenol.[4]) After 5 – 10 min, the reaction mixture had acquired a red colour. After 2 h, water (400 ml) was added, the mixture was extracted with dichloromethane, and the products were separated as above.

The reaction products are given below in the order of their elution from the silica gel column.

Oxidation of 2,3-dimethylphenol (4). (A) With $NaIO_4$ in H_2O-EtOH; t = 21 h. The dichloromethane extract obtained from the reaction mixture was extracted with saturated aqueous sodium bicarbonate. The bicarbonate phase was acidified and extracted with dichloromethane. The dried extract on evaporation gave crystals of *5-methyl-6-oxo-2,4-heptadienoic acid* (4h), m.p. 114 – 117 °C (15 %, based on consumed *4*, see below), after recrystallization from isopropyl ether m.p. 118 – 119 °C. (Found: C 62.30; H 6.55. Calc. for $C_8H_{10}O_3$: C 62.32; H 6.54). Parent mass, found: 154.0631; calc. for $C_8H_{10}O_3$: 154.0630. UV 282 (4.42). IR 3000 – 2400 and 1698 (COOH), 1667 (conj. CO), 1623 and 1587 (C=C). NMR δ 1.97 (d, 3 H, CH_3-5), 2.42 (s, 3 H, CH_3-7), 6.00 (d, 1 H, H-2), 7.13 (t, 1 H, H-3), 8.30 (d, 1 H, H-4). The doublets of H-2 and H-4 are further split by allylic coupling. $J_{2,3} = J_{3,4} = 11$ Hz, $J_{H-4,CH_3-5} = 1$ Hz.

The dichloromethane solution remaining after the extraction with bicarbonate, when dried and evaporated, gave a residue which afforded the following products on silica gel chromatography:

(a) A brownish yellow crystalline product, which on TLC with benzene as eluent gave two spots, the R_F values of which were identical with those found for starting phenol *4* and *p*-quinone *4d*. The NMR spectrum of the crude product showed the signals expected for these two compounds, and the integrals of their CH_3 signals indicated that the product was composed of about 85 % of *4* and 15 % of *4d*. The amount of phenol present corresponded to 33 % of the amount used. The yields of products given below are based on consumed phenol.

For identification of the *p*-quinone, a solution of the crude fraction in ethyl acetate was passed through a column (4 × 5 cm) of Al_2O_3 (neutral, Woelm) which retained the phenol *4*; the filtrate gave *2,3-dimethyl*-p-*benzoquinone* (4d), m.p. 54 – 55 °C (8 %), identical by m.p. and mixed m.p. with an authentic sample.[20]

(b) 10-Ethoxy-1,4,4a,8a-tetrahydro-1,6,7,10-tetramethyl-1,4-ethanonaphthalene-5,8,9-trione (4f). The ethyl acetate solution of the crude material was purified with Al_2O_3 (see above) and then gave faintly yellow needles, m.p. 160 – 161 °C (ethyl acetate); yield, 5 %. (Found: C 71.45; H 7.29. Calc. for $C_{18}H_{22}O_4$: C 71.50; H 7.33). UV 203 (3.88), 252 (3.94), sh 315 (2.23), 365 (2.02); *cf.* Ref. 9. IR 1728 s, 1663 s, 1628 w. NMR δ 1.10 (t, 3 H, OCH_2CH_3), 1.25, 1.40 (s, 3 H each, CH_3-1, CH_3-10), 1.95 (s, 6 H, CH_3-6, CH_3-7), 3.3 – 3.8 (m, 5 H, OCH_2CH_3, H-4, H-4a, H-8a), 6.0 (m, 2 H, H-2, H-3).

(c) 6-Ethoxy-5,6-dimethyl-2,4-cyclohexadienone (4b), yellow oil, purified by distillation at 50 °C/1 mmHg; yield, 8 %. Parent mass, found: 166.0986; calc. for $C_{10}H_{14}O_2$: 166.0993. UV 316 (3.66), sh 385 (2.66); the former λ_{max} value ($\pi \rightarrow \pi^*$) is in accord with the value (320 nm) calculated according to the extended Woodward rules.[21] IR 1678 vs, 1640 s, 1570 m; for the IR spectra of 2,4-cyclohexadienones, see Refs. 1, 22 – 24.

(d) 1,4,4a,8a-Tetrahydro-10-hydroxy-1,6,7,10-tetramethyl-1,4-ethanonaphthalene-5,8,9-trione (4e); yield, 27 %; faintly yellow prisms, m.p. 146 – 148 °C (isopropyl ether). (Found: C 69.98; H 6.65. Calc. for $C_{16}H_{18}O_4$: C 70.05; H 6.61). UV 203 (3.88), 252 (4.01), sh 315 (2.49), 360 (2.10); *cf.* Ref. 9. IR 3470, 1740, s, 1660 s, 1620 m. NMR, recorded on a Bruker WH 270 instrument: δ 1.23, 1.31 (s, 3 H each, CH_3-1, CH_3-10), 1.90, 1,96 (s, 3 H each, CH_3-6, CH_3-7), 2.55 (s, 1 H, OH-10, exchangeable with D_2O), 3.30 (dd, 1 H, H-4a), 3.60 (d, 1 H, H-8a), 3.68 (four doublets, 1 H, H-4), 6.00 (dd, 1 H, H-2), 6.12 (dd, 1 H, H-3). $J_{3,4} = 6$ Hz, $J_{4,4a} = 3$ Hz, $J_{2,4} = 1.5$ Hz, $J_{2,3} = 8$ Hz, $J_{4a,8a} = 9$ Hz.

(e) 1,4,4a,8a-Tetrahydro-9-hydroxy-4,7,8,9-tetramethyl-1,4-ethanonaphthalene-5,6,10-trione (4g); yield, 7 %; yellow needles of m.p. 150 – 151 °C (isopropyl ether). (Found: C 69.63; H 6.60. Calc. for $C_{16}H_{18}O_4$: C 70.05; H 6.61). UV 273 (3.79), sh 308 (3.29), 418 (1.87). IR ($CHCl_3$)

1730, 1715, 1675 (three CO groups), 1625 (C=C); in KBr: 3440 (OH), 1725 (broad), 1680, 1628. These UV and IR characteristics are similar to those of the adduct between 6-hydroxy-4,6-dimethyl-2,4-cyclohexadienone and 3,5-dimethyl-1,2-benzoquinone.[25] NMR, recorded on a Bruker WH 270 instrument: δ 1.23, 1.30 (s, 3 H each, CH_3-4, CH_3-9), 1.83, 2.07 (s, 3 H each, CH_3-7, CH_3-8), 2.32 (broad s, 1 H, OH, exchangeable with D_2O), 3.34 (d with broadened signals, 1 H, H-8a), 3.40 (d, 1 H, H-4a), 3.46–3.53 (m, 1 H, H-1), 6.00 (dd, 1 H, H-2), 6.05 (dd, 1 H, H-3). $J_{1,2}=6$ Hz, $J_{1,3}=1.5$ Hz, $J_{2,3}=7.5$ Hz, $J_{4a,8a}=8$ Hz.

*(B) With HIO_4 in CH_3OH. (a) 5-Methoxy-2,3-dimethyl-*p*-benzoquinone (4i)*, 2 % of yellow needles, m.p. 104–108 °C which was raised to 112–113 °C (lit.[26] m.p. 110–111 °C) by recrystallization from methanol. NMR δ 2.03 (s, 6 H, CH_3-2, CH_3-3), 3.80 (s, 3 H, OCH_3-6), 5.85 (s, 1 H, H-5).

(b) 6-Methoxy-5,6-dimethyl-2,4-cyclohexadienone (4c), 12 % of a yellow oil, which crystallized on cooling, m.p. 29–31 °C after distillation at 40 °C/15 mmHg. (Found: C 70.64; H 8.14; OCH_3 20.52. Calc. for $C_9H_{12}O_2$: C 71.02; H 7.95; OCH_3 20.39). The UV and IR spectra of *4c* were almost identical with those of *4b* (see above).

Oxidation of 6-hydroxy-5,6-dimethyl-2,4-cyclohexadienone (4a). The *o*-quinol[12] (600 mg) was treated for 6 h with aqueous-ethanolic $NaIO_4$ under the conditions given above for the oxidation of phenols. The reaction was terminated by the addition of ethylene glycol, and the mixture extracted with chloroform. Evaporation of the extract left 560 mg (84 %) of *4h*.

Oxidation of 2,3,5-trimethylphenol (5). (A) With $NaIO_4$ in H_2O-EtOH, $t=18$ h (cf. Ref. 7). The following products were obtained:

*(a) 2,3,5-Trimethyl-*p*-benzoquinone (5e)*; yield, 21 %. M.p. 33–34 °C (lit.[27] 34 °C).

(b) 6-Ethoxy-3,5,6-trimethyl-2,4-cyclohexadienone (5b), yellow oil; yield, 4 %. Parent mass, found: 180.1154; calc. for $C_{11}H_{16}O_2$: 180.1150.

(c) 6-Hydroxy-3,5,6-trimethyl-2,4-cyclohexadienone (5a), yellow crystals, m.p. 35–38 °C (lit.[12] 42 °C), in 35 % yield. The UV data were in agreement with those given in Ref. 28 for a compound wrongly designated as "mesityl-*o*-quinol", *i.e.*, *o*-quinol *1e* which, however, is not stable as monomer (see also Ref. 7). IR 3430 m, 1670 vs, 1642 s, 1570 m.

In a separate experiment, in which the purification of the crude product mixture on Al_2O_3 was omitted, the silica gel chromatography afforded the lactone *5d* (see below) as an additional product in a yield of 6 %.

(B) With HIO_4 in CH_3OH. (a) p*-Benzoquinone* 5e (see above); yield, 7 %.

(b) 4,4-Dimethoxy-2,3,5-trimethyl-2,5-cyclohexadienone (5f), crystals of m.p. 70–75 °C in 4 % yield. After sublimation at 35 °C/0.1 mmHg and recrystallization from isopropyl ether, m.p. 74–75 °C. (Found: C 67.45; H 8.29; OCH_3 31.84. Calc. for $C_{11}H_{16}O_3$: C 67.32; H 8.22; OCH_3 31.63). UV 230 (4.17), 278 (3.28), sh 340 (1.88). IR 1679, sh 1640, 1632. NMR δ 1.93 (s, 9 H, 3 CH_3), 3.02 (s, 6 H, 2 OCH_3), 6.31 (q, 1 H, H-6). $J_{CH_3\text{-}5,H\text{-}6}=1.5$ Hz.

(c) 6-Methoxy-3,5,6-trimethyl-2,4-cyclohexadienone (5c), yellow oil in 12 % yield, distillable at 60 °C/15 mmHg. Parent mass, found 166.0991; calc. for $C_{10}H_{14}O_2$: 166.0993. UV spectrum very similar to that of *4c*. IR 1668 vs, 1650 s, 1580 m. In the NMR spectrum, the signal of the OCH_3 group appears at δ 3.00.

Oxidation of 6-hydroxy-3,5,6-trimethyl-2,4-cyclohexadienone (5a). The *o*-quinol (890 mg) was treated with $NaIO_4$ in H_2O-EtOH (conditions see "Oxidation procedures") for 63 h. Extraction with chloroform gave an oil, which on silica gel chromatography provided 270 mg of unchanged *o*-quinol *5a* (30 %) and 29 % of *4-methyl-5-(1-methyl-2-oxopropyl)-2(5H)-furanone* (5d) as a colourless oil which distilled at 40 °C/0.1 mmHg. Parent mass, found: 168.0798; calc. for $C_9H_{12}O_3$: 168.0786. UV 210 (4.14). IR 1765 (α,β-unsat. γ-lactone), 1715 (2-oxo group), 1645 (C=C). NMR δ 1.24 (d, 3 H, $CHCH_3$, $J=7$ Hz), 2.08 (d, 3 H, CH_3-4, $J=1.5$ Hz), 2.23 (s, 3 H, $COCH_3$), 3.09 (q, split into doublets with $J=3.5$ Hz, 1 H, $CHCH_3$), 5.24 (d, 1 H, H-5, $J=3.5$ Hz; further split by coupling with H-3), 5.98 (m, 1 H, H-3).

Oxidation of 2,3,6-trimethylphenol (6). (A) With $NaIO_4$ in H_2O-EtOH; $t=3$ h. Since no *o*-quinone is formed in this case, the aluminium oxide step could be omitted. The dichloromethane extract was dried and evaporated, leaving a partly crystalline product which on treatment with cold hexane gave a yellow solution and as a residue:

(a) 1,4a,5,8a-Tetrahydro-5,9-dihydroxy-1,2,5,-7,8,9-hexamethyl-1,4-ethanonaphthalene-6,10-(4H)-dione (6e) in a yield of 44 %; colourless crystals of m.p. 191–192 °C (ethyl acetate). (Found: C 70.97; H 7.95. Calc. for $C_{18}H_{24}O_4$: C 71.03; H 7.95). UV 203 (3.80), 253 (3.86), sh 310 (2.36). IR 3390, 1725, 1658, 1628. NMR δ 1.20 (s, 3 H) and 1.26 (s, 6 H), due to the CH_3 groups at C-1, C-5 and C-9; 1.46 (d, 3 H, CH_3-2), 1.85 (s, 3 H, CH_3-8), 2.02 (d, 3 H, CH_3-7), 2.8–3.2 (m, 2 H, H-4a, H-8a), 3.36 (dd, 1 H, H-4), 3.06 and 4.20 (s, 1 H each, OH-5, OH-9, exchangeable with D_2O), 6.00 (dq, 1 H, H-3). $J_{CH_3\text{-}2,H\text{-}3}=1.5$ Hz, $J_{3,4}=7$ Hz, $J_{4,4a}=2$ Hz, $J_{CH_3\text{-}7,H\text{-}8a}$ about 1 Hz.

The above-mentioned hexane solution was evaporated and the resulting oil chromatographed on silica gel to give two further products:

*(b) 2,3,5-Trimethyl-*p*-benzoquinone (6d)*; yield, 26 %.

(c) 6-Hydroxy-2,5,6-trimethyl-2,4-cyclohexadienone (6a), yellow oil (18 %), distillable at 40 °C/1 mmHg. Parent mass, found: 152.086; calc. for $C_9H_{12}O_2$: 152.084. UV 317 (3.46), sh 362 (2.98). IR 3436 m, 1664 vs, 1642 s, 1588 m.

(B) With HIO_4 in CH_3OH. Three products

were obtained:

(a) 2,3,5-Trimethyl-p-*benzoquinone* (6d); yield, 15 %.

(b) 6-Methoxy-2,5,6-trimethyl-2,4-cyclohexadienone (6c), yellow crystals of m.p. 47–51 °C (17 % yield), after recrystallization from hexane m.p. 51–52 °C. (Found: C 72.04; H 8.53; OCH_3 18.82. Calc. for $C_{10}H_{14}O_2$: C 72.25; H 8.49; OCH_3 18.67). UV 321 (3.68), sh 390 (2.82). IR 1667 vs, 1652 s, 1598 m.

(c) 1,4a,5,8a-Tetrahydro-5,9-dimethoxy-1,2,5,-7,8,9-hexamethyl-1,4-ethanonaphthalene-6,10-(4H)-dione (6f), colourless crystals of m.p. 149–152 °C (yield, 12 %), prisms of m.p. 153–154 °C from ethyl acetate. (Found: C 71.94; H 8.67; OCH_3 18.98. Calc. for $C_{20}H_{28}O_4$: C 72.25; H 8.49; OCH_3 18.67). UV 206 (3.79), 252 (3.91), 311 (2.50). IR 1718, 1680, 1634. The NMR spectrum of *6f* is very similar to that of *6e*; the signals of the OCH_3 groups in the former compound appear at 3.34 and 3.52 ppm.

The dimethyl ether 6f was also obtained by methylation of *dimer 6e* with CH_3I/Ag_2O [29] as described in Part XVII.[4] Silica gel chromatography of the crude reaction product gave, in addition to *6f* (46 %), 24 % of *the monomethyl ether* (*6e*, R = CH_3, R′ = H), m.p. 105–106 °C (isopropyl ether). (Found: C 71.80; H 8.18; OCH_3 10.10. Calc. for $C_{19}H_{26}O_4$: C 71.68; H 8.23; OCH_3 9.75). UV 208 (3.82), 252 (3.91), 305 (2.86). IR 3458, 1716, 1660, 1625. The position of the peak due to the unconjugated CO (1716) of the monomethyl ether is very close to the corresponding peak of the dimethyl ether *6f* (1718), but differs from that of the nonmethylated dimer *6e* (1725), whereas the peak due to the α,β-conjugated CO (1660) is very close to that of *6e* (1658), but differs by 20 cm^{-1} from that of *6f* (1680). This indicates that the methoxyl group is located at C-9, *i.e.*, adjacent to the unconjugated CO. In the NMR spectrum, which is very similar to the spectra of *6e* and *6f*, a signal for a single OCH_3 group is found at 3.34 ppm.

Oxidation of 3-methoxy-2-methylphenol (7). *(A) With $NaIO_4$ in H_2O-EtOH*, $t = 21$ h. The following two products were isolated:

(a) 3-Methoxy-2-methyl-p-*benzoquinone* (7d), yellow oil [30] (11 %). The IR data of the compound were in agreement with those reported in the literature.[31]

(b) 6-Ethoxy-5-methoxy-6-methyl-2,4-cyclohexadienone (7b); yield, 11 %. Yellow crystals of m.p. 100–101 °C (hexane.) (Found: C 65.91; H 7.80. Calc. for $C_{10}H_{14}O_3$: C 65.91; H 7.74). UV 347 (3.70), sh 362 (3.60). IR 1669 vs, 1632 s, 1550 vs. NMR δ 1.17 (t, 3 H, OCH_2CH_3), 1.42 (s, 3 H, CH_3-6), 3.32 (q, 2 H, OCH_2CH_3), 3.80 (s, 3 H, OCH_3), 5.42 (d, 1 H, H-4), 5.83 (d, 1 H, H-2), 7.10 (dd, 1 H, H-3). $J_{2,3} = 10$ Hz, $J_{3,4} = 7$ Hz.

(B) With HIO_4 in CH_3OH. The following three products were obtained:

(a) 2-Iodo-5,6-dimethoxy-6-methyl-2,4-cyclohexadienone (7e); yield, 2 %. Yellow prisms of m.p. 183–184 °C (ethyl acetate-hexane). (Found: C 36.51; H 3.68; OCH_3 20.80; I 43.11. Calc. for $C_9H_{11}O_3I$: C 36.76; H 3.77; OCH_3 21.10; I 43.15). UV 388 (3.75). IR 1668 vs, 1625 s, 1527 vs. NMR δ 1.48 (s, 3 H, CH_3-6), 3.02 (s, 3 H, OCH_3-6), 3.83 (s, 3 H, OCH_3-5), 5.38 (d, 1 H, H-4), 7.82 (d, 1 H, H-3). $J_{3,4} = 7.5$ Hz.

(b) 5,6-Dimethoxy-6-methyl-2,4-cyclohexadienone (7c); yield, 17 %. Light-yellow rods, m.p. 109–110 °C (hexane). (Found: C 64.24; H 7.02; OCH_3 36.91. Calc. for $C_9H_{12}O_3$: C 64.27; H 7.19; OCH_3 36.90). UV and IR spectra closely similar to those of *7b*.

Treatment of *7c* with zinc dust in 50 % aqueous acetic acid at room temperature for 30 min regenerated phenol *7*.

(c) 1,4a,5,8a-Tetrahydro-2,5,5,8,9,9-hexamethoxy-1,7-dimethyl-1,4-ethanonaphthalene-6,10-(4H)-dione (7f); yield, 3 %. Colourless needles of m.p. 159–160 °C (ethyl acetate-hexane). (Found: C 60.82; H 6.97; OCH_3 46.53. Calc. for $C_{20}H_{28}O_8$: C 60.59; H 7.12; OCH_3 46.97). UV 270 (4.03) (α,β-enone, calc.[21] 267), sh 315 (3.08). IR 1740 (CO), 1680 (conj. CO), 1640 and 1629 (CH_3O-substituted C=C bonds), all peaks strong. NMR δ 1.25 (s, 3 H, CH_3-1), 1.82 (s, 3 H, CH_3-7), singlets at 3.00, 3.26, 3.40, 3.41, 3.44, 3.75 (6 OCH_3) overlapping a multiplet (3 H, H-4, H-4a, H-8a), 4.84 (d, 1 H, H-3). $J_{3,4} = 7.5$ Hz.

Oxidation of 2,3-dimethoxyphenol (8) [32] *with HIO_4 in CH_3OH.** The crystalline product obtained after passage through the Al_2O_3 column was separated on silica gel into two compounds.

(a) 2-Iodo-5,6,6-trimethoxy-2,4-cyclohexadienone (8d); yield, 3 %. After purification by preparative TLC and recrystallization from hexane, yellow crystals of m.p. 85–87 °C. (Found: C 34.90; H 3.56; OCH_3 29.65; I 40.56. Calc. for $C_9H_{11}O_4I$: C 34.86; H 3.58; OCH_3 30.02; I 40.92). UV 394 (3.73). IR 1680 s, 1668 s, 1634 vs, 1531 vs. NMR δ 3.32 (s, 6 H, 2 OCH_3 at C-6), 3.80 (s, 3 H, OCH_3-5), 5.29 (d, 1 H, H-4), 7.70 (d, 1 H, H-3). $J_{3,4} = 7.5$ Hz.

(b) 5,6,6-Trimethoxy-2,4-cyclohexadienone (8c); yield, 39 %. Yellow needles, m.p. 77–78 °C (hexane). (Found: C 58.61; H 6.63; OCH_3 50.58. Calc. for $C_9H_{12}O_4$: C 58.68; H 6.57; OCH_3 50.54). UV 358 (3.66). IR 1670 vs, 1638 s, 1555 vs. NMR δ 3.37 (s, 6 H, 2 OCH_3 at C-6), 3.83 (s, 3 H, OCH_3-5), 5.45 (dd, 1 H, H-4), 5.84 (dd, 1 H, H-2), 7.07 (dd, 1 H, H-3). $J_{2,4} = 1$ Hz, $J_{2,3} = 10$ Hz, $J_{3,4} = 7$ Hz.

Treatment with zinc dust in 50 % aqueous acetic acid (room temperature, 15 min) reduced the *o*-quinone ketal *8c* to the phenol *8*.

Heat treatment of 2,4-cyclohexadienones. (a) Solutions of the *o*-quinol methyl ethers *4c–7c*, of the ethyl ether *7b*, and of the *o*-quinone ketal *8c* (10 mg each) in toluene (2 ml) were heated in sealed tubes at 110 °C for 17 h. In all cases,

* Experiment performed by H. Thomelius.

TLC gave only a single spot revealing the presence of unchanged monomer.

(b) *o*-Quinone ketal *8c* was heated first at 120 °C for 3 h and then at 150 °C for 1.5 h. After both heating periods, TLC gave an intense single spot (*8c*) located within a continuous band. When heating was continued at 120 °C for 11 d, the intensity of the spot due to *8c* gradually decreased, whereas that of the band increased. Attempts to isolate a defined reaction product by column chromatography (silica gel, benzene/ethyl acetate 1:1) were unsuccessful.

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The Reaction between Thiophosgene and Hydrazines. Formation of Trithiocarbonates with *N,S*-Ambident 1,3,4-Thiadiazolines as Intermediates

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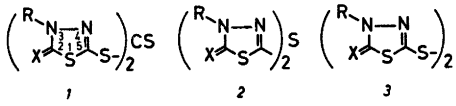
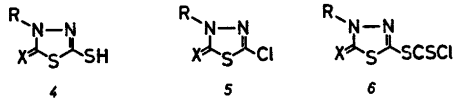
The reaction between substituted hydrazinium salts and thiophosgene in water has been reinvestigated. Previous suggestions that the products are *N*-isothiocyanatoamines have been disproved. If methyl- or phenylhydrazinium chloride are employed in the reactions 5,5-thiocarbonyldithiobis[3-methyl-1,3,4-thiadiazol-2(3*H*)-one] (*1a*) and 5,5-thiocarbonyldithiobis[3-phenyl-1,3,4-thiadiazol-2(3*H*)-one] (*1c*), respectively, are formed as the main products. Several by-products and one key intermediate were isolated. Each was characterized by comparison to samples prepared by alternative synthetic methods and by information obtained from ^{13}C NMR, and IR spectrometry. A mechanism involving 5-mercapto-1,3,4-thiadiazol-2(3*H*)-ones and thiones as intermediates is proposed and the literature relating to *N,S* ambident reagents reviewed.

In 1937 Beckett and Dyson [1] found that the reaction between thiophosgene and hydrazines in general leads to the formation of thiocarbonohydrazides. Subsequent work has questioned this result only in few cases, *cf.*, *e.g.*, Hayes [2] and Sherman.[3]



1 *2* *3*

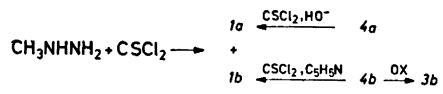


4 *5* *6*

	R	X
a:	Me	O
b:	Me	S
c:	Ph	O
d:	Ph	S

When the reaction was performed in hydrochloric acid Beckett and Dyson obtained products which were formulated as *N*-isothiocyanatoamines. However, our previous investigations concerning *N*-isothiocyanatoalkylamines have disclosed methods of preparation,[4–6] properties,[7–12] and tendency to dimerization [13] characteristic of *N*-isothiocyanatoamines which appear to be quite different from those described by Beckett and Dyson. This initiated a study of the reaction between thiophosgene and various hydrazine derivatives which has led to an elucidation[14–16] of the structures of several products claimed to be *N*-isothiocyanatoamines. In the present paper a reinvestigation of the reaction between methyl- or phenylhydrazinium salts and thiophosgene is presented.

Ambiguous results were obtained by Beckett and Dyson [1] on reacting methylhydrazinium sulfate with thiophosgene. The proposed structure for the reaction product was based on its reaction with aniline, claimed to give 1-methyl-4-phenylthiosemicarbazide. However, the melting point given [1] for this compound is quite different from that of authentic 1-methyl-4-phenylthiosemicarbazide,[17] but agrees well with that of 2-methyl-4-phenylthiosemicarbazide.[17]



Scheme 1.

The above controversy has now been settled by a chromatographic separation of the main constituents of the product formed by the reaction between an aqueous solution of methylhydrazinium sulfate and thiophosgene. The compounds were identified as trithiocarbonates by independent syntheses as outlined in Scheme 1. The formulation of *1a* and *1b* as trithiocarbonates is based upon the assumption that thiophosgene attacks sulfur in preference to nitrogen (*cf.* discussion later).

Both *1a* and *1b* formed 1,3-diphenylthiourea and the anilinium salts of 4a and 4b on boiling with a solution of aniline in benzene. The formation of the methylphenylthiosemicarbazide found by Beckett and Dyson, must be explained by the presence of a small amount of unreacted thiophosgene and/or methylhydrazine in the crude mixture, since no trace of this compound was found when the reaction was performed with aniline and the purified reaction product *1a* or *1b*.

The formation of 1,3,4-thiadiazoline derivatives in the reaction between methylhydrazinium sulfate and thiophosgene led us to reinvestigate the reaction between phenylhydrazinium chloride and thiophosgene. According to Beckett and Dyson this reaction results in the formation of 1,2,4-triisothiocyanatobenzene and phenylisothiocyanate. We obtained a compound with the same melting point as given for the alleged triisothiocyanatobenzene but with a different elemental analysis. Physical and chemical evidence (discussed in connection with Scheme 2) identified this compound as the trithiocarbonate *1c*. Only very small amounts of phenylisothiocyanate were formed.

The product obtained by refluxing *1c* with aniline was identified as a mixture of two salts of 5-mercapto-3-phenyl-1,3,4-thiadiazol-2(3*H*)-one, *4c*. One of the salts is, as expected, the anilinium salt, the other is the 1,3-diphenylformamidinium salt (*cf.* the discussion given in connection with Scheme 3). Treatment of *1c* with ammonia similarly gave the ammonium salt of *4c* in addition to ammonium thiocyanate. On boiling with ethanol *1c* formed the disulfide *3c*, the oxidation product of the initially formed thiol. The products obtained by Beckett and Dyson had approximately the same melting points as the above products but were formulated quite differently.

These results show that the reaction between hydrazinium salts and thiophosgene generally results in the formation of 1,3,4-thiadiazoline derivatives. To elucidate how this ring system is formed the crude product has been separated by column chromatography with the purpose of isolating and identifying its minor constituents. In addition to traces of phenylisothiocyanate and *ca.* 35 % unidentified, presumably high molecular weight substances, the following 5 fractions, given in the order of decreasing flow velocity, were isolated (*cf.* Scheme 2).

6c: 5-[(Chlorothiocarbonyl)thio]-3-phenyl-1,3,4-thiadiazol-2(3*H*)-one.

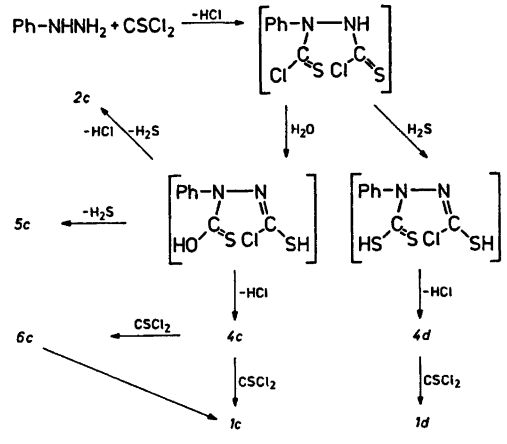
5c: 5-Chloro-3-phenyl-1,3,4-thiadiazol-2(3*H*)-one.

1d: 5,5-Thiocarbonyldithiobis[3-phenyl-1,3,4-thiadiazole-2-(3*H*)-thione].

1c: 5,5-Thiocarbonyldithiobis[3-phenyl-1,3,4-thiadiazol-2(3*H*)-one].

2c and *3c*: A mixture of 5,5-thio and 5,5-dithiobis[3-phenyl-1,3,4-thiadiazol-2(3*H*)-one].

The identity of *1c* was established by comparison to an authentic specimen prepared from 5-mercapto-3-phenyl-1,3,4-thiadiazol-2(3*H*)-one (*4c*) and thiophosgene. Substance *4c* was prepared according to Busch [18] and characterized by oxidation to the disulfide *3c*.

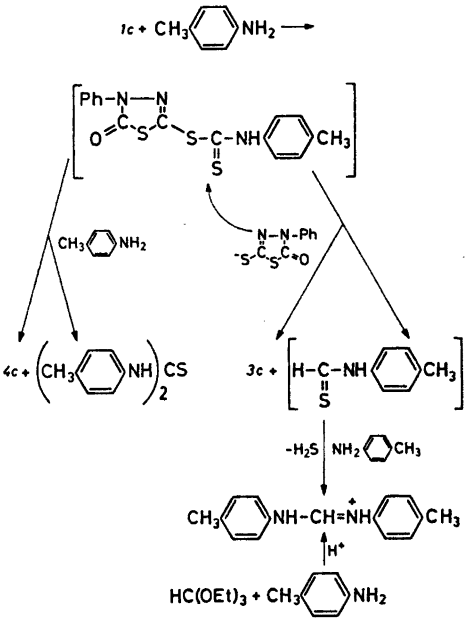


Scheme 2.

The identity of *1d* was also confirmed by comparison to authentic material prepared from the potassium salt of 5-mercapto-3-phenyl-1,3,4-thiadiazole-2(3H)-thione (*4d*) and thiophosgene according to the directions given by Runge *et al.*[19] The structure of *4d* was characterized by oxidation to the disulfide *3d*.

A logical scheme to encompass the formation of the products *5c*, *6c*, *2c*, *1c* and *1d* is shown in Scheme 2. The reaction between methylhydrazinium sulfate and thiophosgene discussed above (Scheme 1) proceeds by a similar route. By analogy with the acylation of derivatives of hydrazine, which almost invariably [20] gives 1,2-diacylhydrazines, the initial steps probably involve attack of thiophosgene on both nitrogen atoms in phenylhydrazine. The bis(thioacid chloride) formed undergoes rapid hydrolysis in the aqueous medium and the resulting intermediate can either release hydrogen sulfide with formation of *5c* or eliminate hydrogen chloride to give the 1,3,4-thiadiazoline ring. Further attack by thiophosgene gives *6c* and *1c*. The latter is very probably formed *via* *6c* as an intermediate. The formation of *1d* can be explained by a similar mechanism from the initially formed bis(thioacid chloride) and hydrogen sulfide. Hydrogen sulfide either originates from hydrolysis of thiophosgene or is a by-product in the formation of *5c*.

An unusual pattern was noted in the reaction between *1c* and aniline. In order to distinguish clearly between the phenyl group originating from *1c* and that from aniline we chose to investigate instead the reaction between *1c* and *p*-toluidine. The results are shown in Scheme 3. From *1c* four products were obtained in approximately equal yields. Two of the products, 1,3-di(*p*-tolyl)thiourea and the *p*-toluidinium salt of *4c*, arise from the straightforward reaction between *1c* and an excess of *p*-toluidine. The remaining products, the disulfide *3c* and the 1,3-di(*p*-tolyl)formamidinium salt of *4c*, can be explained by invoking the reaction sequence shown to the right in Scheme 3. Nucleophilic attack of *p*-toluidine on the trithiocarbonate group of *1c* initially gives a dithiocarbamate and, in addition, the thiolate of *4c*. The strongly nucleophilic character of the sulfur atom in this anion apparently results in its attack on the dithiocarbamate

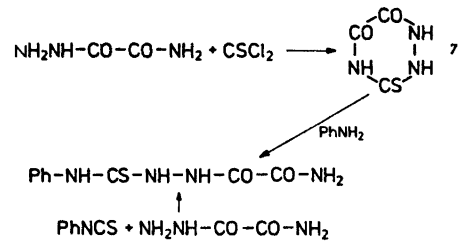


Scheme 3.

competitive to the expected attack of *p*-toluidine. The proposed attack on sulfur is analogous to the reaction of related selenium compounds.[21] Also a similar attack was recently reported for thiols and isothioureas at elevated temperatures.[22] In the present case, the attack leads to the formation of the disulfide *3c*, and in a well known type of reaction [23] the *N*-thioformyl-*p*-toluidine simultaneously formed is converted by excess *p*-toluidine into 1,3-di(*p*-tolyl)formamidine, isolated as the salt of *4c*. This salt was identical to material prepared from the authentic formamidine (Backer and Wanmaker [24]) and 5-mercapto-3-phenyl-1,3,4-thiadiazol-2(3*H*)-one (*4c*).

The reaction between 1,2-dimethylhydrazinium chloride and thiophosgene gives a mixture of 3,4-dimethyl-5-thioxo-1,3,4-thiadiazolidin-2-one, *4e*, and 3,4-dimethyl-1,3,4-thiadiazolidine-2,5-dithione, *4f* in the ratio 2:1. This result parallels very closely that discussed above and supports the proposal (Scheme 2) that hydrolysis/thiolysis of the initially formed bis(thioacid chloride) takes place prior to the formation of the trithiocarbonate.

Finally we have studied two reactions which



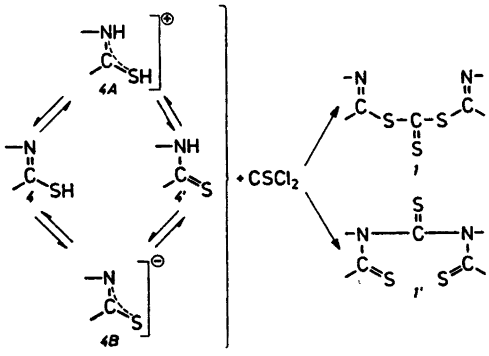
Scheme 4.

according to Beckett and Dyson also should give rise to isothiocyanatoamines. In the reaction between thiophosgene and oxamohydrazide ("semioxamazide"), the reaction product was identified as 3-thioxohexahydro-1,2,4-triazine-5,6-dione (*7*) (Scheme 4). It undergoes ring-opening on treatment with aniline and forms 1-oxamoyl-4-phenylthiosemicarbazide

The other reaction is that between *p*-nitrophenylhydrazine and thiophosgene. The crude product was shown by column chromatography to consist of several compounds. The main fraction was identified as the 5,5-thiocarbonyldithiobis[3-(4-nitrophenyl)-1,3,4-thiadiazol-2(3*H*)-one] (*1e*).

SULFUR vs. NITROGEN ATTACK ON AMBIDENT HETEROAROMATIC COMPOUNDS

From the results described above it can be concluded that the reaction between hydrazines and thiophosgene in aqueous hydrochloric acid proceeds *via* mercaptothiadiazolines, *4* (Scheme 5) to give trithiocarbonates, *1*, rather than thioureides, *1'*.



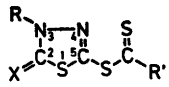
Scheme 5.

In order to clarify the factors responsible for the preferential attack of sulfur, the nature of the reacting species *4* must be considered. Tautomerisation gives the thione structure, *4'*, which could, however, only be established for *4a* in the solid state by infrared spectroscopy. Infrared spectra of the thiols *4b* – *4d* in KBr and *4a* – *4d* in chloroform solution did not show detectable amounts of the thione isomers *4a'* – *4d'*. Provided the reaction of *4* with thiophosgene occurs in aqueous solution, prototropic equilibria are set up leading to formation of thiolate ions, *4B*, and the protonated species, *4A*. Kjellin and Sandström [25] have recently investigated the thione-thiole protonation equilibria of a series of oxazoline-, thiazoline-, and imidazoline-2-thiones. Since the pK_{HA} of compounds containing structure *4A* was found to be < 0, and since thiophosgene is in general much less susceptible to electrophilic than to nucleophilic attack [26] it is expected, that the presence of *4A* in the reaction mixture is insignificant. The reaction accordingly proceeds by attack of one or more of the three ambident nucleophilic species *4B*, *4*, and *4'* on thiophosgene.

It is usually assumed [19,27–30] that alkylation or acylation of ambident nucleophiles containing competing *N* and *S* points of attack yields the *S*-derivatives. Cases are known in which *S*-attack is unambiguously proved by formation of a cyclic trithiocarbonate.[31] However, evidence has been presented [32–36] to show that *S*-attack is not necessarily the rule and should be confirmed in each case. Furthermore, cases have been reported in which S to N acyl migrations occur [37] as have compounds in which N to S migrations occur instead.[38]

The factors determining nitrogen *vs.* sulfur attack have been discussed by several authors.[39] In a recent study of glucosidation of 2,5-dimercapto-1,3,4-thiadiazole it was concluded that the rules derived by Kornblum *et al.*[40] were followed to give *S*-glycosides preferentially when the reaction proceeds *via* an S_N2 mechanism. By contrast *N*-glycosides are obtained if mercury salts, known to promote the S_N1 pathway, are present.[41] Halasa and Smith [32] were able to predict the conditions for *N- vs. S*-attack of the ambident anion of benzothiazoline-2-thione by using the oxibase scale. Since however the result is determined at least

Table 1. ^{13}C NMR chemical shifts (δ) in (3*H*)-thiadiazoles downfield from TMS.



Compound	C(2)	C(5)	R′C=S	R(3)[a]
1a	170.3	138.8	211.1	34.9
1b	188.3	144.9	208.0	39.1
1c	168.8	140.3	209.9	137.1, 128.0, 129.2, 122.1
1d	188.1	146.1	207.2	137.8, 129.7, 129.2, 125.8
3b	187.2	152.6		39.1
3c	167.8	147.5		137.0, 127.2, 128.8, 121.3
3d	187.2	153.7		138.0, 129.5, 129.1, 125.6
4a	169.8	140.1		34.3
4b	186.6	148.4		38.8
4c	168.2	142.1		137.3, 127.1, 128.9, 121.5
4d	186.3	149.4		138.0, 129.1, 128.9, 125.5
4e	163.6	177.9		35.1,[b] 33.4[b]
4f	180.1	180.1		36.7[b]
5c	167.1	137.9		137.3, 127.5, 129.1, 121.7
6c	168.6	142.3	189.8	137.2, 128.0, 129.2, 122.1

[a] For R = Ph the numbers given are in order of increasing peak area (1:1:2:2). [b] Me(3) and Me(4).

by the pH[37] and the temperature[32,42] unambiguous criteria were needed in order to distinguish between the possible final products, *1* and *1′* (Scheme 5) arising from the reaction between thiophosgene and hydrazines.

^{13}C NMR spectroscopy was found to be a most convenient method for the distinguishing between *1* and *1′*. Also considerable IR spectroscopic evidence substantiating the suggested structure of the compounds *1*–*6* was collected during the investigations. The two categories of data are described separately in the following sections.

^{13}C NMR SPECTRA

The capability of ^{13}C NMR to differentiate between the trithiocarbonate and the thioureide structure (*1* and *1′* Scheme 5) becomes evident by comparison of ^{13}C NMR shieldings of the compounds examined in this study and appropriate model compounds. The chemical shift data and assignments are summarized in Table 1. For the thiols *4a*–*d* and the disulfides *3b*–*d* the assignments are straightforward. Thus, in addition to the resonances for R (CH_3 or Ph, see Table 1), which are readily identified, the spectra contain only two peaks. The low field peak is assigned to C(2) because of the large downfield shift (~20 ppm) observed when oxygen is replaced by sulfur at this carbon atom (*e.g.* when going from *4a* to *4b* see Table 1). The high field peak is consequently assigned to C(5), and it is only slightly shifted by the substitution.

The ^{13}C shieldings of C(5) for *4a*–*d* show that the compounds in chloroform exist predominantly in the thiol form *4* (see Scheme 5). First the large chemical shift difference of more that 30 ppm between C(2) and C(5) in the spectra of *4b* and *4d* is not compatible with a thione structure, *4′*, in which these two carbon atoms would be expected to be nearly equivalent. Furthermore, the ^{13}C NMR spectra of the disulfides *3b*–*d* are very similar to those of compounds *4a*–*d*.

The spectra of the trithiocarbonates *1a*–*d* display in addition to the peaks for the R group three peaks with very different chemical shifts (see Table 1). The C(2) resonance may be identified in a manner similar to that used for compounds *3* and *4*. The shielding of C(2) is seen to be nearly the same in the corresponding compounds with structures *1*, *3*, and *4*. The two remaining peaks in the spectra of *1a*–*d* are found at approximately 210 and 140 ppm,

neither of which can correspond to the thioureide structure (*1'*). In the latter case both peaks should represent thiocarbonyl carbons where one [C(5)] should be very similar to C(5) in *4e* and *4f* ($\delta \sim 180$) and the other R'*C*=S would be expected to fall around δ 170–180; *cf.* thiocarbonyl shieldings in the model compounds thiocarbonohydrazide: δ 181.3; 1,1-' thiocarbonyldiimidazole: δ 172.8, 1,3-diphenylthiourea: δ 179.9. On the other hand, the presence of two peaks around 210 and 140 ppm is what would be expected for the trithiocarbonate structure, *1*. Here, a chemical shift value of ~140 ppm is expected for C(5) by comparison with compounds of types *3* and *4*. The peak at δ 210 is then ascribed to R'C=S, in good agreement with the value of δ 224 found for the thiocarbonyl carbon in diphenyl trithiocarbonate. The evidence is thus conclusive that the compounds *1a–d* possess the trithiocarbonate structure *1*.

The ^{13}C chemical shifts for the chlorine compounds *5c* and *6c* included in Table 1 fit the pattern shown by the other compounds examined. A small upfield shift is observed at C(5) in compound *5c* relative to the other oxygen-containing compounds. In contrast, the chlorine atom causes a large upfield (~20 ppm) shift for the adjacent carbon atom in compound *6c*. Similarly, a large difference in shielding of the thiocarbonyl carbon is found for diphenyl trithiocarbonate (δ 224) and phenyl chlorodithioformate (δ 197).

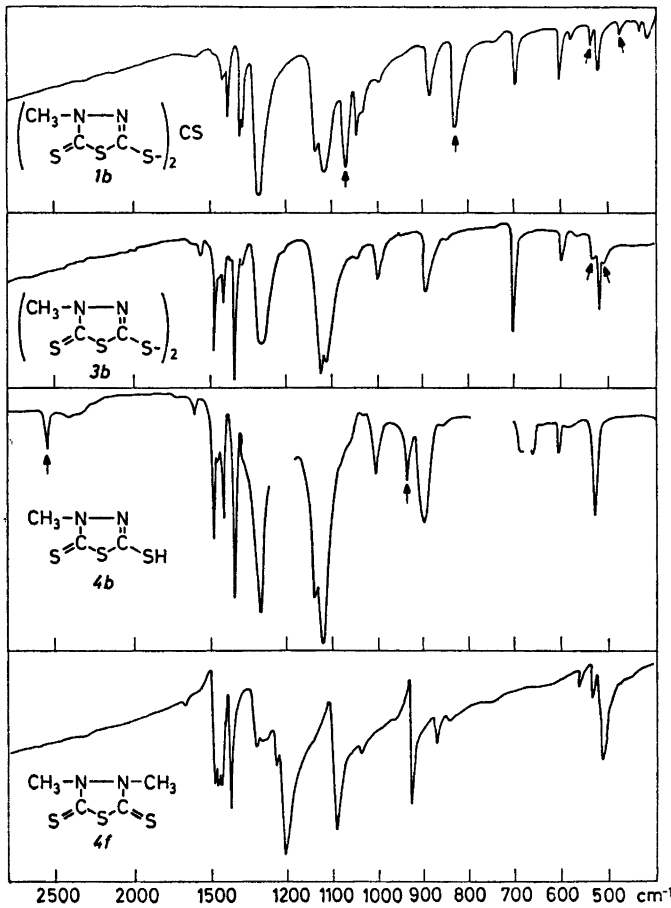


Fig. 1. Infrared spectra of 1*b*, 3*b*, 4*f* (KBr) and 4*b* ($CHCl_3$) in the range 400–2700 cm^{-1}. The bands discussed in the text are indicated by arrows.

INFRARED SPECTRA

Infrared spectroscopy provides evidence that the products of the reaction between thiophosgene and hydrazinium salts are trithiocarbonates, *1*, rather than thioureides, *1'*. The method can be exemplified by considering the infrared spectra shown in Fig. 1. The dithione *4f* (KBr) has necessarily the structure shown since the thione-thiol tautomerism is effectively prevented by the two methyl groups. Comparison with the infrared spectra of related compounds indicates that this compound displays features characteristic of a thione structure *4'*. On the other hand, the thiol *4b* ($CHCl_3$), the disulfide *3b* (KBr), and the trithiocarbonate *1b* (KBr) are derived from the tautomeric thiol structure *4*. Thus we might expect the infrared spectra of these compounds to exhibit a pattern typical of this structure. Of course, since electron delocalisation will tend to diminish the apparent differences expected from the formulas given in Fig. 1, only experiments can show whether the infrared spectra of compounds with a *4'* and *4* structure can in fact be used to distinguish between these structures.

From Fig. 1 it is seen that the spectra of all four compounds are similar in the range 1250 – 1500 cm^{-1}, while the spectrum of *4f* is different from the spectra of *1b*, *3b*, and *4b* in the region between 400 and 1250 cm^{-1}. The compounds with structure *4* are characterized by the following nine bands (approximate location, cm^{-1}): 1120 (doublet), 1050w, 1000w – m, 895m, 700m, 600m, 575vw, and 525m. Though some of these bands are also seen in the spectrum of *4f*, the general pattern is clearly different. The same is true when the changes originating in the presence of an extra methyl group are taken into account.

The nine bands characteristic of the 3-methyl-1,3,4-thiadiazole-2(3*H*)-thione moiety are essentially independent of the changes in the substituent in the series *4b*, *3b*, and *1b*. Accordingly structure *4* can be recognized by investigating the infrared region between 400 and 1250 cm^{-1}.

The interpretation of the differences in the spectra of *4b* and *3b* (indicated by arrows) is facilitated by the data available for benzenethiol [43] and for diaryldisulfides.[44] The band at 2560 cm^{-1} in *4b* is then satisfactorily established as the S – H stretching vibration and the band at 936 cm^{-1} as originating mainly in C – S – H bending. On oxidation of the thiol *4b* to the disulfide *3b* both these bands disappear and are replaced by two weak bands at 507 and 530 cm^{-1}. The latter absorption is ascribed to S – S stretching which is generally only observed as very weak absorption in the infrared, although it is usually prominent in the Raman spectrum.[44]

In a similar way we can explain the differences between the spectra of *3b* and *1b* (indicated by arrows) as a replacement of the S – S stretching absorption in *3b* by bands characteristic of the CS_2 group. The corresponding 6 bands have positions which can be readily estimated from the results obtained by a complete vibrational analysis of ethylene trithiocarbonate.[45] The three stretching vibrations of the trithiocarbonate group have been found near 1060 cm^{-1} (very strong), 830 – 880 cm^{-1} (strong), and 500 cm^{-1} (medium) and compare favourably with those observed in the spectrum of *1b* at 1075, 834, and 480/540 cm^{-1}. Of the remaining vibrations of the trithiocarbonate group only the out-of-plane deformation vibration is expected within the region investigated, probably between 400 and 500 cm^{-1} where three weak bands are observed in *1b*. The remaining bands are attributed to over- and combination tones of the lower fundamentals. In our opinion this analysis of the infrared spectrum of *1b* proves it to be a trithiocarbonate provided of course that the spectral data are incompatible with those of the thioureide structure *1b'*. Thus thioureides exhibit [46] a strong band in the range 1400 – 1600 cm^{-1} which is not found in the spectrum of *1b*. Since the region in question is devoid of other strong absorption the possibility that the latter band is masked by other absorption can be excluded.

The method works equally well within the aromatic series as evidenced by the spectra shown in Fig. 2 (CCl_4 solution). It is seen that the spectra of *4c* and *5c* are very similar apart from the two bands at 2560 cm^{-1} (S – H stretching) and 933 cm^{-1} (C – S – H deformation). Since the structure of *5c* was not confirmed by independent synthesis this serves to verify the presence of the 3-phenyl-

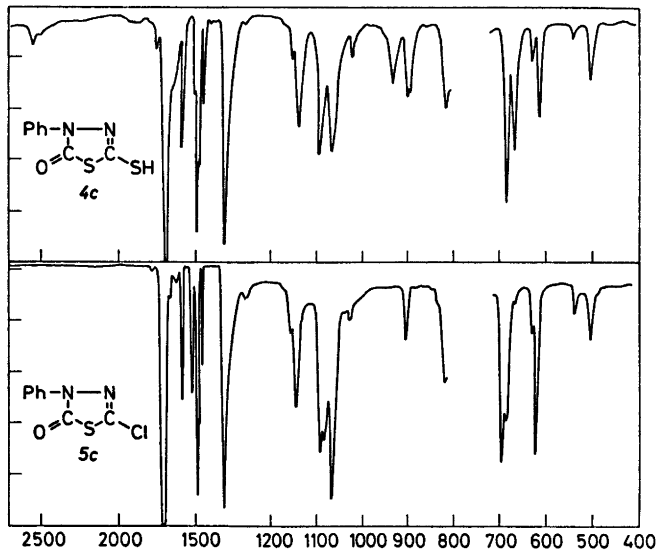


Fig. 2. Infrared spectra of *4c* and *5c* (CCl_4) in the range 400 – 2650 cm^{-1}.

1,3,4-thiadiazol-2(3*H*)-one group in *5c* and, in connection with results obtained from elemental analysis and mass spectrometry, establishes the formula given.

EXPERIMENTAL

General. Microanalyses were carried out in the Microanalysis department of this laboratory. Satisfactory elemental analyses were obtained for all new compounds and key intermediates. Melting points were determined on a Büchi melting point apparatus and are not corrected. The separations by preparative layer chromatography (PLC) were performed with portions of 0.5 – 1.0 g using 20 × 100 cm plates with silica gel (2.5 mm, Merck $PF_{254+366}$). The plates were developed 2 – 4 times with a mixture of benzene, chloroform, and hexane and the fractions isolated by continuous extraction with chloroform in a Soxhlet tube. For preparative-scale separations by column chromatography, columns were constructed from 30 cm lengths of 20 mm i.d. glass tubing slurry-packed with silica gel (Merck, 0.040 – 0.063 mm) or cationotropic aluminium oxide (Merck 90).

Spectra. Noise-decoupled ^{13}C NMR spectra were determined with a Bruker WH 90 system (22.63 MHz) operating in the Fourier transform mode. All samples were examined as 5 – 15 % (w/v) solutions in $CDCl_3$ and shieldings were measured relative to internal TMS. For the compounds *1a* – *1d* a trace of tris(2,4-pentanedionato)chromiumIII) was added to decrease the relaxation time of the trithiocarbonate carbon atom.[47] This was found to be without influence (< 0.05 ppm) on the chemical shift values.

The infrared spectra in the range 400 – 4000 cm^{-1} were recorded on a Perkin-Elmer model 337 grating infrared spectrophotometer.

Mass spectra were obtained using an AEI MS-902 mass spectrometer operating at 70 eV. The source temperature was kept between 120 and 180 °C. Exact mass measurements were performed at a resolution of 5 000 (10 % valley definition) using heptacosafluorotributylamine to provide reference masses.

The electronic spectra were recorded using 1 cm cells on a Unicam SP 1800 Ultraviolet Spectrophotometer in $CHCl_3$ solvent.

Identification of products. In general, the fractions obtained from the chromatographic separations were identified in the following way: A pure product was obtained from the center cut of a band or by recrystallization from an appropriate solvent. The formula was indicated by analysis (C, H, Cl, N, S) and the structure from the combined information obtained from infrared, NMR, and mass spectrometry. Comparison with authentic specimens included comparison of spectroscopic data and mixed melting point determination whenever practicable. In most instances (exluding, *e.g.*, some salts) the compounds were provisionally identified by their behaviour on thin layer chromatography (colour, R_F-value, colour on irradiation with ultraviolet light).

The reaction between methylhydrazinium sulfate and thiophosgene. Following the directions given by Beckett and Dyson,[1] a solution of methylhydrazinium sulfate (14 g) in water

(700 ml) was shaken vigorously with thiophosgene (15 g) added in one portion at room temperature. A slightly exothermic reaction took place and an orange solid separated. In order to ensure complete reaction the mixture was allowed to stand for an additional 2 h with occasional shaking and the solid was filtered off. Thin layer chromatography showed it to consist of several components of which the two major ones have been identified. The crude product was extracted with ethyl acetate and the extract chromatographed on a cationotropic aluminium oxide column. The column was eluted with a chloroform-carbon disulfide mixture (1:3), and the two major fractions were collected. The first was identified as 5,5-thiocarbonyldithiobis(3-methyl-1,3,4-thiadiazole-2(3*H*)-thione) (*1b*) by comparison with an authentic sample (see below) and constituted from 8 – 20 % of the total amount of these two fractions. The second (80 – 90 %) was identical to 5,5-thiocarbonyldithiobis[3-methyl-1,3,4-thiadiazol-2(3*H*)-one] (*1a*) prepared by a different route as described below.

*5-Mercapto-3-methyl-1,3,4-thiadiazole-2(3*H*)-thione (4b).* Methylhydrazine was treated with an excess of carbon disulfide in pyridine solution according to the method described for 1,3,4-thiadiazole-2,5-dithiol by Sandström.[48] An almost quantitative yield of the colourless crystalline pyridinium salt of *4b*, m.p. 134 – 135°C, was obtained. The pyridinium salt was dissolved in a minimum amount of water. The solution was filtered and poured into an excess of 6 N hydrochloric acid at 0 °C. Colourless crystals with a m.p. 68 – 69 °C [49] were obtained in 70 % yield.

*5,5-Dithiobis[3-methyl-1,3,4-thiadiazole-2(3*H*)-thione]* (*3b*). To an aqueous ethanolic (1:1) solution of the pyridinium salt of *4b* was added the equivalent amount of iodine. After addition of water, the precipitated ochre-yellow powder was filtered off and dried. Recrystallization from ethyl acetate gave the pure compound, m.p. 139 – 140 °C. The yield was almost quantitative.

*5,5-Thiocarbonyldithiobis(3-methyl-1,3,4-thiadiazole-2(3*H*)-thione) (1b).* Thiophosgene (0.02 mol) was added dropwise to a stirred solution of the pyridinium salt of *4b* (0.04 mol) in water (25 ml). The precipitate was collected and washed with water. Recrystallization four times from ethyl acetate gave orange coloured crystals of m.p. 139 – 140 °C. Yield 60 %.

*5-Mercapto-3-methyl-1,3,4-thiadiazol-2(3*H*)-one (4a).* Crude ethyl 2-methylcarbazate (0.1 mol, prepared according to Sasse) [50] was dissolved in ethanol (150 ml) and carbon disulfide (0.1 mol) was added followed by a solution of potassium hydroxide (0.1 mol) in ethanol (100 ml). After refluxing for 30 min the solution was allowed to stand overnight. The colourless, crystalline *potassium 3-ethoxycarbonyl-3-methyldithiocarbazate* was filtered off, washed with a little ethanol; 70 % yield; m.p. 204 – 205 °C (dec.). The crude product was boiled in abs. ethanol for 10 h, and the solution taken to dryness *in vacuo*. Recrystallization from 50 % aqueous ethanol furnished the slightly impure *potassium salt of 4a* of m.p. 270 – 275 °C (dec.) in 50 % yield. Acidification as outlined for the preparation of *4b* afforded *4a* as colourless crystals, m.p. 82 – 83 °C; yield 70 %.

*5,5-Thiocarbonyldithiobis(3-methyl-1,3,4-thiadiazol-2(3*H*)-one (1a).* Thiophosgene (0.02 mol) was added dropwise with stirring to a solution of the potassium salt of *4a* (0.04 mol) in water (25 ml). The precipitate was filtered off and recrystallized from a chloroform-pentane mixture to give *1a*, an orange crystalline material, m.p. 186 – 187 °C, in 70 % yield.

Reaction between 1b *and aniline.* Aniline (0.004 mol) was added to *1b* (0.001 mol) dissolved in benzene (25 ml). The orange colour of the solution faded rapidly followed by formation of a colourless crystalline product. This was filtered off and recrystallized from benzene-chloroform (1:1) to a m.p. of 99 – 100 °C. The product was identical to the *anilinium salt of* 4b prepared from *4b* and aniline in diethyl ether. The yield was almost quantitative. Evaporation of the mother liquor furnished almost the expected amount of 1,3-diphenylthiourea (m.p. 153 – 154 °C) identical to an authentic specimen prepared from aniline and phenyl isothiocyanate. When the reaction was carried out in ethanol instead of benzene the orange colour of the solution faded on boiling *1b* (0.001 mol) with aniline (0.004 mol) for a few minutes. The solution was evaporated to dryness. The residue consisted of 1,3-diphenylthiourea and starting material (recovery *ca.* 10 %), which were separated by fractional recrystallization.

Reaction between 1a *and aniline.* The procedure outlined above for the reaction of *1b* with aniline was followed. The *anilinium salt of* 4a, m.p. 111 – 112 °C, precipitated as a colourless crystalline compound. Again 1,3-diphenylthiourea was isolated in almost quantitative yield from the benzene solution by evaporation to dryness.

Reaction between phenylhydrazine and thiophosgene. Phenylhydrazine (10 g) and thiophosgene (15 g) were allowed to react in hydrochloric acid as described by Beckett and Dyson.[1] The resultant orange-coloured, sticky solid was extracted with benzene to give upon evaporation an orange-red oil (10.5 g). The residue from the benzene extraction was phenylhydrazinium chloride (*ca.* 2 g) and water.

Separation of the main components was accomplished by the following method. Triturating the oil with pentane (250 ml) in 25 ml portions gave, after evaporation of the pentane, an orange-coloured oil (4 g) consisting mainly of *5c* and *6c*, but some phenyl isothiocyanate and *1c* was also present. The residue from the pentane extraction was suspended in acetone

leaving *1c* (1 g). Evaporation of the acetone gave a residue (5.5 g) containing *1c* as the main product. It was found advantageous to separate the crude product into these pentane- and acetone-soluble fractions prior to the complete separation of each fraction by column chromatography. The elutions began with a 1:1 mixture of CCl_4/benzene, proceeded to pure benzene and concluded with pure chloroform.

We may summarize the information obtained from the chromatographic separations as follows: The first band to emerge was identified as *phenyl isothiocyanate* (100 mg). We did not establish whether this compound originated from an aniline impurity in the phenylhydrazine used for the reaction. The second band consisted of an unidentified mixture of at least three compounds (60 mg). Next the yellow crystalline *5-[(chlorothiocarbonyl)thio]-3-phenyl-1,3,4-thiadiazol-2(3H)-one, 6c*, was eluted (2.30 g); m.p. 42 – 43 °C from pentane. The fourth component consisted of a yellow oil (0.30 g) identified as *5-chloro-3-phenyl-1,3,4-thiadiazol-2(3H)-one, 5c*. The two compounds *5c* and *6c* were not compared to authentic materials, but the structures proposed above are consistent with recorded IR, MS, and ^{13}C NMR spectra.

5,5-Thiocarbonyldithiobis[3-phenyl-1,3,4-thiadiazole-2(3H)-thione] (1d) was isolated next (100 mg) and identified by comparison with an authentic sample (*vide infra*).

Then we obtained 2 g of *5,5-thiocarbonyldithiobis-[3-phenyl-1,3,4-thiadiazol-2(3H)-one] (1c)* characterized by comparison with an authentic sample prepared as described below. The following fraction (150 mg) was identified by mass spectrometry to be a mixture of *5,5-thiobis[3-phenyl-1,3,4-thiazol-2(3H)-one], 2c, and 5,5-dithiobis[3-phenyl-1,3,4-thiadiazol-2(3H)-one], 3c*, the elemental composition of which was confirmed by high resolution MS. The ensuing fractions were eluted with pure chloroform and consisted of unidentified reddish, oily products.

5-Mercapto-3-phenyl-1,3,4-thiadiazol-2(3H)-one (4c). This compound was prepared according to the directions given by Busch [18] in 51 % yield. It was purified by dissolution in sodium hydroxide and reprecipitation with hydrochloric acid. Recrystallization from pentane gave colourless crystals. M.p. 84.5 – 85.5 °C (Busch:[18] 86 – 87 °C).

5,5-Dithiobis[3-phenyl-1,3,4-thiadiazol-2(3H)-one] (3c). Adding an equivalent amount of iodine in abs. ethanol to a solution of *4c* in benzene gave, after precipitation with pentane, a 75 % yield of yellow crystals, m.p. 77 – 77.5 °C (lit.[18] 78 – 79 °C).

5,5-Thiocarbonyldithiobis[3-phenyl-1,3,4-thiadiazol-2(3H)-one] (1c). Mixing *4c* with the calculated amount of thiophosgene in benzene solution gave *1c*, in good yield. It was recrystallized from benzene and CCl_4 to orange-yellow crystals; m.p. 156 °C. An identical compound could be obtained in poor yield by mixing the reactants in aqueous hydrochloric acid, *i.e.* under the general conditions used by Beckett and Dyson [1] in their experiments. The electronic spectrum of this compound shows maxima at $\lambda = 249$ nm ($\varepsilon = 17\,600$), $\lambda = 275$ nm ($\varepsilon = 18\,800$), and $\lambda = 296$ nm ($\varepsilon = 19\,000$).

5-Mercapto-3-phenyl-1,3,4-thiadiazole-2(3H)-thione (4d). The potassium salt of *4d* [18] was dissolved in a minimum amount of water. The filtered solution was poured into 6 N hydrochloric acid at 0 °C. The yield of colourless crystals was 70 %. M.p. 76 – 77 °C.

5,5-Dithiobis[3-phenyl-1,3,4-thiadiazole-2(3H)-thione] (3d). The potassium salt of *4d* [18] was dissolved in aqueous ethanol (1:1). The equivalent amount of iodine in ethanol was added and *3d* separated immediately as yellow crystals in almost quantitative yield, m.p. 117 – 118 °C (from ethanol).

5,5-Thiocarbonyldithiobis(3-phenyl-1,3,4-thiadiazole-2(3H)-thione) (1d) was prepared according to Runge *et. al.*[19] After recrystallization from ethyl acetate the m.p was 150 – 151 °C and C, H, N, and S analysis confirmed the results of Runge *et. al.* The electronic spectrum of this compound shows maxima at $\lambda = 242$ nm ($\varepsilon = 20\,000$); $\lambda = 275$ nm ($\varepsilon = 18\,500$), $\lambda = 305$ nm ($\varepsilon = 21\,000$) and $\lambda = 332$ nm ($\varepsilon = 19\,000$).

1,3-Di-p-tolylformamidine was prepared according to the directions given by Backer and Wanmaker;[24] m.p. 141.5 – 142.5 °C from pentane, (lit.[24] 143 – 144 °C).

Action of p-toluidine on 1c. By adding *p*-toluidine (1.33 g) dissolved in benzene (5 ml) to the orange-coloured solution of *1c* (1 g) in benzene (20 ml) at 50 °C the reaction mixture was immediately decolorized. The solution was refluxed for 3 h and allowed to cool for an additional 3 h. The colourless crystalline *p-toluidinium salt of 4c* (0.41 g) was collected, m.p. 167 – 167.5 °C. The compound was identified by comparison with an authentic specimen, prepared in nearly quantitative yield by mixing equimolar amounts of *p*-toluidine and *4c* in benzene; colourless needles, m.p. 164.5 – 165.5 °C from benzene. Furthermore, the salt liberated *p*-toluidine and *4c* on successive treatment with sodium hydroxide and hydrochloric acid.

From the mother liquor the colourless crystalline *1,3-di-p-tolylformamidinium salt of 4c* precipitated (0.36 g); m.p. 165.5 – 167 °C after recrystallization from benzene. The identity of this compound was established by comparison with authentic material precipitated in almost quantitative yield as colourless crystals by mixing equimolar amounts of the appropriate formamidine and *4c* in benzene; m.p. 165.5 – 167.5 °C. Furthermore, successive treatment of the salt with sodium hydroxide and hydrochloric acid liberated *1,3-di-p-tolylformamidine* and *4c*, respectively, both identified by comparison with authentic samples.

On concentrating the remaining mother liquor almost to dryness, 0.25 g *1,3-di-p-tolyl-*

thiourea, m.p. 178–179 °C, separated. This was identified by elemental analysis and by comparison with an authentic sample. The remaining material was separated by chromatography on a 2,3 × 20 cm column of silica gel eluting first with a chloroform-hexane mixture (1:3) and finally with pure chloroform. The major component was identified as *3c*, (0.2 g) with a m.p. (after recrystallization from pentane) of 76–76.5 °C.

Action of ammonia on 1c. Dry ammonia was passed through a solution of *1c* in benzene. After a few minutes the colour faded and a colourless product precipitated. This was identified as a mixture of ammonium thiocyanate and the *ammonium salt* of *4c*. The latter compound was identical to a sample prepared as follows. Dry ammonia was passed through a solution of *4c* in benzene. The reaction mixture was heated to the boiling point, and colourless crystals precipitated in almost quantitative yield. M.p. *ca* 191 °C.

Action of ethanol on 1c. A mixture of *1c* (0.001 mol) and ethanol (12 ml) was refluxed for 12 h. The precipitated yellow oil was dissolved by adding 5 ml of boiling ethanol to the mixture, which upon cooling and scratching afforded a 75 % yield of *3c*.

Action of thiophosgene on 1,2-dimethylhydrazine. An aqueous solution (20 ml) of 1,2-dimethylhydrazinium chloride (0.01 mol) was shaken for 1 h with thiophosgene (0.02 mol). 1 g of the isolated solid was separated by PLC into two fractions. *3,4-Dimethyl-1,3,4-thiadiazolidine-2,5-dithione* (*4f*) (300 mg) was obtained as colourless crystals, m.p. 164–165 °C from pentane. Previously m.p. of 163–164 °C [51] and 168–169 °C [52] were reported for the same compound. The other fraction was identified as *3,4-dimethyl-5-thioxo-1,3,4-thiadiazolidin-2-one* (*4e*), 600 mg. Light yellow crystals of m.p. 112–113 °C after recrystallization from pentane.

3-Thioxo-hexahydro-1,2,4-triazine-5,6-dione (*7*). Semioxamazide hydrochloride and thiophosgene were allowed to react in aqueous suspension following the procedure described by Beckett and Dyson.[1] The product (65 % yield) was purified by recrystallization from nitrobenzene as recommended to give 27 % of yellowish crystals, m.p. 219–223 °C (dec.). (Found: C 24.63; H 2.04; N 28.90; S 21.74. Calc. for $C_3H_3N_3O_2S$: C 24.83; H 2.08; N 28.97; S 22.06). Beckett and Dyson obtained a compound with almost identical m.p. (223 °C) but with a sulfur content of 29.25 %.

The compound *7* was treated with aniline according to the directions given by Beckett and Dyson, and it was confirmed that *1-oxamoyl-4-phenylthiosemicarbazide* was formed. This was identical to a sample prepared from semioxamazide and phenyl isothiocyanate (Scheme 4).

5,5-Thiocarbonyldithiobis[3-(4-nitrophenyl)-1,3,4-thiadiazol-2(3H)-one] (*1e*). 4-Nitrophenylhydrazine was shaken with thiophosgene following the directions given by Beckett and Dyson.[1] 1 g of the dark brown crude material was dissolved in chloroform and chromatographed on a column of neutral aluminium oxide. The main fraction (700 mg) was collected and *1e* precipitated with pentane. Orange crystals with m.p. 60–61 °C.

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Short Communications

Separation of Isoenzymes of Acid Phosphatase from Needles of *Pinus silvestris* by Partition in Aqueous Polymer Two-Phase Systems.

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The partition of a protein in aqueous polymer two-phase systems is mainly dependent on its net charge and its relative solubility in the two phases. The effect of charge on the partition of a protein is enhanced by introducing into the phase system a polymer which carries a covalently-bound ionizable group.[1] One such polymer is the positively-charged trimethylamino-poly(ethylene glycol) (TMA–PEG). Phase systems containing this polymer have been used to separate the components of various isoenzyme systems.[2] If two components of an isoenzyme system show only small differences in partition behaviour, a multistage procedure such as counter-current distribution (CCD) is needed. On the other hand, if the difference in partition behaviour is large, only a few extractions are necessary for a complete separation.

This paper describes the separation of isoenzymes of acid phosphatase from a crude extract of pine needles by CCD. A two-step batch procedure, which separates the two main components of enzyme activity, electrophoretically distinct from each other, is also described.

Materials and methods. Dextran 500 (Mw 500 000) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and poly(ethylene glycol), (PEG) (Mw 6000) was obtained from Union Carbide (New York, U.S.A). TMA–PEG was prepared from PEG as described by Johansson [3] and contained about 10 mequiv. trimethylamino groups per g polymer. Triton X-100 was obtained from KEBO (Stockholm, Sweden). Other chemicals used were of analytical grade quality. The water used was distilled in a quartz apparatus. The source of acid phosphatase was needles of *Pinus silvestris* taken from the same tree.

Enzyme assay. The phosphatase activity was measured essentially according to Sigma Technical Bulletin No. 104. Only relative activities have been calculated.

Pine needle extract. Fresh pine needles, 1.5 g, were chopped into 1 mm pieces and homogenized with an Ultra-Turrax (Junke & Kunkel, Stauven, Germany) for 30 s in 7.5 ml 750 mM TRIS-citrate buffer pH 5.4, 2 % (w/w) TMA–PEG, and 1 %(w/w) Triton X-100. The homogenate was then incubated at +4 °C for 3 h and centrifuged at 40 000 *g* for 30 min. The supernatant was dialysed at +4 °C overnight against a 20 mM Tris-citrate buffer pH 5.4 containing 0.15 %(w/w) Triton X-100. The dialysate, after removal of precipitated material, was used as pine needle extract.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out in 6 × 100 mm glass tubes as described by Hjerten *et al.*[4]

The gel was composed of 0.375 %(w/v) *N,N'*-methylenbisacrylamide, 7.125 %(w/v) acrylamide (Cyanogum 41 from Merck, Darmstadt, Germany) and 100 mM TRIS, 250 mM boric acid, and 30 mM ethylenediamine tetraacetic acid, pH 7.2. The gels were selectively stained for acid phosphatase activity according to the method described by Shaw and Prasad.[5]

Counter-current distribution. The two phase system was composed of 6.6 %(w/w) dextran, 6.4 %(w/w) TMA–PEG, 0.15 %(w/w) Triton X-100 and 10 mM TRIS-citrate buffer pH 4.1. The CCD was carried out in an automatic thin-layer CCD apparatus described by Albertsson.[6] A plate with 60 cavities numbered 0–59 was used. The settling time was 8 min and the shaking time 40 s. After 58 transfers the content of each cavity was analysed for acid phosphatase activity.

Two-step extraction. The extraction procedure is described in Fig. 3. The upper phase of phase system 1 was extracted twice with fresh lower phase (Phase systems 3 and 5) and the lower phase of system 1 was extracted twice with fresh upper phase (Phase systems 2 and 4). The degree of partition was expressed as the partition ratio G:

$$G = \frac{\text{(Amount of enzyme activity in upper phase)}}{\text{(Amount of enzyme activity in lower phase)}}$$

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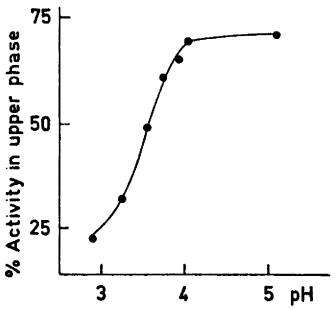

Fig. 1. Extraction of acid phosphatase from needles of *Pinus silvestris* into the upper phase of a phase system containing 6.6 % dextran, 6.4 % TMA–PEG. The pH was raised by titration with TRIS under continuous stirring.

Extraction profile. The extraction of acid phosphatase at different values of pH was performed as described previously.[7]

Gel filtration. The gel filtration was performed in a 1.5 × 70 cm column, filled with Sephadex G-200, (Pharmacia Fine Chemicals, Uppsala, Sweden). The elution buffer was 0.1 M TRIS-citrate, pH 7.5, and the flow rate was 7 ml h^{-1}.

Results and discussion. Acid phosphatase is mainly localized in the lysosomes of the cell. The enzyme was difficult to solubilize, probably due to the membranes surrounding the lysosomes. By addition of Triton X-100 and TMA–PEG to the homogenizing buffer, the extracted enzyme activity increased up to 100 times.

The extraction profile of acid phosphatase is shown in Fig. 1. It is seen that a plateau in enzyme activity is reached at about pH 4. At pH 5 only 70 % of the enzyme activity is found in the upper phase, while 30 % remains in the lower phase. This indicates that the enzyme is composed of at least two components. To investigate this heterogeneity of the enzyme, CCD was used.

Fig. 2 shows the counter-current distribution of acid phosphatase activity. Two peaks were obtained when the enzyme activity was plotted as a function of cavity number. The left peak of enzyme activity was broad and could be fitted to the sum of two theoretical peaks, as shown by the use of the computer program described elsewhere.[8] The material corresponding to each of the two experimental peaks of enzyme activity in Fig. 2 was subjected to gel filtration. No difference in elution volume could be detected. This means that the three fractions possess similar molecular weight. It is therefore probable that the three fractions of enzyme activity obtained by CCD reflect true acid phosphatase isoenzymes.

If the *G*-values of two components greatly differ they may be separated from each other by only two consecutive extractions. The extraction procedure used is shown in Fig. 3. The *G*-value of 12.4 for the isoenzyme component in the upper phase (system 5) shows that this isoenzyme has been highly purified from the component which prefers the lower phase. This latter component has a *G*-value of 0.13 (system 4).

Fig. 4 shows the electrophoretic pattern of acid phosphatase at different stages of the extraction procedure. The electrophoretically fast moving main component, which preferred the upper phase, was found to be homogeneous and in phase-system 5 distinctly separated from the electrophoretically slow-moving main component. This latter component, which preferred the lower phase, was heterogeneous and distinctly separated from the former compo-

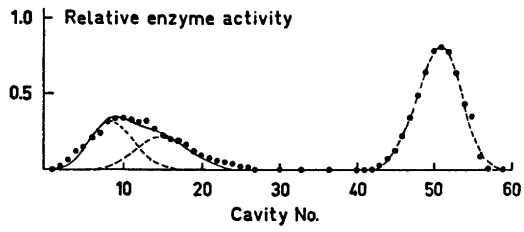

Fig. 2. Distribution of acid phosphatase activity after CCD in identical biphasic systems as used in Fig. 1. The experiment was performed at pH 4.1 and +4 °C. ●, experimental points; – – –, theoretical curves: ———, the sum of theoretical curves. *G*-values calculated according to Hecker[9] were found to be 10.5 and 0.24 for the right and the left main peaks of enzyme activity.

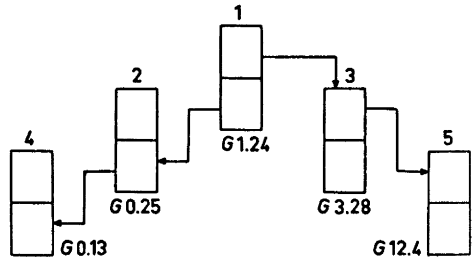

Fig. 3. Extraction of acid phosphatase from pine needles. The upper and lower phase of system 1 is extracted twice with fresh lower and upper phase, respectively. The same phase system as in Fig. 1 was used. The pH of the phase system was 4.0. The *G*-values were calculated for each system and are given in the figure.

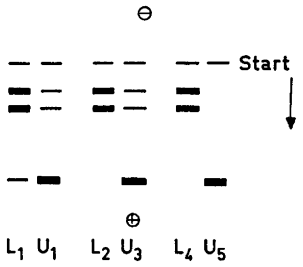

Fig. 4. The electrophoretic pattern of acid phosphatase after polyacrylamide gel electrophoresis. Samples were taken from the phase systems of Fig. 3. L and U correspond to lower and upper phase of the phase systems, respectively. The indices refer to the phase systems of Fig. 3.

nent in phase system 4. The results obtained by electrophoresis shown in Fig. 4, agree with the CCD pattern shown in Fig. 2.

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Substituent Dependent Mass Spectrometric Fragmentation of Monosubstituted Phenyl Propargyl Ethers

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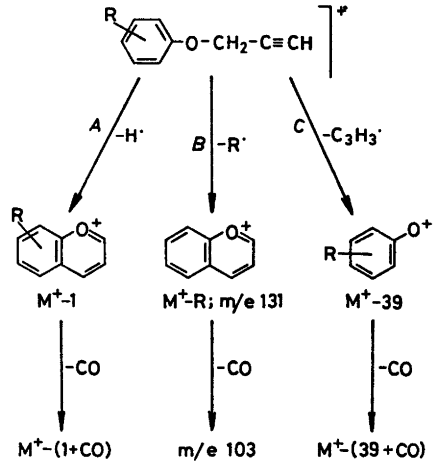
N-Propargylaniline moieties generated by electron impact fragmentation from several different types of molecular ions undergo an intramolecular cyclization to the corresponding quinoline ion.[1] It is reasonable to assume that phenyl propargyl ethers may rearrange in a similar manner giving rise to stable chromanonium ions (*cf.* Scheme 1, routes *A* and *B*). However, from a structural point of view, it is evident that this type of compounds should also be able to decompose by an ether cleavage at an activated benzylic or propargylic[2] site (route *C* in Scheme 1). We have studied a series of monosubstituted phenyl propargyl ethers (compounds *1–15*) and found that their fragmentation patterns follow the proposed routes as outlined in Scheme 1. The relative abundance of the peaks associated with these fragmentations are collected in Table 1. The spectra of compounds *11* and *13* exhibit prominent peaks corresponding to ions formed by fission of the aryl-oxygen bond, a behaviour that may be explained on the basis of an *ortho* effect,[3] which process is exemplified in Scheme 2. Compound *11* also fragments by an unusual route involving a



Scheme 1.

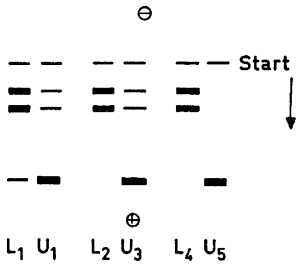


Fig. 4. The electrophoretic pattern of acid phosphatase after polyacrylamide gel electrophoresis. Samples were taken from the phase systems of Fig. 3. L and U correspond to lower and upper phase of the phase systems, respectively. The indices refer to the phase systems of Fig. 3.

nent in phase system 4. The results obtained by electrophoresis shown in Fig. 4, agree with the CCD pattern shown in Fig. 2.

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Substituent Dependent Mass Spectrometric Fragmentation of Monosubstituted Phenyl Propargyl Ethers

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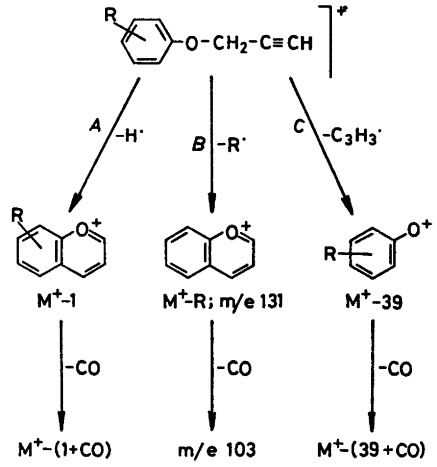
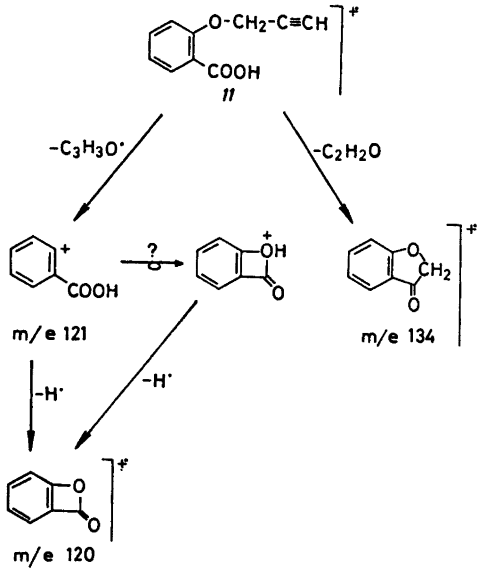
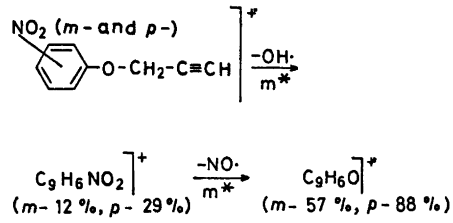
N-Propargylaniline moieties generated by electron impact fragmentation from several different types of molecular ions undergo an intramolecular cyclization to the corresponding quinoline ion.[1] It is reasonable to assume that phenyl propargyl ethers may rearrange in a similar manner giving rise to stable chromanonium ions (*cf.* Scheme 1, routes *A* and *B*). However, from a structural point of view, it is evident that this type of compounds should also be able to decompose by an ether cleavage at an activated benzylic or propargylic[2] site (route *C* in Scheme 1). We have studied a series of monosubstituted phenyl propargyl ethers (compounds *1–15*) and found that their fragmentation patterns follow the proposed routes as outlined in Scheme 1. The relative abundance of the peaks associated with these fragmentations are collected in Table 1. The spectra of compounds *11* and *13* exhibit prominent peaks corresponding to ions formed by fission of the aryl-oxygen bond, a behaviour that may be explained on the basis of an *ortho* effect,[3] which process is exemplified in Scheme 2. Compound *11* also fragments by an unusual route involving a



Scheme 1.



Scheme 2.



Scheme 3.

complex rearrangement with expulsion of a C_2H_2O fragment (*cf.* Scheme 2). The *m*- and *p*-isomers of the nitro derivatives (compounds *14* and *15*) behave in an unexpected manner with consecutive loss of OH and NO as outlined in Scheme 3. This fragmentation is consistent with the metastable peaks found in the mass spectra of *14* and *15* and with the results of high resolution measurements on crucial fragments. No analogous reactions seem to have been reported in the literature.[4] It is evident from the data in Table 1 that the dominating mode of fragmentation for the phenyl propargyl ethers is governed by the electronic properties of the substituent. The unsubstituted derivative *1* as well as those with a strongly electron attracting group (compounds *11*–*15*) completely lack fragments arising from fission at the propargylic bond (route *C* in Scheme 1) whereas this cleavage dominates the fragmentation pattern of the compounds having an electron donating group in *ortho* or *para* position (compounds *2*, *4*, *5*, *7*). Thus there is also a considerable difference in the mass spectrometrical behaviour between position isomers, since the corresponding *meta* isomers (compounds *3* and *6*) break down mainly by routes *A* and *B*.

Table 1. Mass spectra of compounds *1*–*15*. Relative abundance of selected peaks.

Compound No.	R	M+	M+−1	M+−(1+CO)	M+−39	M+−(39+CO)	M+−R	M+−(R+CO)	M+−C_3H_3O
1	H	52	100	48	—	—	—	—	20
2	*o*-CH_3	48	45	43	93	45	50	12	24
3	*m*-CH_3	50	78	50	19	48	100	20	50
4	*p*-CH_3	56	45	33[a]	100	67	50	12	15
5	*o*-CH_3O	54	4	—	100	65	3	—	—
6	*m*-CH_3O	72	100	5	—	18	19	8	—
7	*p*-CH_3O	50	6	—	100	13	—	—	—
8	*o*-Cl	88	78	18	13	76	100	63	—
9	*m*-Cl	16	25	—	—	16	100	31	3
10	*p*-Cl	71	16	16	67	80	100	53	6
11[b]	*o*-COOH	11	11	22	—	—	65[a]	29[a]	84
12	*p*-COOH	25	16	7	—	4	100	25	4
13[c]	*o*-NO_2	24	5	4	—	—	26	14	98
14[d]	*m*-NO_2	14	4	6	—	—	30	27	—
15[e]	*p*-NO_2	29	29	13	—	—	79	50	4

[a] Fragment examined by high resolution measurement. [b] *m/e* 120 100 %, *m/e* 134 74 %. [c] *m/e* 63 100 %, *m/e* 122 98 %. [d] *m/e* 64 100 %, *m/e* 63 90 %. [e] *m/e* 63 100 %, *m/e* 77 94 %.

Substituent dependent fragmentation is a well-known phenomenon and different explanations involving both qualitative and quantitative aspects have been presented.[4] It seems adequate to explain our results on the basis of the charge localization theory,[5] since this is a widely accepted concept in the interpretation of organic mass spectral data. The phenyl propargyl ethers contain two principal centra where the charge may be localized. Electron withdrawing groups facilitate the localization on the aromatic ring, favouring loss of the substituent and formation of the stable *m/e* 131 ion.[6] On the other hand, electron donating groups in *ortho* and *para* positions favour the ether oxygen as a site of the charge, which triggers the ether cleavage.

Experimental. General. Mass spectra were recorded on an AEI MS 30 mass spectrometer at 70 eV. The temperature of the ion source was kept at 250 °C. High resolution measurements on crucial fragments were performed at the laboratory of Dr. R. Ryhage, Karolinska Institutet, Stockholm, using an Atlas SM 1 instrument. Stable isotope studies with deuterium labelling of the acetylenic hydrogen[3] are in agreement with the data presented. PMR and IR spectra were routinely recorded and found to be in accordance with the proposed structures. Melting points were determined in open capillary glass tubes in an electrically heated metal block and are uncorrected. Elemental analyses were performed at the laboratories of Dr. A. Bernhardt, Mülheim, West Germany.

Syntheses. Compounds *1*, *5–10*, *13–15*,[7] *3*,[8] *4*,[9] and *12*[10] were all prepared as described in the literature cited.

o-*Tolyl propargyl ether.* This substance (compound *2*) was prepared from *o*-cresol, 10.8 g (0.10 mol), propargyl bromide, 11.9 g (0.10 mol), and anhydrous K_2CO_3, 16.6 g (0.12 mol) in refluxing acetone (40 ml) according to a general method.[7] The product was purified by distillation *in vacuo*, b.p. 52–53 °C (0.4 mmHg). Yield 10.2 g (69 %), n_D^{25} 1.5330. (Calc. for $C_{10}H_{10}O$: C 82.16; H 6.90. Found: C 82.23; H 7.01).

Methyl o-*propargyloxybenzoate.* Methyl salicylate, 15.2 g (0.10 mol), dissolved in 50 ml of dry methanol was added to 100 ml of a 1 M sodium methoxide solution followed by propargyl bromide, 11.9 g (0.10 mol) in one portion. The mixture was refluxed for 10 h and then cooled in an ice-bath. To the reaction mixture was added 1 M HCl to make it faintly acidic whereupon the solvent was evaporated. The residue was taken up in 200 ml of ether, and the ethereal solution was washed consecutively with 3 × 50 ml of 1 M NaOH, 100 ml of 0.5 M H_2SO_4, and 100 ml of water. The crude product obtained after removal of solvent from the dried ($MgSO_4$) ether solution was fractionated *in vacuo*, b.p. 109–110 °C (1 mmHg). Yield 10.0 g (52 %), n_D^{25} 1.5440. (Calc. for $C_{11}H_{10}O_3$: C 69.47; H 5.30. Found: C 69.29; H 5.33).

o-*Propargyloxybenzoic acid.* (Compound *11*). Methyl *o*-propargyloxybenzoate, 4.8 g (0.025 mol), was warmed with 40 ml of 1 M NaOH at 70 °C for 1 h. The reaction mixture was cooled in an ice-bath and then acidified. The yellowish crystalline precipitate was filtered off, and refluxed with decolourizing charcoal in 25 ml of 50 % ethanol. After two recrystallizations from 50 % ethanol, 3.6 g (82 %) of product, m.p. 88–89 °C, was obtained. (Calc. for $C_{10}H_8O_3$: C 68.18; H 4.58. Found: C 68.29; H 4.64).

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5-Acylthio-4-phenyl-1,2-dithiole-3-thiones; Thia Analogues of α-(1,2-Dithiol-3-ylidene) Ketones

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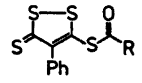
The reaction of dimethylammonium 4-phenyl-3-thioxo-1,2-dithiole-5-thiolate [1,2] with carboxylic acid chlorides results in the formation of 5-acylthio-4-phenyl-1,2-dithiole-3-thiones (*1*).



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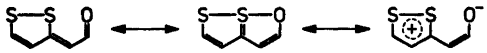
Although dialkylation of the 4-phenyl-3-thioxo-1,2-dithiole-5-thiolate ion has been described [1] diacylation has never been observed even with excess of acyl halide.

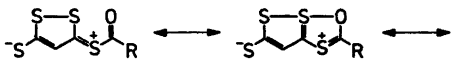
Compounds of type (*1*) may be considered as analogues to α-(1,2-dithiol-3-ylidene) ketones (*2*) for which the resonance structures (*3*) and (*4*) have to be taken into consideration due to the absence of absorption bands in IR above



2 3 4

1600 cm^{-1} [3] where the carbonyl absorption is normally observed. The lack of a normal carbonyl group in these compounds is also reflected in the O 1*s* binding energy observed in the ESCA spectra. An average value of 531.1 eV [4] has been found, which is considerably more negative than normal for carbonyl oxygen.

A series of analogous resonance structures (*5*), (*6*) and (*7*) can be written down for (*1*).



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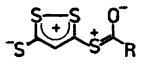

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As seen from Table 1 these compounds show a normal carbonyl vibration in the 1600 – 1700 cm^{-1} region. This indicates that there is no conjugation through the exocyclic sulfide sulfur, *i.e.* no interaction between the dithiole ring sulfur and the carbonyl oxygen is present. From model considerations it is obvious that the carbonyl group has to be *cis* with respect to the dithiole sulfurs due to the sterical interaction of the 4-phenyl group.

This means that the resonance forms (*6*) and (*7*) only play a minor role in the description of these compounds.

Table 1. 5-Acylthio-4-phenyl-1,2-dithiole-3-thiones.

R	ν_{CO}/cm^{-1} (KBr)	M.P.[a]/°C	Yield/%
CD_3	1700	131 – 132	69
CCl_3	1725	118 – 119	55
CH_3	1699	155 – 156	63
CH_3CH_2	1700	83 – 84	48
CH_2CH_2CH	1681	100 – 101	55
$CH_3CH_2CH_2$	1688	79 – 80	51
$C(CH_3)_3$	1684	98 – 99	53
C_6H_5	1670	178 – 179	58
$4\text{-}CH_3OC_6H_4$	1652	189 – 190	55

[a] All compounds show satisfactory elemental analysis.

Experimental. 5-Acylthio-4-phenyl-1,2-dithiole-3-thiones. (Table 1). Dimethylammonium 4-phenyl-3-thioxo-1,2-dithiole-5-thiolate [1] (0.01 mol) in acetonitrile (50 ml) was mixed at room temperature with a solution of the acid chloride (0.01 mol) in acetonitrile (50 ml). The mixture was stirred for 5 min; if no precipitate had occurred, the solution was cooled in an ice/acetone mixture. The precipitates were recrystallized from benzene or mixtures of benzene and ether (1:1).

The mass spectra of the compounds all exhibited abundant peaks corresponding to $[M-RCO]^+$ as well as $[RCO]^+$.

Acknowledgements. The authors are indebted to The Danish Science Research Council for support and to the Danish Government for a grant to N. L.

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The Relative Ease of Oxidation of Phenols and Phenolic Ethers. A Comparison of Reversible Redox Potentials

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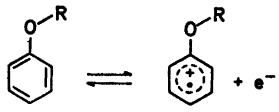
The oxidation processes of phenols are of great interest in both organic and biological chemistry.[1] It is a common observation that phenols are more easily oxidized than the corresponding ethers. The latter is not what one would predict by comparing the ability of an alkyl group with a hydrogen atom to inductively supply electrons to the phenoxy moiety. Here we report reversible redox potentials of some simple phenols and their corresponding methyl ethers and present conclusive evidence that E° for reaction (1) is in general more positive for a phenol (R = H) than for the ether (R = Me). The only reversible redox potentials previously



(1)

reported for phenolic compounds involved special structural features, the α-tocopherol model compound [2] and bridged biphenyls.[3]

Voltammetric data for several phenols and the corresponding methyl ethers are summarized in Table 1. In dichloromethane at −50 °C all of the phenols gave irreversible oxidation peaks while two of the ethers, 1,4-dimethoxybenzene and 4-methoxybiphenyl gave reversible cyclic voltammograms. The first column in the table summarizes peak potentials in dichloromethane at −50 °C. The second column shows data from the same solutions after adding $HFSO_3$ (10 %).[4] The fact that the peak potentials for the two ethers showing reversible behaviour did not change upon addition of the acid indicates that the changes in peak potentials for the phenols given in the third column are the differences between the irreversible and reversible oxidation potentials and do not arise from changes in the solvent or in the reference electrode. The data are exemplified by the cyclic voltammograms of 4-hydroxybiphenyl in dichloromethane (Bu_4NBF_4 = 0.2 M) shown in Fig. 1. In the absence of acid (Fig. 1a) a single irreversible oxidation peak was observed at +1.48 V.[5] In

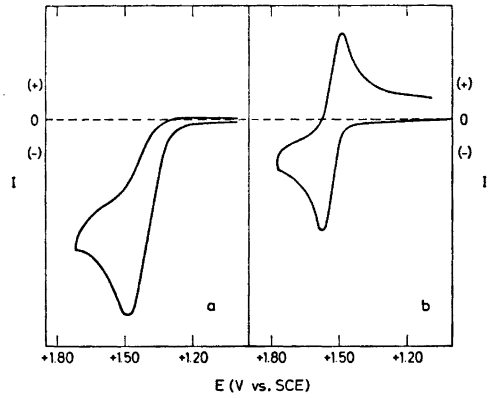


Fig. 1. Cyclic voltammetry at a platinum button electrode of 4-hydroxybiphenyl (2×10^{-3} M) in dichloromethane (Bu_4NBF_4 = 0.2 M) at −50 °C and 86 mV/s. (a) in the absence of acid, (b) in the presence of FSO_3H (10 %).

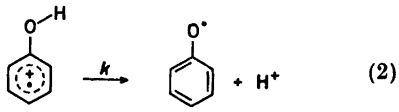
Table 1. Reversible oxidation potentials of phenols and phenol ethers.

Compound	E_p^{ox} (V *vs.* SCE) CH_2Cl_2 [a]	CH_2Cl_2-$HFSO_3$ [b]	Shift (mV) [c]	k (s^{-1}) [d]
4-Methoxyphenol	1.20	1.49	290	2.1×10^{10}
1,4-Dimethoxybenzene	1.38	1.39		
4-Hydroxybiphenyl	1.48	1.59	110	1.8×10^{4}
4-Methoxybiphenyl	1.55	1.56		
2,4,6-Trimethylphenol	1.33	1.65	320	2.2×10^{11}
1-Methoxy-2,4,6-trimethylbenzene	1.70	1.68		

[a] [Bu_4NBF_4] = 0.2 M, −50 °C, voltage sweep rate = 86 mV/s. [b] Acid concentration = 10 % by volume. [c] Differences in reversible and irreversible peak potentials. [d] Estimated first order rate constants for deprotonation of the cation radicals at −50 °C.

the presence of $HFSO_3$ (10 %) the voltammogram (Fig. 1b) showed that the cation radical was stable during the time scale of the experiment. Further addition of acid had no effect upon the reversible potetial (+1.59 V) indicating that kinetic processes were no longer operative and that the value approximates E^0.

In the cases studied, the irreversible potentials measured in the absence of acid were always considerably lower for the phenols than for the ethers while those measured from reversible voltammograms, except for 2,4,6-trimethylphenol, showed the opposite order, *i.e.* the phenol potentials were more positive than those of the ethers. Differences in voltammetric peak potentials measured under reversible and irreversible conditions are due to the occurrence of chemical reactions coupled to the electron transfers.[6] The source of the large differences between the irreversible and reversible potentials for the phenol oxidations is no doubt connected to the deprotonation of the cation radical (2) accompanied by the generation of the phenoxy radical which is more easily oxidized than the phenol. Further electron transfer then occurs generating the phenoxonium ion (3), a species very susceptible to attack by any nucleophiles present in the medium.[1] From the differences in the potentials between the reversible and irreversible voltammograms, the rate constants for steps (2) can be calculated by the method of Nicholson and



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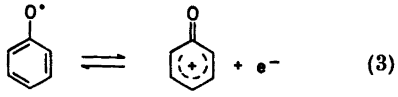

(3)

Shain.[6] Estimates of the rate constants for the deprotonation of the cation radicals are given in the last column of the table.[7]

It is of interest to note that the reversible potential for 2,4,6-trimethylphenol was less positive than that of the corresponding ether. A similar effect has been observed during a comparison of ionization potentials of phenols and phenol ethers.[8] The gas phase ionization potential of 2,6-dimethylphenol was found to be 0.25 eV less than 2,6-dimethylanisole while several other methyl phenols were found to have ionization potentials about 0.2 eV higher than the corresponding ethers. The reason for the increased difficulty of oxidation of 2,6-dimethylanisoles is apparently due to the fact that the ether methyl group is hindered from occupying a position in the same plane as the aromatic ring and thus conjugation of the oxygen lone pair of electrons is diminished in the cation radical. It has previously been shown that such conjugation inhibition gives rise to unusual reactivity of phenol ether cation radicals.[9]

The results presented here are the first for the reversible oxidation of simple phenols. These, along with data from the corresponding ethers and gas phase ionization potentials [8] allow us to draw the conclusion that the E_0 values for phenols in general are more positive than those for the ethers.

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2-*C*-Methyl-erythritol, a New Branched Alditol from *Convolvulus glomeratus*

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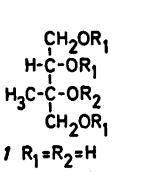
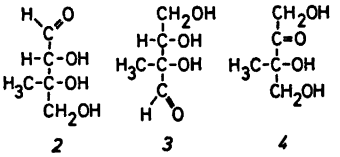
The Convolvulaceae family is known to contain a number of resin glycosides.[1] Their building blocks are short chain volatile acids, long chain hydroxy-fatty acids and carbohydrates. The carbohydrates isolated so far are D-glucose, L-rhamnose, D-fucose, D-quinovose and presumably 6-deoxygulose. Fractionation of extracts of *Convolvulus glomeratus* Choisy afforded in addition to some hydrocarbons and steroids which were not further characterized, 2-*C*-methyl-D- or L-erythritol (*1*) which was identified by ^{1}H and ^{13}C NMR spectroscopy. Biosynthetically the alditol *1* may arise from isoprenoid or amino acid precursors. Carbohydrate precursors such as the aldoses *2* or *3* or the ketose *4* are also possible.

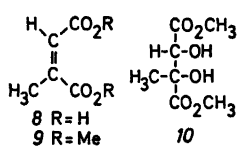
The molecular formula of *1* was established as being $C_5H_{12}O_4$ on the basis of the proton decoupled ^{13}C NMR spectrum which reveals the presence of five carbon atoms, and the



1 $R_1=R_2=H$
5 $R_1=R_2=SiMe_3$
6 $R_1=Ac$ $R_2=H$
7 $R_1=R_2=Ac$



2 *3* *4*



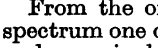
8 R=H
9 R=Me

10

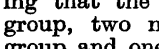
elemental analysis. The mass spectrum of the tetra-*O*-trimethyl-silylether (*5*) shows no molecular ion peak. However, characteristic peaks due to the ions $[M-{\cdot}CH_2OSiMe_3]^+$ and $C_9H_{23}Si_2O_2^+$ are of high abundance.

From the off-resonance decoupled ^{13}C NMR spectrum one quartet, two triplets, one doublet, and one singlet are clearly visible thus showing that the alditol *1* contains one methyl group, two methylene groups, one methine group and one fully substituted carbon atom. The ^{1}H NMR spectrum was not very informative, except for the resonance of the methyl group (δ 1.12). However, on addition of europium nitrate to the solution one AB-pattern and one ABX-pattern became clear. On acetylation the alditol *1* furnished a tri- (*6*) and a tetraacetate (*7*). The ^{1}H NMR spectrum of the triacetate is particularly informative. Beside the resonances of the methyl group (1.23) and the three acetyl groups (2.02, 2.09, 2.09), the AB (ν_A = 4.15, ν_B = 3.91, J_{AB} = 11.5 Hz) and the ABX patterns (ν_A = 4.57, ν_B = 4.16, ν_X = 5.20, J_{AB} = 12.0, J_{AX} = 3.0, J_{BX} = 7.5 Hz) were seen.

The two spin patterns established the presence of one $-CH_2-OH$ group and one



group in the molecule and thus confirmed the conclusion based upon the ^{13}C NMR spectrum. Moreover, the resonance of the methyl group was shifted downfield in the tetraacetate and this suggested



the structural element. This established the constitution of *1* as that of a 2-*C*-methyltetritol, but did not distinguish between the *erythro* or *threo* configuration.

This information could possibly be sought in the lanthanide induced shift ^{1}H NMR spectra, since *erythro* forms are reported to give much smaller induced shifts than the *threo* forms.[2,3] However, in order to draw safe conclusions on this background it seemed necessary to investigate the shift curves of both the *threo* and the *erythro* form.

To answer the stereochemical question we carried out a synthesis of racemic *1* from citraconic acid (*8*).

Methylation gave dimethylcitraconate (*9*)[4] which on oxidation with potassium permanganate furnished a dihydroxy diester (*10*). This was converted into *1* by lithium aluminium hydride reduction. The ^{1}H NMR spectrum of the synthetic compound and its triacetate were identical with those of *1* and its triacetate.

Further studies on the lanthanide induced shifts and synthetic compounds related to *1* are in progress.

Experimental. The NMR spectra were recorded on Varian A-60A (^{1}H, 60 MHz) and Jeol PS-100 (^{13}C, 25.1 MHz, PFT). Chemical shifts are given in ppm relative to TMS in $CDCl_3$ or dioxane solutions and relative to sodium 3-(trimethylsilyl)-propane sulfonate in D_2O.

Mass spectra were obtained with AEI MS 902. Combustion analysis was performed by Alfred Bernhardt, Elbach über Engelskirchen, Germany.

Isolation of the alditol 1. *Convolvulus glomeratus* (roots and shoots) was collected in April and May in Sind, Pakistan. The plant was cut into small pieces, dried and ground. The ground material (12 kg) was refluxed with light petroleum for 6 h. The extract was filtered and evaporated to dryness under vacuum to yield a thick dark green oil (113 g, 1 %) which was shown mainly to consist of hydrocarbons.

The residue was dried and refluxed with

ethanol (95 %) for 6 h. The extract was filtered and evaporated to dryness under vacuum to yield a dark green gum (1125 g, 9.4 %). This material was mixed with filter-aid and successively Soxhlet-extracted with light petroleum, benzene, chloroform, ethyl acetate, and ethanol to yield five extracts; 91.5, 19.5, 312, 151.5, and 645 g, respectively. The light petroleum extract was found to consist mainly of steroids. So far no further compounds have been identified from the benzene, chloroform and ethanol extracts. The ethyl acetate extract was applied to an alumina column and eluted with chloroform – ethyl acetate (1:1), ethyl acetate and ethanol. The green mass obtained from the ethyl acetate fractions was shaken with hexane which removed most of the coloured material. The semi-solid material was left at room temperature for a few months and the alditol *1* crystallized. Repeated recrystallizations from hot acetone gave colourless crystals (12.5 g, m.p. 82 – 83 °C, $[\alpha]_D^{22}$ = 21.4° (H_2O *c* 7.0). (Found: C 44.87; H 8.86. Calc. for $C_5H_{12}O_4$: C 44.11; H 8.89). ^{1}H NMR (D_2O): δ 1.12 (3 H, *s*, tertiary methyl), 3.45 and 3.57 (AB pattern J_{AB} = 11.0 Hz, $-CH_2OH$), 3.5 – 4.0 (ABC pattern, HO – CH – CH_2OH). ^{13}C NMR (dioxane): δ 20.0 (q, methyl carbon), 63.5 and 67.8 (both *t*, two methylene carbons), 75.6 (*s*, quaternary carbon), 76.4 (*d*, methine carbon).

Acetylation. The alditol *1* yields both a tri- and a tetraacetate on treatment with acetic anhydride and pyridine. The relative yields of the two acetates depended on the conditions of acetylation. The two acetates were easily separable on TLC. In 65 % ether in light petroleum the R_F-values were 0.28 (triacetate) and 0.66 (tetraacetate).

Triacetate (6). The alditol *1* (178 mg) was dissolved in a mixture of 2 ml pyridine and 2 ml acetic anhydride and left at room temperature for 3 h. After evaporation to dryness and purification on TLC it furnished a colourless oil (320 mg), $[\alpha]_D^{20}$ = +18.1° ($CHCl_3$, *c* 18.4). ^{1}H NMR (CCl_4): δ 1.23 (3H *s*, tert. methyl), 2.02, 2.09 and 2.09 (all 3H and *s*, three acetyl groups), 4.15 and 3.91 (AB pattern, J_{AB} = 11.5 Hz, $-CH_2-OAc$), 4.57 (A), 4.16 (B) and 5.20 (X) (ABX pattern, J_{AB} = 12.0, J_{AX} = 3.0 and J_{BX} = 7.5, AcO – CH – CH_2OAc), 2.76 (*s*, OH conc. dependent). MS: M^+ 262 absent, *m/e* 189 (6 %, M – $\cdot CH_2OAc$), (Calc. for $C_8H_{13}O_5$ 189.0762; found 189.0771), 159 (55 %, M – 60 – 43), 129 (90 %, *m/e* 189 – 60, *m** 88.0), 117 (100 %, $AcOCH_2-C(CH_3)=^+OH$).

Tetraacetate (7). The alditol *1* (183.5 mg) was dissolved in a mixture of 1.8 ml pyridine and 2 ml acetic anhydride and the mixture was refluxed for 3 h. Evaporation to dryness yielded a yellow oil (321 mg) which was purified on TLC to yield pure oily tetraacetate. ^{1}H NMR (CCl_4): δ 1.51 (*s*, tert. methyl), 2.01, 2.01, 1.99 and 1.99 (all 3 H and *s*, four acetyl groups, 4.45 and 4.30 (AB pattern, J_{AB} = 12.0 Hz, $-CH_2OAc$), 4.48 (A), 4.05 (B) and 5.36 (X) (ABX pattern, J_{AB} = 12.0, J_{AX} = 3.0 and J_{BX} = 7.5, AcO – CH – CH_2OAc). MS: M^+ 304 absent, *m/e* 231 (24 %, M – $\cdot CH_2OAc$); (Calc. for $C_{10}H_{15}O_6$ 231.0869, found 231.0863), 189 (14 %, 231 – 42, *m** 154.5), 159 (43 %, 231 – 72, *m** 109.6, and $AcOCH_2-C(CH_3)=^+OAc$), 129 (100 %, 189 – 60, *m** 88.0).

Tetra-O-trimethylsilyl ether (5). To a solution of *1* (40 mg) in pyridine (5 ml) was added hexamethyldisilazane (0.5 ml) and trimethylchlorosilane (0.5 ml). The mixture was left overnight and evaporated in vacuum. The residue was dissolved in carbon tetrachloride (1 ml), filtered and dried over sodium sulfate. The product was used directly for mass spectroscopy. M^+ 424 (absent), *m/e* 409 (0.7 %, M – Me), 321 (7.9 %, M – $\cdot CH_2OSiMe_3$), 219 (100 %, $Me_2SiOCH_2-C(CH_3)=^+OSiMe_3$), (Calc. for $C_9H_{23}Si_2O_2$ 219.1236 as found), 73 (52.2 %, $^+SiMe_3$).

Dimethyl citraconate (9). To citraconic acid (*8*) (20 g) was added an excess of thionyl chloride (*ca.* 50 ml) and the mixture was kept at 50 °C for 3 h. Excess of thionyl chloride was distilled off under reduced pressure and to the resulting citraconoyl dichloride methanol (10 ml) was added without further purification. This mixture was kept at 50 °C for 2 h. Excess methanol was removed and the crude dimethyl citraconate was distilled under vacuum (12 mm, 96 – 98 °C, yield 13.4 g oily diester). ^{1}H NMR ($CDCl_3$) δ 2.04 (*d*, *J* = 1.7 Hz, olefinic methyl), 3.78 and 3.66 (both *s*, two methoxyls), 5.89 (*q*, *J* = 1.7 Hz, olefinic H). ν_{max} ($CHCl_3$) 1725 cm^{-1}.

Dimethyl 2-C-methyl-erythro-tartrate (10). A solution of dimethyl citraconate (10 g, 0.063 mol) in water (200 ml) was kept at 5 °C and pH 13. Potassium permanganate (10 g, 0.063 mol) in water solution (300 ml) was added and the mixture was stirred for 20 min. The solution was decolourized by addition of sodium hydrogen sulfite and after saturation with sodium chloride it was extracted with ethyl acetate three times. The combined extracts were dried over sodium sulfate and evaporated to dryness (yield 1.8 g). Recrystallization from hot ethyl acetate gave colourless crystals (1.5 g, m.p. 93 °C). ^{1}H NMR ($CDCl_3$) δ 1.53 (*s*, quaternary methyl), 3.75 and 3.81 (both *s*, two methoxyls), 3.80 (*s*, two hydroxyls), 4.40 (*s*, methine H), ν_{max} (KBr) 1740 and 3370 cm^{-1}. MS: M^+ absent, *m/e* 133 20 %, ($M - CO_2CH_3$), *m/e* 90 100 %, (McLafferty rearr. $C_3H_6O_3^+$).

2-C-Methyl-DL-erythritol (racemic 1). A solution of the ester *10* (1.5 g) in dry tetrahydrofuran was added to a solution of lithium aluminium hydride (1.1 g) in dry tetrahydrofuran (20 ml). The mixture was stirred at room

temperature for 2 h and then poured into water (60 ml). The solid material was removed in a centrifuge. The solution was evaporated almost to dryness, mixed with methanol–water (1:1, 50 ml) and solid carbon dioxide was added. The residue that appeared was filtered off and the filtrate evaporated to dryness to yield a solid material (0.5 g) which was difficult to crystallize. The NMR spectra of this compound and its triacetate were identical with the corresponding spectra of the alditol *1* and its triacetate, respectively.

Acknowledgements. We are grateful to NORAD for a fellowship to S.W. Shah and to JEOL Skandinaviska AB for the ^{13}C NMR spectra.

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N-Vinyl as N–H Protecting Group. A Convenient Synthesis of Myosmine

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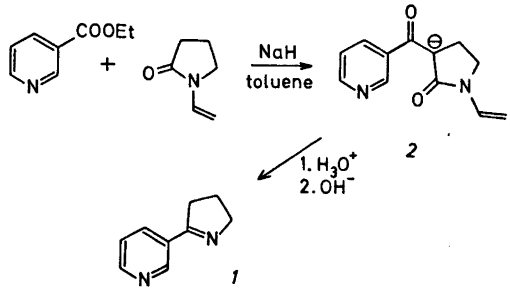
We here describe a convenient synthesis of the minor tobacco alkaloid myosmine (*1*), starting with a condensation of *N*-vinylpyrrolidone with ethyl nicotinate. The condensation product 2 is treated with boiling concentrated hydrochloric acid without prior purification and, after extraction of bases and distillation, *1* is obtained in a 63 % yield.

This synthesis of myosmine is similar to previous ones in which the N–H group in 2-pyrrolidone was protected as an *N*-acyl [1] or *N*-trimethylsilyl [2] derivative, but preparation of



the N–H protected pyrrolidone is here avoided by using the commercially available *N*-vinylpyrrolidone. Reports on the use of vinylic groups as protective groups for N–H are scanty. 2-Acyl-1-methylvinyl groups have for instance found application in peptide syntheses.[3]

Experimental. A solution of freshly distilled *N*-vinylpyrrolidone (Fluka) (20 g, 0.18 mol) and ethyl nicotinate (25.0 g, 0.17 mol) in dry toluene (200 ml) was added to a stirred suspension of sodium hydride (0.26 mol, introduced as 10.4 g of a 60 % suspension in mineral oil) in dry toluene (100 ml). The mixture was then refluxed for 1.5 h. A light green precipitate was formed at the beginning of the heating. The cooled reaction mixture was poured under stirring into dilute hydrochloric acid (100 ml of conc. acid + 180 ml of water). After 5 min the pH was adjusted to 4 with concentrated sodium hydroxide solution, the toluene layer was separated, and the aqueous layer was extracted with chloroform-ethanol (3:2, 3 × 200 ml). The organic layers were combined and dried (Na_2SO_4), and the solvent was evaporated giving a residue which was treated with refluxing concentrated hydrochloric acid (250 ml, 14 h). The tar formed was discarded and the remaining solution was made alkaline (pH 10) with concentrated sodium hydroxide solution and then extracted with chloroform (3 × 150 ml). After drying (Na_2SO_4), concentration and distillation, *1* was obtained as a pale yellow liquid (15.1 g, 63 %), b.p. 113–115 °C (0.4 kPa), which solidified in the receiver, m.p. 39–42 °C. These values, as well as the IR absorption max at 1618 cm^{-1} (film), agree well with those previously given for myosmine.[1]

Acknowledgement. This work has been supported by the Swedish Tobacco Company.

1. Korte, F. and Schulze-Steinen, H.-J. *Chem. Ber. 95* (1962) 2444.
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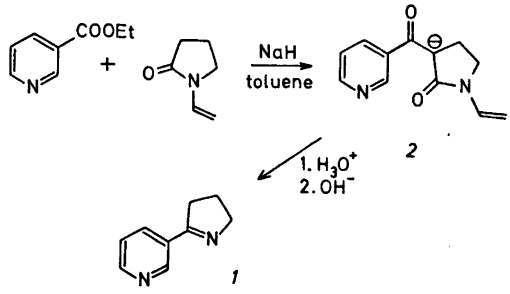

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Experimental. A solution of freshly distilled *N*-vinylpyrrolidone (Fluka) (20 g, 0.18 mol) and ethyl nicotinate (25.0 g, 0.17 mol) in dry toluene (200 ml) was added to a stirred suspension of sodium hydride (0.26 mol, introduced as 10.4 g of a 60 % suspension in mineral oil) in dry toluene (100 ml). The mixture was then refluxed for 1.5 h. A light green precipitate was formed at the beginning of the heating. The cooled reaction mixture was poured under stirring into dilute hydrochloric acid (100 ml of conc. acid + 180 ml of water). After 5 min the pH was adjusted to 4 with concentrated sodium hydroxide solution, the toluene layer was separated, and the aqueous layer was extracted with chloroform-ethanol (3:2, 3 × 200 ml). The organic layers were combined and dried (Na_2SO_4), and the solvent was evaporated giving a residue which was treated with refluxing concentrated hydrochloric acid (250 ml, 14 h). The tar formed was discarded and the remaining solution was made alkaline (pH 10) with concentrated sodium hydroxide solution and then extracted with chloroform (3 × 150 ml). After drying (Na_2SO_4), concentration and distillation, *1* was obtained as a pale yellow liquid (15.1 g, 63 %), b.p. 113–115 °C (0.4 kPa), which solidified in the receiver, m.p. 39–42 °C. These values, as well as the IR absorption max at 1618 cm^{-1} (film), agree well with those previously given for myosmine.[1]

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Crystal Structures of Synthetic Analgetics. V. Dextromoramide

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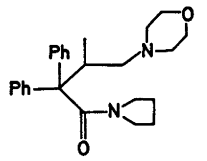
The molecular and crystal structure of dextromoramide has been determined by X-ray methods. The crystals are orthorhombic, space group $P2_12_12_1$ with unit cell dimensions $a = 9.720(4)$ Å; $b = 12.226(3)$ Å; $c = 18.381(3)$ Å. The structure was determined by direct methods and the model refined to an R-value of 0.036 for 1788 observed reflections. The mean e.s.d.'s in bond lengths and angles are 0.004 Å and 0.3°, respectively.

The morpholine moiety is nearly in *anti* position relative to the quaternary carbon atom C6, the pertinent angle C6 – C7 – C9 – N2 being −159.4°. This conformation is similar to that previously reported for the bitartrate of the title compound. The pyrrolidine ring has the *envelope* conformation and the amide group is strictly planar.

The conformations of some acyclic analgetics are discussed.

Dextromoramide is a synthetic analgetic related to methadone,[1] and the present X-ray



crystallographic investigation was undertaken as a part of a research program in this laboratory on compounds exerting analgesic activity.

Acyclic synthetic analgetics related to methadone exhibit pharmacological activity similar to that of morphine and their activity may be dependent on a specific conformation, as proposed by Beckett *et al.*[2-3]

Recently the proposed molecular conformation was confirmed by X-ray crystallographic investigations on methadone.[4-5] The conformational flexibility of these compounds is exemplified by the totally different conformation of methadone in the crystal structures of the hydrobromide [6] and of the free base.[4] This has encouraged the author in studying crystal structures of analgetics with the parent molecules in different environments.

In a previous paper the crystal structure of the bitartrate of the title compound [7] has been reported and here the X-ray analysis of the free base is presented.

EXPERIMENTAL

Dextromoramide was prepared from the commercial dextromoramide bitartrate and crystallized readily at room temperature by slow evaporation from a diethyl ether solution. A single crystal of dimensions 0.2 mm × 0.3 mm × 0.4 mm was used for the experiments.

The crystals are orthorhombic and systematically absent reflections $h00$, $0k0$ and $00l$ for odd indices are compatible with space group $P2_12_12_1$. Unit cell dimensions were determined on a Syntex $P\bar{1}$ diffractometer with graphite crystal monochromated Mo$K\alpha$-radiation ($\lambda = 0.71069$ Å).

Three-dimensional intensity data were collected applying the $2\theta - \theta$ autocollection program with variable scan rate (2 – 8° min^{-1}). The scan range was from 1.0° below 2θ (α_1) to 1.0° above $2\theta(\alpha_2)$ and the background was counted for 0.7 times the scan time. The intensities of three standard reflections were measured periodically during the collection of data; they did not show any systematic variation. 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 2238 independent reflections with $\sin\theta/\lambda < 0.60$ were recorded; 1788 had a net count larger than 2.5 σ_I.

The data were corrected for Lorentz and polarization effects.

All calculations were performed on a CYBER-74 computer utilizing the programs in Ref. 8, except for the phase determination.[9] Atomic form factors were those of Hanson *et al.*[10] for O, N and C and of Stewart *et al.*[11] for H.

CRYSTAL DATA

Dextromoramide, $C_{25}H_{32}N_2O_2$, orthorhombic. $a = 9.720(4)$ Å, $b = 12.226(3)$ Å, $c = 18.381(3)$ Å. $V = 2184.3$ Å^3, $M = 392.55$, $Z = 4$. $D_{obs} = 1.20$ g cm^{-3} (flotation), $D_{calc} = 1.19$ g cm^{-3} Systematic absences: $h00$, $0k0$, $00l$ for odd indices; space group $P2_12_12_1$.

STRUCTURE DETERMINATION

A preliminary scale factor and an overall isotropic temperature factor ($B = 3.7$ Å^2) were derived by Wilson's statistical method and used for calculation of normalized structure factors. The phases of 400 reflections ($E > 1.25$) were determined by MULTAN.[9] Only nine out of 35 peaks in the "best" E-map could be associated with reasonable atomic positions, and this fragment was the basis for another phase determination as proposed by Karle.[12] Successive fragment refinements including an increased number of atoms, eventually gave an E-map where all the 29 non-hydrogen atoms could be recognized. Anisotropic full-matrix least-squares refinements gave an R-factor of 0.10 and approximate positional parameters of all the 32 hydrogen atoms were calculated from stereochemical considerations. All the hydrogen parameters were refined, including the isotropic temperature factors, although atoms bonded to the same carbon atom were ascribed common B-values.

The refinement converged at $R = 0.036$ ($R_w = 0.036$) for the 1788 observed reflections.

Table 1. Positional and thermal parameters for the heavy atoms (10^5) and the hydrogen atoms (10^4) with e.s.d.'s in parentheses.

ATOM	X	Y	Z	B11	B22	B33	B12	B13	B23
C1	72617(31)	44926(25)	37884(17)	1109(38)	822(26)	458(12)	239(57)	-284(35)	244(38)
C2	81374(38)	54930(30)	39887(21)	1181(41)	1045(32)	783(18)	-121(70)	-392(46)	484(42)
C3	75965(39)	63343(29)	34172(21)	1673(54)	869(28)	646(16)	-675(65)	-899(51)	346(38)
C4	61578(34)	60653(23)	32489(18)	1273(42)	588(22)	529(15)	-226(52)	-162(43)	353(29)
C5	48737(38)	42653(21)	34178(14)	1158(36)	429(19)	283(9)	115(46)	-77(33)	61(23)
C6	35224(25)	47138(18)	38663(13)	987(32)	482(17)	231(8)	+7(42)	-86(27)	55(21)
C7	24096(28)	37767(28)	38743(13)	1126(35)	447(17)	234(8)	-95(42)	-33(31)	18(28)
C8	11897(32)	39804(23)	25742(14)	1314(41)	692(22)	297(9)	-399(54)	-185(35)	99(25)
C9	18615(38)	34771(21)	38294(14)	1212(37)	528(19)	263(9)	-67(49)	-25(32)	-22(22)
C10	22791(31)	19378(23)	39427(16)	1099(37)	592(21)	425(12)	8(52)	227(36)	145(27)
C11	16269(35)	4117(24)	38641(20)	1497(46)	596(22)	567(15)	-277(59)	429(48)	117(31)
C12	-4611(33)	11429(38)	42537(18)	1149(48)	1168(33)	398(12)	-513(64)	174(38)	380(35)
C13	1484(31)	22243(23)	43369(16)	1181(38)	702(23)	333(10)	216(53)	285(38)	285(26)
C14	20628(26)	56918(19)	35828(13)	945(31)	423(17)	242(8)	-187(42)	-8(30)	94(20)
C15	33694(29)	58487(21)	42256(14)	1183(36)	542(19)	256(9)	-60(48)	8(31)	56(23)
C16	27983(35)	66519(26)	46542(15)	1627(48)	734(24)	282(10)	-375(63)	151(38)	-85(27)
C17	18175(35)	73584(24)	43887(18)	1478(44)	651(22)	417(13)	145(59)	278(42)	-269(29)
C18	13867(32)	72198(23)	36692(19)	1218(42)	631(22)	475(14)	274(53)	89(40)	7(29)
C19	19585(38)	63986(22)	32396(14)	1178(36)	548(20)	294(10)	183(50)	-155(33)	-3(23)
C20	39183(27)	49153(19)	22582(14)	945(32)	442(18)	263(9)	-118(43)	-84(30)	3(21)
C21	37878(29)	58636(21)	18883(14)	1181(35)	514(19)	274(9)	-252(46)	-83(32)	45(23)
C22	46776(32)	59535(24)	11448(16)	1321(48)	741(24)	265(10)	-534(56)	-93(35)	121(27)
C23	45375(34)	50468(29)	7741(16)	1508(44)	974(30)	258(10)	-685(62)	123(37)	10(31)
C24	47428(35)	40833(26)	11399(17)	1558(47)	809(26)	325(11)	-374(62)	369(39)	-137(30)
C25	44449(32)	40238(22)	18741(16)	1387(42)	585(21)	354(11)	-118(51)	171(36)	23(25)
N1	59844(23)	49184(17)	34619(13)	929(27)	535(16)	378(8)	139(38)	-184(28)	198(20)
N2	12513(22)	23769(16)	38127(11)	988(28)	532(16)	252(7)	-141(37)	182(25)	52(18)
O1	49424(23)	33218(15)	36243(12)	1497(29)	475(14)	588(9)	69(36)	-434(29)	
O2	5385(22)	2748(17)	43718(12)	1442(29)	789(16)	475(9)	-294(36)		

ATOM	X	Y	Z	B
H1C1	7772(38)	3866(22)	3427(14)	6,3(,5)
H2C1	6987(29)	4111(22)	4284(15)	6,3(,2)
H1C2	9867(37)	5399(29)	3935(18)	10,1(,7)
H2C2	7685(39)	5676(29)	4453(19)	10,1(,3)
H1C3	8858(44)	6187(34)	2948(20)	11,1(,8)
H2C3	7839(43)	7087(32)	3474(20)	11,1(,2)
H1C4	5642(36)	6477(28)	3562(18)	9,1(,7)
H2C4	5888(38)	6289(26)	2819(19)	9,1(,2)
H67	2949(22)	3181(17)	2894(10)	3,8(,5)
H1C8	1415(27)	4158(23)	2855(14)	5,9(,4)
H2C8	668(28)	4644(21)	2691(14)	5,9(,2)
H3C8	523(27)	3319(23)	2616(14)	5,9(,2)
H1C9	1187(21)	3999(17)	3979(10)	3,1(,3)
H2C9	2597(22)	3543(17)	4173(10)	3,1(,2)
H1C10	2687(25)	1648(28)	4452(12)	4,6(,4)
H2C10	3837(25)	1617(19)	3592(12)	4,6(,2)

ATOM	X	Y	Z	B
H1C11	2237(35)	-75(28)	3965(17)	8,9(,6)
H2C11	1189(34)	268(26)	3389(17)	8,9(,6)
H1C12	-1196(27)	1806(21)	4613(13)	5,5(,4)
H2C12	-724(27)	1818(21)	3748(13)	5,5(,9)
H1C13	523(28)	2292(23)	4844(13)	5,4(,5)
H2C13	-819(26)	2739(21)	4268(13)	5,4(1,1)
HC15	4879(22)	5316(18)	4434(11)	3,6(,5)
HC16	3133(27)	6738(21)	5182(12)	5,8(,6)
HC17	1387(26)	7935(21)	4696(13)	5,8(,7)
HC18	656(29)	7730(22)	3459(14)	6,3(,7)
HC19	1611(21)	6389(16)	2735(11)	2,8(,5)
HC21	3458(21)	6529(17)	2131(11)	3,8(,5)
HC22	3978(24)	6694(19)	896(11)	4,2(,5)
HC23	4745(26)	5112(21)	238(13)	5,3(,6)
HC24	5171(29)	3364(24)	888(14)	6,8(,7)
HC25	4615(22)	3368(18)	2128(11)	3,6(,5)

Table 2. Interatomic distances (Å) and angles (°).

DISTANCE	(Å)	DISTANCE	(Å)	DISTANCE	(Å)
C1 - N1	1,474(4)	C1 - C2	1,507(4)	C2 - C3	1,466(4)
C3 - C4	1,473(5)	C4 - N1	1,478(3)	C5 - N1	1,345(3)
C5 - O1	1,218(3)	C5 - C6	1,502(4)	C6 - C7	1,576(3)
C7 - C8	1,521(4)	C7 - C9	1,531(4)	C9 - N2	1,470(3)
N2 - C10	1,462(3)	C10 - C11	1,522(4)	C11 - O2	1,420(4)
C12 - O2	1,420(4)	C12 - C13	1,501(4)	C13 - N2	1,453(3)
C6 - C14	1,538(3)	C14 - C15	1,399(3)	C15 - C16	1,386(4)
C16 - C17	1,378(4)	C17 - C18	1,383(4)	C18 - C19	1,398(4)
C19 - C14	1,395(4)	C6 - C20	1,552(3)	C20 - C21	1,377(3)
C21 - C22	1,384(4)	C22 - C23	1,376(4)	C23 - C24	1,371(4)
C24 - C25	1,382(4)	C25 - C26	1,399(4)		

ANGLE	(°)	ANGLE	(°)
C1 - N1 - C4	110,7(2)	N1 - C1 - C2	104,4(2)
C1 - C2 - C3	106,8(3)	C2 - C3 - C4	108,6(3)
C3 - C4 - N1	105,1(3)	C1 - N1 - C5	119,4(2)
C4 - N1 - C5	129,9(2)	N1 - C5 - O1	120,8(3)
N1 - C5 - C6	119,4(2)	O1 - C5 - C6	120,5(3)
C5 - C6 - C7	108,5(2)	C5 - C6 - C14	110,8(2)
C5 - C6 - C20	104,3(2)	C7 - C6 - C14	108,5(2)
C7 - C6 - C20	106,9(2)	C14 - C6 - C20	117,4(2)
C6 - C7 - C8	114,2(2)	C6 - C7 - C9	114,9(2)
C8 - C7 - C9	108,4(2)	C7 - C9 - N2	109,9(2)
C9 - N2 - C10	111,5(2)	C9 - N2 - C13	113,6(2)
C13 - N2 - C10	107,9(2)	N2 - C10 - C11	109,7(2)
C10 - C11 - O2	110,8(3)	C11 - O2 - C12	109,8(2)
O2 - C12 - C13	111,4(2)	C12 - C13 - N2	109,9(2)
C6 - C14 - C15	119,7(2)	C6 - C14 - C19	123,4(2)
C14 - C15 - C16	121,2(3)	C15 - C16 - C17	121,2(3)
C16 - C17 - C18	118,9(3)	C17 - C18 - C19	120,1(3)
C18 - C19 - C14	121,9(3)	C19 - C14 - C15	116,6(2)
C6 - C20 - C21	126,7(2)	C6 - C20 - C25	116,7(2)
C20 - C21 - C22	121,9(3)	C21 - C22 - C23	120,8(3)
C22 - C23 - C24	119,8(3)	C23 - C24 - C25	119,6(3)
C24 - C25 - C26	122,8(3)	C25 - C26 - C21	116,5(2)

Table 3. Torsional angles (°).

DIHEDRAL ANGLE	(°)	DIHEDRAL ANGLE	(°)
C1 - C2 - C3 - C4	23,8(4)	C1 - N1 - C5 - O1	-2,3(4)
C4 - N1 - C5 - O1	179,1(3)	C1 - N1 - C5 - C6	-179,6(2)
C4 - N1 - C5 - C6	1,7(5)	N1 - C5 - C6 - C14	-61,8(3)
N1 - C5 - C6 - C20	65,4(3)	C5 - C6 - C14 - C15	-28,6(3)
C5 - C6 - C20 - C25	55,7(3)	C15 - C14 - C6 - C7	98,5(3)
C21 - C20 - C6 - C7	121,1(3)	C14 - C6 - C7 - C8	75,4(3)
C20 - C6 - C7 - C8	-52,2(3)	C14 - C6 - C7 - C9	-50,9(3)
C20 - C6 - C7 - C9	-178,4(2)	C6 - C7 - C9 - N2	-159,4(2)
C7 - C9 - N2 - C13	67,8(3)	C7 - C9 - N2 - C13	-158,8(2)
C9 - N2 - C10 - C11	-175,7(2)	C9 - N2 - C13 - C12	177,8(2)
N2 - C10 - C11 - O2	-68,8(3)	N2 - C13 - C12 - O2	58,8(3)
C10 - C11 - O2 - C12	56,6(3)	C11 - O2 - C12 - C13	-58,9(3)

The final parameters are listed in Table 1, where the anisotropic temperature factor is given by

$$\exp -(B11h^2 + B22k^2 + B33l^2 + B12hk + B13hl + B23kl)$$

A complete list of the observed and calculated structure factors may be obtained from the author on request.

DISCUSSION

Interatomic distances and bond angles are listed in Table 2. Table 3 gives some dihedral angles. The e.s.d.'s are calculated from the correlation matrix.

Fig. 1 shows the dextromoramide molecule as seen along the *a*-axis.

The pyrrolidine ring has the *envelope* conformation with C2 being displaced by 0.31 Å out of a plane through the other ring atoms. This is about half the displacement found in moramide bitartrate (0.61 Å),[7] both values being in the range of displacements reported for proline-peptides.[13] A considerable thermal motion of the ring atoms C2 and C3 is probably the main reason for the short C – C single bonds of the ring.

The peptide unit –C6–C5(O1)–N1–(C1, C4) is strictly planar. Suggestions were made upon the influence of intramolecular repulsions being responsible for the slightly non-planar peptide group in the structure of dextromoramide bitartrate.[7] However, the planarity of this group in the present structure indicates that effects from crystal packing may

Table 4. Torsional angles (τ) and absolute configurations at C_α or C_β of the most active enantiomer of some acyclic analgetics.

	Compound	τ (°)	Configuration	Ref.
1.	(+)-Propoxyphene [a]	−176.6	*R* (C_β)	16
2.	(+)-Moramide [b]	−166.5	*R* (C_β)	7
3.	(+)-Propoxyphene	−174.2	*R* (C_β)	17
4.	(+)-Moramide	−159.4	*R* (C_β)	Present work
5.	(−)Isomethadone [a]	−152.5	*S* (C_β)	18
6.	(−)-Methadone	−68.5	*R* (C_α)	4
7.	(−)-Methadone	−69.8	*R* (C_α)	5
8.	(−)-Methadone [c]	−146.3 [d]	*R* (C_α)	6
9.	(+)-Methadol [a]	−116.1 [d]	*R* (C_α) [e]	18
10	(−)-Acetylmethadol [a]	−146.2	*R* (C_α)	18

[a] Hydrochloride. [b] Bitartrate. [c] Hydrobromide.
[d] The sign has been changed to perform a direct comparison with the other values. [e] The configuration of the less active enantiomer.

also be responsible for the non-planarity of the bitartrate salt. The small lengthening of the C5−N1 bond, 1.356(3) Å, as compared to the accepted value (1.325 Å),[14] is in agreement with earlier reported values for similar peptide units.[7,15] The asymmetry of the bond angles around N1 agrees well with that of the bitartrate.

Earlier structure investigations on diphenylpropylamine analgetics have established the significance of non-bonded interactions on bond distances and angles.[4,7,16–18] The molecular distortions at the quaternary carbon atom (C6) (see Table 2) are similar in the present structure. C−C single bonds involving C6 are increased by 0.04 Å as compared to their normal values,[19–20] and the C−C6−C bond angles vary from 104.3(2) to 117.4(2)°.

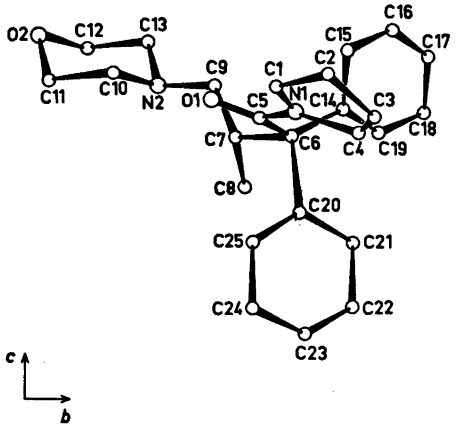


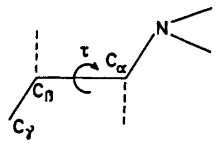
Fig. 1. The dextromoramide molecule with the numbering of the atoms indicated.

C6 is displaced by 0.12 Å and 0.11 Å from the planes of the two phenyl rings, respectively. These planes form an angle of 40.6° which is far below the values found in methadone (80.6°) and moramide bitartrate (55.2°). The rather large angles C14−C6−C20 (117.4°), C6−C14−C19 (123.4°) and C6−C20−C21 (126.7°) are effecting a separation of the two rings to compensate non-bonded interactions between the rings. The hydrogen atoms at C19 (HC19) and C21 (HC21) are only 2.12 Å apart and there is another short intramolecular distance between the hydrogen atoms bonded to C7 and C25 (2.16 Å).

The propylamine chain with the morpholine moiety has the *extended* form, the dihedral angle C6−C7−C9−N2 being −159.4°. This conformation is similar to the one found in the bitartrate, where the corresponding angle is −166.5°. Thus the free base of moramide has a conformation different from that of methadone, which has a "folded" propylamine chain in the solid state.

An interesting conformational feature of the various acyclic analgetics studied by X-ray crystallographic methods so far, is the variation of the torsional angle, τ, as defined below.

This torsional angle defines the position



of the amino group with respect to the quaternary carbon atom C_γ (C6 in the present paper). At present, there are ten crystal analyses available, which are listed in Table 4. The torsional angles refer to the analgetically more potent enantiomer, except for methadol which displays inversion of configurational selectivity at the receptor.[21-22] The compounds may be divided into two distinct classes, those having the *s*-Me group at C_β (Nos. 1–5, the isomethadone series) and the second class comprising those being substituted at the α carbon atom (the methadone series).

The table reveals that the torsional angles may be divided into the same two classes. The variation of τ among the first five compounds is comparatively small (roughly $-165 \pm 12°$) and thus shows the molecules in this class to be more rigid than the others. For the second group, including methadone, the table indicates a lack of conformational similarity. The difference in conformational flexibility of the compounds in the two classes confirms earlier suggestions.[17,23]

The crystal structure of dextromoramide is visualized in Fig. 2 and the crystal packing may be compared to that in the bitartrate. The moramide layers along the *b*-axis in the present structure resemble the chains parallel to the *c*-axis in the latter structure. The difference is mainly an expansion of about 8 Å in the *c* repeat unit of the bitartrate. Roughly,

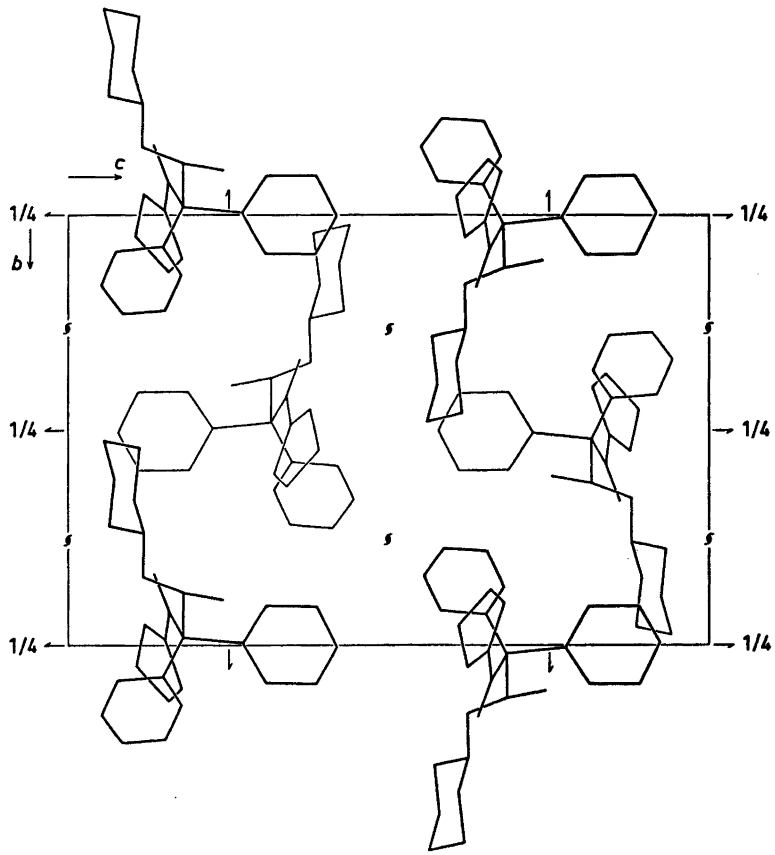


Fig. 2. The crystal structure of dextromoramide as seen along the *a*-axis.

this expansion seems to be the only modification necessary when comparing the structure of the moramide with that of the moramide bitartrate, to satisfy the crystal space requirements of the infinite chains of bitartrate ions along the *a*-axis.

There are no exceptionally short intermolecular distances in the present structure.

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2-Hydroxyselenophenes. I. Tautomeric Properties and Alkylation of the Selenolene-2-one and 5-Methyl-selenolene-2-one Systems

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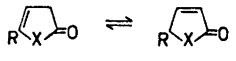
The unsubstituted 2-hydroxyselenophene system was prepared by hydrogen peroxide oxidation of 2-selenopheneboronic acid, while the 5-methyl-substituted system was prepared by acid-catalysed dealkylation of 2-*tert*-butoxyselenophene. Both systems exist mainly as 3-selenolene-2-ones, and for the 5-methyl derivative it was possible to isolate the β,γ-unsaturated form and follow the tautomeric isomerisation. The activation parameters thus obtained are compared with those previously obtained for the corresponding furan and thiophene systems.

Upon alkylation by using an ion-pair extraction method, the 5-methyl-substituted selenolene-2-one system gives mainly *C*-alkylation with the soft acid methyl iodide and mainly *O*-alkylation with the hard acid dimethyl sulfate.

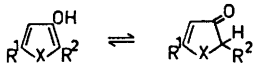
For the hydroxy derivatives of the five-membered heterocycles furan, thiophene and selenophene, it has been shown that the 5-alkyl substituted 2-hydroxy compounds exist in an equilibrium between the two unsaturated γ-lactones A and B [1–3] undergoing carbon-



A B

I X = O
II X = S
III X = Se

carbon tautomerism, while the 2,5-dialkyl substituted 3-hydroxy derivatives exist in a oxo-enol equilibrium (C and D),[4,5] with possibilities for carbon-oxygen tautomerism. For the 5-methyl- and 5-*tert*-butylthiolene-2-one systems (II), the mechanism of tautomerisation has been studied in more detail in methanol

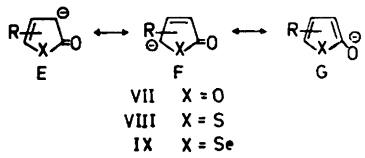


C D

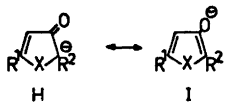
IV X = O
V X = S
VI X = Se

as solvent and by using pyridines as base catalysts. Under these conditions, the isomerisation was found to follow a two-step mechanism of the classical type, obeying both parts of the Hughes-Ingold's rule.[7] In a previous paper,[6] the rates of the tautomeric rearrangement of 5-methyl- and 5-*tert*-butyl-β,γ-butenolide (IA) were compared with those of their sulfur analogues, the thiolene-2-ones (IIA). In benzene solution with triethylamine as base, the butenolides were found to tautomerise at rates three to four powers of ten slower than those of the thiolene-2-ones.[7] In this paper, the study of the influence of the heteroatom on the rates of isomerisation will be extended to that of selenium.

During our current investigation of the hydroxy derivatives of the five-membered heterocycles, we have also studied the reactions of their trident (E–G) and ambident (H,I) anions with alkylating reagents. For these experiments, the ion-pair extraction method has been found to be the superior one, and the alkylating agents used have been methyl iodide (soft) and dimethyl sulfate (hard).[5,8,9] It has been shown that the product distribution is not very sensitive to the counterion, but to the position and size of the substituent [9] and to the alkylating reagent used. Methyl iodide favours *C*-alkylation and dimethyl sulfate *O*-



VII X = O
VIII X = S
IX X = Se



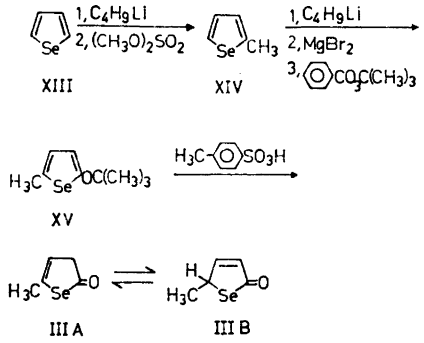
X X = O
XI X = S
XII X = Se

alkylation.[8] However, the butenolide anion (VII E-G) showed no oxygen reactivity, in contrast to the sulfur analogue.[8] In the 3-hydroxy substituted series, on the other hand, even the furan system (X H, I) showed oxygen reactivity, but still the reaction at carbon was the predominant one. In this series, the selectivity was higher for both dimethyl sulfate in *O*-alkylation and methyliodide in *C*-alkylation for the 3-hydroxyselenophene system (XII H, I) compared to the thioselenophene system XI H, I).[5] The influence of the selenium atom in the 2-hydroxy series will be discussed in this paper.

RESULTS AND DISCUSSION

Preparation. The two methods most frequently used for preparation of the hydroxy derivatives of the five-membered heterocycles are oxidative cleavage of boronic acids or esters with hydrogen peroxide, and acid-catalysed dealkylation of *tert*-butoxy derivatives. In our previous work, we have preferred to use the oxidative cleavage method because it is very mild. In the preparation of the 5-alkylsubstituted thiolene-2-ones, for instance, the kinetically controlled β,γ-unsaturated compounds (II A) were isolated. In the furan series, this is really an advantage as these isomers cannot be obtained by alkaline work up.[7] However, this method also has limitations, since the boronic acids, influenced by electron-donating substituents, easily undergo deboronation, and the dealkylation method can therefore be a useful complement.

The unsubstituted, as well as the 5-methylselenolene-2-one systems (III A – B) have been prepared by Morel *et al.*[3] by the acid-catalysed dealkylation of the 2-*tert*-butoxy derivatives according to a procedure developed by Lawesson and coworkers.[10] When we used the oxidative cleavage method for the preparation of these compounds, only the unsubstituted compound was obtained and the yield was rather low (31 %). In the 5-methylsubstituted case, the deboronation became the main reaction. However, by repeating the reaction route of Morel *et al. via* 5-methyl-2-*tert*-butoxythiophene (XV) the 5-methylselenolene-2-one



system was obtained in acceptable yield (61 %), but under these conditions a side reaction also took place. Besides the described 5-methyl selenolene-2-one system a considerable amount of 3-*tert*-butyl-5-methyl-3-selenolene-2-one was obtained. This side reaction was not observed by Lawesson *et al.* in the thiophene series [10] or by Morel *et al.* in their preparation of the 5-methylselenolene-2-one system.[3] However, when we repeated these reactions we found transalkylation in both cases. Alkylation of 3-thiolene-2-one with isobutylene under the same conditions also gives 3-alkylated products. Under the preparative conditions 3- and 5-alkylated and 3,5-dialkylated products are formed in 17, 1 and 2 % yield, respectively.

Indirectly, both the unsubstituted and the 5-methylsubstituted selenolene-2-one systems show enolic properties, as they could be transformed to the acetoxy derivatives when treated with acetyl chloride. At equilibrium the 3-selenolene-2-one form (III B) dominates in both systems. Due to hyperconjugation of the methyl substituent, 7 % of the 4-selenolene-2-one form (III A) could be observed for the 5-methyl-substituted derivative in benzene

solution. In the 3-*tert*-butyl-5-methyl-substituted compound, this effect was over-compensated by the stabilization of the *tert*-butyl group with the α,β-double bond, as only the 3-selenolene-2-one form (XVII B) could be observed.

Under the rather vigorous conditions used for the preparation of the selenolene-2-one system, the thermodynamic equilibrium mixture is obtained. However, the thermodynamically more stable α,β-conjugated form (III B) can be converted into the β,γ-unsaturated form (III A) by dissolving the tautomeric mixture in cold alkali and acidifying. This is in agreement with Hughes-Ingold's rule, which states that when a proton is added to the mesomeric anion (IX E – G) of a weakly dissociating tautomer, the thermodynamically less stable tautomer (III A) is first formed. It is interesting to note that in the thiophene series the anion could be generated by extraction with sodium hydroxide solution, while in the selenophene series we were only successful by using tetrabutylammonium hydroxide (*cf.* Experimental part). The more unstable enol form, obtained by protonation of IX G, could, however, not be isolated, which is also the case in the thiophene series.

Tautomeric rearrangement. Since the ^1H absorption bands of the ring and the side-chain protons have different δ-values (*cf.* Tables 6, 8) for the two tautomeric forms of 5-methylselenolene-2-one, NMR could be used as an analytical tool for the kinetic experiments. When benzene was used as solvent and pyridine as base, first-order kinetics with half-lives suitable for this type of NMR determination were obtained for both 5-methyl-4-selenolene-2-one and 5-methyl-4-thiolene-2-one. Another solvent-base system, methanol-pyridine, which was suitable for the study of thiolene-2-ones, caused ring-opening leading to methyl levulinate and loss of selenium in the case of 5-methyl-4-selenolene-2-one.

Table 1. Values for $k_A + k_B$ in benzene solution.

Temp. °C	Pyridine conc. M	$(k_A+k_B)\times 10^4$ (min^{-1})	r
5-Methylthiolene-2-one			
20	0.60	2.28 ± 0.04	0.998
30	0.60	5.2 ± 0.1	0.996
40	0.60	9.9 ± 0.2	0.998
40	0.48	7.1 ± 0.1	0.998
40	0.24	3.18 ± 0.06	0.998
50	0.60	19.8 ± 0.3	0.999
5-Methylselenolene-2-one			
20	0.60	4.7 ± 0.1	0.997
30	0.60	10.3 ± 0.2	0.998
40	0.60	22.7 ± 0.4	0.998
40	0.48	16.1 ± 0.3	0.998
40	0.24	7.0 ± 0.1	0.999
50	0.60	38.6 ± 0.8	0.998

Table 2. Equilibrium constants and specific rate constants at different base concentrations in benzene solution at 40 °C.

K	Pyridine conc. M	$k_A\times 10^4$ (min^{-1})	$k_A\times 10^4$ (min^{-1})
5-Methylthiolene-2-one			
4.1 ± 0.2	0.60	7.9 ± 0.1	2.0 ± 0.1
4.1 ± 0.2	0.48	5.7 ± 0.1	1.39 ± 0.09
4.1 ± 0.2	0.24	2.56 ± 0.05	0.62 ± 0.04
5-Methylselenolene-2-one			
14.5 ± 0.7	0.60	21.2 ± 0.4	1.5 ± 0.1
14.7 ± 0.7	0.48	15.1 ± 0.3	1.03 ± 0.07
14.4 ± 0.7	0.24	6.5 ± 0.1	0.45 ± 0.03

The rearrangement was performed at three different base concentrations, 0.60, 0.48 and 0.24 M, and four temperatures, 20, 30, 40 and 50 °C. The experimental results are collected in Table 1. The specific rates of isomerisations were found to be proportional to the base concentration (Table 2), showing that the rearrangement is first-order with respect to the base. The results in all the isomerisation experiments satisfied eqn. (1), corresponding to

$$\ln \frac{AK - B}{A_0K - B_0} = -(k_A + k_B)t \qquad (1)$$

a reversible process (eqn. 2).

$$A \underset{k_B}{\overset{k_A}{\rightleftharpoons}} B \qquad (2)$$

The least-squares method was used for obtaining the best fit of the experimental results to eqn. (1). The algebraically calculated values for (k_A+k_B) and their standard deviations are given in Table 1. Since the quotient k_A/k_B can

Table 3. Equilibrium constants and specific rate constants at different temperatures in benzene solution. (Base conc. 0.60 M.)

Temp. °C	K	$k_A \times 10^4$ (min^{-1})	$k_B \times 10^4$ (min^{-1})
5-Methylthiolene-2-one			
20	4.4 ± 0.2	1.86 ± 0.03	0.42 ± 0.03
30	4.3 ± 0.2	4.2 ± 0.1	0.98 ± 0.07
40	4.1 ± 0.2	7.9 ± 0.1	2.0 ± 0.1
50	3.9 ± 0.2	15.8 ± 0.2	4.0 ± 0.3
5-Methylselenolene-2-one			
20	15.7 ± 0.8	4.4 ± 0.1	0.28 ± 0.02
30	15.6 ± 0.8	9.7 ± 0.2	0.62 ± 0.05
40	14.5 ± 0.7	21.2 ± 0.4	1.5 ± 0.1
50	14.1 ± 0.7	36.0 ± 0.8	2.6 ± 0.2

Table 4. Enthalpies, free energies and entropies of activation in benzene solution at 25 °C.

	5-Methyl-selenolene-2-one	5-Methyl-thiolene 2-one
Assoc. with k_A		
$\Delta H^{\ddagger}$ (kJ mol^{-1})	54 ± 2	53 ± 1
$\Delta G^{\ddagger}$ (kJ mol^{-1})	102 ± 5	103 ± 2
$\Delta S^{\ddagger}$ (J K^{-1} mol^{-1})	−161 ± 7	−169 ± 3
Assoc. with k_B		
$\Delta H^{\ddagger}$ (kJ mol^{-1})	57 ± 3	56 ± 1
$\Delta G^{\ddagger}$ (kJ mol^{-1})	108 ± 5	107 ± 2
$\Delta S^{\ddagger}$ (J K^{-1} mol^{-1})	−172 ± 8	−171 ± 3

be derived from the composition of the equilibrium mixture, it is possible thereby also to determine the individual rate constants at different base concentrations and temperatures. These data are given in Tables 2 and 3.

The activation parameters are determined in accordance to Frost and Pearson [11] and are collected in Table 4.

The equilibrium constants at different temperatures were found to satisfy function (3).

$$\ln K = -(\Delta H/RT) + C \quad (3)$$

The heat of reaction, ΔH, was determined by the method of least-squares and the thermodynamic constants were found to be $\Delta H = -3.6 \pm 0.5$ kJ mol^{-1}, $\Delta G = -7 \pm 1$ kJ mol^{-1} and $\Delta S = 11 \pm 2$ J K^{-1} mol^{-1} for 5-methylselenolene-2-one at 25 °C and $\Delta H = -3.2 \pm 0.2$ kJ mol^{-1}, $\Delta G = -3.6 \pm 0.5$ kJ mol^{-1} and $\Delta S = 1.5 \pm 0.3$ J K^{-1} mol^{-1} for 5-methylthiolene-2-one at 25 °C. These values are in good agreement with those obtained as differences of the activation parameters in Table 4.

In order to obtain information about the nature of the mechanism of this rearrangement, a benzene solution, 5 M in CD_3OD, 0.62 M in lactone and 0.6 M in pyridine was examined. As in the cases of 5-methyl-4-thiolene-2-one [12] and of 5-methyl-β,γ-butenolide,[7] it was found that the unconjugated isomer of 5-methylselenolene-2-one undergoes hydrogen-deuterium exchange more rapidly than the conjugated form. Furthermore, exchange of the methylenic protons for deuterium was complete before any tautomerisation of the unconjugated form or ring-opening of the lactone was observed. Since the hydrogen exchange in the 3-position is more rapid than isomerisation, nothing can be said about the degree of the intramolecular mechanism for the rearrangement except that the 5-proton is involved in the rate-determining step. During the last decade it has been shown that besides the intermolecular mechanism, there exists a competing mechanism which under certain conditions becomes predominant. In this mechanism the proton-transfer is intramolecular and involves ion pairs as intermediates. Bergson and Weidler [13] and Cram *et al.*,[14] the pioneers in this field, elegantly established this mechanism for tautomerisation in a series of investigations.[15]

From Table 3 it is seen that the rate for replacement of the proton in the 5-position of

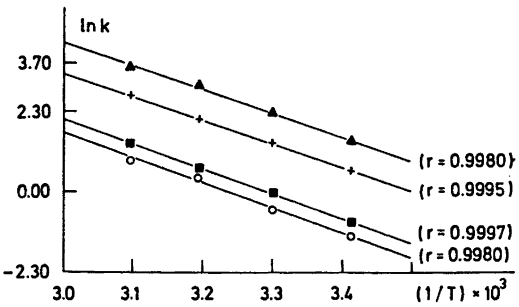


Fig. 1 Arrhenius plots for 5-methyl-thiolene-2-one and 5-methyl-selenolene-2-one in benzene solution ▲ = k_A^{Se}; + = k_A^{S}; ■ = k_B^{S}; and ○ = k_B^{Se} (*cf.* Table 3).

the selenophene system is more rapid than that of the thiophene system and that the rate for deprotonation is somewhat slower in the selenophene system than in the thiophene system.

The activation enthalpies for the replacement of the proton in the 5-position are the same in both systems, as shown by the fact that ln k_A^S parallels ln k_A^{Se} in Fig. 1, and thus the difference in the free energy is due to a contribution from the activation entropy term, which is somewhat lower in the selenophene system (*cf.* Table 4). The similarity between the activation parameters for the proton abstraction step is reflected in Fig. 1, where the lines for ln k_B^S and ln k_B^{Se} are parallel and very close to each other.

Alkylation reactions. The results of the alkylation experiments of the 5-methyl-2-hydroxyselenophene system with the ion pair extraction method using methyl iodide and dimethyl sulfate as alkylating reagents are collected in Table 5. For convenience, the results obtained under the same conditions for the 5-methyl-2-hydroxythiophene system [8] are also presented. Again it is demonstrated that the soft methyl iodide favours *C*-alkylation and the hard dimethyl sulfate *O*-alkylation. However, the selectivity of the mono alkylated selenophene system is lower than that of the thiophene system, which can be seen from the amount of dialkylated products formed. This increase in reactivity may be due to a higher lipophilicity of the selenophene system or to the lower stability of the anion in the selenophene system compared to the thiophene system. So far only the dissociation constants of the latter system are known, and the CH acidity was found to be of the same magnitude as the OH acidity of the corresponding phenols.[16] From the data in Table 5 it is interesting to note that the tendency for *O*-alkylation is somewhat more pronounced in the thiophene system than in the selenophene system, indicating that the former system is the more aromatic.

The ^{1}H NMR data for the starting materials characterized as acetoxy derivatives and the

Table 5. Alkylation of the 5-methyl-2-hydroxyselenophene and 5-methyl-2-hydroxythiophene system with methyl iodide and dimethyl sulfate.

1, $R^1=R^2=H$ 3, $R^1=H$
2, $R^1=H$: $R^2=CH_3$ 5, $R^1=CH_3$
4, $R^1=R^2=CH_3$

Substrate system	5-Methyl-2-hydroxyselenophene		5-Methyl-2-hydroxythiophene	
Reagent	CH_3I	$(CH_3O)_2SO_2$	CH_3I[a]	$(CH_3O)_2SO_2$[a]
Yield (%)	37	44	53	89
Products (relative yields, %)				
X	Se	Se	S	S
1	5	–	–	–
2	38	6	86	4
3	4	76	12	90
4	50	3	2	2
5	3	15	–	4

[a] After addition of $[Eu(FOD)_6]$.

Table 6. IR and ^{1}H NMR data for 3-R^1-5-R^2-3-selenolene-2-ones. The NMR parameters were obtained in deuteriochloroform solutions at 60 MHz.

R^1	R^2	δ				J				C=O
		R^1	R^2	4-H	5-H	4-R^1 (Hz)	5-R^1 (Hz)	5-R^2 (Hz)	45 (Hz)	cm^{-1} film
H	H	6.31	–	7.66	4.40	6.4	2.0	–	2.9	1700
H	CH_3	6.20	1.75	7.31	4.81	6.4	2.0	7.8	2.7	1690
CH_3	CH_3	1.91	1.72	7.03	4.65	1.4	2.0	7.4	2.8	1680
$C(CH_3)_3$	CH_3	1.27	1.75	7.07	4.58	–	–	7.4	2.7	1685

Table 7. ^{1}H NMR data for 2-R^1-3R^2-5-R^3-2-oxyselenophenes. The NMR parameters were obtained in deuteriochloroform solutions at 60 MHz.

R^1	R^2	R^3	δ				J			
			R^1	R^2	R^3	4-H	4-CH_3 (Hz)	34 (Hz)	45 (Hz)	35 (Hz)
OCH_3	H	CH_3	3.86	5.98	2.41	6.43	1.3	4.0	–	–
OCH_3	CH_3	CH_3	3.84	1.98	2.44	6.42	1.3	–	–	–
$OC(CH_3)_3$	H	CH_3	1.36	6.21	2.44	6.47	1.3	3.9	–	–
O_2CCH_3	H	H	2.18	6.74	7.48	6.96	–	4.1	6.2	1.5
O_2CCH_3	H	CH_3	2.14	6.54	2.39	6.54	1.3*[a]	4.1*[a]	–	–

Taken from Ref. 8.

Table 8. IR and ^{1}H NMR data for 3,3-R^1-5-methyl-selenolene-2-ones. The NMR parameters were obtained in deuteriochloroform solutions at 60 MHz.

R^1	δ			J			C=O (cm^{-1}) film
	R^1	CH_3	4-H	4-CH_3 (Hz)	3-CH_3 (Hz)	34 (Hz)	
H	3.52	2.27	5.55	1.70	2.50	2.70	1725
CH_3	1.21	2.22	5.48	1.70	–	–	1715

products isolated from the alkylation experiments are collected in Tables 6 – 8.

EXPERIMENTAL

The 3-selenolene-2-one system. Butyllithium in hexane (33 ml; 1.66 M) was added dropwise to 6.55 g (0.050 mol) of selenophene [17,18] in 150 ml of dry ether with stirring under nitrogen. The addition was made at such a rate that gentle reflux was maintained. When the addition was complete, refluxing and stirring were continued for another 15 min, whereupon the temperature was decreased to −70 °C and the selenienyllithium was treated with 7.3 g (0.050 mol) of triethylborate in 100 ml of dry ether. After stirring at −70 °C for 4 h, the reaction mixture was allowed to warm to room temperature and then treated dropwise with 10.1 ml of 30 % hydrogen peroxide solution. After complete addition, the reaction mixture was refluxed for 1 h with vigorous stirring. The reaction mixture was cooled, the layers separated and the aqueous phase extracted with ether. The combined ethereal phases were washed with cold water until the separating water did not oxidize ferrous ammonium sulfate, dried over magnesium sulfate and distilled. 2.3 g (31 %) of the product, b.p. 96 °C/9 mmHg, $n_D^{20} = 1.5955$ (lit. value [3] b.p. 121 °C/20 mmHg), was obtained. For IR and NMR data see Table 6.

2-Acetoxyselenophene (procedure A). 11.4 g (0.0776 mol) of the 3-selenolene-2-one system was treated with an excess of acetyl chloride.

The reaction mixture was refluxed and the reaction followed by gas chromatography (Reoplex 400, 5 %, 3 m × 3 mm). After 45 h all of the starting material had disappeared. The excess of acetyl chloride was evaporated and the residue dissolved in ether. The ether solution was washed with 2 M sodium hydroxide and then with water until neutral reaction, whereupon it was dried over magnesium sulfate. The ether was removed and the residue distilled, giving 8.3 g (57 %) of the product, b.p. 62 – 64 °C/0.8 mmHg, $n_D^{20} = 1.5575$. IR spectrum (film): C=O = 1755 cm^{-1}. For NMR data see Table 7. [Found: C 38.19; H 3.24; Se 41.10; M.wt. 190. Calc. for $C_6H_6O_2Se$: C 38.11; H 3.19; Se 41.76; M.wt. 189.07.]

*5-Methyl-2-*tert*-butoxyselenophene* was prepared according to Ref. 10 from 34.2 g (0.236 mol) of 2-methylselenophene,[5] 162 ml of 1.48 N butyllithium in hexane, 7.5 g (0.31 mol) of magnesium, 13 ml (0.24 mol) of bromine and 39 ml (0.19 mol) of *tert*-butyl perbenzoate. Distillation gave 33.1 g (80 %) of the product, b.p. 61 – 62 °C/1.5 mmHg, $n_D^{20} = 1.5195$. (Lit. value[3] b.p. 58 °C/1.3 mmHg.) For NMR data see Table 7.

The 5-methyl-2-hydroxyselenophene system. 5-Methyl-2-*tert*-butoxyselenophene (16.0 g; 0.073 mol) and 0.1 g of *p*-toluenesulfonic acid were placed in a distillation flask while a vigorous stream of nitrogen was passed through the reaction mixture, the flask was heated with an oil bath at 155 °C, whereupon the reaction started with isobutylene evolution. After 5 min, the gas evolution had ceased and the oil bath was removed. The residue was distilled under nitrogen at reduced pressure, giving 11.00 g of a fraction with b.p. 85 – 89 °C/5.0 mmHg containing 82 % of the 5-methyl-2-hydroxyselenophene system and 18 % of the 5-methyl-3-*tert*-butyl-2-hydroxyselenophene system. Redistillation gave, after a forerun containing both 5-methyl-3- and 4-selenolene-2-one, a fraction containing 7.2 g (61 %) of 5-methyl-3-selenolene-2-one, b.p. 93 °C/10 mmHg, $n_D^{20} = 1.5789$. For IR and NMR data, see Table 6. The second fraction (2.9 g) contained comparable amounts of the 5-methyl-2-hydroxyselenophene and 5-methyl-3-*tert*-butyl-2-hydroxyselenophene systems, b.p. 95 – – 103 °C/10 mmHg.

*5-Methyl-3-*tert*-butyl-3-selenolene-2-one* was obtained pure by preparative gas chromatography (OV 17, 15 %, 2.7 m × 9 mm) of the second fraction described above, $n_D^{20} = 1.5358$. For IR and NMR data, see Table 6. [Found: C 49.76; H 6.68; Se 35.40; M.wt. 218. Calc. for $C_9H_{14}OSe$: C 49.77; H 6.50; Se 36.46; M.wt. 217.17.]

Alkylation of 3-thiolene-2-one with isobutene. 3-Thiolene-2-one (2.0 g; 0.020 mol) and 0.05 g of *p*-toluenesulfonic acid were mixed in a distillation flask. The mixture was heated to 150 – 155 °C and isobutene was bubbled in. The reaction was followed on GLC (OV 17, 3 %, 3 m × 3 mm). No starting material remained after 90 min.

The alkylated products were distilled, giving 0.61 g, b.p. 65 – 80 °C/0.8 mmHg, of a mixture of 3-*tert*-butyl-3-thiolene-2-one (17 %), 5-*tert*-butyl-3-thiolene-2-one (1 %) and 3,5-di-*tert*-butyl-3-thiolene-2-one (2 %). [NMR, GLC.]

The mixture was eluted on silica gel 60 with chloroform and hexane (1:1) as solvent. The elution was followed on GLC.

*3-*tert*-Butyl-3-thiolene-2-one* and *5-*tert*-butyl-3-thiolene-2-one* had the same spectroscopic data as given in the literature.[2,9]

*3,5-Di-*tert*-butyl-3-thiolene-2-one:* IR (film) C=O = 1685 cm^{-1}. NMR ($CDCl_3$): δ 1.24 (3*t*-Bu), 1.03 (5*t*-Bu), 7.04 (4-H), 4.04 (5-H, J_{45} 2.4 Hz. MS: $M^+ - 56$.

5-Methyl-2-acetoxyselenophene was prepared according to procedure A from 1.62 g (0.010 mol) of the 5-methyl-2-hydroxyselenophene system. After 8 h, the reaction was over and distillation gave 1.3 g (63 %) of the product, b.p. 56 – 57 °C/0.6 mmHg. Recrystallization from ethanol gave white crystals, m.p. 34.0 – – 34.5 °C. IR (KBr): C=O = 1755 cm^{-1}. For NMR data, see Table 7. [Found: C 41.43; H 3.93; Se 38.81; M.wt. 204 Calc. for $C_7H_8O_2Se$: C 41.40; H 3.97; Se 38.87; M.wt. 203.10.]

5-Methyl-4-selenolene-2-one. After isomerisation, the pure 4-selenolene form was obtained by dissolving 5.88 g (0.037 mol) of the tautomeric mixture in cold ether and extracting the ethereal phase three times with a cold solution of 12.5 g (0.037 mol) of tetrabutylammonium hydrogen sulfate, 3.0 g (0.075 mol) of sodium hydroxide and 25 ml of water. Immediately after separation, the alkaline phase was poured into an ice-cooled 1 M sulfuric acid solution covered with ether. The ether phase was separated and the water phase extracted twice with ether. The combined ether phases were washed with water until neutral reaction of the separating water was obtained, dried over magnesium sulfate and distilled; b.p. 75 – 76 °C/9 mmHg, $n_D^{20} = 1.5706$. The yield of this procedure was 1.84 g (31.3 %). For IR and NMR data, see Table 8.

5-Methyl-4-thiolene-2-one was prepared as described in Ref. 2.

Alkylation of the 5-methyl-2-hydroxyselenophene system with methyl iodide. A freshly prepared solution of 7.5 g (0.022 mol) of tetrabutylammonium hydrogen sulfate, 1.80 g (0.044 mol) of sodium hydroxide and 25 ml of water was added dropwise to 3.6 g (0.022 mol) of the 5-methyl-2-hydroxyselenophene system and 6.4 g (0.045 mol) to methyl iodide in 25 ml of chloroform. During the addition, the temperature was kept at 30 °C and after the addition was complete the stirring was continued for another 10 min. The reaction mixture was acidified with 2 M hydrochloric acid, the phases were separated and the water phase was extracted with chloroform. The combined chloroform phases were evaporated,

and when the residue was treated with ether, tetrabutylammonium iodide precipitated. The solid phase was filtered off and washed with ether, whereupon the filtrate was washed with water, dried over magnesium sulfate and evaporated. The residue was distilled and the fraction with b.p. 75 – 102 °C/10 mmHg was collected. The crude product and the distilled fraction were analysed by gas chromatography (OV 17, 3 %, 3 m × 3 mm) and shown to have the same proportions of the different products, which are given in Table 5. After separation by preparative gas chromatography (OV 17, 15 % and BDS, 20 %, 2.7 m × 9 mm), the structures of the products were determined by IR, NMR and mass spectroscopy. The IR and NMR data are given in Tables 6 – 8.

3,5-Dimethyl-3-selenolene-2-one. $n_D^{20} = 1.5630$.

5-Methyl-2-methoxyselenophene. $n_D^{20} = 1.5543$. [Found: C 41.06; H 4.54; Se 44.81; M.wt. 176. Calc. for C_6H_8OSe: C 41.15; H 4.60; Se 45.09; M.wt. 175.09.]

3,3-Dimethyl-5-methyl-4-selenolene-2-one. $n_D^{20} = 1.5285$.

3,5-Dimethyl-2-methoxyselenophene. $n_D^{20} =$ $= 1.5517$. [Found: C 44.16; H 5.40; Se 41.63; M.wt. 189. Calc. for $C_7H_{10}OSe$: C 44.45; H 5.33; Se 41.75; M.wt. 189.12.] The IR and NMR data are given in Tables 6 – 8.

Alkylation of the 5-methyl-2-hydroxyselenophene system with dimethyl sulfate was performed as decribed above by using 2.7 g (0.021 mol) of dimethyl sulfate instead of methyl iodide. Distillation gave 1.6 g (44 %) of a fraction with b.p. 70 – 85 °C/10 mmHg. The proportions of the four components formed are given in Table 5. After separation of the products by preparative gas chromatography, the retention times and spectroscopic data were found to be in accordance with those described above.

The NMR spectra for the kinetic experiments were obtained with a Jeol MH 100 high resolution spectrometer equipped with a vt-3c variable temperature accessory and integration unit. During the rearrangement experiments, the samples were kept in thermostats at different temperatures, and the time required for the integrations was neglected.

The tautomerisation was followed by observing the increase in intensity of the absorption band of the component formed, and the decrease in intensity of the band due to the component consumed. The intensities were determined through integration of the side-chain methyl absorptions. In the runs performed, the sums of the integrals of the increasing and decreasing bands were constant within the limit of accuracy of the instrument, which shows that no side reactions had taken place during the time reaction kinetics were studied.

The numerical treatment of the rate data was carried out on a Hewlett-Packard 9820A desk calculator equipped with a 9832A calculator plotter.

The other NMR spectra were obtained with a Varian A-60 spectrometer. The IR spectra were recorded on a Perkin-Elmer Model 257 instrument. The mass spectra were recorded on an LKB 9000 mass spectrometer, using an ion-source voltage of 70 eV. The gas chromatographs used were a Perkin-Elmer 900 analytical instrument and a Perkin-Elmer F21 preparative instrument. The quantitative analyses were corrected for the differences in the sensitivity of the detector for the different compounds.

The elemental analyses were carried out by Dornis and Kolbe, Mikroanalytisches Laboratorium, Mülheim/Ruhr.

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Algal Carotenoids. XIV.* Structural Studies on Peridinin. Part 1. Structure Elucidation

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The characteristic pigment of the dinoflagellates, peridinin, has been studied as a joint project in our four laboratories. Based on detailed spectroscopic and chemical evidence peridinin has been assigned the structure 5′,6′-epoxy-3,5,3′-trihydroxy-6,7-didehydro-5,6,5′,6′-tetrahydro-12,13,20-trinor-β,β-caroten-19′,11′-olide 3-acetate (*1*).

Dinoflagellates (class, Dinophyceae; division, Pyrrophyta) together with the diatoms are the major producers of organic matter in the sea.[1] These planktonic algae are the most frequent cause of "red tide", natural blooms in which the cells are sufficiently abundant to confer a distinct reddish tinge to the water.[2] The dinoflagellate chloroplast is typically brown in colour and contains relatively large amounts of carotenoid pigments. The major one, peridinin, constitutes 70 – 80 % of the total [3] and functions as an auxiliary light harvesting pigment for photosynthesis.[4,5]

Peridinin was first isolated and named by Schütt in 1890.[6] The source of this crude preparation was a harvest of mixed marine dinoflagellates, principally *Ceratium tripos*, and its name was derived from Peridineen (peridinians), a collective term for the dinoflagellates. Peridinin, as a homogeneous pigment, has since been isolated from several different dinoflagellate sources, [7–10] and it is now recognized as an important taxonomic marker for this class of algae.[11]

A pigment obtained from the sea anemone *Anemonia sulcata*, and named sulcatoxanthin,[12] has since been shown to be identical to peridinin and the actual source of the pigment to be the endozoic dinoflagellate symbionts, zooxanthellae, of the sea anemone.[13] Peridinin has also been obtained from other marine sources, namely the zooxanthellae of various corals, clams [3] and sea anemones.[13]

Together with fucoxanthin (*2*), the major xanthophyll of brown algae and diatoms,[8] peridinin is one of the dominant carotenoid pigments in Nature.

Previous investigations of peridinin have disclosed some of its physical and chemical properties. Thus combustion analysis indicated a molecular composition of $C_{40}H_{52}O_8$ (Mw = 660),[12] whereas high resolution mass spectrometry [14] gave a composition $C_{39}H_{50}O_7$ (Mw = 630) for the highest observable ion.

The visible light absorption spectrum exhibited pronounced fine-structure in non-polar solvent (λ_{max} 454, 484 nm, hexane)[10] but a broad round-shaped spectrum in polar solvents (λ_{max} 472 nm, ethanol, E(1 cm, 1 %) = 1325).[3] This dramatic loss of fine-structure is somewhat unusual and the observed extinction coefficient is exceptionally low.

In the presence of alkali peridinin is rapidly decolourized [10,13] and reduction with $LiAlH_4$ gives a mixture of products which have essentially pentaene chromophores.[10]

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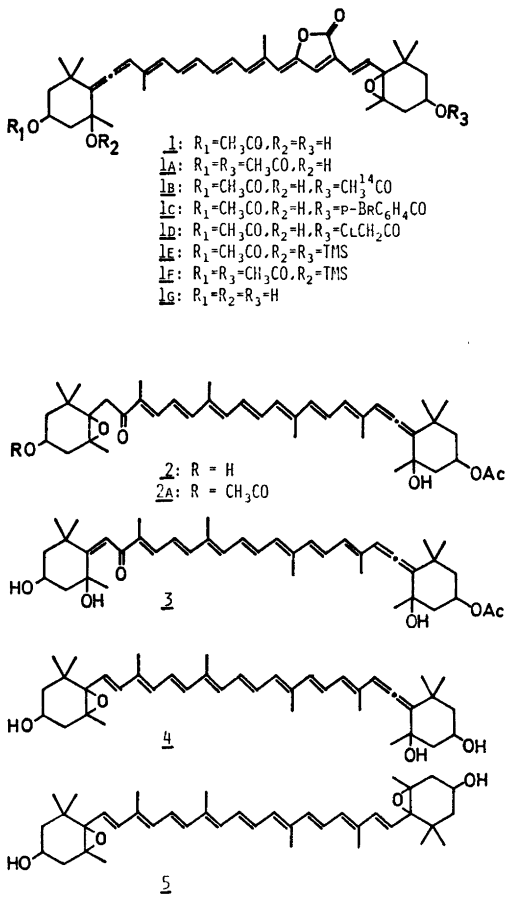
With acid peridinin is slowly transformed, giving products with visible light absorption maxima shifted hypsochromically by 20 nm,[10] suggesting a 5,6-epoxide to 5,8-furanoxide rearrangement.[10,15]

The present co-operative investigation in our four laboratories led to a proposed structure for peridinin (*1*), communicated briefly in a priority note.[16] More detailed evidence is now presented.

The main cultivations were carried out at La Jolla; isolation and physical characterization at Argonne, Berkeley and La Jolla; chemical and physical studies at Berkeley and Trondheim.

For convenience these studies will be presented in two separate parts. The present paper contains the necessary data for the structure elucidation, whereas the second part [17] gives supporting evidence in terms of the proposed structure.



Scheme 1.

RESULTS AND DISCUSSION

Peridinin (*1*) was obtained from several different sources, namely from a natural bloom containing more than 95 % *Gonyaulax polyedra*, from the zooxanthellae of the Pacific coast sea anemone *Anthopleura* (previously *Bunodactis*) *xanthogrammica* and from unialgal cultures of *Cachonina niei*.

The isolation involved extraction of the fresh, frozen, or freeze-dried algal material with acetone or methanol and chromatography on sucrose columns or reversed phase chromatography on polyethylene columns, followed by further purification on calcium carbonate, alumina or silica. Homogeneous fractions were then crystallized from suitable solvents.

Peridinin (*1*, Scheme 1) was obtained as purple crystals of m.p. 128 – 132 °C from ether-hexane. Analysis of a carefully purified sample gave C = 73.15 % and H = 7.68 % fitting best for $C_{41}H_{52}O_8$ (Mw = 672), requiring C = 73.18 % and H = 7.79 %. The high resolution mass spectrum on the other hand had the highest observable ion at *m/e* 630.3553 corresponding to $C_{39}H_{50}O_7$ (calc. 630.3555). These results differ by C_2H_2O which could be an element of ketene. This raises some doubt as to whether the molecular ion is observed on electron impact. However, loss of ketene from acetates is usually not favoured over the loss of acetic acid.[18,19]

Attempts to distinguish between the two alternatives by measuring the specific activity of peridinin ^{14}C-acetate (*1b*), prepared by acetylation with ^{14}C-acetic anhydride of known activity, were unsuccessful.

The presence of at least one secondary acetoxy function follows from the IR absorptions at 1745 and 1250 cm^{-1}, a methyl singlet at δ 2.01 and a methine multiplet at δ 5.4 in the ^{1}H NMR spectrum and hydrolysis to peridininol (*1g*).

Attempts to determine the number of acetate functions by acid hydrolysis of peridinin ^{14}C-acetate (*1b*) and determination of the specific activity of the liberated acetic acid as acetanilide did not give reliable results.

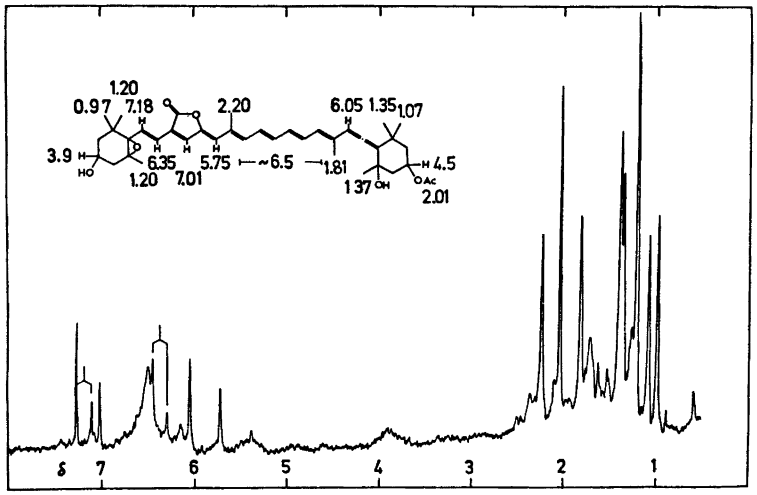


Fig. 1. ^{1}H NMR spectrum (100 MHz) of peridinin (*1*) in $CDCl_3$ solution.

Unspecific determination of ester functions by colourimetry of the hydroxamic acid-ferric ion complex gave 1.7 ester functions per molecule of peridinin. Under the same conditions fucoxanthin (*2*) and fucoxanthin acetate (*2a*) gave 0.9 and 1.7 ester groups, respectively. Moreover, the ^{13}C NMR spectrum of peridinin *p*-bromobenzoate (*1c*, Fig. 2) exhibited three signals at -42.0, -40.1 and -36.2 ppm relative to benzene, in the region where ester and lactone carbonyls normally resonate.[20]

In an attempt to determine the carbon skeleton, peridinin was hydrogenated, the reaction mixture reduced with KBH_4, and the products converted to the hydrocarbons by the method of Cope *et al.*[21] Mass spectrometry gave the highest molecular ion at *m/e* 510, corresponding to $C_{37}H_{66}$, suggesting that peridinin has a C_{37}-skeleton with five rings. However, similar treatment of fucoxanthin (*2*) gave a compound with the molecular ion at 6 mass units lower than that expected for the fully saturated compound, thus allowing no conclusions as to the number of rings in peridinin.

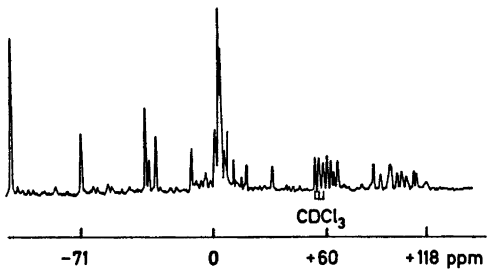


Fig. 2. ^{13}C NMR spectrum of peridinin *p*-bromobenzoate (*1c*) in $CDCl_3$.

Since all derivatives prepared from peridinin gave presumed molecular ions consistent with *m/e* 630 being the molecular ion for peridinin, it may now be concluded that peridinin has a C_{37}-skeleton, is a natural monoacetate ($C_{39}H_{50}O_7$), and in addition contains an unknown ester function.

Examination of the peridinin ^{1}H NMR spectrum (Fig. 1) shows that only nine methyl groups are present, six of which are in saturated environments. Of the remaining three, one is at an unusually low field (δ 2.20) compatible with a methyl ketone or an aromatic methyl.[22] However, no support for these assignments is found in the ^{13}C NMR spectrum of peridinin *p*-bromobenzoate (*1c*, Fig. 2)[20] or in the IR spectrum (Fig. 3) of peridinin itself.

Under standard conditions peridinin readily forms a monoacetate (*1a*) a di-TMS(=trimethylsilyl) ether (*1e*) and a monoacetate-mono-TMS ether (*1f*) as well as a chloroacetate (*1d*) and a *p*-bromobenzoate (*1c*). This suggests that peridinin has two free hydroxy functions. One of these is secondary as supported by a one proton multiplet at δ 3.9 and the other is tertiary.

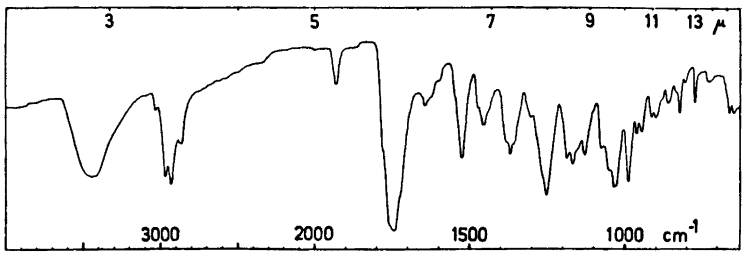


Fig. 3. IR spectrum (KBr) of peridinin (*1*).

The IR spectra of peridinin (Fig. 3) and its derivatives show a medium strength, characteristic absorption at 1930 cm^{-1} indicating that peridinin is an allenic compound.[19] Supporting evidence is found in the ^{13}C NMR spectrum of the *p*-bromobenzoate (*1c*) which has a signal at −74.6 ppm relative to benzene, assigned to the central allenic carbon,[23] and also in the mass spectrum of peridinin itself where an abundant hydrocarbon fragment ion at *m/e* 197.1325 ($C_{15}H_{17}$) is observed. Similar fragments (by composition) are observed in the mass spectra of both fucoxanthin (*2*) [24] and isofucoxanthin (*3*),[25] where they are most likely derived from the allenic end groups.

The ^1H NMR spectrum of peridinin exhibits signals which coincide with all the signals assigned to the allenic end group A of fucoxanthin (*2*).[24] These include the acetate at δ 2.01 and the methyl group next to the allene at δ 1.81 and the allenic proton at δ 6.05, Table 1.

In parallel experiments with fucoxanthin (*2*), addition of the $Eu(dpm)_3$ shift reagent [26,27] to the ^1H NMR samples caused, in both spectra, virtually parallel shifts of all the signals which coincide in the original spectra, Table 2. Also the induced shifts of the other saturated methyl signals of peridinin agree fairly well with those of the epoxy end group signals of fucoxanthin (*2*), Table 2, indicating that peridinin and fucoxanthin also have the other end group in common. The probable presence of an epoxidic end group B, such as in neoxanthin (*4*) and violaxanthin (*5*), is substantiated by the ^1H NMR data for *4* and *5*, Table 1.

Ozonolysis of peridinin *p*-bromobenzoate (*1c*) led to the isolation, by TLC, of an allenic methyl ketone (*6*) with properties identical to that obtained on ozonolysis of fucoxanthin (*2*).[24,25]

Further evidence for the second end group B is found in the peridinin mass spectrum with the base peak at *m/e* 181.1218 ($C_{11}H_{17}O_2$), Scheme 3. Abundant ions of this composition are usually encountered for hydroxylated 5,6-epoxidic or 5,8-furanoxide carotenoids.[27] This fragment ion suggests that peridinin is a 5,6-epoxide as already indicated by comparative ^1H NMR data, Tables 1 and 2.

Additional evidence for end group B comes

Table 1. ^1H NMR signals of peridinin and some model carotenoids.

Compound	Methyl groups at C-1	C-5	C-9	C-13	C-13′	C-9′	C-5′	C-1′	Other acetate	H-8′
Fucoxanthin *(2)*	9.03 8.97	8.80	8.08	8.04	8.04	8.21	8.63	8.93 8.67	8.00	3.95
Peridinin *(1)*	9.03 8.80	8.80		7.80		8.19	8.63	8.93 8.65	7.99	3.95
Neoxanthin *(4)*	9.04 8.86	8.83	8.09	8.06	8.06	8.21	8.67	8.94 8.63		
Violaxanthin *(5)*	9.05 8.88	8.84	8.11	8.08	8.08	8.11	8.84	9.05 8.88		

Table 2. Induced chemical shifts observed for peridinin *(1)* and fucoxanthin *(2)* with 0.0 and 1.0 molar ratios of $Eu(dpm)_3$.

A. Signals of methyl groups.

Molar ratio substr./ $Eu(dpm)_3$	Carbon No. 1	1	1′	1′	5	5′	9	9′	13	13′
Peridinin *(1)*										
0	9.03	8.80	8.93	8.65	8.80	8.63	—	8.19	7.80	—
1	8.00	8.30	8.30	8.30	7.86	7.68	—	8.05	7.57	—
Δ	1.03	0.50	0.73	0.35	0.94	0.95	—	0.14	0.23	—
Fucoxanthin *(2)*										
0	9.03	8.97	8.93	8.67	8.80	8.63	8.08	8.21	8.04	8.04
1	7.96	8.30	8.30	8.30	7.86	7.72	7.72	8.05	7.86	7.92
Δ	1.07	0.67	0.73	0.37	0.94	0.91	0.40	0.16	0.18	0.12

B. Signals of other hydrogens.

Molar ratio substr./ $Eu(dpm)_3$	Carbon No. 7	8	8′	10	12	14—10′	Acetate
Peridinin *(1)*							
0	2.82	3.65	3.95	2.99	4.25	3.5	7.99
1	1.95	3.25	3.85	2.86	4.17	3.45	7.57
Δ	0.87	0.40	0.10	0.13	0.08	0.05	0.42
Fucoxanthin *(2)*							
0	—	—	3.95	—	—	—	7.99
1	—	—	3.85	—	—	—	7.57
Δ	—	—	0.10	—	—	—	0.42

from the behaviour of peridinin towards acids. Under mild acidic conditions peridinin and peridinin acetate *(1d)* are both slowly isomerized to two new products (Part 2 [17]) with identical visible light absorptions shifted 20 nm hypsochromically and less polar than the starting compounds, compatible with the formation of two epimeric (at C-8) 5,8-furanoxides.[29] Epimers of this type are normally readily separated.[28] The low rate of reaction is, however, unusual.[30]

The position of the hydroxy substituent in this presumed six-membered end group is evident from the ^1H NMR multiplet at δ 3.9, assigned to a methine proton at C-3 rather than C-2 or C-4, since otherwise distinct triplet coupling patterns could have been observed.[31]

It is inferred therefore, that peridinin has the second end group in common with fucoxanthin *(2)*, neoxanthin *(4)* and violaxanthin *(5)*. End group B explains the acid catalysed rearrangement of peridinin.

Assignment of the two end groups A and B, Scheme 2, leaves a $C_{13}H_{11}O_2$ central structural element unaccounted for.

The visible light absorption spectrum of peridinin in hexane (Fig. 4) exhibits maxima at 454, 484 nm with a very pronounced fine-structure (% III/II [32] = 80). However, on changing the solvent to methanol only one broad maximum at 468 nm is observed. This extreme behaviour indicates that peridinin is a conjugated carbonyl compound.[19] In agreement with cross-conjugation, the low-temperature electronic spectrum (Fig. 4) exhibits pronounced fine-structure in the first overtone band (~300 nm), as observed for some cross-conjugated

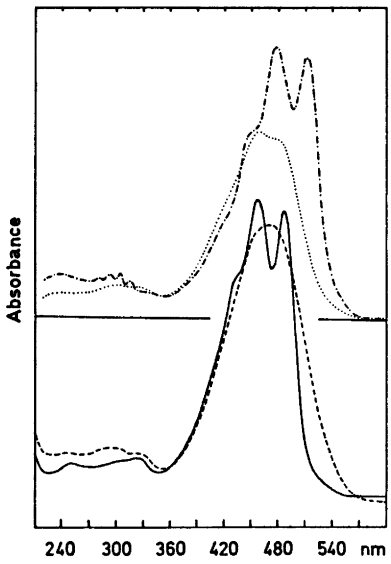


Fig. 4. Electronic spectra of peridinin (*1*) in hexane (——), methanol (– – –) and EPA at room temperature (···) and liquid nitrogen temperature (– · –).

carotenoids.[33] From the ^{13}C NMR data of the *p*-bromobenzoate (*1c*) the conjugated carbonyl function must be of an ester type, since only two of the three ester carbonyl signals at −42.0, −40.1 and −36.2 ppm are accounted for by the acetate and the *p*-bromobenzoate carbonyls and there is no signal in the region for keto carbonyls (*ca.* −50 ppm).[20] No aldehyde signal in the ^1H NMR spectrum and no IR absorption in the usual 1600−1730 cm^{-1} region rule out the presence of keto or aldehyde functions.

With complex metal hydrides peridinin is reduced to a number of compounds [17] which have essentially aliphatic pentaene chromophores. The large hypsochromic shifts in the visible light absorptions upon reduction, *ca.* 110 nm, cannot be explained by simple reduction of the conjugated carbonyl group and must represent a break of conjugation.

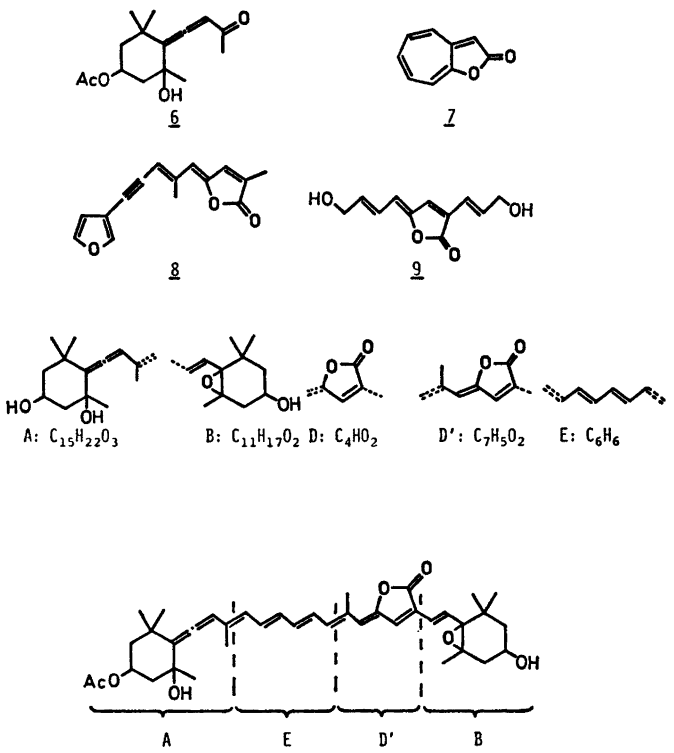
The olefinic region of the peridinin ^1H NMR spectrum is unusually simple, consisting of three singlets at δ 5.75, 6.05 and 7.01, the one at δ 6.05 being ascribed to the allenic proton, Table 1, Fig. 1. Two doublets at δ 6.35 and 7.18 with *trans*, $J = 16$ Hz, couplings were made apparent upon addition of $Eu(dpm)_3$. Approximately six protons resonate at *ca.* δ 6.5.

An unprecedented loss of CO_2 on electron impact and IR absorptions at 1745 (broad) and 1525 (sharp) cm^{-1} may be explained by a conjugated lactone, assuming that the lactone carbonyl vibration is superimposed on that of the acetate. Lactones are, however, so far unknown among carotenoids, and their properties must therefore be extrapolated from model compounds. Normally substituted α-pyrones have carbonyl IR frequencies in the 1725−1735 cm^{-1} region and seven-membered lactones at even lower frequencies.[33] Butenolides, however, have carbonyl absorptions in the 1740−1775 cm^{-1} region,[33] for example, the model compound *7* absorbs at 1740 cm^{-1}.[34] It is therefore implied that the lactone moiety represents a butenolide in conjugation with the main chromophore of the peridinin molecule. The unusual IR absorption at 1525 cm^{-1} is also attributed to the butenolide moiety D, since in other butenolides similar absorptions have been ascribed to a vibration of the butenolide ring.[35,36]

A conjugate butenolide moiety explains adequately both the reduction data [17] and the remaining ester carbonyl signal in the ^{13}C NMR spectrum of the *p*-bromobenzoate *1c*.[20] Furthermore, a ^{13}C NMR signal at −17.5 ppm may be assigned to an enolic carbon.[20]

The remaining C_9H_{10} element must contain a vinylic methyl group in unusual magnetic surroundings to account for the ^1H NMR signal at δ 2.20, and it is reasonable to assume that the strong deshielding is caused by the close proximity to the lactone. For example, the sesquiterpene lactone freelingyne (*8*) has a central methyl group which resonates at δ 2.33 and the endocyclic and one exocyclic olefinic proton give rise to signals at δ 7.02 and 5.62, respectively,[37] in good agreement with the olefinic singlets at δ 7.01 and 5.75 in the peridinin spectrum. It is therefore likely, also considering the biogenetic isoprene rule,[38] that peridinin contains a structural element of the same type as freelingyne (*8*).

Assuming that the structural element D′ (Scheme 2) is present, the remaining C_6H_6, as a conjugated polyene system (E, Scheme 2), would then account for the rest of the mole-



Scheme 2.

cule, supported by the six proton ^1H NMR signal at δ 6.5 already mentioned.

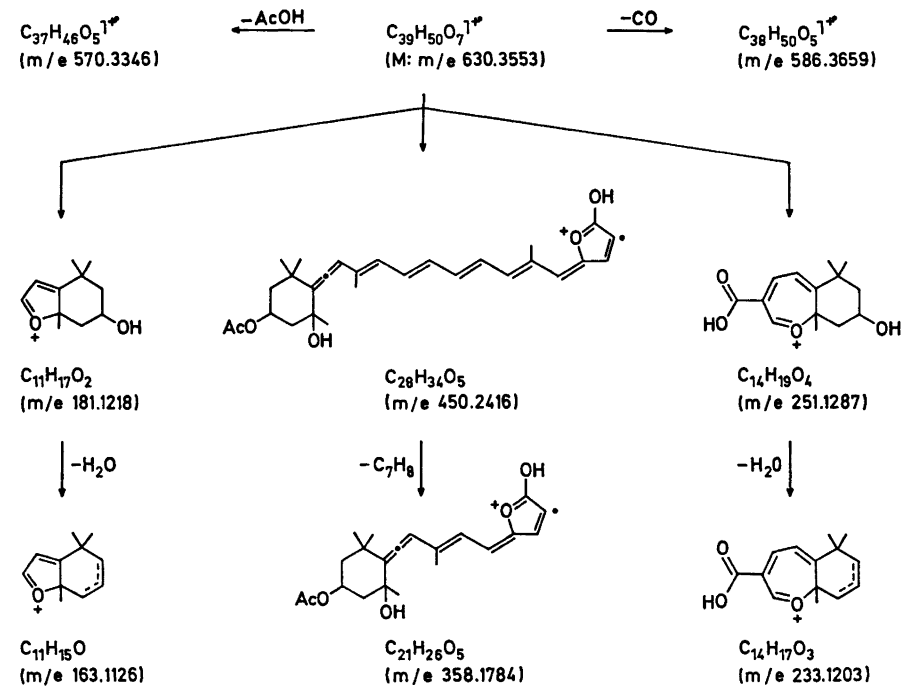
It now remains to combine the various structural elements A,B,D′,E in a way which adequately explains the properties of peridinin.

Examination of the high resolution mass spectrum [17,43] (Scheme 3) shows that the smallest O_5-fragment has a composition $C_{21}H_{26}O_5$ (*m/e* 358.1784). Another O_5-fragment, $C_{28}H_{34}O_5$ (*m/e* 450.2416, differs from the first by C_7H_8, which may be an element of toluene containing E, Scheme 2. Since toluene (C_7H_8) is also lost from the molecular ion and other fragments, it is reasonable to assume that the two fragments are interconnected by C_7H_8 and that the larger one represents the true molecular boundaries for the five oxygen atoms. The fragment $C_{28}H_{34}O_5$ must, by necessity, contain the allenic end group A plus E, Scheme 2, to fulfill the carbon and oxygen requirements.

On the other hand, the smallest O_4-fragment, $C_{14}H_{19}O_4$ (*m/e* 251.1287), cannot be derived from the allenic end group, since ozonolysis revealed only three oxygen atoms within a C_{15} element. Still disregarding the possibility of gross skeletal rearrangements, this fragment must therefore be derived from the epoxidic end of the molecule including the lactone oxygens. A similar fragment, $C_{14}H_{17}O_3$ (*m/e* 233.1203), has obviously lost an element of water, whereas no corresponding fragment representing a loss of acetic acid was observed

One may consequently assume that the lactone moiety is located within fourteen carbons containing the epoxidic end group and within twenty-six carbons containing the allenic end group, excluding the acetate. One then arrives at structure *1* which satisfies all the relevant data. Taking biosynthetic considerations into account, this implies that the methyl group at C-9′ of a traditional C_{40}-skeleton is oxidized to give the lactone, and the C_3-element lost from the skeleton must include the methyl group at C-13.

Regarding the visible light absorption of peridinin, extrapolation of the absorption



Scheme 3.

maximum of the antibiotic lactone tetrenolin (*9*), λ_{max} 340 nm in methanol,[39] to that of peridinin, λ_{max} 468 nm in methanol, is not successful. However, the unique chromophore of the proposed structure contains eight spectroscopically efficient double bonds, expected λ_{max} *ca.* 425 nm in hexane,[19] cross-conjugated with the lactone carbonyl. Cross-conjugation is known to cause substantial bathochromic shifts in related polyenes.[40,41]

Tetrenolin (*9*) also has an exocyclic double bond in a similar position to peridinin, the olefinic protons of which give rise to signals at δ 6.90 and 7.38 [39] in fair agreement with the two doublets at δ 6.35 and 7.18 in the peridinin ^{1}H NMR spectrum.

Additional information which supports the proposed structure is presented in the following paper.[17]

Regarding the biosynthesis of peridinin, hypotheses regarding the formation of the lactone and the loss of the C_3-unit have already been advanced.[16] A somewhat modified mechanism for the expulsion of the C_3-unit, taking into account the principle of conservation of orbital symmetry,[42] proceeding *via* a cyclobutene derivative and scission of the C_3-unit as an acetylenic element, has subsequently been proposed.[43] Coupling of the two geranylgeranyl residues [44] in an unusual way facilitating the expulsion of the C_3-element, or even a totally independent biosynthesis, may also be considered. Since, however, dinoflagellates also contain normal C_{40}-carotenoids, it seems likely that peridinin would have a normal carotenoid precursor, although no obvious precursor has been found so far.[45]

The peridinin structure contains six chiral centers, so far of undetermined configuration. Work on the stereochemistry of peridinin (*1*) will be pursued.

The currently used definition of carotenoid [46a] has recently been changed [46b] in order to include peridinin (*1*) and related nor-carotenoids.[45]

EXPERIMENTAL

Materials and methods employed in the Norwegian laboratory were as described elsewhere.[47]

Biological material. Gonyaulax polyedra was harvested at La Jolla during a "red tide" in which this species represented about 99 % of the phytoplankton enumerated by microscopic examination. The harvests were freeze-dried and held at −10 °C.

Anthopleura xanthogrammica was collected at Halt Moon Bay (Pillar Point), California.

Cachonina niei (Indiana Culture Collection No. 1564) provided the major starting material used in the isolation of peridinin. Large scale culture of the alga was grown in 10 l bottles or in 180 l polyethylene drums at 18−20 °C in an enriched sea-water medium.[48,49] Illumination was provided by "cool white" fluorescent lamps at a light intensity of 500−800 cd. The cultures were bubbled lightly with air during growth and were harvested either directly by centrifugation or after initially floculating the cells with alum. Yields ranged from 0.2−0.4 g wet weight/l after 10 d growth. Harvests were freeze-dried and held at −10 °C.

Isolation. Peridinin was isolated at La Jolla as described previously,[3] and at Argonne by the following procedure:

The sea anemones (2 kg wet weight) were cut into pieces and extracted with methanol and ether until the extracts were colourless. The pigments were transferred into ether by dilution with aq. NaCl.

Freeze-dried *Cachonina niei* (10−20 g) was extracted alternately with methanol and ether in a column containing a layer of Celite. The filtrate was washed with aq. NaCl solution and evaporated.

The pigments were dissolved in ether-petroleum ether (1:4) and chromatographed on columns of powdered sugar using 1.5 % propanol in petroleum ether as developer. The major zone (peridinin) was cut out and eluted with ethanol-petroleum ether. Peridinin was rechromatographed on sugar columns developed with 2, 2.25, and 2.5 % propanol in petroleum ether as above. The eluate was washed with water, dried and evaporated.

Homogeneous peridinin was crystallized from chloroform-lipopure hexane (1:15) and the crystals collected by centrifugation; approximate yield 1.5 mg peridinin/g cells.

Recrystallization was effected from the same solvent system or methanol-water.

Peridinin (*1*). [43,50] Crystallization of chromatographically homogeneous fractions from ether-hexane afforded peridinin as purple crystals; m.p. 128−132 °C. Found: C 73.15; H 7.68. $C_{41}H_{52}O_8$ (Mw = 672) requires C 73.18; H 7.79; $C_{39}H_{50}O_7$ (Mw = 630) requires C 74.25; H 7.99. λ_{max} (hexane) 454, 484 nm; % III/II [32] = 80; λ_{max} (acetone) 466 nm; λ_{max} (ethanol) 475 [E(1 cm, 1 %) = 1350] nm; ν_{max} (KBr) 3450 (bonded OH), 3040−2800 (CH), 1930 (C=C=C), 1745 (broad, C=O), 1525 (C=C), 1450 (CH_2), 1365 (CH_3), 1250 (C−O−, acetate), 1190-1110 (C−O−), 1080−1010 (C−O−), 985 (*trans*-CH=CH−), 960, 942, 913, 900, 860, 820 and 770 cm^{-1}; δ ($CDCl_3$) 0.97 (s, 3 H, CH_3 at C-1′), 1.07 (s, 3 H, CH_3 at C-1), 1.20 (s, 2 × 3 H, CH_3 at C-1′ and C-5′), 1.35 (s, 3 H, CH_3 at C-1), 1.37 (s, 3 H, CH_3 at C-5), 1.81 (s, 3 H, CH_3 at C-9), 2.01 (s, 3 H, CH_3, acetate), 2.20 (s, 3 H, CH_3 at C-13′), 3.9 (m, 1 H, H-3′), 5.4 (m, 1 H, H-3), 5.75 (s, 1 H, H-12′), 6.05 (s, 1 H, H-11), 6.35 (d, 1 H, J = 16 Hz, H-8′), *ca.* 6.5 (m, 6 H, olefinic), 7.01 (s, 1 H, H-10′), and 7.18 (d, 1 H, J = 16 Hz, H-7′); for Eu(dpm)$_3$ shift experiments [43] see Table 2. *m/e* (high resolution) 630.3553 (M = $C_{39}H_{50}O_7$, calc. 630.3555), 612 (M − H_2O), 594 (M − 2 × H_2O), 586 (M − CO_2), 570 (M − AcOH, m*), 568 (M − H_2O − CO_2), 552 (612 − AcOH, *m**), 538 (M − C_7H_8), 534 (M − 2H_2O − AcOH), 520 (M − H_2O − C_7H_8), 508 (M − H_2O − AcOH − CO_2), 478 (538 − AcOH, *m**), 450 ($C_{28}H_{34}O_5$), 397 ($C_{28}H_{29}O_3$), 358 ($C_{21}H_{26}O_5$), 257 ($C_{17}H_{23}O_3$), 251 ($C_{14}H_{19}O_4$), 234 ($C_{14}H_{18}O_3$), 233 ($C_{14}H_{17}O_3$), 223 ($C_{17}H_{19}$), 212 ($C_{16}H_{20}$), 197 ($C_{15}H_{17}$, 75 %), 181 ($C_{11}H_{17}O_2$, 100 %), 167 ($C_{10}H_{15}O_2$), 163 ($C_{11}H_{15}O$), 149 ($C_{10}H_{13}O$), and 125 ($C_7H_9O_2$).

Peridinin acetate (*1a*).[51] Peridinin (*1*, 25 mg) and acetic anhydride (0.5 ml) in dry pyridine (5 ml) were reacted at room temp. for 16 h. Ether (50 ml) was added and the reaction mixture extracted twice with water, then with 0.05 N HCl, 10 % $NaHCO_3$ and dried over Na_2SO_4. The solvent was evaporated and the residue crystallized from boiling hexane; yield 19 mg (71 %); λ_{max} (hexane) 455, 486 nm, λ_{max} (methanol) 472 nm; ν_{max} (CCl_4) 1930 (C=C=C), 1760, 1740 (C=O) cm^{-1}; *m/e* 672 (M, consistent with $C_{41}H_{52}O_5$), 610 538, 223, 216 and 197.

Peridinin ^{14}*C-acetate* (*1b*).[51] ^{14}C-acetic anhydride was prepared from sodium ^{14}C-acetate and unlabelled acetic anhydride by exchange and distillation of the product. The specific activity was 3.83×10^5 dpm/mmol counted as acetanilide.

Peridinin (*1*, 10 mg) and ^{14}C-acetic anhydride (100 µl) was treated as for peridinin acetate (*1a*) above. The product was purified by chromatography on $CaCO_3$ and sucrose columns. Crystallization twice from ether-hexane gave 5 mg (47 %) peridinin ^{14}C-acetate (*1b*).

Two samples (1 mg each) were dissolved in scintillation grade toluene, ozonized at −78 °C until colourless, scintillation solution added and the samples counted to 549 and 551 dpm/mg, respectively, corresponding to a molecular weight of 696 for *1b*.

Acetate estimation by dilution of activity.[51] Peridinin ^{14}C-acetate (*1b*, 20 mg), prepared with ^{14}C-acetic anhydride of acyl activity 3.071×10^6 dpm/mmol as above, was hydrolyzed in 12 N H_2SO_4:dioxane (1:1,10 ml) and refluxed for 16 h. Steam distillation liberated 2.24 mg (1.25 equiv.) as estimated by titration. The neutralized liberated acid was freeze-dried and the residue suspended in ether (10 ml). The *m*-iodoanilide was prepared by addition of oxalyl chloride (0.1 ml), stirring for 1 h and addition

of *m*-iodoaniline (0.5 ml). After 1 h at room temperature ether was added, the suspension filtered and the filtrate washed with dilute HCl and 10 % $NaHCO_3$, dried over Na_2SO_4 and the solvent evaporated. Isolation by TLC and sublimation twice gave 2 mg *m*-iodoacetanilide, homogeneous by TLC. Two samples were counted giving specific activities of 3.70×10^3 and 4.12×10^3 dpm/mg corresponding to dilutions of the original activity by 3.2:1 and 2.9:1, respectively.

Peridinin p-*bromobenzoate* (*1c*).[51] Peridinin (*1*, 50 mg) in dry pyridine (5 ml) and *p*-bromobenzoyl chloride (200 mg) in dry pyridine (5 ml) were pooled at 0 °C and reacted at room temperature for 20 h. The reaction mixture was diluted with ether, washed with water, 0.2 N HCl and 10 % $NaHCO_3$. Evaporation of the solvent and crystallization of the residue twice from ether-hexane gave 53 mg (82 %) *p*-bromobenzoate (*1c*).

Peridinin chloroacetate (*1d*).[51] Peridinin (*1*, 5 mg) in acetone (10 ml) and chloroacetyl chloride (50 µl) in dry pyridine (1 ml) were pooled at 0 °C and reacted at room temp. for 12 h. The reaction mixture was diluted with ether (25 ml), washed with water, dilute HCl and finally with 10 % $NaHCO_3$. The solvent was removed and the residue crystallized twice from ether-hexane. The chloroacetate (4 mg, 71 %), homogeneous on TLC, had *m/e* 706 ($M = C_{41}H_{51}{}^{35}ClO_8$).

Peridinin di-TMS ether (*1e*).[50] Peridinin (*1*, 1 mg) was treated with hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.1 ml) in dry pyridine (1 ml) at room temp. for 2 h. Excess reagents were removed by evaporation and the residue chromatographed on $CaCO_3$ and eluted with 4 % acetone in hexane. The di-TMS ether (0.6 mg, 60 %), homogeneous on TLC, had λ_{max} (hexane) 454, 484 nm, λ_{max} (methanol) 472 nm; *m/e* 774 ($C_{45}H_{66}O_7Si_2$).

Peridinin acetate-TMS ether (*1f*).[50] Peridinin acetate (*1a*, 0.3 mg) was silylated as above for 5 h and the product chromatographed on silica plates developed with 30 % acetone in petroleum ether. The homogeneous TMS ether (0.28 mg, 93 %) had λ_{max} (petroleum ether) 457, 487 nm; % $III/II^{32} = 60$; *m/e* 744 ($M = C_{44}H_{60}O_8Si$).

Peridininol (*1g*).[50] Peridinin (*1*) was saponified as described in the following paper.[17] Peridininol (3.4 mg, 6.5 %) had λ_{max} (acetone) 464 nm; *m/e* 588 ($M = C_{37}H_{48}O_6$), 570 ($M - H_2O$), 552 ($M - 2H_2O$).

Upon acetylation peridininol (*1g*) gave a diacetate (M = 672), inseparable from peridinin acetate (1a), and two monoacetates (M = 630), one of which could not be separated from peridinin (*1*).

Acid treatment of peridinin (*1*).[50] See a following paper.[17]

Acid treatment of peridinin acetate (*1a*).[51] Peridinin acetate (*1a*, 1 mg) in CH_2Cl_2 (5 ml) was treated with 2 drops of CH_2Cl_2 saturated with HCl gas at room temperature for 2 h during which period the visible light absorption shifted from λ_{max} 467, 483 nm to λ_{max} 446, (463) nm. Two products, both less polar than *1a* on TLC (silica), were observed.

Hydrogenation of peridinin.[51] Peridinin (*1*, 25 mg) in ethanol (96 %, 10 ml) was hydrogenated at atmospheric pressure over PtO_2 catalyst (10 mg) for 4 h. The H_2-uptake was 9.8 equivalents per mol peridinin. Filtration and evaporation of the solvent gave 23 mg (93 %) crude hydrogenation product; λ_{max} (hexane) 267, 273 nm; ν_{max} (liq) 1750–1730 (C=O) cm^{-1}; δ (60 MHz, $CDCl_3$) 0.8–1.1 (*ca.* 15 H), 1.1–1.4 (*ca.* 30 H), 1.4–1.8 (*ca.* 20 H), 2.02 (s, 3 H), 2.5 (m, 1 H) and 5.2 (m, 1 H).

TLC (silica-$CHCl_3$) revealed the presence of at least six products.

Perhydrogenation of peridinin.[51] The crude hydrogenation product (22 mg) in methanol (25 ml) was reduced with KBH_4 (100 mg) by refluxing for 1 h. Excess KBH_4 was destroyed with 1 N HCl, the solvent evaporated and the residue redissolved in ether. The solution was washed with 10 % $NaHCO_3$, dried over Na_2SO_4 and evaporated to give 19 mg (83 %) of crude reduction product which had no detectable UV absorption and no carbonyl absorption in IR.

The above crude product (18 mg), freshly distilled HI (55 %, 3 ml) and red phosphorus (100 mg) in glacial acetic acid (9 ml) were refluxed for 24 h. The acetic acid was removed *in vacuo*, the aqueous phase extracted with ether, the extract washed with 10 % $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated to give 14 mg (75 %) of a complex mixture of products as demonstrated by TLC (silica-CCl_4). The non-polar material ($R_F = 0.8 - 0.95$) had no significant C=C absorption in the IR. Mass spectrometry of this fraction gave the highest observable molecular ion at *m/e* 510 corresponding to a hydrocarbon $C_{37}H_{66}$.

After similar treatment fucoxanthin (*2*) gave the highest molecular ion at *m/e* 552 ($C_{40}H_{72}$), 6 mass units less than expected (*m/e* 558) for $C_{40}H_{78}$. Similar treatment of β-carotene gave one fraction with the molecular ion at the expected *m/e* 558.

Hydroxamic acid test for ester functions.[51] Peridinin (*1*, 2.5 mg) in ethanol (96 %, 2 ml), 7.0 % hydroxylamine hydrochloride (1 ml), and 10 % NaOH (1 ml) were incubated at 37 °C for 15 min. 0.1 M Citrate buffer (pH 1.4, 1 ml) and water (2 ml) were added, the solution extracted with benzene (3 × 3 ml) and the benzene phase removed. A 0.3 M solution of $Fe(NH_4)_3(SO_4)_4.24H_2O$ (1 ml) was added to the water phase and the volume adjusted to 10 ml in a volumetric flask. An absorption of 0.59 at 495 nm, as read against a blank and standards of isopropyl acetate (0.002 and 0.004 mM, absorbance 0.18 and 0.36, respectively), corresponded to 1.7 ester functions per molecule of *1* (for Mw = 630).

Similar treatment of fucoxanthin (*2*, 4.5 mg) and fucoxanthin acetate (*2a*, 3.0 mg) gave absorbances of 0.54 and 0.68, corresponding to 0.9 and 1.7 ester functions per molecule of *2* and *2a*, respectively.

Ozonolysis of peridinin p-*bromobenzoate* (*1c*).[51] Peridinin *p*-bromobenzoate (*1c*, 25 mg) in methanol-chloroform (3:1, 50 ml) was ozonized at −78 °C until the solution had a light yellow colour. Dimethyl sulfide (2 ml) was added and the solution allowed to reach ambient temp. within 2 h. Evaporation of the solvent and TLC (silica, benzene-acetone 4:1) gave one homogeneous fraction ($R_F = 0.3$). Purification on an alumina (grade 3) [52] column, eluting with 1 % acetone in benzene, gave a colourless viscous oil (*6*, 3 mg) with λ_{max} (hexane) 225 nm, λ_{max} (ethanol) 233 nm; ν_{max} (liq) 3450 (OH), 1945 (C=C=C), 1730 (C=O, acetate) and 1675 (C=O, ketone) cm^{-1}; δ (CCl_4) 1.14 (3 H), 1.41 (2 × 3 H), 1.96 (3 H), 2.10 (3 H) and 5.76 (1 H); *m/e* 266.1514 ($M = C_{15}H_{22}O_4$, calc. 266.1518), 223 ($M - C_2H_3O$), 206 ($M - C_2H_4O_2$).

The other TLC fractions all contained *p*-bromobenzoic acid when checked by mass spectrometry.

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Decarboxylation of *S*-Adenosyl-L-methionine in Vitamin B_6 Deficiency in Rat Liver

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The effect of a prolonged diet deficient in B_6-vitamin on the activity of adenosylmethionine decarboxylase from rat liver was investigated. In contrast to an earlier report,[12] adenosylmethionine decarboxylase activity was not found to be decreased under these restricted nutritional conditions. Moreover, the addition of pyridoxal phosphate into the standard incubation mixture did not stimulate the activity of adenosylmethionine decarboxylase from the livers of the rats fed the B_6-vitamin deficient diet. It is suggested that the contradictory results of Sturman and Kremzner [12] might be based on an artificial liberation of carbon dioxide from *S*-adenosyl-L-methionine in the presence of pyridoxal phosphate. The results of this communication do not support the view that pyridoxal phosphate acts as the prosthetic group of rat liver adenosylmethionine decarboxylase.

Adenosylmethionine decarboxylase (Ado-Met DC; EC 4.1.1.50) appears to play a regulatory role in the biosynthesis of spermidine and spermine in eucaryotic tissues.[1-3] The mechanism of the enzymic decarboxylation of *S*-adenosyl-L-methionine (Ado-Met) is not fully understood and there exists some controversy as to whether pyridoxal phosphate (PLP) or some other carbonyl compounds function as the prosthetic group of the putrescine-activated mammalian decarboxylase. Primarily based on experiments with 4-bromo-3-hydroxybenzyloxyamine, a compound known to inhibit PLP requiring histidine decarboxylase (EC 4.1.1.22) from rat fetus,[4] it has been suggested that PLP also acts as the coenzyme of mammalian Ado-Met DC.[5-7] On the other hand, common inhibitors of enzymes requiring PLP inhibited the activity of Ado-Met DC from different mammalian tissues only slightly, if at all, although some other carbonyl reagents rapidly inactivated the decarboxylase.[1,8-10] Furthermore, an inclusion of PLP in the incubation mixture did not stimulate Ado-Met DC from rat liver purified to electrophoretic homogeneity.[11] Very recently Sturman and Kremzner [12] reported that undialyzed liver cytosol fraction from rats maintained on a diet deficient in B_6-vitamin exhibited decreased Ado-Met DC activity. Full activity was reportedly restored by the addition of PLP to the incubation mixture.[12]

Because of the apparent controversy concerning the possible participation of PLP in the enzymic decarboxylation of Ado-Met in mammalian tissues, the effect of a B_6-vitamin deficient diet on the activity of Ado-Met DC from rat liver was reinvestigated in more detail. In addition to Ado-Met DC, the activities of some other soluble enzymes, *i.e.* spermidine synthase (EC 2.5.1.16), tyrosine aminotransferase (EC 2.6.1.5) and lactate dehydrogenase (EC 1.1.1.27), were assayed using undialyzed liver cytosol fraction as the source of the enzymes.

The activity of Ado-Met DC was not shown to be influenced by the vitamin B_6 deficiency nor was it stimulated by an inclusion of PLP to the standard incubation mixture.

EXPERIMENTAL

1-^{14}C-DL-Methionine (sp.act. 5.65 mC/mmol) and 1,4-^{14}C-putrescine (sp.act. 19.5 mC/mmol) were purchased from the New England Nuclear Corp. PLP, pyridoxine hydrochloride, py-

ruvate, and α-ketoglutaric acid were obtained from E. Merck, Darmstadt. NADH was a product of Boehringer, Mannheim. Dithiothreitol (DTT) and unlabelled putrescine were supplied by Calbiochem. The synthesis of Ado-Met from either L-methionine or radioactive DL-methionine has been described elsewhere.[5] Methylthioadenosylhomocysteamine (decarboxylated adenosylmethionine) was prepared as described earlier.[13] All other reagents were of analytical grade of purity.

Animals and preparation of tissue extracts. Male albino rats of the Wistar strain weighing 45–60 g were divided in two groups of five animals. The first group was fed a vitamin B_6 deficient diet (Nutritional Biochemicals Corp.) and the control group was fed the same diet supplemented with 50 mg/kg pyridoxine hydrochloride.[12] The animals were allowed to eat and drink *ad libitum*. After five weeks the rats were killed. The livers were rapidly removed and homogenized with two volumes of 25 mM phosphate buffer, pH 7.2, containing 0.1 mM EDTA and 0.5 mM DTT (Buffer A). The homogenates were centrifuged at 75 000 g_{max} for 60 min and the supernatant fractions were used for the enzyme assays. Solid ammonium sulfate (Mann/Schwarz special enzyme grade) was used for the fractionation of pooled cytosols. The proteins precipitated between 0.35 and 0.60 were collected, dialyzed overnight against Buffer A and used for the determination of Ado-Met DC activity.

Enzyme assays. The standard incubation mixture for Ado-Met DC contained 100 mM phosphate buffer, pH 7.4, 3.3 mM DTT, 0.2 mM carboxyl labelled Ado-Met (sp.act. 0.91 mC/mmol), 2.5 mM putrescine (if present) and the enzyme in a final incubation volume of 0.15 ml. One unit is defined as the liberation of (1 pmol of $^{14}CO_2$ from Ado-Met)/(30 min) (mg protein) at 37 °C. The incubation conditions for spermidine synthase has been described earlier.[3] One unit is expressed as the production of 1 pmol of spermidine from radioactive putrescine (sp.act. 5.0 mC/mmol) in the presence of (decarboxylated adenosylmethionine)/(30 min) (mg protein) at 37 °C. Each Ado-Met DC and spermidine synthase assay was accompanied by blanks where the active protein was replaced either by water or boiled enzyme. Lactate dehydrogenase activity was determined as described by Lindy.[14] One unit is defined as the oxidation of (1 μmol of NADH)/(1 min) (mg protein) at 25 °C. The activity of tyrosine aminotransferase was measured by the method of Diamondstone, as modified by Hayashi *et al.*[15] One unit is expressed as the formation of (1 nmol of *p*-hydroxybenzaldehyde)/(1 min) (mg protein) at 37 °C. All enzyme assays were carried out in duplicate. The concentration of PLP (if present) in the incubation mixtures was 0.05 mM.

Protein was measured by the method of Lowry *et al.*[16] using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

The rats fed the B_6-vitamin deficient diet rapidly developed typical deficiency symptoms (see Ref. 17) including a clear retardation of growth ($p < 0.05$, Table 1). In contrast to the results obtained by Sturman and Kremzner,[12] liver Ado-Met DC activity was not decreased

Table 1. Effect of vitamin B_6 deficient diet on the activities of adenosylmethionine decarboxylase, spermidine synthase, tyrosine aminotransferase and lactate dehydrogenase from rat liver. Undialyzed cytosols were used as the source of the enzymes and the activities are expressed as units (±SEM for adenosylmethionine decarboxylase) (for incubation conditions and definition of units see Experimental). The activity of adenosylmethionine decarboxylase was assayed separately from each liver, other enzyme activities are means of two separate pools, two and three livers in each, from either group. The concentration of pyridoxal phosphate (PLP), if present in the incubations, was 0.05 mM. Ado-MetDC, adenosylmethionine decarboxylase; SpdS, spermidine synthase; TAT, tyrosine aminotransferase; LDH, lactate dehydrogenase.

Diet	$-B_6$ $-$PLP	+PLP	$+B_6$ $-$PLP	+PLP
Body wt. (g)	121±6		170±8	
Liver wt. (g)	4.8±0.3		6.7±0.3	
Enzyme activities (units)				
Ado-Met DC				
−Putrescine	15.9±3.3	18.4±2.7	17.1±2.9	18.5±2.2
+Putrescine	150±7.5	161±8.8	193±19.5	171±13.0
SpdS	922	1049	1348	1148
TAT	0.6	23.9	1.5	17.5
LDH	5.2	5.2	5.7	5.7

Table 2. Adenosylmethionine decarboxylase activity (units) in dialyzed liver cytosols from rats kept on vitamin B_6 deficient or pyridoxine hydrochloride supplemented diets. Each value represents the mean determined from two separate pools from either group after dialyzing the cytosols against Buffer A (see Experimental). Each enzyme assay was carried out in duplicate accompanied by blanks where the enzyme was omitted. Pyridoxal phosphate (PLP) concentration, if present in the incubation mixture, was 0.05 mM.

Diet	−putrescine		+putrescine	
	−PLP	+PLP	−PLP	+PLP
$-B_6$	27.0	23.0	409	426
$+B_6$	21.6	16.9	320	318

in rats maintained on the vitamin B_6 deficient diet, nor was the activity stimulated by the addition of PLP to the incubation mixture. Only minor changes were found in the activities of spermidine synthase and lactate dehydrogenase (Table 1). As indicated in Table 1, the activity of tyrosine aminotransferase (when assayed in the presence of PLP) was somewhat increased in liver cytosols of rats fed the vitamin B_6 deficient diet supporting the suggestion that under these conditions the amount of the apoenzyme might be increased.[18] The activity of Ado-Met DC was not stimulated by the addition of PLP to the standard incubation mixture when either dialyzed liver cytosols (Table 2) or dialyzed ammonium sulfate fractions (not tabulated) from either group was used as the enzyme source. The dialysis of the cytosol fraction, however, increased the activity of Ado-Met DC, especially when assayed in the presence of putrescine. In fact using partially purified Ado-Met DC from rat liver (DEAE step, Ref. 2, producing 5000 units $^{14}CO_2$ from Ado-Met), an addition of 25 μl of the pooled undialyzed cytosol (about 1 mg protein) into the routine incubation mixture reduced the activity of the purified decarboxylase by 50 % (vitamin B_6 deficient group) and by 35 % (control group). No inhibition was produced by a similar addition of dialyzed cytosol from either group, thus indicating the presence of small molecular weight inhibitor(s) in the undialyzed cytosols.

There is a strong disagreement between the results obtained by Sturman and Kremzner [12] and those tabulated in Table 1, with regard to the stimulation of Ado-Met DC by the exogenous addition of PLP to the incubation mixture. To study possible reasons for this inconsistency, some experiments were carried out in incubation conditions used by Sturman and Kremzner [12] (DTT was omitted from the incubation mixture). In agreement with these authors, when undialyzed cytosols were used as the enzyme, the addition of PLP to the incubation medium (in the absence of putrescine) increased the decarboxylation of Ado-Met from 14.2 units to 28.8 units in livers of B_6-vitamin deficient rats, but had no effect on the decarboxylation rate in the control group. However, when dialyzed cytosols (dialysis overnight against 25 mM phosphate buffer, pH 7.2; Buffer B) were used as the enzyme, the inclusion of PLP to the incubation mixture (in the absence of DTT and putrescine) resulted in increases in CO_2 production from carboxyl labelled Ado-Met in the vitamin B_6 deficient- and the control groups of 80 % and 40 %, respectively. Furthermore, when the decarboxylation of Ado-Met was catalyzed by the dialyzed ammonium sulfate fractions (redialysis overnight against Buffer B), in the absence of DTT and putrescine, the additional PLP in the incubation mixture increased the liberation of $^{14}CO_2$ by about 40 % in either group. These results suggest that the incubation conditions employed by Sturman and Kremzner [12] make Ado-Met susceptible to nonenzymic decarboxylation by PLP, as also indicated earlier.[19] It should be noted that in the presence of putrescine in the incubation mixture (containing no DTT) the addition of PLP did not increase the liberation of carbon dioxide from Ado-Met by any of the preparations used as the enzyme. This is consistent with the report of Sturman and Kremzner [12] concerning Ado-Met decarboxylation in rat brain. It is suggested that the previous report of the decreased activity of Ado-Met DC from undialyzed liver cytosols of rats maintained on the vitamin B_6 deficient diet [12] cannot be considered by any means as a proof of the dependence of the enzymic decarboxylation of Ado-Met upon PLP. The B_6-vitamin deficiency appears to lead to the

accumulation of small molecular weight compound(s) (or the diet contains some impurities) which inhibit(s) the activity of Ado-Met DC in rat liver, complicating the interpretation of the results obtained with undialyzed liver cytosols. The results of this communication do not support the view that PLP acts as the coenzyme for Ado-Met DC from rat liver.

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A Comparison of Crystallographically Independent Iminodiacetic Acid Residues

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The dimensions of the iminodiacetic acid residues in six different crystal structures, which have been determined by X-ray crystallography, are compared by the method of half-normal probability plots. The bond distances (except C–O) within the organic molecule are independent of the crystallographic environment, with N–C and C–C distances of 1.488(3) and 1.505(3) Å, respectively. Bond angles are more dependent on the crystallographic environment and have different values in different crystal structures. The molecular residue $N-C-C\langle_{O}^{O}$ is shown to have a preferred planar conformation in the solid state. The double bond character in the C–OH bond is estimated to be in the range 10–20 %.

General information about the conformations of chemical groups can be of value in the interpretation of physical observations and for predicting the most probable spatial arrangements in larger molecules. For example the knowledge of the geometry of the peptide group was of great importance in deducing the stable conformations of polypeptide chains.[1] A determination of the geometry of a molecule in the solid state using X-ray diffraction methods cannot give any detailed information about the energy of different conformations. However, if one particular conformation is observed in a number of crystal structures with different packing arrangements, one may conclude that the intermolecular forces here are of less importance than the intramolecular ones and hence the conformation observed is the one with the lowest energy.[2] One aim of the present investigation is to search for preferred conformations in the solid state of the iminodiacetic acid group (Fig. 1).

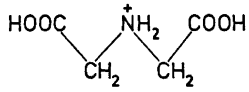


Fig. 1. The positively charged iminodiacetic acid ion.

A prerequisite for a quantitative conformational analysis based on classical mechanical principles is the access to accurate, strain free bond distances and angles. No compound is really "strain free" but if the same bond distances and angles for a molecule are observed in several crystal structures, one may conclude that the strain factors involved in these quantities are quite small. The set of "normal" structural parameters given in this paper are calculated from six different crystal structures.[3–7]

HALF-NORMAL PROBABILITY PLOT ANALYSIS

De Camp [8] and Albertsson and Schultheiss [9] have shown that the use of normal probability plot analysis [10] can be extended to the comparison of independently determined molecular geometries. This method is convenient in detecting minor differences in the residues compared. In the present paper half-normal probability plots are used. Observed ranked values

$$\delta d_i = |d(1)_i - d(2)_i| / [\sigma^2 d(1)_i + \sigma^2 d(2)_i]^{1/2}$$

are plotted *versus* the quantiles ζ_i expected for a normal distribution of errors.[11] The quantities $d(1)_i$ and $d(2)_i$ are the corresponding intramolecular distances with estimated stand-

ard deviations $\sigma d(1)_i$ and $\sigma d(2)_i$ in the structures 1 and 2. To make the analysis cover a comparison of all bond distances, bond angles and conformational angles in an organic molecule, all independent intramolecular distances up to about 4.65 Å should be included in the calculation of δd_i. A cut-off at about 2.55 Å is equivalent to a comparison of bond distances and bond angles only. Besides information about differences in the compared molecular geometries these plots also give information about the reliability of the standard deviations assigned to the interatomic distances, as discussed by Abrahams and Keve [10] in their original paper on the method. A correct match between the measured and assumed error distributions in two equal molecules will result in a linear plot with a slope of unity and zero

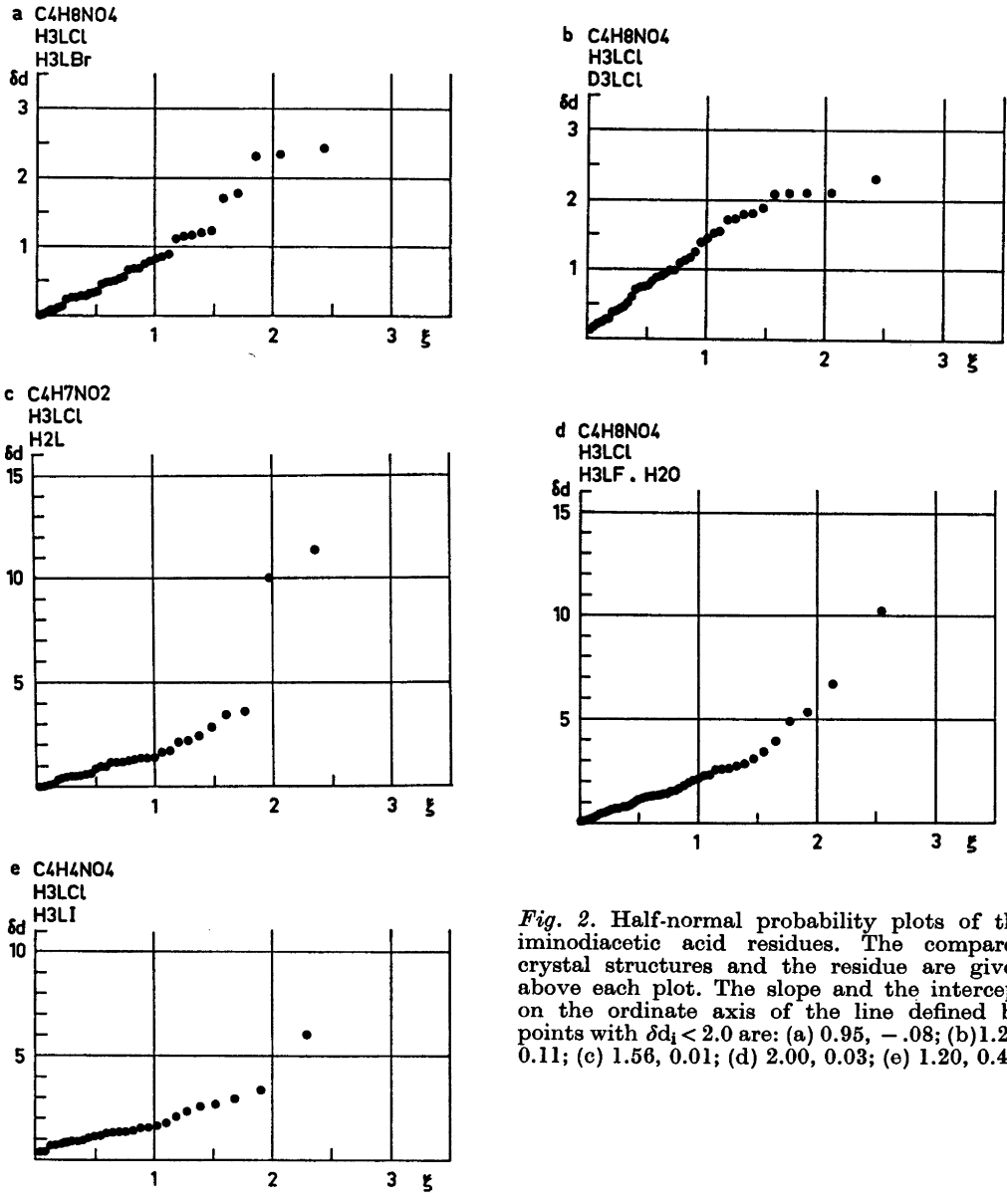


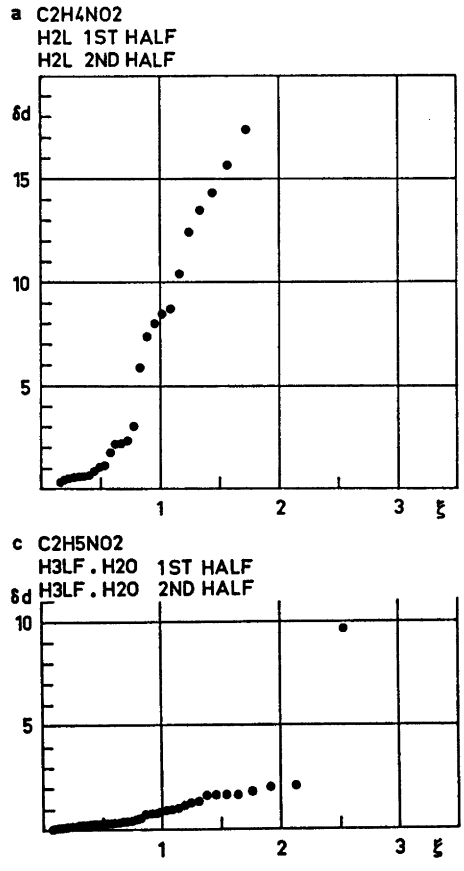
Fig. 2. Half-normal probability plots of the iminodiacetic acid residues. The compared crystal structures and the residue are given above each plot. The slope and the intercept on the ordinate axis of the line defined by points with $\delta d_i < 2.0$ are: (a) 0.95, −.08; (b)1.28, 0.11; (c) 1.56, 0.01; (d) 2.00, 0.03; (e) 1.20, 0.46.

intercept. Systematic differences in the two compared geometries normally give rise to a curved plot but can also show up as a non-zero intercept. A linear plot with a slope s different from unity, may be an indication of uniform under or overestimation in $\sigma d_i = [\sigma^2 d(1)_i + \sigma^2 d(2)_i]^{1/2}$ by a constant factor s. The plots discussed below were obtained by the program PPCMOL.[12]

The structures of H_3LCl ($L^{2-} = HN(CH_2COO^-)_2$, H_3LBr and D_3LCl are closely isostructural.[4-5] A comparison of the ion $H_2\overset{+}{N}(CH_2COOH)_2$ in the chloride and bromide salts using interatomic distances less than 4.65 Å gave a linear plot (Fig. 2a) with an intercept close to zero and a slope of 0.95 suggesting that in these two structures the errors may have been correctly estimated. Therefore the structure of H_3LCl was chosen as a reference structure for checking the presence of systematic differences between the iminodiacetic acid geometries and the reliability of the standard deviations in the other structures.

Only distances less than 2.55 Å were included in the comparisons with the other structures, since the conformation of the organic molecule in these structures is different from that of H_3LCl. The results are given in Figs. 2c–e. The plots are only linear up to about $\delta d = 2$ and with slopes in the range 1.2–2.0. In Fig. 2c the eight points with $\delta d > 2$ represent two C–C distances, two C–O distances and four bond angles. The two C–C distances are the points closest to $\delta d = 2$. In Fig. 2d, the twelve points with $\delta d > 2.5$ represent three C–O distances and nine bond angles. In Fig. 2e, the seven points with $\delta d > 2$ represent two O–H distances and five bond angles. The intercepts are close to zero except for H_3LI (0.4). This deviation is probably caused by a systematic underestimation of the standard deviations in that structure.

The two halves of the iminodiacetic acid group are crystallographically independent in H_2L, $H_3LF.H_2O$ and H_3LI and can therefore be compared using this method. Since the half-normal probability plots in these cases are not based upon two independent sets of data the original obtained standard deviations were



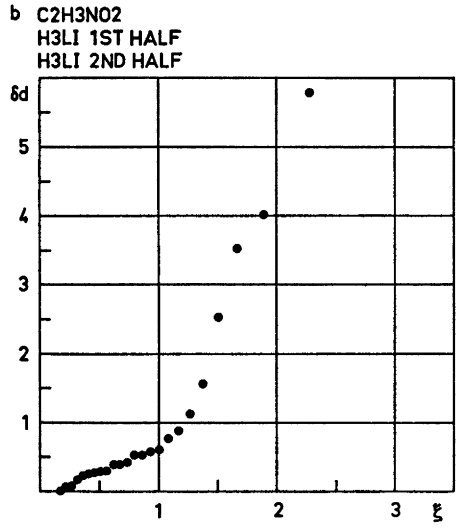


Fig. 3. Half-normal probability plots of the independent halves of the iminodiacetic acid residues in H_2L, $H_3LF.H_2O$ and H_3LI.

multiplied by the factor s obtained by the comparison with H_3LCl for this particular structure (Fig. 2). Interatomic distances less than 4.65 Å have been used. The results are shown in Fig. 3. For H_2L a non-linear plot is expected because of the different states of protonation of the carboxylic groups. In H_3LI the points falling off the straight line represent a bond angle, the conformation across the C–C bonds and the conformation involving a hydrogen atom in the methylene group. In $H_3LF.H_2O$ the plot is judged as linear.

A detailed study of the half-normal probability plots clearly shows that the differences in the bond distances (except C–O) are normally distributed and that such a conclusion is untrue for the bond angles.

BOND DISTANCES AND ANGLES

The N–C and C–C bonds. From the half-normal probability plots it was concluded that the N–C and C–C distances are independent of the crystallographic environment. The values of these distances in $H_3LF.H_2O$ are not significantly different from the values obtained in the other structures, in spite of the fact that the data set was collected at −100 °C for this compound. Therefore it seems reasonable to assume that differences in the iminodiacetic acid residue in $H_3LF.H_2O$ as compared to the other structures should not primarily be attributed to the temperature difference.

The average values of the bond distances and angles are collected in Table 1. Only the N–C and C–C distances are assumed to be of general validity in different types of iminodiacetic acid structures. The N–C distance is the same as the average value [1.488(2) Å] calculated from 13 neutron structure determinations of α-amino acids.[13] The C–C distance is not significantly different from the average value found in oxydiacetates [1.515(3) Å].[14] Several authors have pointed out that bond lengths should vary with hybridization.[15–17] The carbon atoms in the C–C bond are sp^3 and sp^2 hybridized in the iminodiacetates and oxydiacetates, so that a C–C distance slightly smaller than the pure aliphatic C–C single bond length found in gas phase (1.533 Å)[18] is to be expected in these compounds.

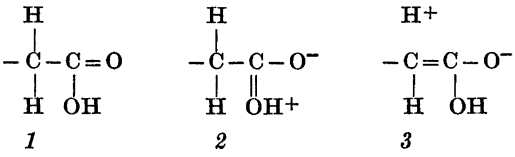
Table 1. Bond distances and angles in the iminodiacetic acid residue. Only the N–C and C–C distances are assumed to be of general validity.

	Distance/Å		Angle/°
N–C	1.488(3)[a]	C–N–C	114.0
C–C	1.505(3)	N–C–C	109.6
C–OH	1.315	C–C–O	110.8
C=O	1.200	C–C=O	123.5
		O–C=O	125.7

[a] Figures within parentheses represent standard deviations in the least significant digit.

Bond angles. A majority of the points falling off the straight lines in the half-normal probability plots represent bond angles. Therefore these quantities are dependent on the environment and cannot be assigned same values in different structures. The same situation was found for the oxydiacetates.[14] This is not surprising since angular deformation constants are much smaller than those for bond lengths and hence the variation of bond angles will be greater than the range of values for bond lengths.

The carboxylic groups. The dimensions of the carboxylic group are dependent on the environment, why the distances given in Table 1 cannot be used uncorrected in other structures. In terms of valence bond theory the following structures can be used to describe the unionized carboxyl group:



1 *2* *3*

Bond length – bond number curves were derived with the use of the relation

$$r_x = r_1 - (r_1 - r_2) \times 1.84(x-1)/(0.84x + 0.16)$$

where x is the bond number, r_x is the actual bond length and r_1 and r_2 are the pure single and double bond lengths, respectively.[19] For the C–O bond Hahn has proposed the value 1.185 Å for r_2 and the value 1.395 Å as an upper limit for r_1.[20] A value of r_1 was found by holding r_2 constant at 1.185 Å and calculat-

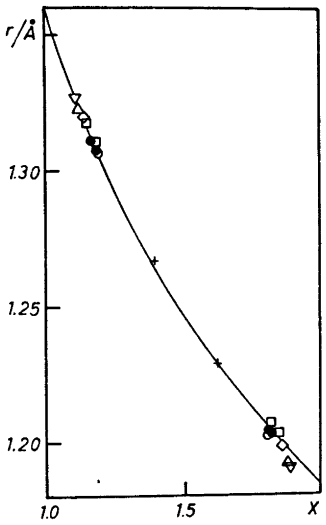


Fig. 4. A Bond length-bond number curve for C–O bonds calculated with $r_1 = 1.360$ Å and $r_2 = 1.185$ Å. The symbols are experimental points and refer to H_2L, carboxylic group (○), H_2L, carboxylate group (+), $H_3LF.H_2O$ (□), H_3LCl (△), D_3LCl (▽), H_3LBr (◇) and H_3LI (●).

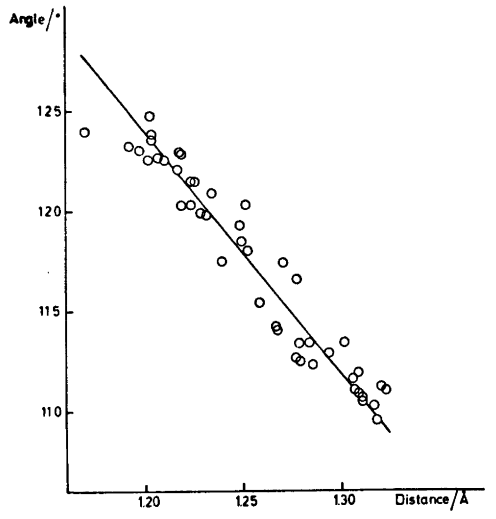


Fig. 5. The graph illustrates the C–C–O angle as a function of the corresponding C–O distance in some compounds containing the C–C(=O) group. The data are taken from Refs. 3–7, 28–31, Thomas, J. O.: *Acta Crystallogr. B 28* (1972) 2073, *B 29* (1973) 1767, Nahringbauer, I.: *Acta Crystallogr. 23* (1967) 956, *Acta Chem. Scand. 23* (1969) 1653, and Abdel Hady, S., Nahringbauer, I. and Olovsson, I. *Acta Chem. Scand. 23* (1969) 2764.

ing x for different values of r_1. The initial value was 1.395 Å and this was decreased in steps of 0.005 Å. If there is no hyperconjugation (structure *3*) the total bond number in C=O should be three. A pure single bond length of 1.360 Å results in an average bond number close to three in the investigated compounds and was therefore accepted as the value for r_1. Fig. 4 shows a bond length bond number curve calculated with $r_1 = 1.360$ Å and $r_2 = 1.185$ Å and it is seen that the contribution of form *2* is only about 10–20 %. The points from the carboxylate group in H_2L have been included in Fig. 4 and it would seem that the same relation is valid for the ionized carboxylate group as for the un-ionized carboxylic group. Hahn [20] has suggested that there is a linear relationship between bond-number and bond-angles C–C–O in carboxylic acids, with end points 109.5 and 125.25° for C–C–O and C–C=O, respectively. This is not found in the present investigation and it is not to be expected according to the previous statement that bond angles are more sensitive to packing forces than bond distances. However, there is roughly a linear relationship between the bond angle C–C–O and the bond distance C–O (Fig. 5), but the spread of the points is so large that the error in the bond angle may be as large as 4° if that curve is used for predicting bond angles from bond lengths. Although the C–O and C=O bond lengths depend upon the environment a χ^2 test shows, with a probability of 0.95, that their sum does not. The average value of the sum, 2.515(1) Å, is not significantly different from the value 2.52 Å found by Speakman *et al.*[21]

A contribution of structure *3* would imply a shorter C–C bond, a longer C–O^- bond and a smaller O–C–O angle compared to compounds where form *3* has zero weight. All these three criteria must be fullfilled if structure *3* is of any importance. The average value of the O–C–O angle is 125.7° in the investigated compounds, which is larger than the expected value 125.25°. The short C–C bond length found is in agreement with the different state

Table 2. Dihedral angles (°) about the C–C bond. The angle is defined as positive, if going from C–O to Cα–N one describes a right-handed screw.

Compound	N–Cα–C–O″	N–Cα–C=O′
H_3L	−169.5	17.3
	−177.1[a]	4.7[a]
$H_3LF.H_2O$	−173.1	6.7
	179.5	0.0
$[Pr_2(H_2O)_4(HL)_2L]Cl_2.3H_2O$	178[a]	−2[a]
	175[a]	−5[a]
	−162[a]	12[a]
Compound	N–Cα–C–O″	N–Cα–C=O′
H_3LCl	180.0	0.0
D_3LCl	180.0	0.0
H_3LBr	180.0	0.0
H_3LI	−177.4	2.6
	180.0	0.0
$[NdL(H_2O)_3]Cl$	158[a]	−36[a]
	153[a]	−24[a]

[a] Carboxylate group.

Table 3. Deviations (Å) from the least-squares plane through the group N–Cα–C(=O′)–O″. This group is situated in a mirror plane in H_3LCl, D_3LCl and H_3LBr.

Compound	N	C	C	O′	O″
H_3L	−.112	0.165	0.027	0.035	−.081
	−.024	0.036	−.004[a]	0.011[a]	−.015[a]
$H_3LF.H_2O$	0.043	−.060	−.011	−0.016	0.040
	−.003	0.003	0.003	0.000	−.003
H_3LI	0.000	0.007	−.002	−.003	−.002
	0.025	−.032	−.004	−.009	0.020
$[NdL(H_2O)_3]Cl$	0.193	−.261	0.034[a]	0.134[a]	−.101[a]
	−.181	0.218	0.055[a]	−.156[a]	0.064[a]
$[Pr_2(H_2O)_4(HL)_2L].$	0.035	−.040	−.018[a]	−.010[a]	0.033[a]
$Cl_2.3H_2O$	0.030	0.002	−.039[a]	0.023[a]	−.016[a]
	−.124	0.055	0.136[a]	0.046[a]	−.113[a]

[a] Carboxylate group.

of hybridization of the carbon atoms. Therefore it is concluded that form *3* does not contribute to the resonance energy of the carboxylic group.

CONFORMATION

The dihedral angles about the Cα–C bond is a suitable parameter if one wants to describe the relative positions of N and O in N–Cα–C(=O′)–O″–H″. The atoms are designated according to IUPAC recommendations.[22] Dihedral angles collected from the previously discussed structures and from two rare earth compounds [23,24] are given in Table 2. It is evident that the group N–Cα–C(=O)–O has a

preferred planar arrangement in the solid state, independent of the state of protonation of the carboxylic group. The largest deviation from planarity (Table 3) is found in $[NdL(H_2O)_3]Cl$, where this group forms a chelate with the metal ion. The shape of the coordination polyhedron in this compound is determined by three factors (if one disregards restrictions imposed by the packing arrangement of the polyhedra): (a) the C_{4v} or D_{3h} configuration, adopted by nine identical monodentate ligands; (b) a preferred planar arrangement of the group $N-C\alpha-C(=O)-O$; (c) a strong coordination bond between the nitrogen and the metal ion. The resulting polyhedron might be a compromise between the influence from these factors. Therefore the chelates in $[NdL(H_2O)_3]Cl$ are formed at the expence of an energetically more stable conformation of the organic ligand.

The double-bonded oxygen in the carboxylic group is in a position *cis* to the nitrogen in all studied structures. The two carbon-oxygen distances in the carboxylate group in H_2L are significantly different, with the oxygen participating in the shortest C–O bond *cis* to the nitrogen.

The group $C\beta-C\alpha-C(=O')-O''-H''$ has also been found to have a preferred planar arrangement in the solid state.[25–27] This feature has often been ascribed to non-bonded interactions $C\beta\cdots O$. Non-bonded distances $N\cdots O'$ and $(N-)H\cdots O'$ obtained in this study are given in Table 4. The average value of the $N\cdots O'$ distance, 2.69(4) Å, may be compared with the calculated non-bonded distances for the two planar structures. The parameters in Table 1 imply a $N\cdots O$ distance of 2.70 Å with O′ in a position *cis* to the nitrogen and 2.47 Å with O″ in this position. On the assumption that the planar conformation is stabilized by non-bonded interactions the potential energy curve describing the interactions $NH_2\cdots O$ should have a minimum around 2.7 Å. This relatively small value is not surprising since it is well known that intramolecular non-bonded distances may be smaller than the sum of the corresponding van der Waals radii. In one case (H_2L: $H\cdots O'$ = 2.16 Å) there might be a contribution to the potential well from an intramolecular hydrogen bond. However, the group $R-C\alpha-C(=O)-O$, where R = O or S, is also planar with O′ in a position *cis* to R.[28–33] Since these residues cannot be stabilized by intramolecular hydrogen bonding, one may assume that the contribution of this factor is of minor importance in stabilizing the planar iminodiacetic acid residue.

From the preceding discussion it may be concluded that a planar arrangement of the group $-R-C-C(=O)-O$, seems to be a general feature for a great variety of R. Furthermore, if the C–O bonds have different lengths, the arrangement with O′ *cis* to R is the preferred one.

I am indebted to Dr. Jörgen Albertsson for useful discussions and many valuable suggestions. This work is part of a research project supported by the Swedish Natural Science Research Council.

Table 4. Non-bonded distances (Å) $N\cdots O'$ and $(N-)H\cdots O'$ in the studied structures. H is the hydrogen atom in the NH_2 group, which is closest to O′.

Compound	N···O′	(N–)H···O′	Compound	N···O′	H···O′
H_2L	2.660(3)	2.55(2)	H_3LI	2.75(1)	2.4(1)
	2.675(2)	2.16(2)		2.68(1)	2.3(1)
$H_3LF.H_2O$	2.744(3)	2.60(2)	$[NdL(H_2O)_3]Cl$	2.74(3)	
	2.693(2)	2.60(3)		2.72(4)	
H_3LCl	2.660(3)	2.55(2)	$[Pr_2(H_2O)_4(HL)_2L]Cl_2.$	2.73(3)	
D_3LCl	2.651(8)	2.49(3)		2.65(3)	
H_3LBr	2.666(4)	2.60(3)	$3H_2O$	2.60(3)	

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Phosphoramides. I. Hexamethylphosphoric Triamide as a Reagent in a New Synthesis of 2,4-Bis(dimethylamino)quinolines

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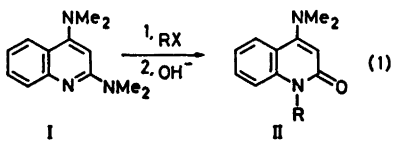
6-Methyl-, 6-methoxy-, 8-methoxy-, 6-chloro-, 7,8-dimethyl-, and unsubstituted 2,4-bis(dimethylamino)quinoline have been prepared in 23 – 30 % yield by heating the appropriate aniline and diethyl malonate in hexamethylphosphoric triamide (HMPT) to reflux temperature.

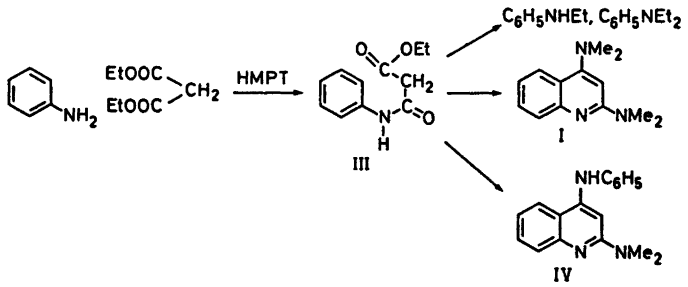
The carbostyrils II have been prepared[1] by heating 2,4-bis(dimethylamino)quinoline (I) with a primary alkyl halide, and treating the resulting 1-alkyl quaternary ammonium compound with dilute aqueous alkali, eqn. 1. They are claimed to have analgesic, antiinflammatory, and antipyretic activity, and may be used



in the treatment of rheumatic disorders.[1] Unfortunately, I is only available by a multistep synthesis; it was prepared by heating the corresponding 2,4-dichloroquinoline in an ethanolic solution of dimethylamine. This paper states that 2,4-bis(dimethylamino)quinolines are easily available by a one step synthesis from very simple starting materials.

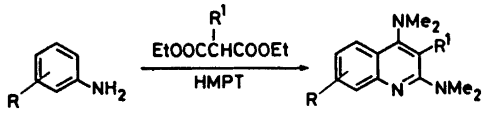
RESULTS AND DISCUSSION

Recently, HMPT has been used as a cyclization reagent in a new synthesis of 2-dimethylaminoquinolines.[2] It was therefore conceivable, that the quinoline I could be produced by heating the amide III in HMPT. I and not the corresponding 4-hydroxycarbostyril should be expected, because HMPT is known to replace potential hydroxy-groups with dimethylamino-groups.[3] As carboxamides may be prepared by heating appropriate esters with amines, it was thus attempted to prepare I simply by heating aniline and ethyl malonate in HMPT. For those anilines investigated in which steric hindrance could be excluded, the corresponding 2,4-bis(dimethylamino)quinolines were produced in yields of 23 – 30 % (Table 1). Steric hindrance seems to play an important role



Scheme 1.

Table 1. Yields of 2,4-bis(dimethylamino)quinolines.



R	R^1	Quinoline (%)
H	H	30
p-Me	H	25
p-OMe	H	26
o-OMe	H	25
p-Cl	H	29
2,3-di-Me	H	23
2,5-di-Me	H	< 1
H	Et	5

in the success of the ring closure reaction. Thus, the yield of the corresponding quinoline was only 5 %, when diethyl ethylmalonate was heated with aniline in HMPT. A methyl group in the 5-position of the aniline ring gave rise to almost complete steric hindrance, so that in the reaction of 2,5-dimethylaniline the yield of the corresponding 2,4-bis(dimethylamino)quinoline was estimated to be less than 1 % and it was in fact not isolated in the pure state. Steric hindrance is evident in the case of 2,5-dimethylaniline, as the isomeric 2,3-dimethylaniline produced the corresponding quinoline in a yield of 23 %. These findings are also in accordance with the general rule of electrophilic aromatic substitution saying that a third substituent does not enter the 2-position of 1,3-disubstituted benzenes.

In all the investigated reactions of anilines with diethyl malonate in HMPT at reflux temperature, a lower boiling fraction could be isolated. This fraction was, in the case of aniline, separated by preparative TLC, and *N*-ethyl- and *N*,*N*-diethylaniline could be isolated in pure state, In fact for all other anilines the NMR spectra of the low boiling fractions also showed a mixture of the corresponding *N*-ethyl- and *N*,*N*-diethylanilines. The alkylating power of diethyl malonate in HMPT at high temperature was confirmed by heating *N*-methylaniline in this medium at reflux temperature, whereupon *N*-ethyl-*N*-methylaniline was produced in 67 % yield. In the reactions of anilines with diethyl malonate in HMPT a very high boiling fraction of a glassy substance was isolated in all cases. When the cyclization was carried out with aniline the glassy substance could be induced to crystallize and was identified as 4-anilino-2-dimethylaminoquinoline (IV). Similarly, crystalline 4-(*p*-anisidino)-2-dimethylamino-6-methoxyquinoline was isolated, when *p*-anisidine was reacted with diethyl malonate and HMPT.

In order to increase the yield of the quinoline I, attempts were made using methyl instead of ethyl malonate, and also the malonic ester and aniline were heated in HMPT in the ratios 1:2, 1:1, and 2:1, but in all cases 25 – 30 % yields of I were obtained. It was then expected that the quinoline I would be produced in high yield by heating in HMPT the amide III, which was thought to be an intermediate in the reaction. However, the quinoline I was formed in the same yield (29 %) as before, but a mixture of *N*-ethyl- and *N*,*N*-diethylaniline, and the quinoline IV were also isolated. This strongly indicated that very fast equilibria exist at the prevalent reaction temperature of ~230 °C, so that the use of aniline and malonic ester or the amide III as starting materials in the quinoline synthesis has almost no effect on the product distribtution.

EXPERIMENTAL

In all experiments commercial HMPT (Pierrefitte-Auby) was used. The microanalyses were performed by the Microanalytical Laboratory, University of Copenhagen. IR-spectra were recorded on a Perkin Elmer Model 457, UV spectra on a Beckmann ACTA III, and NMR spectra on a Jeol C-60HL spectrometer.

2,4-Bis(dimethylamino)quinoline. Aniline (9.3 g), ethyl malonate (16 g) and HMPT (50 ml) were heated in a distillation flask on a silicone oil bath at 210 °C for 1 h and then at 250 °C for 2. During the reaction EtOH distilled off. The residue, allowed to cool to 100 °C, was then poured into ice and 200 ml 2 N NaOH, and extracted with 4 × 100 ml ether. The organic phase was washed with 50 ml H_2O, dried over K_2CO_3, and the ether was stripped off. Distillation gave:

1. 1.4 g of a fraction, b.p. 80 – 100 °C/10 mmHg. From this fraction were isolated, by PLC using siliga gel as the supporting material and elution with ether-light petroleum (1:9), *N*-ethylaniline, $n_D^{20} = 1.5534$, lit.[4] $n_D^{20} = 1.55397$, and *N*,*N*-diethylaniline, $n_D^{20} = 1.5412$, lit.[4] $n_D^{20} = 1.5418$. Both compounds gave NMR

spectra identical with spectra of authentic samples.

2. A fraction, b.p. 100 – 150 °C/0.05 mmHg, which on subsequent recrystallization from petroleum ether gave 6.5 g (30 %) of 2,4-bis(dimethylamino)quinoline, m.p. 77 – 78 °C, lit. m.p. 78 – 78.5 °C [5] and 68 – 70 °C;[1] NMR δ ($CDCl_3$): 2.83 (s, 6 H), 3.08 (s, 6 H), 6.10 (s, 1 H), 6.8 – 7.9 (m, 4 H).

3. A fraction, b.p. 180 – 220 °C/0.03 mmHg, which crystallized by treatment with boiling benzine (80 – 100 °C) to give 1,9 g of 4-anilino-2-dimethylaminoquinoline, m.p. 121 – 125 °C (subl.); NMR δ ($CDCl_3$): 3.05 (s, 6 H), 6.48 (s, 2H), 6.8 – 7.8 (m, 9 H); UV (EtOH): λ_{max} = 243 nm (log ε = 4.51) 320 nm (log ε = 4.08). (Found: C 77.40; H 6.63; N 16.02. $C_{17}H_{17}N_3$ requires: C 77.53; H 6.51; N 15.96).

2,4-Bis(dimethylamino)-6-methoxyquinoline. *p*-Anisidine (12.3 g), ethyl malonate (16 g), and HMPT (50 ml) were heated as above and the mixture was then worked up in a similar way. Distillation gave:

1. A fraction, b.p. 120 – 180 °C/0.05 mmHg, which on subsequent recrystallization from petroleum ether gave 6.4 g (26 %) of the title compound, m.p. 96 – 98 °C; NMR δ ($CDCl_3$): 2.91 (s, 6 H), 3.15 (s, 6 H), 3.86 (s, 3 H), 6.25 (s, 1 H), 6.9 – 7.3 (m, 2 H), 7.4 – 7.7 (m, 1 H); UV (EtOH): λ_{max} = 240 nm (log ε = 4.58), 267 nm (log ε = 4.45), 360 nm (log ε = 3.67). (Found: C 68.77; H 8.39; N 17.06. $C_{14}H_{19}N_3O$ requires: C 68.54; H 7.81; N 17.13).

2. A fraction b.p. 220 – 270 °C/0.05 mmHg, which crystallized by treatment with boiling benzine (80 – 100 °C) to give 2.3 g of 4-(*p*-anisidino)-2-dimethylamino-6-methoxy-quinoline, m.p. 144 – 148 °C; NMR δ ($CDCl_3$): 3.00 (s, 6 H), 3.77 (s, 6 H), 6.19 (broad singlet, 2 H), 6.6 – 7.3 (m, 6 H), 7.4 – 7.7 (m, 1 H); UV (EtOH): 240 nm (log ε = 4.52), 315 nm (log ε = 3.99), 340 nm (sh). (Found: C 70.10; H 7.12; N 12.96. $C_{19}H_{21}N_3O_2$ requires: C 70.56; H 6.55; N 13.00).

2,4-Bis(dimethylamino)-8-methoxyquinoline. *o*-Anisidine (12.3 g), ethyl malonate (16 g), and HMPT (50 ml) were heated as above and the mixture was then worked up in a similar way. Distillation 100 – 200 °C/0.04 mmHg and subsequent recrystallization from petroleum ether gave 6.2 g (25 %) of the title compound, m.p. 104 – 106 °C; NMR δ ($CDCl_3$): 2.90 (s, 6 H), 3.19 (s, 6 H), 3.97 (s, 3 H), 6.20 (s, 1H), 6.7 – 7.5 (m, 3H); UV (EtOH): 263 nm (log ε = 4.49), 346 nm (log ε = 3.67). (Found: C 68.40; H 7.98; N 16.98. $C_{14}H_{19}N_3O$ requires: C 68.54; H 7.81; N 17.13).

2,4-Bis(dimethylamino)-6-methylquinoline. *p*-Toluidine (10.7 g), ethyl malonate (16.0 g), and HMPT (50 ml) were heated as above and the mixture was then worked up in a similar way. Distillation 100 – 170 °C/0.1 mmHg and subsequent recrystallization from light petroleum gave 5.8 g (25 %) of the title compound m.p. 75 – 77 °C; NMR δ ($CDCl_3$): 2.42 (s, 3 H), 2.90 (s, 6 H), 3.14 (s, 6 H), 6.18 (s, 1 H), 6.9 – 7.6 (m, 3 H); UV (EtOH): 247 nm (log ε = 4.52), 302 nm (log ε = 3.75), 348 (log ε = 3.76). (Found: C 73.15; H 7.93; N 18. 26. $C_{14}H_{19}N_3$ requires: C 73.52; H 8.35; N 18.33).

7,8-Dimethyl-2,4-bis(dimethylamino)quinoline. 2,3-Dimethylaniline (12.1 g), ethyl malonate (16.0 g), and HMPT (50 ml) were heated as above, and the mixture was then worked up in a similar way. Distillation 100 – 200 °C/0.1 mmHg and subsequent recrystallization from petroleum ether gave 5.6 g (23 %) of the title compound m.p. 116 – 117 °C; NMR δ ($CDCl_3$): 2.39 (s, 3 H), 2.60 (s, 3 H), 2.86 (s, 6 H), 3.13 (s, 6 H), 6.14 (s, 1 H), 6.89 (d, J = 9 Hz, 1 H), 7.53 (d, J = 9 Hz, 1 H); UV (EtOH): 255 nm (log ε = 4.58), 345 nm (log ε = 3.71). (Found: C 73.80; H 8.83; N 17.18. $C_{15}H_{21}N_3$ requires: C 74.03; H 8.70; N 17.27).

2,4-Bis(dimethylamino)-5,8-dimethylquinoline. 2,5-Dimethylaniline (12.1 g), ethyl malonate (16 g), and HMPT (50 ml) were heated as above and the mixture was worked up in a similar way. For a fraction (1.3 g), b.p. 145 – 150 °C/0.05 mmHg an NMR spectrum was obtained with big singlets at 2.60 and 3.05, and a small singlet at δ 6.15 ($CDCl_3$), which should be expected for the title compound. From this fraction 0.4 g of an oil with the same characteristic NMR signals as the title compound was obtained by preparative TLC, using acetone-ether (2:3) for eluation and silica gel as supporting material. As this fraction also was very impure no further purification was attempted.

6-Chloro-2,4-bis(dimethylamino)quinoline. *p*-Chloroaniline (12.8 g), ethyl malonate (16.0 g), and HMPT (50 ml) were heated as above, and the mixture was then worked up in a similar way. Distillation 100 – 200 °C/0.1 mmHg and subsequent recrystallization from petroleum ether gave 7.3 g (29 %) of the title compound m.p. 106 – 107 °C; NMR δ ($CDCl_3$): 2.87 (s, 6 H), 3.12 (s, 6 H), 6.15 (s, 1 H), 7.1 – 7.8 (m, 3 H); UV (EtOH): 244 nm (log ε = 4.43), 274 nm (log ε = 4.42), 355 nm (log ε = 3.74). (Found: C 62.60; H 7.07; N 16.88. $C_{13}H_{16}ClN_3$ requires: C 62.52; H 6.46; N 16.86).

2,4-Bis(dimethylamino)-3-ethyl-quinoline. Aniline (9.3 g), diethyl ethylmalonate (18.8 g), and HMPT (50 ml) were heated as above, and the mixture was then worked up in a similar way. Distillation 100 – 160 °C/0.05 mmHg gave a fraction which was subjected to separation on a silica gel column. Elution with ether gave 1.2 g (5 %) of the title compound, which was then distilled at 0.05 mmHg to give the analytically pure compound; NMR δ ($CDCl_3$): 1.05 (t, J = 7Hz, 3H), 2.80 [q, J = 7Hz; this signal overlaps the singlets of the two dimethylamino groups at 2.83 and 2.97 (a total of 14 H)], 6.5 – 7.9 (m, 4 H); UV (EtOH): 252 nm (log ε = 4.37), 327 nm (log ε = 3.85). (Found: C 74.10; H 8.72; N 16.40. $C_{15}H_{21}N_3$ requires; C 74.03; H, 8.70; N, 17.27).

N-*Ethyl*-N-*methylaniline*. *N*-Methylaniline (10.7 g), ethyl malonate (16.0 g), and HMPT (50 ml) were heated in a distillation flask on a silicone oil bath (250 °C) for 3 h and a low boiling material was allowed to distill off. The residue was allowed to cool to 100 °C, and was then poured into ice and 200 ml 2 N NaOH and extracted with 4 × 100 ml ether. The organic phase was washed with 50 ml H_2O, dried over K_2CO_3, and the ether was stripped off. Distillation 82 – 83 °C/11 mmHg, gave 9.0 g (67 %) of the title compound, $n_D^{20} = 1.5459$ lit.[6] $n_D^{20} = 1.5450$; NMR δ ($CDCl_3$): 0.98 (t, $J = 7$ Hz, 3 H), 2.70 (s, 3 H), 3.22 (q, $J = 7$ Hz, 2 H), 6.3 – 7.3 (m, 5 H).

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2-Butyl Propenyl Disulfides from Asafetida: Separation, Characterization, and Absolute Configuration*

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2-Butyl propenyl disulfide, the major volatile sulfur compound of asafetida oil, is separated into *E*- and *Z*-isomers by pressure liquid chromatography; the *E*,*Z*-ratio is 7:3.

(*R*)-Configuration of the predominant, levorotatory enantiomers and an enantiomeric purity of about 75 % of the natural disulfides in the present study follow from a described, enantiospecific synthesis of a 47:53 mixture of levorotatory *E*- and *Z*-isomers of (*R*)-2-butyl propenyl disulfide with an enantiomeric purity supposedly exceeding 93 %, departing from (*S*)-2-butanol.

A possible terpenoid origin of the disulfides is briefly discussed.

Asafetida, the oleogum resin derived from certain *Ferula* species, such as *F. alliacea* Boiss., *F. foetida* Regel, and *F. narthex* Boiss., native to Iran and Afghanistan, contains volatile sulfur compounds, the composition, quantities and properties of which have been studied in connexion with food problems (*cf.* Ref. 1 and literature cited therein). The characteristic smell of asafetida is largely due to volatile disulfides with a long chemical history. In 1936, Mannich and Fresenius [2] identified the levorotatory, major disulfide, $C_7H_{14}S_2$, previously described by Semmler,[3] as 2-butyl propenyl disulfide *1*, of unspecified stereochemistry. Recently, a similar disulfide fraction, obtained from an asafetida of Afghan origin ('Patheni Hing'), was established as a levorotatory mixture of the *E*- and *Z*-isomers of *1*, with the former predominating.[4,5] A second, and minor, constituent $C_7H_{14}S_3$, was identified, here [5] and elsewhere,[4] as an *E*,*Z*-mixture of the disulfide *2*. Finally, a third sulfur component, $C_8H_{16}S_3$, was recognized as the allylic disulfide *3*, again occurring as a mixture of diastereomers.[4] Chemical syntheses of racemic *E*,*Z*-mixtures of *1*,[6] as well as of *2* and *3*,[7] have been recently reported.

Et-CH(Me)-S-S-CH=CH-Me
1

MeS-CH(Et)-S-S-CH=CH-Me
2

Et-CH(Me)-S-S-CH_2CH=CH-Me
3

Marked differences in the optical rotation of the major disulfide *1* obtained from gum resins of different origin, combined with uncertainty as to its biogenetic derivation, chirality, and propensity to undergo stereomutation, prompted the present, more detailed investigation, aimed at the separation of the various stereoisomers of *1*. The study was conducted on the levorotatory major fraction of the essential oil of asafetida ('Hing oil'), isolated and separated by inverted dry column and liquid chromatography. The distilled fraction (b.p. 105 °C/30 mmHg), consisting, according to GLC and ^{1}H NMR-spectroscopy, of 62 %

* To be regarded as Part III of a series of papers on asafetida from the Indian laboratory; Part II: Raghavan, B., Abraham, K. O., Shankaranarayana, M. L. and Natarajan, C. P. *Flavour Ind.* *5* (1974) 179.

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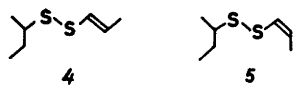
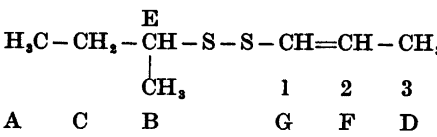


4 *5*

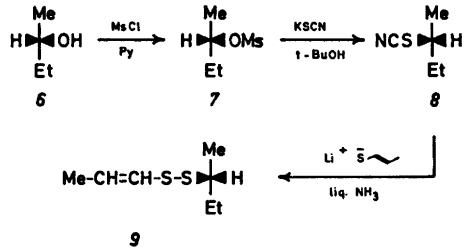
of the *E*-isomer *4*, 28 % of the *Z*-isomer *5*, and 10 % of a third compound, probably a monosulfoxide, exhibited physical data, including optical rotation, which differed significantly from those previously reported for similar fractions.[2-4] No isomerization was observable on distillation or GLC-analysis. Upon pressure liquid chromatography, the mixture was separated into virtually homogeneous *E*-isomer *4*, and a specimen of the *Z*-isomer *5*, containing *ca.* 10 % of *4* as the sole contaminant. Physical data and ^{1}H NMR-characteristics for the isomers are summarized in Table 1.

In order to establish the chirality and optical purity of *4* and *5*, a chemical synthesis of a mixture of the two was performed, departing from (*S*)-2-butanol and following the route designed for preparation of the diastereomeric

Table 1. Physical data and ^{1}H NMR characteristics for the *E*- and *Z*-isomers of 2-butyl propenyl disulfide (*4* and *5*).



racemates.[6] (*S*)-2-Butanol *6*, with an enantiomeric purity > 93 %, was converted into the mesylate *7* in a yield of 80 %, *i.e.* considerably higher than the 50 % previously reported for the production of the enantiomeric mesylate under different conditions.[8] In a carefully conducted displacement reaction (see Experimental), accompanied by inversion,[9] the mes-



ylate *7* afforded a 65 % yield of distilled (*R*)-2-butyl thiocyanate *8*, a sensitive compound undergoing partial isomerization to the isothiocyanate as well as further decomposition on storing. Its chemical and enantiomeric purity appears superior to that of a previously described specimen.[9] Conversion of *8* into a mixture of the *E*- and *Z*-isomers of (*R*)-2-butyl propenyl disulfide *9* was accomplished essentially as described for the racemic series.[6] According to GC-analysis, the *E*,*Z*-ratio of the synthetic product was 47:53, significantly smaller than that prevailing in the naturally derived mixture (*ca.* 70:30). The specific rotation of a 47:53 *E*:*Z*-mixture of the latter, with a value of −36°, calculated on the basis of the specific rotation of the individual isomers (Table 1), was significantly lower than the −46°, observed for the synthetic mixture with the same composition. On the reasonable assumptions (i) that the enantiomeric purity of the (*S*)-2-butanol used in the synthesis in fact exceeds 93 %, and (ii) that all steps in the sequence *6*→*9* proceed with complete stereochemical integrity, an expected maximum rotation of −49° for the mixture permits calculation of the specific rotation of the homogeneous (*R*),*E*- and (*R*),*Z*-2-butyl propenyl disulfides, as −52 and −46° (in EtOH), respectively. Hence, the enantiomeric purity of the natural *E*- and *Z*-isomers in the present study did not exceed 75 %. Since racemization during isolation can be precluded, the large differences in rotation values for

E-isomer (*4*).
$[\alpha]_D^{20}$ −38.4° (*c* 0.95, EtOH);[a] n_D^{25} 1.5153.[a] (Found: C 51.6; H 8.75: S 39.0. Calc. for $C_7H_{14}S_2$: C 51.8; H 8.69; S 39.5.)
^{1}H NMR ($CDCl_3$): δ 0.98 (A, t, *J* 7 Hz), 1.31 (B, d, *J* 7 Hz), 1.59 (C, m), 1.77 (D, d, *J* 5 Hz), 2.82 (E, m), [b] 5.89 (F, d, *J* 15 Hz), 6.10 (G, d, *J* 15 Hz).

Z-isomer (*5*) (containing 10 % of *4*).
$[\alpha]_D^{20}$ −33.8° (*c* 0.85, EtOH); [a] n_D^{25} 1.516. [a,c] (Found: C 51.6; H 8.96; S 39.0.)
^{1}H NMR ($CDCl_3$): δ 0.98 (A, t, *J* 7 Hz), 1.31 (B, d, *J* 7 Hz), 1.59 (C, m), 1.77 (D, dd, $J_{3,2}$ 7 Hz, $J_{3,1}$ 1.5 Hz), 2.79 (E, m), [b] 5.67 F, 2q, $J_{2,1}$ 9 Hz, $J_{2,3}$ 7 Hz), 6.10 (G, dd, $J_{3,2}$ 9 Hz, $J_{1,3}$ 1.5 Hz).

[a] Reported for natural mixture of *4* and *5*: $[\alpha]_D^{25}$ −15.95° (*c* 1.25, EtOH),[4] n_D^{22} 1.5343.[4] [b] Reported (CCl_4): δ 2.73 (s),[6] and ($CDCl_3$): δ 2.29 (sext.).[4] [c] Determined by extrapolation to 100 % *5*.

2-butyl propenyl disulfide reported in the various asafetida studies [α −12.5° (neat),[3] α −17.62° (neat),[2] and $[\alpha]_D^{25}$ −15.95° (EtOH)[4]], suggest that the enantiomeric composition may vary greatly with the origin of the gum resin.

The above assignment of (*R*)-chirality to the levorotatory *E*- and *Z*-2-butyl propenyl disulfide is in keeping with the previously described formation of levorotatory 2-butanethiol ($[\alpha]_D^{25}$ −14.6°) upon reduction of the naturally derived (−)-disulfide(s) with zinc,[2] combined with the recent finding that (*S*)-2-butanethiol is dextrorotatory, with a reported $[\alpha]_D^{25}$ of +34.44° for a specimen considered enantiomerically homogeneous.[10]

The (*R*)-chirality, predominant in the disulfides of natural origin, renders isoleucine, with its (3*S*)-configuration, an unlikely biogenetic donor of the 2-butyl grouping. By way of conjecture, an *in vivo* derivation from terpenoid progenitors appears conceivable, especially in view of the substantial quantities of monoterpenes, such as α-pinene, β-pinene, and, possibly, ocimene and alloocimene, encountered in this laboratory in the essential oil from asafetida gum resins.[5]

EXPERIMENTAL

Gas chromatography was performed on an F & M 810 instrument equipped with FID and packed columns (3 mm, 180 cm) containing OV-225 on Chromosorb G, or OV-17 on Chromosorb W as the stationary phase; carrier gas: He (20 ml/min); injection port: 250 °C; column temperature: isothermal in the range 90−120 °C. ^{1}H NMR spectra were recorded on a 90 MHz Bruker HX-90E instrument. Microanalyses were performed by Mr. G. Cornali and his staff.

Asafetida samples. TLC and GC analysis of the essential oil, procured from a variety of asafetida specimens of Afghan and Iranian origin, revealed the presence of from four to eight individual compounds. Two of these were disulfides, present in all samples, while a third disulfide only occurred in some of the specimens. The present study was conducted on asafetida samples of the Khadda (Iranian) or Hadda (Afghan) type, containing all three disulfides.

Isolation and fractionation of the volatile oils. The inverted dry column chromatographic technique[11] (silica gel: TLC grade, binder-free, NCL, Poona: 230 g. Distance run: 22 cm, solvent: hexane; mild suction (55−60 mmHg)) served to separate the crude oil (1.5 g), in the course of 3.5 h, into three fractions: a, 100 mg; b, 90 mg; and c, 600 mg. Each of these was further purified by column chromatography (silica gel, hexane), followed by vacuum distillation, b.p.: a, 130 °C/4 mmHg, b, 125 °C/10 mmHg, and c, 105 °C/30 mmHg.

Separation of E- *and* Z-*2-butyl propenyl disulfide of asafetida oil.* By GLC, ^{1}H NMR, and MS, the three fractions were found to consist of the disulfides *1*, *2*, and *3*, yet partly contaminated with each other and with unidentified impurities. The present work is concerned solely with the major disulfide fraction, b.p. 105 °C/30 mmHg, containing the disulfides *1*, n_D^{25} 1.5152 [lit. n_D^{22} 1.5343[4]]; $[\alpha]_D^{20}$ −36.9° (*c* 1, EtOH) [lit. α −12.5° neat;[3] α −17.62° (neat),[2] and $[\alpha]_D^{25}$ −15.95° (*c* 1.25, EtOH)[4]]. The UV-, MS-, and ^{1}H NMR spectra were virtually identical with those reported for an *E*,*Z*-mixture of *1*.[4] On GLC, using the OV-225 column, the mixture appeared as a simple two-component system, present in the ratio 70:30, the minor component having the smallest retention time. However, chromatography on the OV-17 column (100 °C isothermal) revealed the presence of about 10 % of a third component, with a slightly higher retention time than both isomers (see below).

Isolation of virtually homogeneous *E*- and *Z*-isomers from the mixture was achieved by pressure chromatography on silica gel (Merck, prepacked columns, size B, *ca.* 2 ato), with petroleum ether (< 60 °C) as the solvent, the intermediate fractions, containing more than one constituent, being recycled. After one passage, the major component, identified as the *E*-isomer by ^{1}H NMR, was obtained in a purity of > 99 %, whereas the *Z*-isomer, even after a second column passage, contained about 10 % of the *E*-isomer according to GLC. Analytical specimens were produced by distillation at 1 mmHg onto a 'cold finger'. Analytical data, $[\alpha]_D^{25}$, n_D^{25}, and ^{1}H NMR patterns for the two isomers are presented in Table 1. Only minor differences were noted in their ^{13}C spectra.

Combined GLC/MS served to establish the identity of the third component in the mixture as one or more monosulfoxides, probably with the general structure Et−CH(Me)−S−S(O)−CH=CH−Me, supported by a molecular ion at *m/e* 178, accompanied by abundant fragment ions at *m/e* 122 (loss of butene) and at *m/e* 89 ([S(O)−CH=CH−Me]$^+$), corresponding to *m/e* 106 and 73 ([S−CH=CH−Me]$^+$) in the disulfide. To what extent the sulfoxide is a genuine constituent, or an artefact, remains unknown.

(S)-*2-Butyl methanesulfonate 7.* (*S*)-2-Butanol ($[\alpha]_D^{27}$ +12.48° (neat); highest reported rotation: $[\alpha]_D^{27}$ +13.52° (neat)[12]) (4.1 g), produced by resolution of the racemic alcohol, *via* the acid phthalate, with brucine,[12,13] was mixed with methanesulfonyl chloride (7.9 g) and cooled to 0 °C. During 15 min, 40 ml of an-

hydrous pyridine was added and the mixture was kept at 0–5 °C for 2.5 h, before it was poured into ice-cold 10 % HCl (180 ml). The mesylate was extracted with ether. After drying and removal of the solvent, the mesylate (6.76 g ~ 80 %) remained as a colourless oil exhibiting the expected ^{1}H NMR-spectrum. An analytical specimen was produced by distillation, b.p. 39–40 °C at 1 mmHg, n_D^{25} 1.4237, $[\alpha]_D^{20}$ +17.8° (*c* 2.1, $CHCl_3$) (Lit. [8] n_D^{25} 1.4232; $[\alpha]_D^{25}$ −17.35° (neat).

(R)-*2-Butyl thiocyanate 8.* After much experimentation the following synthetic procedure was found useful: (*S*)-2-butyl mesylate (6.7 g) and KSCN (dried at 90 °C) (6.4 g) was heated to reflux in anhydrous *t*-BuOH (100 ml) for 1.5 h. After cooling, hexane (400 ml) was added and the azeotropic *t*-BuOH-hexane mixture (22:78), b.p. 63.7 °C, distilled slowly off from the mixture through a short column, care being taken not to expose the distillation flask to undue heating. When nearly all solvent had been removed, the thiocyanate was distilled, b.p. 23 °C at 1 mmHg. Water (about 3 ml) and ether were added to the distillate, which, according to ^{1}H NMR, still contained some butanol. The organic phase was thoroughly treated with $CaCl_2$ and the ether was removed at 0 °C *in vacuo*, leaving the thiocyanate (3.3 g ~ 65 %) in virtually pure form. An analytical specimen was produced by short-way distillation at 1 mmHg, n_D^{25} 1.4589, $[\alpha]_D^{20}$ −60.0° (*c* 2.4, EtOH) (Found: C 51.7; H 8.00; N 11.6; S 27.5, Calc. for C_5H_9NS: C 52.1; H 7.87; N 12.1; S 27.8). (Lit. n_D^{25} 1.4621; $[\alpha]_D^{20}$ −24.98° (*c* 5.003, EtOH)).[9]

E(R)- *and* Z(R)-*2-Butyl propenyl disulfide 9.* (*R*)-2-Butyl thiocyanate (2.5 g)was brought into reaction, in liquid NH_3, with lithium propenylthiolate, prepared from Li (0.3 g) and ethyl propenyl sulfide [14] (2.2 g, 3:2 ratio of stereoisomers), as described for the racemic series.[6] The distilled reaction product (2.5 g ~ 72 %) contained trace amounts of ethyl propenyl sulfide and 2-butyl thiocyanate in addition to the isomeric disulfides. The former could be removed on pressure liquid chromatography. A specimen, thus purified, consisted of 47 % *E*- and 53 % *Z*-isomer, according to GLC, n_D^{25} 1.5166 (Lit. [6] n_D^{20} 1.5172), $[\alpha]_D^{20}$ −46° (*c* 1.6, EtOH). Control chromatography of the individual *E*- and *Z*-isomers revealed no tendency to equilibration.

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Copper(I) Catalysed Replacement of Bromine by Chloride Ion in Halonitrobenzenes. Part III.* Fluorine and Alkyl Substituted Bromonitrobenzenes

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The investigation of copper(I) catalysed bromine-chlorine exchange reactions of halonitrobenzenes in an aqueous hydrochloric acid—acetic acid medium, kinetically studied in Parts I and II, has now been extended to 2-bromo-3-fluoro- and 2-bromo-5-fluoronitrobenzenes and 2-bromo-3-methyl-, 2-bromo-3-ethyl-, 2-bromo-3-*t*-butyl- and 2-bromo-5-methylnitrobenzenes. It is further confirmed that chiefly steric but also polar effects account for the activation of the substituent in the 3-position. Some deactivating effect of the alkyl groups, probably of polar character, as compared to halogens of comparable size in the 3-position, is indicated. Activation parameters and mechanistic aspects of the results are given.

In Parts I [1] and II [2] of the present series the copper(I) catalysed bromine-chlorine exchange reactions were studied under homogeneous conditions at 80 – 100 °C in an aqueous hydrochloric acid – acetic acid medium with a chloride ion concentration of 5.25 M and with the experimental conditions described in Ref. 1. The dichlorocuprate(I) ion was found to be the catalysing species in the second-order rate of substitution. The observed large accelerating effect of an *ortho* nitro group and the insensitivity of the rate of substitution to the nature of the *para* substituent, together with the observed effect of halogen in the other position *ortho* to the reaction site, and the large negative entropies (– 173 to – 155 J/K mol) and low enthalpies (45 – 51 kJ/mol)[1–3], indicating a high degree of order in the transition state, led to the suggestion of a tetrahedral intermediate, in which the *ortho* nitro group and the halogen to be replaced interact with the dichlorocuprate(I) ion. Similar observations have been made for other copper-assisted reactions, *e.g.* the Ullmann biaryl synthesis,[4] copper-promoted decarboxylation,[5] and reactions between aryl halides and copper(I) salts [6] or copper(I) acetylides.[7] The entropies of activation found for copper-promoted decarboxylation are – 167 to – 109 J/K mol [5] and for reactions between aryl halides and copper(I) acetylides – 264 to – 121 J/K mol.[7]

The rate for copper(I) catalysed bromine-chlorine exchange was found to be three times larger than the rate of the corresponding iodine-chlorine exchange.[3] In view of this result, it does not seem very likely that the rate-determining step involves breaking of the carbon-bromine and the carbon-iodine bonds in the aryl halides.

The study of *ortho* halogen effects, previously comprising iodine, bromine, and chlorine has now been completed with an investigation of fluorine. A similar study of the effect of an *ortho* alkyl group has also been performed in this work, because it seemed important to compare the effect on the exchange rate of an *ortho* substituent with a negative σ_0-value with the effect of the halogens. The alkyl groups selected for examinations were methyl, ethyl, and *t*-butyl. A methyl group has about the same size (van der Waals volume 13.7 cm³/mol) as chlorine and bromine (12 and 15.1

* For Part II, see Ref. 2.

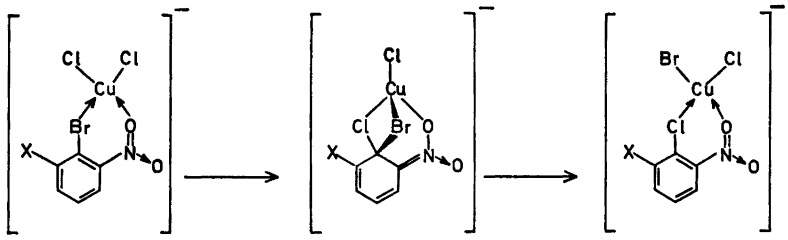

Fig. 1. Bromide – chloride exchange.

cm ^{3}/mol, respectively). The ethyl group has a van der Waals volume of 23.9 cm ^{3}/mol [8] but is not symmetrical and can adjust itself to a more favourable position; its effective size is about the same as that of the methyl group.

RESULTS AND CALCULATIONS

The compounds kinetically studied in this work were 2-bromo-3-fluoronitrobenzene, 2-bromo-5-fluoronitrobenzene, 2-bromo-3-methylnitrobenzene, 2-bromo-5-methylnitrobenzene, 2-bromo-3-ethylnitrobenzene, and in view of the difficulties in its preparation to a limited extent 2-bromo-3-*t*-butylnitrobenzene. The copper(I) catalysed bromine-chlorine exchange of these compounds was studied at 80 – 100 °C.[1] The corresponding 2-chloro compounds were synthesized for calibration purposes.

The exchange mixture was analyzed by GLC, and in the case of 2-bromo-3-fluoronitrobenzene, the starting material and the exchange product 2-chloro-3-fluoronitrobenzene, were isolated together with about 5 % of two by-products which were identified by their mass spectra. The by-products were almost certainly 2-chloro-3-fluoroaniline and 2-bromo-3-fluoroaniline and their identities were indicated by a 3:1 doublet at *m/e* 145 and 147, and by a 1:1 doublet at *m/e* 189 and 191, respectively. Such a reduction of the nitro group has always been observed in the copper(I) catalysed exchange reactions investigated by the present author. Traces at *m/e* 141 revealed fluoronitrobenzene, obviously arising from reductive dehalogenation of 2-bromo-3-fluoronitrobenzene. Reductive dehalogenation has not been noted previously in the bromine-chlorine exchange reactions, but has been detected in the iodine-chlorine exchange mixture.[3] Bacon and Wright [9] observed that in copper(I) catalysed exchange reactions between sodium methoxide and bromo- and iodobenzene derivatives, the iodides were more responsive to reduction than the bromides. Reductive dehalogenation in the presence of a hydrogen donor has also been reported by Fanta [4] and by Nilsson and Björklund.[10] The bromine-chlorine exchange was very slow for 2-bromo-5-fluoronitrobenzene, and the reduction of the nitro group became a competitive reaction. The mass spectrum showed a 1:1 doublet, centered at 190 *m/e*, indicating 2-bromo-5-fluoroaniline.

In the exchange mixture from 2-bromo-3-methylnitrobenzene about 3 % of by-products were detected by GLC, the mass spectra showing a 1:1 doublet at *m/e* 185 and 187 and traces of a 3:1 doublet at *m/e* 141 and 143, due to 2-bromo-3-methylaniline and 2-chloro-3-methylaniline, respectively.

From 2-bromo-5-methylnitrobenzene, about 2 – 3 % of 2-bromo-5-methylaniline was detected in the reaction mixture; the mass spectrum had a 1:1 doublet at *m/e* 185 and 187.

For 2-bromo-3-ethylnitrobenzene the by-products were 2-bromo-3-ethylaniline (2 – 3 %) and traces of 2-chloro-3-ethylaniline as seen from the mass spectra with molecular ions at *m/e* 199 and 201 in the proportions 1:1 and at *m/e* 155 and 157 in the proportions 3:1, respectively.

2-Bromo-3-*t*-butylnitrobenzene was only studied at 98 °C because of the difficulty mentioned above and its poor solubility at lower temperatures in the medium. The compound resulting from the copper(I) catalysed bromine-chlorine exchange of 2-bromo-3-*t*-butylnitrobenzene had to be identified by its

mass spectrum: a 3:1 doublet at *m/e* 213 and 215 was assumed to indicate 2-chloro-3-*t*-butylnitrobenzene.

When the degassed reaction mixture of 2-bromo-3-methylnitrobenzene with copper(I) chloride present was subjected to irradiation at 350 nm for 2 h at about 30 °C, the reduction of the nitro group dominated over the bromine-chlorine exchange, which did not increase its rate compared to an experiment with copper(I) chloride in the dark at 30 °C. When a solution without copper(I) was irradiated, the only observed reaction was a very slight bromine-chlorine exchange and when this solution was stored in the dark neither exchange nor reduction occurred.

The nature of the side reactions, the dehalogenation and the reduction of the nitro group, will be further described in a subsequent paper.[11]

No nuclear coupling products were observed in the reaction mixture within the limits of detection (< 1 %).

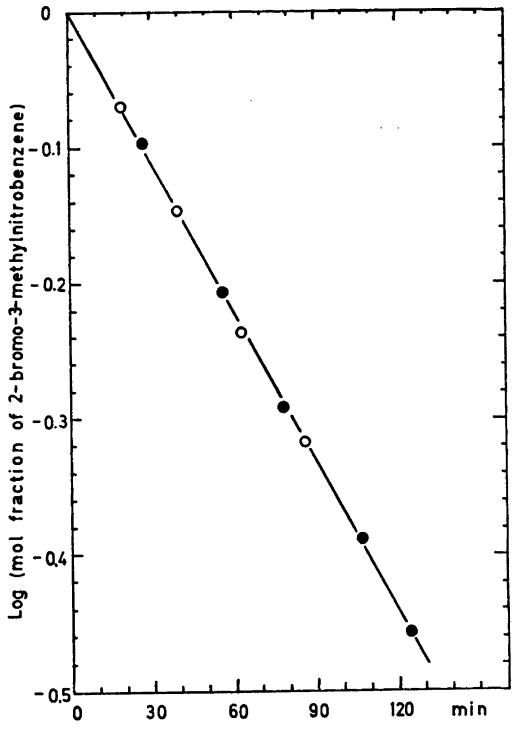

Fig. 2. Two representative runs (● and ○, respectively) of bromine-chlorine exchange in 2-bromo-3-methylnitrobenzene at 98 °C.

The pseudo first-order kinetics are illustrated in Fig. 2 for 2-bromo-3-methylnitrobenzene at 98 °C. The rate constant k_{obs} was calculated from the slope of the lines by the method of least squares and the second-order rate constant k_2 from an estimation of $[CuCl_2^-]$ as described in Ref. 1. The results are summarized in Table 1, together with some values from previous work for comparison.

The activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were calculated from k_2 according to the Eyring equation (see Ref. 1)

$$k_2 = \frac{\varkappa kT}{h} \exp(-\Delta H^{\ddagger}/RT) \exp(\Delta S^{\ddagger}/R)$$

and are presented in Table 2. The transmission coefficient $\varkappa$ is assumed to be unity. The reversibility of the bromine-chlorine exchange was investigated in Ref. 2 and was found not to complicate the interpretation to any marked extent.

DISCUSSION

Steric effects will speed up a reaction if there is a decrease in crowding in going from the reactants to the transition state. The increase of the rate of the exchange is very nicely parallelled by the increasing van der Waals volume of the halogen in the 3-position, as seen from Table 1.

Changes in $\Delta S^{\ddagger}$ are parallelled by changes in $\Delta H^{\ddagger}$ making the resulting effect on reactivity less pronounced. The entropies and enthalpies of activation for the 3-chloro-, 3-bromo-, and 3-iodo-2-bromonitrobenzenes are seen from Table 2 to be very close to one another, while the 3-fluoro compound shows lower values for both parameters.

Steric effects may be small for the fluorine atom with a van der Waals volume of 5.8 cm^3/mol; fluorine requires only slightly more space than a hydrogen atom (van der Waals volume = 3.4 cm^3/mol).[8]

The σ_0-values for chlorine, bromine, and iodine are of the same magnitude, but fluorine displays a lower value due to its larger mesomeric effect, characteristic of second row elements (see Ref. 2). This resonance effect of the fluorine atom is very well demonstrated in the ^1H NMR spectrum of 2-bromo-5-fluoronitrobenzene ($CDCl_3$): δ 7.73 (H-3), 7.19 (H-4),

Table 1. Rate parameters in copper(I) catalysed bromine-chlorine exchange in bromonitrobenzenes. For experimental conditions see Ref. 1.

$[Cu^+]_{tot} = 0.1124$ M, $[Cl^-] = 5.25$ M.

Compound [a]	Temp./ °C [b]	$CuCl_2^-$/ M [c]	k_{obs}/ 10^{-4} s^{-1} [d]	k_2/ 10^{-4} l mol^{-1} s^{-1} [e]	van der Waals [g] volume/cm^3 mol^{-1} of 3-X.
2-Bromo-3-fluoronitrobenzene	79.95	0.056	0.401 ± 0.010	7.13	
2-Bromo-3-fluoronitrobenzene	89.98	0.063	0.662 ± 0.018	10.53	5.8
2-Bromo-3-fluoronitrobenzene	98.05	0.076	1.000 ± 0.010	13.11	
2-Bromo-5-fluoronitrobenzene	89.98	0.063	~0.14 ± 0.006	~2	
2-Bromonitrobenzene [f]	89.98	0.063	~0.28	~4	3.4
2-Bromo-3-chloronitrobenzene [f]	89.98	0.063	1.28	20.4	12.0
2,3-Dibromonitrobenzene [f]	89.98	0.063	1.83	29.2	15.1
2-Bromo-3-iodonitrobenzene [g]	89.98	0.063	2.43	38.7	19.6
2-Bromo-5-chloronitrobenzene [f]	89.98	0.063	~0.22	~3	
2-Bromo-3-methylnitrobenzene	79.95	0.056	0.439 ± 0.000	7.82	
2-Bromo-3-methylnitrobenzene	89.98	0.063	0.878 ± 0.006	13.98	13.7
2-Bromo-3-methylnitrobenzene	98.05	0.076	1.410 ± 0.010	18.49	
2-Bromo-3-ethylnitrobenzene	79.95	0.056	0.393 ± 0.004	6.99	
2-Bromo-3-ethylnitrobenzene	89.98	0.063	0.727 ± 0.005	11.57	23.9
2-Bromo-3-ethylnitrobenzene	98.05	0.076	1.262 ± 0.019	16.54	
2-Bromo-3-*t*-butylnitrobenzene	98.05	0.076	2.602 [h]	34.11	44.3
2-Bromo-5-methylnitrobenzene	89.98	0.063	0.237 ± 0.006	3.78	

[a] 0.562 M. [b] Accuracy ± 0.05. [c] Estimated from the data in Ref. 1. [d] Pseudo first-order rate constant. [e] Second-order rate constant, calculated on substrate and $CuCl_2^-$ as reacting species. [f] Values from Part I.[1] [g] Values from Part II.[2] [h] Values from two runs, which have been combined.

7.58 (H-6). H-6 *ortho* to the fluorine and the nitro group is more shielded than H-3, *ortho* to the bromine atom, and displaced upfield. The corresponding values for 2-bromo-5-chloronitrobenzene: δ 7.72 (H-3), 7.42 (H-4), 7.85 (H-6). The ^1H NMR spectra of 2-fluoro-6-nitroaniline and 2-chloro-6-nitroaniline also demonstrate this resonance effect: δ 7.2 [7.54] (H-3), 6.6 [6.67] (H-4), 7.9 [8.11] (H-5). The values in brackets are for the chloro compound.

2-Bromo-3-fluoronitrobenzene reacts twice as fast as 2-bromonitrobenzene. The difference is probably due to the polar effect of the fluoro atom rather than to a steric effect. 2-Bromo-3-fluoronitrobenzene is five times more reactive than 2-bromo-5-fluoronitrobenzene, probably because of the greater inductive effect of the fluorine atom in the *ortho* position. However, it should be borne in mind that the rates of exchange are influenced by solvation effects, and that the differences in rate are quite small.

Table 2. Activation enthalpies and entropies for 3-substituted 2-bromo-nitrobenzenes.

Compound	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J K^{-1} mol^{-1}
2-Bromo-3-fluoronitrobenzene	34 ± 7 [c]	−211 ± 19 [c]
2-Bromo-3-chloronitrobenzene [a]	45 ± 6	−173 ± 18
2,3-Dibromonitrobenzene [a]	51 ± 4	−155 ± 11
2-Bromo-3-iodonitrobenzene [b]	46 ± 3	−168 ± 10
2-Bromo-3-methylnitrobenzene	49 ± 10	−166 ± 27
2-Bromo-3-ethylnitrobenzene	49 ± 3	−168 ± 8

[a] Values from Part I.[1] [b] Values from Part II.[2] [c] The errors are 3 S.E. from the least-squares method calculations.

The results in Table 1 also demonstrate the deactivating effect of an *ortho* alkyl group on the exchange rate, as compared to an *ortho* halogen atom of comparable size; see above. The electron-releasing effect of an *ortho* alkyl group should make the proposed negatively charged transition state[1] to be energetically less favourable than that for an *ortho* halogen compound.

The polar effects of the methyl and ethyl groups are comparable. This is also true for their steric effects, as discussed above. Both polar and steric effects are reflected in the rates of the bromine-chlorine exchange for 2-bromo-3-ethyl- and 2-bromo-3-methylnitrobenzenes in comparison with the halogen compounds, as seen in Table 1. However, the much larger *t*-butyl group increases the rate of exchange by a factor of two in comparison with methyl and ethyl.

The enthalpies of activation, $\Delta H^{\ddagger}$, and the entropies of activation, $\Delta S^{\ddagger}$, for the 3-methyl- and 3-ethyl-2-bromonitrobenzenes are of the same magnitude as those for the 3-bromo-, 3-chloro- and 3-iodo-2-bromonitrobenzenes; see Table 2.

The mechanisms of copper-promoted reactions, such as the Ullmann biaryl synthesis, copper-catalysed decarboxylation, copper-catalysed halogen exchange and the accompanying reductive dehalogenation, has been the subject of much speculation. Do they have a common intermediate step? It is assumed by Nilsson[12] that the Ullmann biaryl synthesis and copper-promoted decarboxylation proceed *via* a common intermediate, an arylcopper. Reductive dehalogenation often accompanies the Ullmann synthesis, especially in the presence of hydrogen donors. This fact may be taken as evidence for an intermediate arylcopper species.[10]

Cohen *et al.*[13] propose an oxidative addition – reductive eliminition reaction *via* a transient organocopper(III) intermediate in the exchange reactions of aryl halides with salts of copper(I):

$$\mathrm{ArI} + \mathrm{CuCl} \rightarrow \mathrm{ArCuCl^{(III)}I} + 2e^- \quad (1)$$

$$\mathrm{ArCuCl^{(III)}I} + 3\mathrm{CuCl} \rightarrow \mathrm{ArCu} + 2\mathrm{CuCl_2} + \mathrm{CuI} \quad (2)$$

$$\mathrm{ArCu} + 2\mathrm{CuCl_2} \rightarrow \mathrm{ArCu^{(III)}Cl_2} + 2\mathrm{CuCl} \quad (3)$$

$$\mathrm{ArCuCl_2} + 2e^- \rightarrow \mathrm{ArCl} + \mathrm{CuCl} \quad (4)$$

This proposal could account for the exchange reactions taking place in an organic solvent. But do the organocopper(III) intermediates agree with the experience from the investigations by the present author of copper(I) catalysed exchange reactions in aqueous media? Copper(III) complexes have a quadratic or octahedral configuration;[14] complexes with halogen, including fluorine, are known. These configurations of an intermediate complex could hardly explain the observed accelerating effect, correlated to the van der Waals volume, of the substituent *ortho* to the reaction center. In addition, organocopper has the tendency to give coupling products and to be protonated to the arene by carboxylic acids. Biaryl products have never been detected, and dehalogenation products are only obtained as by-products in a few cases in the present series of investigations, see above. On the other hand a tetrahedral intermediate copper(I) – aryl halide complex could explain the reported accelerating effect of an *ortho* substituent, because strain is released, and a transition state of similar kind may also justify the large negative $\Delta S^{\ddagger}$ values (Table 2). The assumption of an intermediate copper(I) complex is also supported by the failure of 3-bromo-2-fluoronitrobenzene to exchange fluorine for chlorine[15] considering the fact that copper(I) fluoride complexes are unknown. Therefore the proposed negatively charged, tetrahedral $CuCl_2^-$-aryl halide complex cannot be rejected as an intermediate and as an approximate model for the transition state until experiments can be shown to support an other mechanism.

One can, however, not exclude the possibility that, when the conditions are altered, some other mechanism could prevail in the halogen exchange reaction. For instance when 2-bromo-3-methylnitrobenzene was irradiated without copper(I) chloride present, traces of the halogen exchange product, 2-chloro-3-methylnitrobenzene, were still observed.

EXPERIMENTAL

Melting points were determined on a Kofler Hot-Stage Microscope. ^{1}H NMR spectra were obtained with a Varian A-60 instrument (TMS as internal standard) and IR spectra with a Beckman IR-9 spectrophotometer. The mass spectra were performed on an LKB A 9000

instrument and the ^{19}F NMR spectrum on a Varian XL-100-15 spectrometer at Instrumentstationen, The Chemical Center, University of Lund.

All calculations were carried out on an Olivetti Programma 101 electronic desk-top computer.

2-Fluoroacetanilide. 2-Fluoroaniline (55.6 g, 0.5 mol, Fluka AG, *purum*) dissolved in 50 ml of benzene, was acetylated with 53 g (0.52 mol) of acetic anhydride. Yield 61.8 g (81 %), m.p. 77.5 – 78.5 °C, lit.[16] 79 °C.

2-Fluoro-6-nitroaniline. 2-Fluoroacetanilide (45.9 g, 0.3 mol) was nitrated according to the method of Franzén and Engel [17] for nitration of 2-bromoacetanilide. The isomeric mononitration products (38 g, 64 %) were separated by agitating the crude material with 450 ml of ice cold "Witt-Utermann solution" as described by Gibson and Johnson.[18] The 2-fluoro-4-nitroacetanilide was only slightly soluble in this solution and was filtered off. After 24 h only 1.5 g of hydrolysed material, 2-fluoro-4-nitroaniline, m.p. 135 – 136 °C, lit.[19] 134 – 135 °C, had precipitated. Treatment of the filtrate with acetic acid gave a precipitate of 9.45 g (16 %) of 2-fluoro-6-nitroacetanilide, m.p. 183 – 184 °C, lit.[20] 182.5 – 183.0 °C. On boiling a solution of this compound in 45 ml of ethanol and 22 ml of hydrochloric acid for 3 h, hydrolysis took place, and 6.65 g (89 %) of 2-fluoro-6-nitroaniline was obtained after dilution with water and filtration; m.p. 75.5 – 76.5 °C, lit.[20] 75 – 76 °C. The yield was 14 % calculated on the 2-fluoroacetanilide.

^1H NMR spectrum in $CDCl_3$: δ 7.2 (H-3, octet), 6.6 (H-4, octet), 7.9 (H-5, octet) 6.12 (NH_2, s). J_{3-4} 7.8 Hz, J_{3-5} 1.5 Hz, J_{4-5} 8.7 Hz, J_{3-F} 10.7 Hz, J_{4-F} 5.5 Hz and J_{5-F} 1.5 Hz.

2-Bromo-3-fluoronitrobenzene was synthesized from the preceding compound by a modification of a procedure by Gunstone and Tucker [21] (see Ref. 2). The 2-fluoro-6-nitroaniline (4.7 g, 0.03 mol) gave a yield after steam distillation of 4.75 g (72 %) of 2-bromo-3-fluoronitrobenzene, m.p. 42 – 43 °C. We have not been able to find the compound in the literature. The mass spectrum showed a 1:1 doublet at *m/e* 219 and 221, as expected for the desired compound.

2-Chloro-3-fluoronitrobenzene was prepared by the procedure described above for 2-bromo-3-fluoronitrobenzene. The 2-fluoro-6-nitroaniline (0.03 mol) gave a yield of 2.7 g (52 %) of 2-chloro-3-fluoronitrobenzene, m.p. 29.5 – 30.5 °C after recrystallization from methanol. Its boiling point is given in the literature [22] but not its melting point. The mass spectrum had a 3:1 doublet centered at *m/e* 176, the molecular weight of 2-chloro-3-fluoronitrobenzene.

4-Fluoroacetanilide. 4-Fluoraniline (22.2 g, 0.2 mol, Fluka AG, *pract.*) dissolved in 30 ml of benzene, was acetylated with 0.21 mol (21.4 g) of acetic anhydride. The yield of 4-fluoroacetanilide was 28.6 g (94 %), m.p. 153.5 – 154.5 °C, lit.[23] 150.5 – 151.5 °C.

4-Fluoro-2-nitroaniline was produced from 4-fluoroacetanilide by the same nitration procedure as described above for 2-fluoro-6-nitroaniline. 4-Fluoroacetanilide (7.65 g, 0.05 mol) gave 8.1 g (82 %) of the crude 4-fluoro-2-nitroacetanilide. After recrystallization from methanol, the m.p. was 72 – 73 °C, lit.[24] 72 – 73 °C. Hydrolysis of the acetanilide and subsequent steam distillation gave 4.7 g of 4-fluoro-2-nitroaniline (61 % calculated on the 4-fluoroacetanilide); m.p. 93 – 94 °C, lit.[25] 93 – 94 °C.

2-Bromo-5-fluoronitrobenzene was synthesized from 4-fluoro-2-nitroaniline by the same procedure as described for 2-bromo-3-fluoronitrobenzene. An amount of 3.12 g (0.02 mol) of the amine yielded 3.35 g of the bromo compound (76 %), m.p. 40 – 41 °C, lit.[26] 39.5 – 40.0 °C. The mass spectrum revealed a 1:1 doublet at *m/e* 219 and 221.

^1H NMR ($CDCl_3$ and $(CD_3)_2CO$ [in brackets]): δ 7.73 [7.9] (H-3, q), 7.19 [7.4] (H-4, oct), 7.58 [7.8] (H-6, q).

$J_{3-4} = 8.9$ Hz [8.95], $J_{4-6} = 2.9$ Hz [2.95], $J_{4-F} = 7.45$ Hz [7.85].

$J_{3-F} = 5.1$ Hz [5.2] and $J_{6-F} = 7.65$ Hz [7.95].

^{19}F NMR spectrum in $(CD_3)_2CO$: $J_{F-H_3} =$ 5.1 Hz and $J_{F-H_6,\ F-H_4} = 7.9$ Hz (octet).

2-Chloro-5-fluoronitrobenzene was prepared from 4-fluoro-2-nitroaniline following the description for 2-chloro-3-fluoronitrobenzene. 1.56 g (0.01 mol) gave after steam distillation a yield of 1.15 g (66 %) of 2-chloro-5-fluoronitrobenzene, m.p. 37.0 – 38.5 °C, lit.[27] 36 °C. The mass spectrum had a 3:1 doublet at *m/e* 175 and 177.

2-Chloro-6-nitroaniline (for NMR study) was synthesized from 2-chloroaniline (Fluka AG, *puriss*) by means of acetylation, nitration and hydrolysis as described above for 2-fluoro-6-nitroaniline; yield 33 % of 2-chloro-6-nitroaniline (calculated on the acetanilide), m.p. 75 – 76 °C, lit.[1] 75.5 – 76.0 °C.

^1H NMR ($CDCl_3$): δ 7.54 (H-3, q), 6.67 (H-4, q), 8.11 (H-5, q), 6.6 (NH_2, s). $J_{3-4} =$ 7.7 Hz, $J_{4-5} = 8.7$ Hz and $J_{3-5} = 1.6$ Hz.

2-Bromo-5-chloronitrobenzene (for NMR study) was prepared as described in Ref. 1.

^1H NMR ($CDCl_3$): δ 7.72 (H-3, d), 7.42 (H-4, q), 7.85 (H-6, d), J_{3-4} 8.4 Hz and J_{4-6} 2.2 Hz.

2-Methylacetanilide. 2-Methylaniline (107 g, 1 mol), dissolved in 100 ml of benzene, was acetylated with 105 g of acetic anhydride (1.03 mol); yield 124.9 g (84 %), m.p. 109 – 110 ° C, lit.[28a] 110 °C.

2-Methyl-6-nitroaniline. The preceding compound (112 g, 0.75 mol) was subjected to the procedure described for the preparation of 2-fluoro-6-nitroaniline. The mononitrated isomers were separated with 3000 ml of Witt-Utermann solution. The 4-nitro-isomer was almost insoluble and was filtered off (35 g

of 2-methyl-4-nitroacetanilide, m.p. 203.5–204.5 °C, lit.[29] 201 °C). The isomer 2-methyl-6-nitroacetanilide (70 g) m.p. 159.0–159.5 °C, lit.[29] 160 °C, was hydrolysed in an ethanol-hydrochloric acid solution (2:1) by boiling for 8 h. After steam distillation 38.6 g of 2-methyl-6-nitroaniline, m.p. 95.5–96.5 °C, lit.[28b] 97 °C, was obtained; yield 34 % based on 2-methylacetanilide.

^{1}H NMR ($CDCl_3$): δ 7.27 (H-3, q), 6.6 (H-4, q), 7.98 (H-5, q), 2.26 (CH_3, s), 5.7 (NH_2, s). $J_{3-4}=6.9$ Hz, $J_{4-5}=8.2$ Hz, and $J_{3-5}=1.5$ Hz.

2-Bromo-3-methylnitrobenzene. 2-Methyl-6-nitroaniline (15.2 g, 0.1 mol) was diazotized and subjected to a Sandmeyer reaction as described for the preparation of 2-bromo-3-fluoronitrobenzene. The yield after steam distillation was 15.15 g of 2-bromo-3-methylnitrobenzene (71 %). After two recrystallizations from methanol the m.p. was 40.0–41.5 °C, lit.[30] 41–42 °C. The mass spectrum showed a doublet, 1:1, at *m/e* 215 and 217, indicative of the desired substance.

^{1}H NMR ($CDCl_3$, Bruker WH 270 instrument): δ 7.43 (H-4, q), 7.33 (H-5, q), 7.50 (H-6, q), 2.48 (CH_3, s). $J_{4-5,\ 5-6}=7.65$ Hz.

2-Chloro-3-methylnitrobenzene. 2-Methyl-6-nitroaniline (15.2 g, 0.1 mol) was subjected to the same procedure as described for the preparation of 2-chloro-3-fluoronitrobenzene. After steam distillation the yield was 12.6 g (74 %). After two recrystallizations from methanol the m.p. was 23–24 °C, lit.[31] 23 °C. The mass spectrum had a 3:1 doublet at *m/e* 171 and 173.

2-Bromo-5-methylnitrobenzene was synthesized from 4-methyl-2-nitroaniline (Fluka AG, *pract.*) as described above for 2-bromo-3-methylnitrobenzene. From 7.6 g (0.05 mol) of the amine the yield was 7.8 g (72 %). After one recrystallization from hexane and two from methanol the m.p. was 31.5–32.0 °C, lit.[32] 31.5–32.5 °C. The mass spectrum showed a 1:1 doublet at *m/e* 215 and 217.

^{1}H NMR ($CDCl_3$): δ 7.58 (H-3, d), 7.23 (H-4, q), 7.64 (H-6, d)), 2.38 (CH_3, s). $J_{3-4}=$ 7.8 Hz and $J_{4-6}=1.7$ Hz.

2-Chloro-5-methylnitrobenzene was synthesized from the same 4-methyl-2-nitroaniline by the method used for the preparation of 2-chloro-3-methylnitrobenzene. The yield was 79 % after steam distillation. After two recrystallizations from hexane and two from methanol the m.p. was 6–7 °C, lit.[33] 7 °C. The mass spectrum had a 3:1 doublet at *m/e* 171 and 173.

2-Ethylacetanilide was prepared from 84.8 g (0.7 mol) of 2-ethylaniline (Fluka AG, *pract.*) in 70 mol of benzene by acetylation with 75 g of acetic anhydride (0.73 mol). The yield of 2-ethylacetanilide was 108 g (95 %), m.p. 113–114 °C, lit.[34] 113–114 °C.

2-Ethyl-6-nitroaniline was prepared from the preceding compound by the method described for the methyl compound. An amount of 40.8 g (0.25 mol) was nitrated, and the mixture of 6- and 4-mono-nitrated isomers was separated by grinding with Witt-Utermann solution. By this procedure 22 g of 2-ethyl-6-nitroacetanilide was obtained (42 %), m.p. 161.5–163.5 °C, lit.[35] 164–166 °C. After hydrolysis in boiling ethanol-hydrochloric acid (2:1) and steam distillation 12.4 (30 %) of 2-ethyl-6-nitroaniline was obtained, m.p. 30.5–31.0 °C, lit.[35] 31–32 °C. 2-Ethyl-4-nitroacetanilide (11 g) was left insoluble in the Witt-Utermann solution. It had m.p. 152.5–154.5 °C, lit.[36] 155 °C, and 6.6 g was hydrolysed and filtered off as 2-ethyl-4-nitroaniline, m.p. 86.5–87.5 °C, lit.[37] 86.0–87.5 °C. Contrary to the report by Hansch ,[35] the separation of the isomers by Witt-Utermann solution proved to be successful.

^{1}H NMR of the 6-nitro compound ($CDCl_3$): δ 7.28 (H-3, q), 6.63 (H-4, q), 8.0 (H-5, q), 1.28 (CH_3, t), 2.57 (CH_2, q), 6.28 (NH_2, s). $J_{3-4}=6.9$ Hz, $J_{3-5}=1.4$ Hz and $J_{4-5}=8.3$ Hz.

2-Bromo-3-ethylnitrobenzene was prepared from 2-ethyl-6-nitroaniline as described for 2-bromo-3-fluoronitrobenzene. A yield of 6 g (75 %) after steam distillation was obtained from 5.8 g (0.035 mol) of starting material. After four recrystallizations from methanol the m.p. was −3.5 to −3 °C. We have not been able to find the compound described in the literature. The mass spectrum showed a 1:1 doublet at 229 and 231 *m/e*.

^{1}H NMR ($CDCl_3$, Bruker WH 270 instrument): δ 7.43 (H-4, q), 7.37 (H-5, q), 7.48 (H-6, q), 1.23 (CH_3, t), 2.83 (CH_2, q). $J_{4-5,\ 5-6}=$ 7.55 Hz and $J_{4-6}=1.8$ Hz.

2-Chloro-3-ethylnitrobenzene was prepared from the same aniline by the method described for 2-chloro-3-fluoronitrobenzene. An amount of 2.35 g (0.014 mol) of the aniline gave 1.8 g (70 %) of 2-chloro-3-ethylnitrobenzene, m.p. 1 °C after steam distillation. Hansch [35] has described the compound as a yellow liquid. The compound was recrystallized twice from methanol. The mass spectrum showed a 3:1 doublet at *m/e* 185 and 187.

^{1}H NMR ($CDCl_3$, Bruker WH 270 instrument): δ 7.45 (H-4, q), 7.32 (H-5, q), 7.58 (H-6, q), 1.24 (CH_3, t), 2.85 (CH_2, q). $J_{4-5,\ 5-6}=$ 7.7 Hz and $J_{4-6}=1.65$ Hz.

2-t-Butylnitrobenzene was prepared from 402 g (3 mol) of *t*-butylbenzene (Fluka AG, *purum*) according to a procedure described by Craig.[38] The crude yield of a mixture of mono-nitrated isomers was 525 g (98 %). From GLC and NMR it was seen that about 15 % was the desired *ortho* isomer and about 75 % was the *para* isomer. In contrast to the report by Craig a third product with a mass spectrum showing the molecular ion at *m/e* 179 was found, indicating the 3-nitro-*t*-butylbenzene. Nelson and Brown [39] also report *o*-, *m*- and *p*-isomers in the proportions 16:11:73 in their nitration of *t*-butylbenzene. The isomeric mixture was distilled twice in a spinning-band column and 40 g of the *ortho* isomer, of 96 % purity, was

isolated, b.p. 109–111 °C at 1.60 kPa. The yield was about 8 %, lit.[39] b.p. 115.5 °C at 1.33 kPa, lit.[40] b.p. 67 °C at 0.053 kPa. The impurity was the *meta* isomer.

2-t-Butylacetanilide. 30.15 g (0.17 mol) of 2-*t*-butylnitrobenzene was reduced with 28.5 g (0.51 mol) of iron powder in 50 % aqueous ethanol, acidified with hydrochloric acid, following a description of Mahood *et al.*[41] The crude yield was 21.5 g (85 %). The crude 2-*t*-butylaniline (15 g, 0.1 mol) dissolved in 20 ml of benzene, was acetylated with 10.5 g of acetic anhydride (0.105 mol); yield 17.2 g (90 %), m.p. 163–164 °C, lit.[42a] 160–161 °C, lit.[42b] 166–168 °C.

2-t-Butyl-6-nitroaniline. The preceding compound (17.2 g, 0.09 mol) was nitrated as described for the methyl compound with a mixture of 23.5 ml of nitric acid ($d = 1.52$) and 12.6 ml of glacial acetic acid at 0 °C. The mixture was left for 48 h at room temperature and was then poured into 300 ml of ice-water. The oily mixture of 6-, 5- (or ev. 3-) and 4-nitro isomers was hydrolysed in hydrochloric acid-ethanol solution (1:2) by boiling for 8 h. Afterwards the amine mixture was carefully steam distilled. The organic layer was dissolved in ether and dried. HCl gas was introduced and most of the 5- (or 3-) and 4-nitroamines were removed as HCl salts. The 6-nitroamine was untouched and was further purified by column chromatography (silica gel < 200 mesh ASTM pH = 7, benzene as eluent). The yield of 2-*t*-butyl-6-nitroaniline was only about 5 % (0.75 g), based on the 2-*t*-butylacetanilide. The m.p. was 63.5–64.5 °C. The compound is not described in the literature. The mass spectrum gave the molecular ion at *m/e* 194.

^{1}H NMR for 2-*t*-butyl-6-nitroaniline ($CDCl_3$): δ 7.38 (H-3, q), 6.63 (H-4, q), 8.05 (H-5, q), 1.47 [$C(CH_3)_3$, s], 6.58 (NH_2, s). $J_{3-4} = 7.4$ Hz, $J_{4-5} = 8.2$ Hz and $J_{3-5} = 1.4$ Hz.

2-t-Butyl-4-nitroaniline was separated from 2-*t*-butyl-5-nitroaniline by thin-layer chromatography (silica gel. benzene as eluent). The mass spectra gave the molecular ion for each of them at *m/e* 194.

^{1}H NMR for 2-*t*-butyl-4-nitroaniline ($CDCl_3$): δ 8.15 (H-3, d), 7.9 (H-5, q), 6.55 (H-6, d), 1.42 [$(CH_3)_3C$, s], 4.55 (NH_2, s). $J_{3-5} = 2.3$ Hz and $J_{5-6} = 8.3$ Hz.

2-Bromo-3-t-butylnitrobenzene was synthesized from 0.58 g (0.003 mol) of 2-*t*-butyl-6-nitroaniline as described above for 2-bromo-3-methylnitrobenzene. The reaction mixture was extracted with ether and then evaporated and purified by column chromatography with the same conditions as for the 2-*t*-butyl-6-nitroaniline. The yield was 0.40 g (52 %). This liquid compound is not described in the literature. The mass spectrum showed a 1:1 doublet at *m/e* 257 and 259 pointing at the desired substance.

Copper(I) chloride was purified according to Keller and Wycoff.[43]

Copper(I) catalysed replacement of bromine by chloride ion. The reactions between the halonitrobenzenes and copper(I) chloride in aqueous hydrochloric acid-acetic acid were performed in an argon atmosphere with the composition of the medium, the apparatus and the method described in Ref. 1. The withdrawn samples were adjusted to pH 7 and then extracted with ether. The ether phase was analysed on a Perkin Elmer F 11 Hot Wire Gas Chromatograph, equipped with a Varian Model 480 Electronic Digital Integrator. A 3 mm o.d. 2 m SE-30 column was used, temp. 105–150 °C, carrier gas He. Calibration curves were made from known mixtures of pure compounds: 2-bromo-3-fluoro- and 2-chloro-3-fluoronitrobenzene, 2-bromo-5-fluoro- and 2-chloro-5-fluoronitrobenzene, 2-bromo-3-methyl- and 2-chloro-3-methylnitrobenzene, 2-bromo-3-ethyl- and 2-chloro-3-ethylnitrobenzene and 2-bromo-5-methyl- and 2-chloro-5-methylnitrobenzene. All the calibration curves were straight lines with unit slope. The reaction mixtures were also analysed by NMR and IR spectroscopy (see Ref. 1).

For the 3-*t*-butylnitrobenzene compounds the calibration curve was assumed to be a straight line with unit slope.

The irradiation of 2-bromo-3-methylnitrobenzene was carried out in a photochemical reactor Rayonet RPR-100, at 350 nm, 30 °C. Otherwise the conditions were the same as described above for the exchange reactions.

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Thermal Properties of Systems Containing Cholesteryl Esters and Triglycerides

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Binary and ternary systems of the three cholesteryl esters, linoleate oleate, and stearate and the two triglycerides, triolein and tristearin were studied in order to determine the phase transitions and the conditions for the cholesteric and smectic mesophases. Phase transitions were determined using differential thermal analysis, melting point determination, and polarizing microscopy.

Of the cholesteryl esters the linoleate-oleate system showed complete miscibility in both the liquid and solid phases. The linoleate-stearate and oleate-stearate systems are of the eutectic type with limited solid solubility. The mesophases are monotropic as to the crystalline state and exist over the entire composition interval in all cholesteryl ester systems studied.

The triglycerides studied showed no mesophase transitions. In mixed systems with cholesteryl esters even low concentrations of the triglycerides removed the cholesteric transition typical for cholesteryl ester systems. At higher concentration of triolein the smectic mesophase was also removed. In systems with tristearin and cholesteryl esters an apparently smectic mesophase with mosaic texture was found. In ternary systems the property of the low melting lipids to depress the melting points of the high melting ones was found to be additive.

Cholesteryl esters and triglycerides belong to the major components of atherosclerotic deposits and serum lipoproteins and the phase conditions of these lipids serve directly to explain the state of the "oil phase" in atherosclerotic deposits and of the lipid core of serum lipoproteins.

Cholesteryl esters and triglycerides are abundant lipids in serum lipoproteins and atherosclerotic lesions.[1,2] Based upon their physical interactions with water both these lipid groups can be classified as insoluble, nonswelling amphiphilic lipids.[3] On the other hand considering their interactions with other lipids there are marked differences. Long chain cholesteryl esters have a very weak polar character, are solubilised at a low concentration in the lamellar mesophase made of egg lecithin and water [4] and do not form miscible films with surface-active lipids.[5] Therefore long chain cholesteryl esters have to be characterized as typical oil phase lipids. Triglycerides show more ambivalent interactions with other lipids. Triolein can be incorporated into the lecithin-water lamellar mesophase in rather high concentration.[6] In mixed films with surface-active lipids triolein forms partly miscible films, while tristearin forms miscible ones.[7] In mixed films with cholesteryl esters and surface-active lipids, the triglycerides together with the cholesteryl esters are squeezed out into an oil bulk phase.[8] The triglycerides then take up a mid-position between oil and interfacial lipids.

An outstanding feature of the physico-chemical properties of long chain cholesteryl esters is their tendency to exhibit thermotropic mesomorphism. In recent years the mesomorphic behaviour of biologically important cholesteryl esters has been intensively investigated.[9,10] Also binary mixtures of cholesteryl esters have been examined.[10,11]

The physico-chemical properties of cholesteryl esters and triglycerides are largely determined by the type of their fatty acid chains. Thus the melting point varies greatly between different esters: *e.g.* cholesteryl stearate 81.8 °C [11] and tristearin 73 °C [12] compared to cholesteryl oleate 50.6 °C [11] and triolein 4 °C.[10]

The physico-chemical state of lipid mixtures in biological systems is not only determined by the physical properties of the individual lipids but also by the interactions between them. An introductory study of the interactions between triglycerides and cholesteryl esters has been done by Small.[10] In order to clarify the role of the physico-chemical properties of lipids for example in the structure of lipoproteins and at the appearance of atherosclerotic plaques, systematic studies of model systems have to be done.

MATERIALS AND METHODS

The cholesteryl linoleate used was prepared by a modified acid chloride method.[13] Cholesteryl oleate and cholesteryl stearate were purchased from E. Merck AG and recrystallized from pentyl alcohol with subsequent washing in an ethanol-water solution. Triolein and tristearin were purchased from Fluka AG. The triolein was purified by Florisil column chromatography and the tristearin by recrystallization from acetone.

Mixtures of the lipids were prepared by dissolving the weighed components in chloroform which was then removed *in vacuo*. Ca 30 mg of sample were weighed in an aluminium pan which was placed in a Fisher Model 370 Differential Thermal Analyzer (DTA). The heating curves were obtained with a scan speed of 10 °C/min. until the sample was entirely melted. The sample was then cooled using a scan speed of 5 °C/min. When examining lipids with unsaturated fatty acid chains an atmosphere of N_2 gas was used.

The melting point values obtained for the lipid samples by DTA measurements were complemented by examinations with a Gallenkamp melting point apparatus. In order to identify the phase changes recorded by the DTA measurements a Wild polarizing microscope equipped with a hot stage was used.

RESULTS

Individual lipids. All three C_{18} cholesteryl esters showed monotropic behaviour; from one solid state they melt directly to an isotropic liquid. Upon cooling the isotropic liquid, cholesteric and smectic mesophases are formed before solidification. The unsaturated esters can exhibit a polymorphism in the solid state [11] but in mixtures of these esters precautions were taken to obtain the stable higher melting modification. The temperatures of the crystal-isotropic, isotropic-cholesteric, and cholesteric-smectic transitions for the three C_{18} cholesteryl esters were found to be 83, 75, 72 (stearate), 50, 44, 39 (oleate), 41, 35, 32 °C (linoleate), respectively. These transition temperatures are close to the values reported by other authors.[9–11]

Triolein melted directly to an isotropic liquid at 4 °C. Tristearin can exhibit a complex phase behaviour on heating.[12] Precautions were, however, taken to bring the tristearin into the triclinic β_L form from which it melted directly to isotropic liquid at 72 °C.

Mixtures of cholesteryl esters. On heating solid cholesteryl linoleate-cholesteryl oleate mixtures only one sharp melting endotherm was registered by DTA over the entire composition region. The phase diagram for mixtures of cholesteryl linoleate and cholesteryl oleate in Fig. 1 shows complete miscibility of the two esters in both the liquid and solid phases. On cooling from the isotropic melt, single homogeneous cholesteric and smectic mesophases were observed (Fig. 2a and b). The smectic-solid transition for mixtures of these cholesteryl esters was slow at low temperatures and could not be recorded by the DTA measurements.

In binary systems with one of the unsaturated cholesteryl esters (linoleate or oleate) and cholesteryl stearate the melting point of stearate is depressed continuously down to an eutectic point at a high concentration of the unsaturated ester (Fig. 3a and b). On heating,

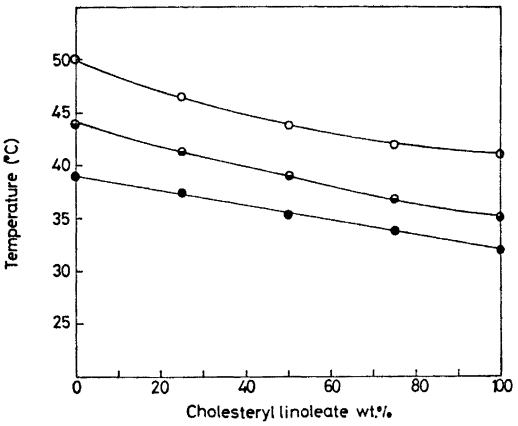

Fig. 1. Cholesteryl linoleate-cholesteryl oleate condensed binary phase diagram. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●.

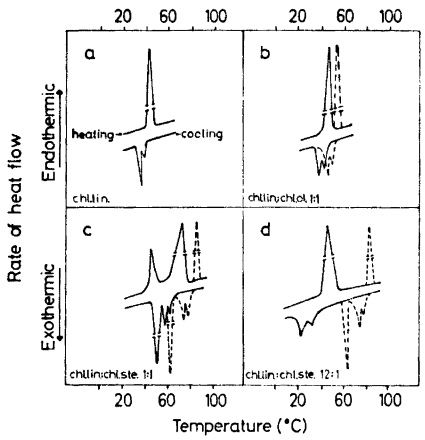

Fig. 2. DTA curves of the three C_{18} cholesteryl esters, linoleate (chl. lin.), oleate (chl. ol.), and stearate (chl. ste.) and mixtures of these. In the figures the first heating curves are above and the cooling curves below. (a) The heating curve shows the sharp melting endotherm and the cooling curve the two mesomorphic transitions (liquid-cholesteric and cholesteric-smectic) of linoleate, (b) the same transitions of oleate (dotted line) and a 1:1 mixture of linoleate and oleate (continuous line). (c) The heating curves show the sharp melting endotherm of stearate (dotted line) and two melting endotherms of a 1:1 mixture of linoleate and stearate (continuous line). The first peak in the curve of the mixture corresponds to the melting of the eutectic composition and the subsequent peak to the melting of the solid solution portion of the blend. On cooling, both stearate and the mixture show two mesomorphic transitions (liquid-cholesteric and cholesteric-smectic) and one crystallization exotherm. (d) DTA curves of a 12:1 mixture of linoleate and stearate (continuous lines) with a single melting endotherm and two mesomorphic transitions (liquid-cholesteric and cholesteric-smectic). The curves of stearate (dotted lines) are shown as a comparison.

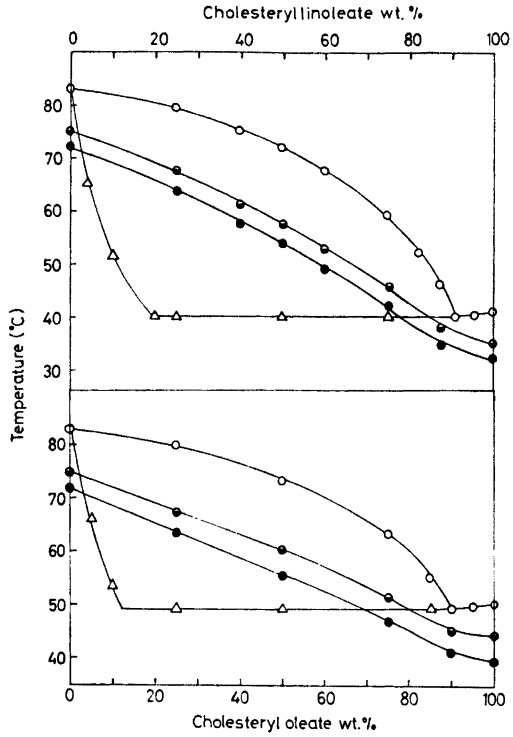

Fig. 3. Condensed binary phase diagrams for the systems (a) cholesteryl linoleate-cholesteryl stearate and (b) cholesteryl oleate-cholesteryl stearate. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, solid-(solid+liquid) △.

the DTA curves for blends with composition within the miscibility gap gave two peaks; the first corresponding to the melting of the eutectic composition and the second one to the melting of the solid portion of the blend (Fig. 2c). At compositions approaching the eutectic point the second peak diminishes (Fig. 2d).

On cooling isotropic melts of binary systems with one unsaturated ester and stearate, single homogeneous cholesteric and smectic mesophases were found to exist over the entire composition interval. On further cooling, crystals were formed from the smectic mesophase. For high concentrations of the unsaturated ester the smectic-solid transition took place slowly and could not be recorded by the DTA measurements.

In ternary systems the cholesteryl esters studied showed the same general features as in the binary systems. The melting point of the higher melting ester is depressed by increasing concentrations of the lower melting esters. This is shown in Fig. 4 where the proportion of cholesteryl stearate is held constant and the mutual concentrations of linoleate and oleate are changed. The linear curve for the solid-liquid transition shows that the melting point depressing property is additive in an ideal manner. On cooling from the isotropic melt, single homogeneous cholesteric and

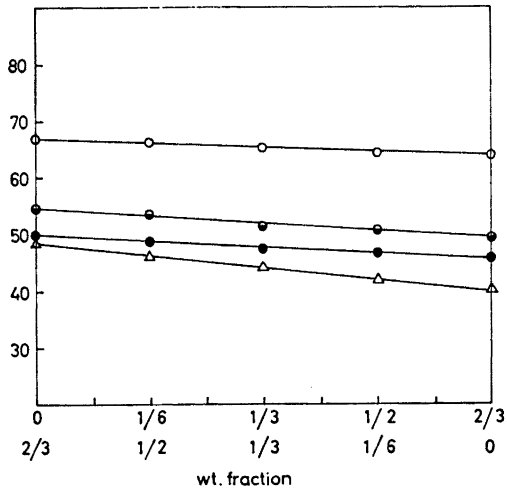

Fig. 4. Phase transitions in ternary systems of the three cholesteryl esters, linoleate, oleate, and stearate. Proportion of stearate is constant at the wt. fraction of 1/3 and the proportions of linoleate (upper abscissa) and oleate (lower abscissa) vary between 0 and 2/3. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, solid-(solid+liquid) △. Ordinate: Temperature (°C).

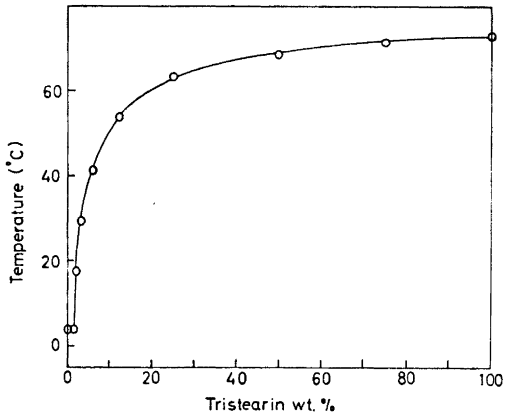

Fig. 5. The liquidus curve for the triolein-tristearin system. Tristearin melts from its $\beta_{I'}$ modification.

smectic mesophases, respectively, are formed. These transitions also show an ideal behaviour.

Mixtures of triglycerides. Tristearin has been reported to exhibit a complex phase behaviour on heating.[12] This property was also noted in mixtures with other lipids. In order to obtain correspondence between different measurements, attention was paid to always having the tristearin in the most stable triclinic $\beta_{I'}$ form. This form melts directly to isotropic liquid.

The melting point of tristearin is depressed by increasing concentrations of triolein (Fig. 5).

Mixtures of cholesteryl esters and triglycerides. The melting points of the cholesteryl esters are depressed by increasing concentrations of triolein down to an eutectic point at high proportion of triolein (Fig. 6).

On cooling from isotropic melt both cholesteric and smectic mesophases were recorded at very low triolein concentrations (Fig. 7a). Between 1 and 3 % triolein the cholesteric mesophase disappears. The smectic transition was recorded by DTA up to about 10 % triolein in mixtures with linoleate and up to about 25 % with oleate and stearate. By microscopic examination the smectic phase was observed at somewhat higher triolein concentrations. For mixtures with more than about 50 % triolein no mesophase can be observed and crystallization takes place directly from the isotropic liquid (Fig. 7b).

As seen in Fig. 8a and b the melting point of tristearin is depressed by increasing proportions of the unsaturated cholesteryl esters down to an eutectic point at high concentration of the cholesteryl ester. In mixtures of cholesteryl stearate and tristearin on the other hand the melting point of the cholesteryl ester, which is higher than that of tristearin, is depressed by increasing concentrations of tristearin down to an eutectic point at about 70 % tristearin (Fig. 8c).

On cooling from isotropic melt both cholesteric and smectic mesophases were exhibited at low tristearin concentrations (Fig. 7c). Between 3 and 6 % tristearin the cholesteric transition disappears. The smectic transition remains at high tristearin concentration but the microscopic texture changes considerably; a mosaic texture phase is formed (Figs. 9 and 10). At the same concentration as the mosaic texture appears, a peak on the DTA curve corresponding to crystallization of tristearin is noticed (Fig. 7d). The direct connection between the typical cholesteryl ester smectic mesophase in the region with low tristearin

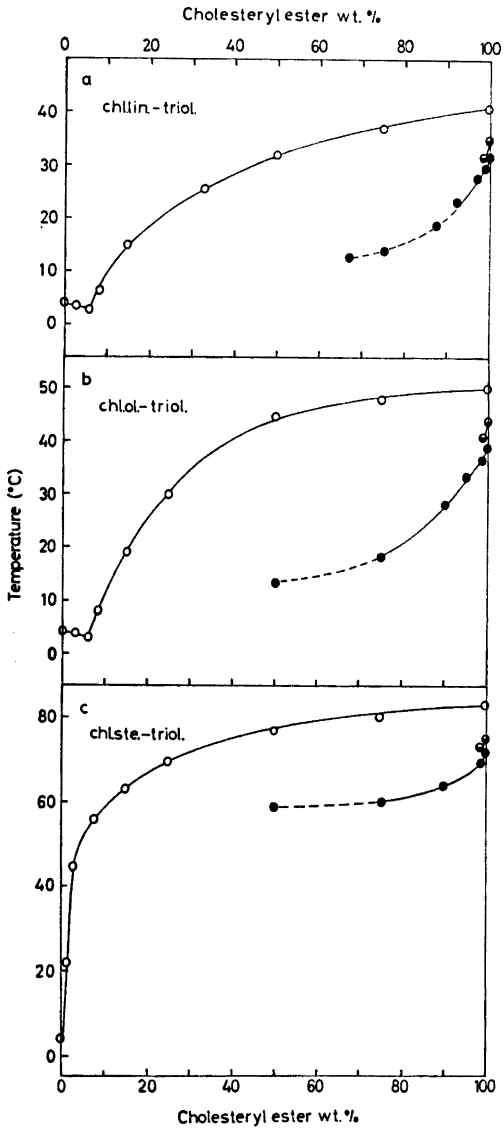

Fig. 6. The liquidus and mesomorphic transitions for the binary systems (a) cholesteryl linoleate-triolein, (b) cholesteryl oleate-triolein, and (c) cholesteryl stearate-triolein. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●.

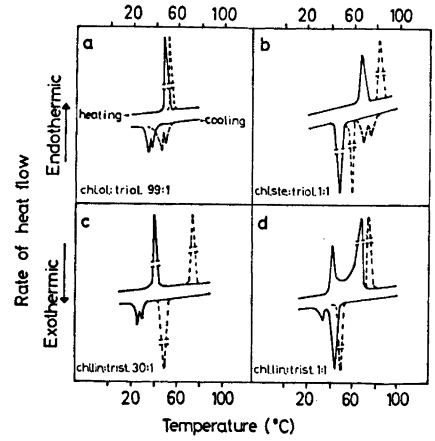

Fig. 7. DTA curves of mixtures of the three C_{18} cholesteryl esters linoleate (chl. lin.), oleate (chl. ol.), stearate (chl. ste.) and the two C_{18} triglycerides triolein (triol.) and tristearin (trist.). In the figures the first heating curves are above and the cooling curves below. (a) The curves with continuous lines show a single melting endotherm and two mesomorphic transitions (liquid-cholesteric, and cholesteric-smectic) of a 99:1 mixture of cholesteryl oleate and triolein. The curves of cholesteryl oleate are shown as a comparison (dotted lines). (b) Melting endotherm and crystallization exotherm of a 1:1 mixture of cholesteryl stearate and triolein (continuous line) compared with the curves of cholesteryl stearate (dotted lines). (c) Melting endotherm and two mesomorphic transitions (liquid-cholesteric and cholesteric-smectic) of a 30:1 mixture of cholesteryl linoleate and tristearin (continuous line). The melting endotherm and the crystallization exotherm of tristearin are shown by dotted lines. (d) the heating curves show the melting endotherm of tristearin (dotted line) and two melting endotherms of a 1:1 mixture of cholesteryl linoleate and tristearin (continuous line). The cooling curves show the crystallization exotherm of tristearin (dotted line) and two exotherms of the mixture (continuous line) the larger of which corresponds to the crystallization of tristearin and the smaller to the mosaic phase transition.

concentration and the mosaic texture phase seems to be plausible but is not structurally confirmed. At low concentration of the unsaturated cholesteryl ester the smectic transition cannot be recorded because of limited calorimeter sensitivity. However, the curve obtained by plotting composition *versus* the transition heat for the smectic transition indicated that this phase exists down to very low cholesteryl ester concentrations.

The interaction of cholesteryl stearate with tristearin is somewhat different from that of the unsaturated cholesteryl esters. At low tri-

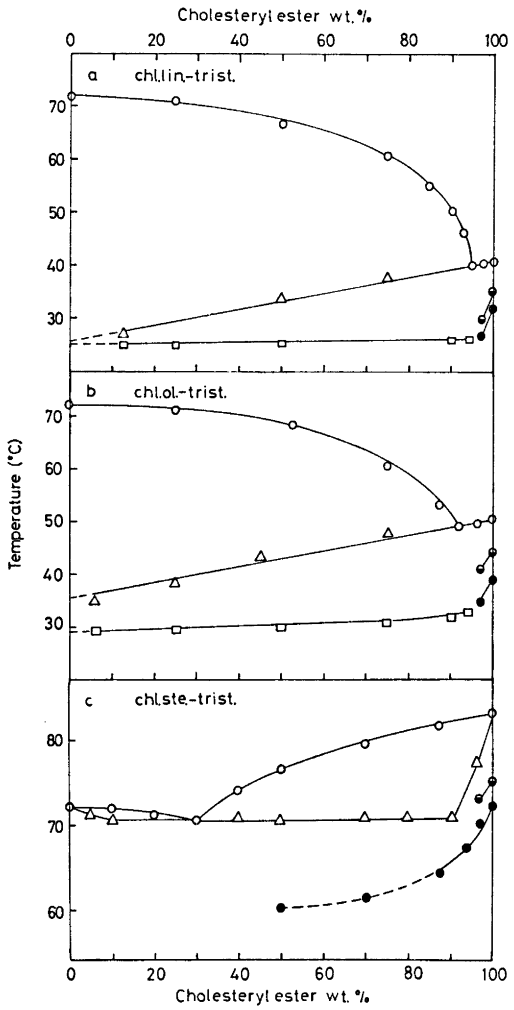

Fig. 8. Condensed binary phase diagrams for the systems (a) cholesteryl linoleate-tristearin, (b) cholesteryl oleate-tristearin, and (c) cholesteryl stearate-tristearin. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, liquid-mosaic smectic □, solid – (solid + liquid) △.

stearin concentrations cholesteric and smectic mesophases are exhibited. However, no mosaic type mesophase is formed at higher tristearin concentrations but the crystallization takes place directly from melt (Fig. 8c).

An addition of triolein to a binary cholesteryl ester mixture depresses the melting points of the esters and removes the mesophase transitions as for the individual cholesteryl esters.

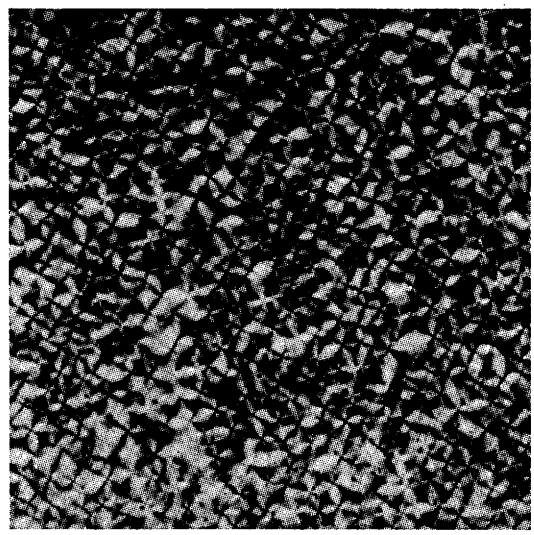

Fig. 9. Spherulitic texture of smectic phase in a system of 3 % tristearin and 97 % cholesteryl oleate. Crossed polars, × 400.

The melting point depressing effect of mixtures of triolein and either of the unsaturated cholesteryl esters on cholesteryl stearate and tristearin was found to be directly additive.

Mixtures of tristearin and the two unsaturated cholesteryl esters showed the same

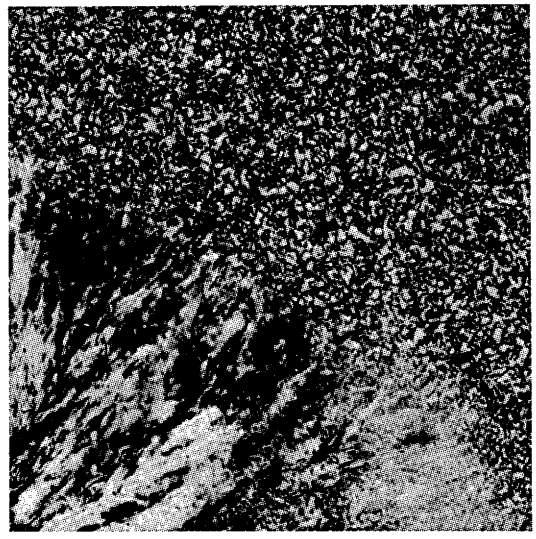

Fig. 10. Mosaic texture of probably smectic phase in the stage of crystallization in a system of 10 % tristearin and 90 % cholesteryl oleate. Crossed polars, × 400.

characteristics as those of tristearin with the individual esters. The melting point depressing effect on tristearin was found to be additive.

BIOLOGICAL CONCLUSIONS

Schematically the arterial tissue and the blood can be divided into oil and water phases which are separated by specific interfaces. The cholesteryl esters and triglycerides are the major oil phase lipids. The physical state of the oil phase is determined by the physical characteristics of the lipids in it and the interaction between them.

The lipids in atherosclerotic deposits can be in a liquid, mesomorphic or solid state.[14] Living organisms require mobility which facilitates, *e.g.*, diffusion, transport, and enzyme function. This requirement for mobility is fulfilled by the liquid state. The viscosity of mesomorphic phases is usually considerably higher than that of the liquid state,[15] but it is still unclear to what extent this quality effects biological metabolization.

The physical characteristics of cholesteryl esters and triglycerides are greatly affected by the fatty acid residues bound in them. Of the lipids studied in this paper triolein is liquid, cholesteryl linoleate cholesteric and the rest solid at body temperature. A typical feature of the interaction between cholesteryl esters and triglycerides is that the low melting ones depress the melting points of the high melting ones. This effect is, however, rather small and an excessive incorporation of high melting cholesteryl esters and triglycerides causes a deposition of solid lipids in the arterial wall.[16]

A remarkable property of the triglycerides is their ability to remove the cholesteric phase in mixtures with cholesteryl esters. This is probably the explanation to why this phase has not been found in atheroschlerotic lesions.[14]

In serum lipoproteins the neutral lipids are supposed to be embedded in the lipid matrix.[17] The physical state of this can be either liquid or smectic. The cholesteric state can be excluded because of too high triglyceride contents. For low-density lipoproteins it can be concluded that the smectic state is the most probable one, because of the high concentrations of cholesteryl linoleate and cholesteryl oleate. For the chylomicrons the situation is different. They contain so much triglycerides that the liquid state is the most probable one. Thus information from model systems can elucidate the physical state of the lipids in biological systems.

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Algal Carotenoids. XV.* Structural Studies on Peridinin. Part 2. Supporting Evidence

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The chemical reactions of peridinin (*1*) towards acids, base and complex metal hydride ($NaBH_4$) have been investigated. In all cases complex mixtures of products were obtained. The physical and chemical properties of these products provide strong support for the structure proposed for peridinin (*1*).

The fragmentation pattern of peridinin (*1*) on electron impact is rationalized in terms of this structure.

The structure of peridinin (*1*), the principal carotenoid pigment of the dinoflagellates, has recently been elucidated.[1,2] With its seven oxygen functions, including a butenolide moiety as part of the chromophore and a C_{37} skeleton, peridinin is a most remarkable carotenoid.

In its chemical behaviour peridinin exhibits some uncommon properties. Simple derivative formations, *e.g.* silylation and ester formation under standard conditions, follow the expected courses and give well defined products.[2] However, previous reports have stated that peridinin is rapidly decolourized by alkali,[3,4] reacts slowly with acids resulting in a 20 nm hypsochromic shift of the visible light absorption spectrum [4] and that reduction with $LiAlH_4$ results in a mixture of products with electronic spectra consistent with pentaene chromophores.[4]

In the present work, a detailed examination of the complex reaction mixtures obtained from peridinin on treatment with acids, base and complex metal hydride ($NaBH_4$), are reported. Preliminary observations on the dehydration with phosphorus oxychloride are also presented.

The physical and chemical properties of these reaction products support the structure (*1*) proposed for peridinin.

The fragmentation pattern on electron impact is further rationalized in terms of the proposed structure.

RESULTS AND DISCUSSION

Acid treatment. Direct comparison of the acid lability of peridinin (*1*) with that of β-carotene diepoxide (*2* = 5,6:5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene) towards 1 % citric acid in methanol showed that *2* was completely isomerized (Δ nm = 40) within 10 h. Peridinin (*1*) was unaffected during the same period. With 1 % HCl in methanol *2* was totally isomerized in 3 min whereas *1* required 1.5 h for complete conversion (Δ nm = 20). Peridinin (*1*) is thus clearly much more stable towards acids than common carotenoid expoxides.[5]

Treatment of peridinin (*1*) with 1 % HCl-methanol resulted in four new products of which the two major ones, while readily separated by TLC, had identical spectral characteristics (λ_{max} 446 nm in acetone; *m/e* 630 = M, 197, 181). The magnitude of the hypsochromic shift in their visible light absorptions relative to peridinin (*1*, λ_{max} 466 nm in acetone) is as

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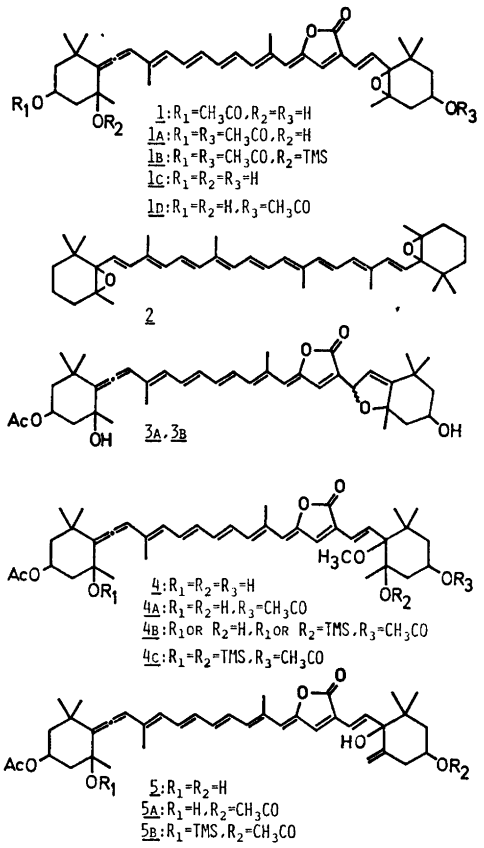


Scheme 1.

expected for a 5,6-epoxide to 5,8-furanoxide rearrangement,[5] and these two products were therefore identified as the epimeric furanoxides *3a* and *3b*.

The two minor products *4* and *5* had identical adsorptive properties and visible light absorptions (λ_{max} 459, (474) nm in acetone) and could only be separated after acetylation. The major acetate (*4a*, *m/e* 704 = M) gave a mono-TMS ether (*4b*, *m/e* 776 = M) and a di-TMS ether (*4c*, *m/e* 848 = M) on silylation, all products having unchanged visible light absorptions. Addition of methanol to peridinin (*1*, Mw = 630) would account for the formation of product *4*. Protonation of the epoxy function followed by substitution (S_N1) at C-6′ with methanol would explain the formation of the new tertiary hydroxy function giving a TMS ether upon silylation, see below. Structure *4* is therefore a likely representation of this product.

The minor acetate (*5a*, *m/e* 672 = M) exhibited the same absorption as the major acetate *4a* in visible light. Upon silylation it formed a mono-TMS ether (*5b*, *m/e* 744 = M) with unchanged spectral properties in visible light. Neither of these products (*5a* and *5b*) co-chromatographed with peridinin acetate (*1a*, *m/e* 672 = M)[2] or the TMS ether of peridinin acetate (*1b*, *m/e* 744 = M).[2] Product *5* is thus an isomer of peridinin (*1*). Acid catalyzed opening of the epoxy group of peridinin followed by elimination of a proton may account for this product, tentatively represented by structure *5* with a hydroxy function at C-6′. Tertiary hydroxy groups in this sterically hindered position are known to be inert towards standard acetylation and silylation conditions.[6] Since no allylic methyl ether was formed, *cf.* lutein,[7] the new double bond is tentatively assigned the exocyclic position.

Base treatment. Reaction of peridinin (*1*) with 0.5 % K_2CO_3 in water-methanol (0.03:1) under conditions similar to those used for fucoxanthin,[8] resulted in severe decomposition of the pigment. After 16 h all peridinin had reacted and recovery of coloured products was only 10 %.

The major product (*1c*, *m/e* 588 = M) had the same visible light absorption spectrum as peridinin (*1*), gave two monoacetates (*1* and *1d*, *m/e* 630 = M) one of which co-chromatographed with peridinin (*1*), and a diacetate (*1a*, *m/e* 672 = M) which co-chromatographed with peridinin acetate (*1a*). Product *1c* was therefore identified as deacetylated peridinin (*1c*), here called peridininol, in analogy with deacetylated fucoxanthin, fucoxanthinol.[8]

One minor product (*6*, *m/e* 620 = M) had λ_{max} 391 nm in acetone solution and gave a diacetate (*6a*, *m/e* 704 = M) on acetylation with unchanged visible light absorption. On silylation the diacetate *6a* gave a di-TMS ether (*6b*, *m/e* 848 = M) with unchanged spectral properties in the visible region. Product *6* thus possesses two primary or secondary and two tertiary hydroxy groups. Assuming that peridinin (*1*) is first deacetylated, formal addition of methanol (630 − 42 + 32 = 620) would account for the molecular weight of 620. However, from the available data no obvious structure may be proposed for this product.

A third product *7*, with λ_{max} 372 nm in acetone, gave upon acetylation an acetate (*7a*, *m/e* 684=M) with unchanged visible light absorption. Assuming that the acetylated product *7a* is a diacetate, the original product *6* must have had a molecular weight of 600 (600+42+42=684). No structural conclusions may be drawn from these data.

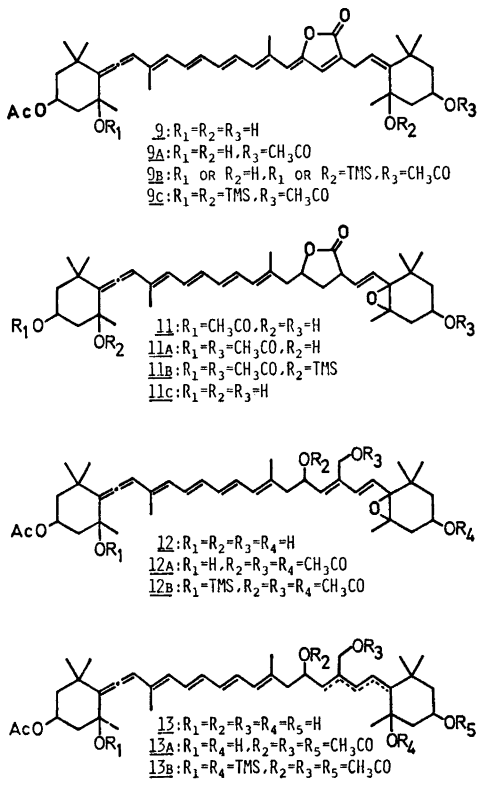
A fourth, acidic product *8* was obtained after acidification of the hypophase. It had λ_{max} 367 nm in methanol and could not be esterified with diazomethane. No conclusions as to structure are drawn for this product.

Dehydration with $POCl_3$. Reaction of peridinin (*1*) with $POCl_3$ under standard conditions[9] gave two monodehydrated (*m/e* 612=M) and two didehydrated (*m/e* 594=M) products. Further studies of this reaction are reported elsewhere.[10]

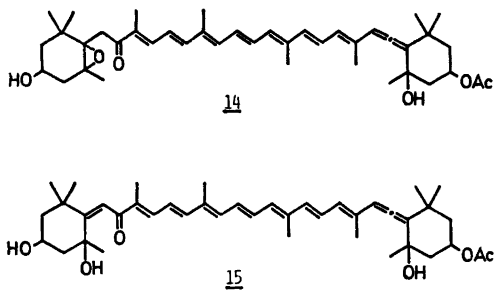
Reduction with $NaBH_4$. Reduction of peridinin (*1*) with $NaBH_4$ at room temperature resulted in only 50 % pigment recovery; unreacted peridinin 35 % and reduction products 15 %, Scheme 2. The major product (*9*) had λ_{max} 433, (452) nm in acetone. Acetylation gave an acetate (*9a*) which on silylation gave a mono-TMS ether (*9b*, *m/e* 746=M) and a di-TMS ether (*9c*, *m/e* 818=M) both with unchanged visible light absorption. Assuming that the acetylated product *9a* is a monoacetate, formation of the reduction product *9* (M=746−72−42=632) involved addition of two hydrogens with the formation of a new tertiary hydroxy group. Considering also the chromophoric change, the reduction has probably occurred as a 1,4-addition to the epoxidic system.

A UV-fluorescent product (*10*, *m/e* 634=M) had λ_{max} 331, 347 and 366.5 nm in ether, compatible with a pentaene chromophore. The IR spectrum showed absorptions for bonded OH (*ca.* 3300 cm^{-1}), allene (1930 cm^{-1}), carbonyl (1725 cm^{-1}, broad) and ester C−O stretch (1250 cm^{-1}). Acetylation gave a triacetate (*10a*, *m/e* 760=M) and silylation of the triacetate *10a* gave a mono-TMS ether (*10b*, *m/e* 832=M). Both derivatives had unchanged UV absorptions. Formation of a triacetate implies that two new acetylable hydroxy functions had been formed. Since total reduction of the lactone requires addition of six hydrogens, whereas only four were added, and partial reduction to a hydroxy ketone should give a coloured enol acetate, no plausible structure is proposed for *10*.

A third, isomeric product (*11*, *m/e* 634=M), also UV-fluorescent, had visible light absorption identical to product *10* above. Saponification gave a product (*11c*, *m/e* 592=M) with unchanged visible light absorption. Acetylation of *11* gave a monoacetate (*11a*, *m/e* 676=M), and silylation of *11* gave a di-TMS ether (*11b*, *m/e* 778=M) both with unchanged chromophores. Addition of four hydrogens to peridinin (*1*) to give a product with pentaene chromophore without formation of new reactive functional groups may be explained in terms of structure *11*. The formation of *11* may involve reduction of the butenolide double bond by 1,4-addition,[11] then opening of the lactone under the weakly alkaline conditions, followed



Scheme 2.



Scheme 3.

by reduction of the keto-enol function and subsequent ring closure.

Two products *12* and *13* had identical visible light absorptions, compatible with a pentaene chromophore, and identical adsorptive properties. They could only be separated after acetylation.

The acetate *12a* (*m/e* 762=M) gave a mono-TMS ether (*12b*, *m/e* 834=M) on silylation. Assuming that *12a* is a triacetate, it may be inferred that *12* is derived from peridinin by addition of six hydrogens with the formation of two new acetylable hydroxy functions and no new tertiary hydroxy group. This result may be explained by assuming complete reduction of the lactone system, Scheme 2.

The other acetate (*13a*, *m/e* 764=M) gave a di-TMS ether (*13b*, *m/e* 908=M) on silylation, consistent with the addition of eight hydrogens and formation of two new acetylable hydroxy functions and one new silylable, tertiary hydroxy group.

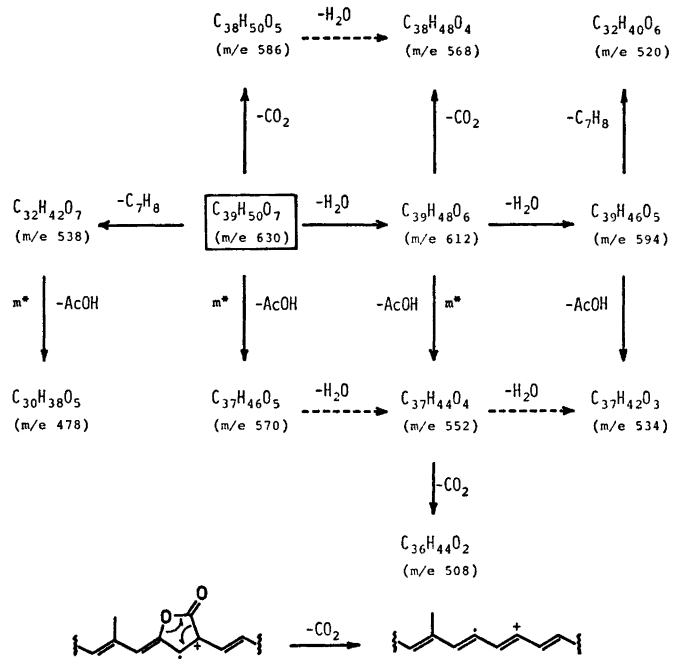
The derivatives *12a*, *12b*, *13a* and *13b* all displayed visible light absorption identical to that of the mixture of *12* and *13*.

Product *13* may be accounted for by assuming total reduction of the lactone moiety as well as reductive opening of the epoxide. The positions of the two double bonds, separated from the main chromophore, are uncertain.

Fragmentations of peridinin induced by electron impact. Important fragments observed in

Table 1. Electron impact induced fragmentation of peridinin, measured with high resolution.

m/e	Composition	Fragment	*m**	Rel.Int.
630.3553	$C_{39}H_{50}O_7$	M		7.9
612.3455	$C_{39}H_{48}O_6$	$M-H_2O$		49.5
594.3350	$C_{39}H_{46}O_5$	$M-2H_2O$		1.7
586.3659	$C_{38}H_{50}O_5$	$M-CO_2$		3.0
570.3346	$C_{37}H_{46}O_5$	M−AcOH	515.5	1.0
568.3550	$C_{38}H_{48}O_4$	$M-H_2O-CO_2$		4.0
552.3229	$C_{37}H_{44}O_4$	$M-H_2O$−AcOH	498	22.3
538.2936	$C_{32}H_{42}O_7$	$M-C_7H_8$		6.1
534.3134	$C_{37}H_{42}O_3$	$M-2H_2O$−AcOH		4.9
520.2856	$C_{32}H_{40}O_6$	$M-H_2O-C_7H_8$		2.7
508.3345	$C_{36}H_{44}O_2$	$M-H_2O$−AcOH$-CO_2$		2.0
478.2712	$C_{30}H_{38}O_5$	$M-C_7H_8$−AcOH	425	3.3
450.2416	$C_{28}H_{34}O_5$	A		1.1
397.2171	$C_{28}H_{29}O_2$	B−H_2O−AcOH		2.3
396.2288	$C_{25}H_{32}O_4$	C		0.5
358.1784	$C_{21}H_{26}O_5$	A−C_7H_8		1.2
257.1654	$C_{17}H_{23}O_3$	D		5.3
251.1287	$C_{14}H_{19}O_4$	E		1.2
234.1256	$C_{14}H_{18}O_3$	F		24.5
233.1203	$C_{14}H_{17}O_3$	E−H_2O		3.3
223.1480	$C_{17}H_{19}$	G−H_2O−AcOH		17.7
212.1572	$C_{16}H_{20}$	H−H_2O−AcOH		43.0
197.1325	$C_{15}H_{17}$	D−H_2O−AcOH		75.0
181.1218	$C_{11}H_{17}O_2$	I		100.0
163.1126	$C_{11}H_{15}O$	I−H_2O		28.0



Scheme 4.

the high resolution mass spectrum of peridinin (*1*) are compiled in Table 1.

In the high mass range losses of water, carbon dioxide, acetic acid and toluene as well as combinations of these are observed, Scheme 4.

Two consecutive losses of water are consistent with the two free hydroxy functions of peridinin. Loss of CO_2 has previously been observed for certain carotenoid carboxylic acids, *e.g.* azafrin,[12] and is readily accommodated with the lactone moiety of peridinin, Scheme 4.

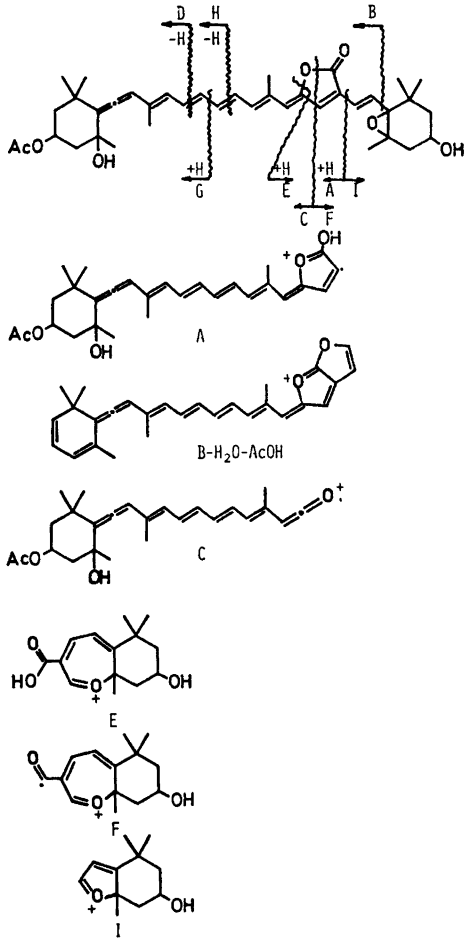
Losses of toluene (C_7H_8), but not of xylene, are observed. In view of the now accepted mechanism for these fragmentations,[13–16] losses of toluene are to be expected for structure *1* whereas loss of xylene is prohibited since no six consecutive carbons of the polyene chain carry the required two methyl groups.

Fragment A, $C_{25}H_{34}O_5$ (*m/e* 450) may be derived by cleavage of the 8′,9′-single bond of peridinin with transfer of the hydrogen at C-7′, resulting in the loss of the epoxy end group as an acetylenic species, Table 1, Scheme 5. Detection of this fragment, although not abundant, is important since it discloses the molecular boundaries for five of the seven oxygen functions of peridinin (*1*).[3] Another fragment, $C_{21}H_{26}O_5$ (*m/e* 358), obviously interconnected with A by an element of toluene (C_7H_8), may be derived by the same process.

Cleavage of the 6′,7′-single bond of peridinin should give rise to a fragment B. This fragment ion was not detected, but fragment $C_{28}H_{29}O_2$ (*m/e* 397) may be derived by this process after initial or subsequent losses of water and acetic acid, Scheme 5.

A fragment C, $C_{25}H_{32}O_4$ (*m/e* 396) may be derived by cleavage of the butenolide ring resulting in a substituted ketene as indicated in Scheme 5. This fragment, although of low abundance, demonstrates the location of the lactone ether oxygen relative to the allenic end group.

A fragment D, $C_{17}H_{23}O_3$ (*m/e* 257), of moderate abundance, is important since it is obviously interconnected with the fragment $C_{15}H_{17}$ (*m/e* 197) below by elements of water and acetic acid. Fragment D may be derived from the allenic end of the peridinin molecule by cleavage of the 11,12-double bond with hydrogen transfer away from the charge, Scheme 5.



Scheme 5.

Three abundant fragments of hydrocarbon composition $C_{17}H_{19}$ (*m/e* 223 = H − H_2O − AcOH), $C_{16}H_{20}$ (*m/e* 212 = G − H_2O − AcOH) and $C_{15}H_{17}$ (*m/e* 197 = D − H_2O − AcOH), may all be derived from the allenic end of the molecule by cleavages of the 13,14-double, 12,13-single and 11,12-double bonds, respectively, with hydrogen transfers as indicated in Scheme 5. No likely structures are, however, proposed for these fragments.

The *m/e* 197 ion is of great diagnostic importance since similar fragments are observed in the mass spectra of both fucoxanthin (*14*) [17] and isofucoxanthin (*15*),[18] Scheme 3. In the latter cases it has been suggested that these fragments are derived from the polyene chain.[17,18] However, to our knowledge this abundant fragment has only been observed for allenic carotenoids. In view of the assignment of fragment D above and the fact that several hydrogen transfers would be required for its formation from the polyene chain of peridinin (*1*) after the initial loss of CO_2, it is more likely to be derived from the allenic end of the molecule as indicated in Scheme 5.

Characteristic fragments of carotenoid 5,6-epoxides and 5,8-furanoxides are the furanoid and homopyryllium ions derived by rearrangements of the oxides and subsequent α-cleavages.[16,19] In the peridinin mass spectrum fragments E ($C_{14}H_{19}O_4$, *m/e* 251), F ($C_{14}H_{18}O_3$, *m/e* 234) and I ($C_{11}H_{17}O_2$, *m/e* 181) as well as the fragments $C_{14}H_{17}O_3$ (E − H_2O, *m/e* 233) and $C_{11}H_{15}O$ (I − H_2O, *m/e* 163) may be accommodated to these general fragmentations, Scheme 5.

Fragment E is of great diagnostic importance since the relative position of the butenolide to the epoxy end group is hereby revealed.[2] Fragment I is identical to that observed for most hydroxylated carotenoid 5,6-epoxides and 5,8-furanoxides.[19] Here its diagnostic importance is obvious since it reveals the epoxidic nature of peridinin.[2]

Several other fragments of the peridinin mass spectrum are of little diagnostic value, but all may be accommodated to structure *1* for peridinin.[20]

The over-all agreement of the twentyfive fragment ions discussed here with structure *1*, strongly support this proposed structure for peridinin.[2]

EXPERIMENTAL

Materials and methods were those commonly employed in the Trondheim laboratory.[21] R_F-values are quoted for Schleicher & Schüll No. 287 (kieselguhr filled) circular paper with mixtures of acetone in petroleum ether (APE) as developer.[22]

Properties of peridinin (*1*) are given in the preceding paper.[2] The high resolution mass spectrum of peridinin (*1*) was obtained with an AEI MS902 instrument with an on line PDP8 data processing unit.[20]

Acid treatment.[23] When dissolved in 1 % citric acid in methanol (3 ml) β-carotene diepoxide (*2*, 0.01 mg) gave a hypsochromic spectral shift of 40 nm after 10 h. Under the same conditions peridinin (*1* 0.1 mg) was quite inert

for the same period. With 15 % citric acid in methanol (5 ml) peridinin (*1*, 0.17 mg) exhibited a hypsochromic spectral shift of 20 nm after 5 h. Treatment of β-carotene diepoxide (*2*, 0.01 mg) in methanol (3 ml) with 0.1 % HCl in methanol (5 drops) resulted in total isomerization within 3 min.

To peridinin (*1*, 5 mg) in methanol (30 ml) was added 0.1 % aq. HCl in methanol (3 ml). After 1.5 h the hypsochromic spectral shift was 20 nm. The reaction mixture was worked up in the usual manner and the products separated by TLC on silica using 50 % APE as developer; pigment recovery was 75 %, including 25 % unreacted peridinin.

The two major products (*3a*, *b*), R_F 0.74 (28 %) and R_F 0.63 (14 %) with 20 % APE, had identical spectral properties: λ_{max} (acetone) 446 nm; *m/e* 630 (M), 612 (M−18), 686 (M−44), 570 (M−60), 552 (M−18−60), 538 (M−92), 234, 223, 212, 197 and 181.

The two minor products (*4* and *5*, 8 %) both had λ_{max} (acetone) 459, (474) nm and R_F 0.41 with 20 % APE. The mixture of *4* and *5* (0.8 mg) in dry pyridine (2 ml) was acetylated with acetic anhydride (0.2 ml) for 19 h and the acetates separated by TLC on silica using 40 % APE as developer. Pigment recovery was 93 %.

The major acetate (*4a*, 0.55 mg, 75 %) had R_F 0.76 with 20 % APE; λ_{max} (acetone) 459, (474) nm; *m/e* 704 (M), 686 (M−18), 672 (M−32), 654 (M−18−32), 644 (M−60), 626 (M−18−60), 612 (M−92), 594 (M−18−60−32), 223, 212, 197 and 149.

Silylation [6] of the acetate *4a* (0.37 mg) at room temperature for 67 h gave a mono-TMS ether [*4b*, R_F 0.67 with 10 % APE, λ_{max} (acetone) 459, (474) nm, *m/e* 776 (M)] and a di-TMS ether [*4c*, R_F 0.98 with 10 % APE, λ_{max} (acetone) 459, (474) nm, *m/e* 848 (M)].

The minor acetate (*5a*, 0.18 mg, 25 %) had R_F 0.77 with 20 % APE; λ_{max} (acetone) 459, (474) nm; *m/e* 672 (M), 654 (M−18), 612 (M-60), 594 (M−18−60), 223, 212, 197 and 149.

Silylation of the acetate *5a* (0.12 mg) at room temperature for 1 h gave a mono-TMS ether (*5b*, 0.11 mg) which had R_F 0.69 with 10 % APE, λ_{max} (acetone) 459, (474) nm, *m/e* 744 (M).

Base treatment.[23] To peridinin (*1*, 52 mg) in methanol (20 ml) was added K_2CO_3 (100 mg) in water (0.6 ml). After 16 h all peridinin had reacted and the reaction mixture was worked up by addition of water and extraction with ether giving 3.9 mg (7.7 %) neutral products. Acidification of the hypophase with 1 % aqueous H_2SO_4 gave 0.93 mg (1.8 %) of an acidic pigment.

The neutral pigments were separated by TLC on silica using acetone as developer.

The major product (*1c*, 3 mg, 5.8 %) had R_F 0.38 with 20 % APE λ_{max} (acetone) 464 nm, *m/e* 588 (M), 570 (M−18), 552 (M−2×18), 544 (M−44), 538 (M−50), 534 (M−3×18), 526 (M−18−44), 508 (M−80), 496 (M−92), 478 (M−18−92), 233, 230, 215, 209, 207, 197, 181 and 167.

Standard acetylation of *1c* (1.3 mg) gave two monoacetates, *1* [R_F 0.68 with 20 % APE, λ_{max} (acetone) 464 nm, *m/e* 630 (M)] which could not be separated from peridinin (*1*) on co-chromatography, and *1d* [R_F 0.58 with 20 % APE, λ_{max} (acetone) 464 nm, *m/e* 630 (M)], and a diacetate *1a* [R_F 0.88 with 20 % APE, λ_{max} (acetone) 464 nm, *m/e* 672 (M)] which could not be separated from peridinin acetate (*1a*) on co-chromatography.

Another neutral product (*6*, 0.83 mg, 1.6 %) had R_F 0.84 with 50 % APE, λ_{max} (acetone) 391 nm, *m/e* 620 (M), gave a diacetate [*6a*, R_F 0.69 with 20 % APE, λ_{max} (acetone) 391 nm, *m/e* 704 (M)] on acetylation and *6a* gave a di-TMS ether [*6b*, R_F 0.90 with 10 % APE, λ_{max} (acetone) 391 nm, *m/e* 848 (M)] on silylation.

A third neutral product (*7*, 0.09 mg, 0.2 %) had R_F 0.42 with 20 % APE, λ_{max} (acetone) 372 nm, *m/e* 600 (M). Acetylation of *7* gave a diacetate with R_F 0.88 with 20 % APE, λ_{max} (acetone) 372 nm and *m/e* 684 (M).

The acidic product *8* (0.93 mg, 1.8 %), purified by TLC on silica using 70 % methanol in acetone as developer, had λ_{max} (methanol) 367 nm and could not be esterified with diazomethane.

Dehydration with $POCl_3$.[24] Peridinin (*1*, 2 mg) in pyridine (2.5 ml) and $POCl_3$ (0.1 ml) were mixed at 0 °C and reacted at room temperature for 4 h. The products were extracted into ether after addition of water, and the extract washed with dilute aq. HCl and $NaHCO_3$ and dried. Evaporation of the solvent gave 1.7 mg (85 %) of oily products which were separated by TLC on silica using chloroform as developer. Four products with molecular ions at *m/e* 612, 612, 594 and 594, respectively, were obtained.

Reduction with $NaBH_4$.[23] Peridinin (*1*, 5.5 mg) in 96 % ethanol (10 ml) was reduced with excess $NaBH_4$ for 2 h at room temperature. The reaction mixture was worked up in the usual manner and the products (2.75 mg, 50 %) separated by TLC on silica using 80 % APE as developer. Unreacted peridinin (1.92 mg, 35 %) was recovered.

The major product (*9*, 0.45 mg, 8.1 %) had R_F 0.11 with 10 % APE, λ_{max} (acetone) 433, (452) nm, and gave a monoacetate, no intermediate, *9a* (R_F 0.28 with 10 % APE, λ_{max} (acetone) 433, (452) nm) on acetylation. Silylation of the acetate *9a* gave a mono-TMS ether *9b* [R_F 0.61 with 10 % APE, λ_{max} (acetone) 433, (452) nm, *m/e* 746 (M)] and a di-TMS ether *9c* [R_F 0.97 with 10 % APE, λ_{max} (acetone) 433, (452) nm, *m/e* 818 (M)].

Another product (*10*, 0.19 mg, 3 %) had R_F 0.15 with 10 % APE, λ_{max} (ether) 331, 347, 366.5 nm, ν_{max} (KBr) 1930 (C=C=C), 1725 (broad, C=O), 1250 (C−O) cm^{-1}, *m/e* 634

(M), 616 (M−18), 598 (M−2×18), 197, 181, 149. Acetylation of *10* gave a triacetate *10a* [R_F 0.55 with 10 % APE, λ_{max} (ether) 331, 347, 366.5 nm, *m/e* 760 (M)] and silylation of *10a* gave a mono-TMS ether *10b* [R_F 0.68 with 5 % APE, λ_{max} (ether) 331, 347, 366.5 nm, *m/e* 822 (M)].

A third product (*11*, 0.066 mg, 1.2 %) had R_F 0.29 with 10 % APE, λ_{max} (ether) 331, 347, 366.5 nm, *m/e* 634 (M), 616 (M−18), 574 (M−60), 556 (M−18−60), 541 (M−93), 523 (M−18−93), 491 (M−143), 251, 243, 223, 207, 200, 197, 185, 181, 163, 143, 125. Saponification of *11* gave a product *11c* [λ_{max} (ether) 331, 347, 366.5 nm, *m/e* 592 (M)]. Acetylation of *11* gave a monoacetate *11a* [R_F 0.44 with 10 % APE, λ_{max} (ether) 331, 347, 366.5 nm, *m/e* 676 (M)] and silylation of *11a* gave a di-TMS ether *11b* [λ_{max} (ether) 331, 347, 366.5 nm, *m/e* 778 (M)].

Two minor products (*12* and *13*) both had R_F 0.20 with 20 % APE, λ_{max} (acetone) 349, 368 nm and could only be separated as the acetates.

The minor acetate (*12a*, 0.05 mg, 0.9 %) had R_F 0.54 with 10 % APE, λ_{max} (acetone) 349, 368 nm, *m/e* 762 (M). Silylation of *12a* gave a mono-TMS ether *12b* [λ_{max} (acetone) 349, 368 nm, *m/e* 834 (M)].

The major acetate (*13a*, 0.074 mg, 1.35 %) had R_F 0.35 with 10 % APE, λ_{max} (acetone) 349, 368 nm, *m/e* 764 (M). Silylation of *13a* gave a di-TMS ether *13b* [λ_{max} (acetone) 349, 368 nm, *m/e* 908 (M)].

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Reduction Reactions of Halonitrobenzenes by Copper(I) in Hydrochloric Acid Medium

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The copper(I) promoted reduction of the nitro group, which always competes to a small extent with the copper(I) catalysed halogen exchange reactions in substituted halonitrobenzenes, studied by the author, has been subject to a closer study in this paper. A common step is suggested for the reduction of the nitro group and for the reductive dehalogenation, which in a few cases also is observed as a by-product in the halogen exchange mixtures.

In the dichlorocuprate(I) ion catalysed iodine-chlorine exchange reactions of 3-halo-2-iodonitrobenzenes in an aqueous hydrochloric acid-acetic acid medium about 5 % of dehalogenation products (ArI→ArH) were detected,[1] but in the bromine-chlorine exchange reactions only traces were obtained and, moreover, only from 2-bromo-3-fluoronitrobenzene.[2] These observations are in full accord with the experience of Bacon and Wright,[3] Fanta,[4] and Nilsson and Björklund [5] in connection with reactions in non-aqueous media. In the copper(I) catalysed exchange reactions of halonitrobenzenes in aqueous media another side reaction is striking, *viz.* the reduction of the *ortho* nitro group, which always accompanies the iodine- and bromine exchanges to an extent of about 2 – 3 %.[1,2] In the case of 3-bromo-2-fluoronitrobenzene this reduction of the nitro group was the only copper(I) promoted reaction.[6] The question is whether these two side reactions have a common intermediate. To test if a free radical mechanism could be prevailing, the 2-bromo-3-methylnitrobenzene was tested with some added initiators and inhibitors and was submitted to UV irradiation at 350 nm. The 3-bromo-2-iodonitrobenzene was also irradiated.

RESULTS

An ESR spectrum of the exchange reaction mixture of 2-bromo-3-methylnitrobenzene gave no radical signal, but this does not exclude the possibility that radicals may have been present in low concentration. The addition of 1,4-benzoquinone to 2-bromo-3-methylnitrobenzene in the molar ratio 2:1, with other conditions the same as in the exchange experiment, seemed to cause no change in the exchange or the reduction rates at 90 °C, nor did 1,4-dihydroxybenzene in the proportions 1:1 to the substrate. *trans*-Azobenzene in the ratio 1:1 apparently destroyed the catalysing copper(I) complex and the halogen exchange was quenched.

When the degassed reaction mixture with copper(I) chloride present was subjected to irradiation at 350 nm for 2 h at about 30 °C, the reduction of the nitro group dominated over the bromine-chlorine exchange, which did not increase its rate compared to an experiment with copper(I) chloride in the dark at 30 °C. Without copper(I) chloride present and in the dark neither exchange nor reduction occurred.

When 3-bromo-2-iodonitrobenzene, which gave 5 % together of 3-bromonitrobenzene and 3-bromoaniline as by-products in its copper(I) catalysed iodine-chlorine exchange reaction,[1] was irradiated for 2 h at 350 nm in the absence of copper(I) chloride, dehalogenation and

traces of halogen exchange also occurred, but without irradiation of this solution no dehalogenation and no exchange were observed.

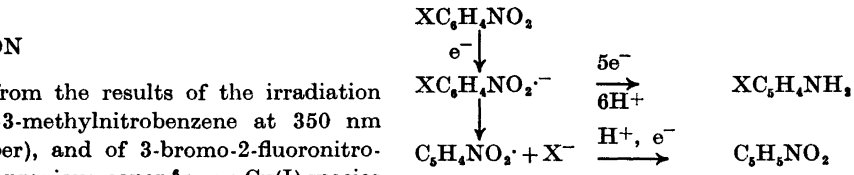
DISCUSSION

Judging from the results of the irradiation of 2-bromo-3-methylnitrobenzene at 350 nm (in this paper), and of 3-bromo-2-fluoronitrobenzene in a previous paper,[6] some Cu(I) species seems to act as the electron donor in the observed reduction of the nitro group, which is accelerated by the irradiation. The aqueous hydrochloric acid – acetic acid medium can serve as the proton donor. The copper(I) catalysed dehalogenation observed in 2-iodonitrobenzenes [1] took also place with irradiation at 350 nm.

These observations suggest a free radical mechanism, but the possibility that the nitrobenzenes in their excited states resulting from absorption of radiation might react *via* an ionic mechanism cannot be excluded.

The photochemical reduction of nitrobenzene to aniline in 2-propanol-hydrochloric acid solution has been described by Hashimoto *et al.*[7] It is proposed that the excited $n\rightarrow\pi^*$ triplet state abstracts a hydrogen from 2-propanol. Ayscough *et al.*[8] have published ESR spectra of radical anions formed by the addition of an electron to the nitro compounds during chemical and photochemical reduction of a number of substituted nitrobenzenes in aqueous alcohols. Lagercrantz and Yhland [9] reported that solutions of electron acceptors such as *m*-dinitrobenzene in donor solvents such as THF exhibit a photoinduced ESR signal. The spectrum is consistent with the formation of negative radical ions. Ito *et al.*[10] reported electron transfer from a copper(I) isonitrile complex to nitrobenzenes, producing radical anions as seen from the ESR spectra. Danen *et al.*[11] used ESR to establish that the lifetime of electrochemically generated anion radicals of halogenated nitrobenzenes sharply decreases with increasing halogen atomic number. The 2-iodonitrobenzene anion radical loses iodide ion very rapidly and is converted into a nitrophenyl radical.

The over-all reduction of nitrobenzene *via* the radical anion to aniline involves six electrons and six protons. A common step is suggested for the copper(I) promoted reduction of the nitro group and of the dehalogenation in 2-halonitrobenzenes in aqueous hydrochloric acid-acetic acid medium.



In this reaction scheme X = I or Br.

EXPERIMENTAL

Mass spectra were obtained with an AEI MS 20 instrument. The irradiation was carried out in a photochemical reactor Rayonet RPR-100 and the GLC with a Perkin Elmer F 11 Hot Wire Gas Chromatograph with a SE 30 column.

2-Bromo-3-methylnitrobenzene was prepared from 2-methyl-6-nitroaniline as described in Ref. 2. M.p. 40.0 – 41.5 °C.

3-Bromo-2-iodonitrobenzene was synthesized from 2-bromo-6-nitroaniline. See Ref. 1. M.p. 120.5 – 121.5 °C.

The reaction of the halonitrobenzenes with copper(I) chloride was performed in the medium and with the method described in Ref. 12.

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Statistical Analysis of the Hammett Equation. III. Evidence against *para* Through-resonance for —M Substituents in Phenylacetic Acids and Phenylpropionic Acids

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Deviation from the Hammett equation for phenylacetic acids and β-phenylpropionic acids in aq. EtOH are studied with statistical methods. The deviations are explained as solvent dependent interactions between the reaction site and *meta* substituents with free electron pairs. No evidence for *para* through-resonance for —M substituents as claimed in literature is found.

The precise definition of σ-values to be used in the Hammett equation is important for several reasons. One important example is the use of σ-values to check the "homogeneity" of an aromatic reaction series. Thus, a reaction in an aromatic reaction series which does not fit a Hammett plot, is usually considered "abnormal". Since the σ-values are the basis for the Hammett equation, their values are crucial for the interpretation of these plots.

A frequently used way to define the σ-value for a particular substituent is to use directly, the pK_a-value for unsubstituted benzoic acid minus the observed pK_a of the corresponding substituted benzoic acid in water. Substituents for which these pK_a-values are ill-defined are customarily given so called secondary σ-values in the following way:

1. Select a well-behaved reaction series where the effect of the particular substituent can be observed.

2. Measure log k (or log K) for (*a*) primary substituents with σ-values defined by the benzoic acid series and (*b*) the new substituent.

3. Define a ϱ-value for the reaction series based on the values of step 2 *a*.

4. Define the secondary σ-values for the substituent as $(\log k - \log k_0)/\varrho$.

In our opinion, this way to define secondary σ-values has serious disadvantages:

(*a*) The analysis is based on the assumption that the standard reaction is free from model errors.

(*b*) A few, mostly *meta*, substituents are assumed to be representative for the Hammett equation as a whole.

However, these assumptions can never be fulfilled exactly. This makes both the ϱ-value and the calculated log k_0-value in a Hammett plot sensitive to unavoidable errors in the primary σ-values. This will, in turn, produce a large uncertainty for the calculated secondary σ-values, especially for reaction series with small ϱ-values and for substituents with large σ-values.

Recently Hoefnagel *et al.*[1,2] used this classical method to determine secondary σ-values for a number of substituents. They concluded that the systems $X-Ph-CH_2-COOH$ (*1*) and $X-Ph-CR_2-CH_2-COOH$ (R= —H or $-CH_3$) (*2*) are unsuitable for the derivation of σ-values (σ^0 or σ^n) for *para* —M substituents. In the Hammett plots, they found large positive deviations for these substituents and explained this as a through-resonance effect. However, we believe the deviations for the *para* —M substituents to be a result of the way they made their data analysis. We claim that no through-resonance effect exists for the systems (*1*) and (*2*). This conclusion

is based on a statistically correct analysis of these systems.

STATISTICAL METHOD AND CALCULATIONS

We have used the method described earlier by us.[3,4] This method makes it possible to analyse the Hammett equation on the form

$$\log k_{ik} = \phi_k + \varrho_k \sigma_i + e_{ik}$$

where e_{ik} are the residuals and ϕ_k is the calculated log k_0-value. The index i varies with the substituent and the index k with the reaction series. By this method, σ-values which best fit the total body of data are calculated. Furthermore, the residuals e_{ik} give information about the fit of both individual reaction series and individual substituents.[3,4]

To investigate whether a −M effect exists in the Hoefnagel data, we have included reaction series of (*1*) and (*2*) (see Table 1) in a data matrix which in addition consisted of about 60 reaction series of σ^0, σ and σ^- data earlier used in similar calculations.[3,4]

The *para* +M substituents in σ series and the *para* −M substituents in σ^- series have been excluded; see further Ref. 4. For each of the two types of series (*1*) and (*2*) two

Table 1. The analysed reaction series of *(1)* and *(2)*.

No.	Reaction	Ref.
Phenylacetic acids		
1a	H_2O[a]	5
1b	H_2O[a]	6,7
1c	10 % EtOH	2
1d	50 % EtOH	2
1e	75 % EtOH	2
β-Arylpropionic acids		
2a	50 % EtOH	1
2b	75 % EtOH	1
β-Arylisovaleric acids		
2c	50 % EtOH	1
2d	75 % EtOH	1

[a] $pK_a = 3.89$ is used for the p-NO_2 compound, see Ref. 2, footnote 37.

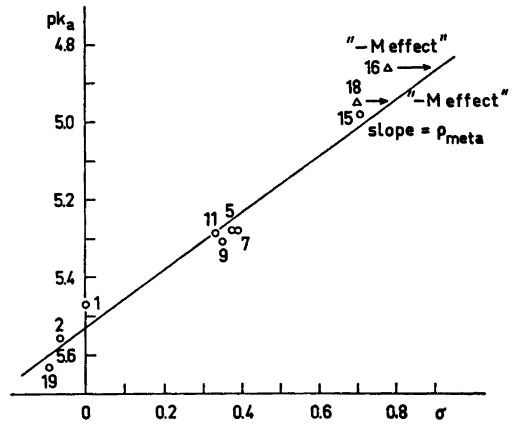

Fig. 1. The analysis of Hoefnagel *et al.* of ionization equilibria of phenylacetic acids in 50 % EtOH. Data for eight selected *meta* substituents, marked with O are plotted against the primary σ-values [1] for these substituents. Substituent numbers see Table 2. No. 19 is 3,5-di-CH_3. Calculated values: $\varrho_m = 0.714$, log $K_{0,calc}$ − log $K_{0,exp} = -0.058$, $s_\varrho = 0.052$ and $s_c = 0.038$. The two −M substituents p-NO_2 (No. 16) and p-CN (No. 18) are plotted against their primary σ-values. The difference between the primary σ-value and the σ-value determined from the equation of the line is explained in Ref. 2 as a "through-resonance" effect.

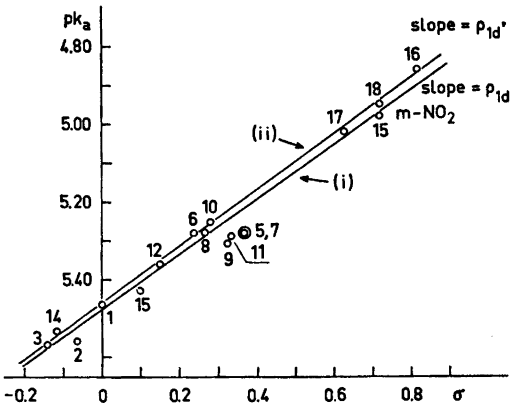

Fig. 2. Our analysis of ionization equilibria of phenylacetic acids in 50 % EtOH. The σ-values used are from Table 2 and are somewhat different from the primary σ-values used by Hoefnagel *et al.* Calculated values: (*i*) with all substituents included; $\varrho_{1d} = 0.712$, log $K_{0,calc}$ − log $K_{0\,exp} = -0.014$, $s_\varrho = 0.030$ and $s_c = 0.036$, (*ii*) without m-OCH_3, m-halogens, and m-NO_2; $\varrho_{1d'} = 0.733$, log $K_{0,calc}$ − log $K_{0,exp} = 0.003$, $s_\varrho = 0.017$ and $s_c = 0.018$.

Table 2. Residuals e_{ik} for each reaction of system (*I*), (*i*) with and (*ii*) without the substituents *m*-OCH_3, *m*-halogens, and *m*-NO_2. For each computation, the ϱ-value, the C_ϱ-value [a] and the residual standard deviation s_e, are also given. Numbering, see Table 1. All values are derived with about 60 additional reactions of σ^0, σ, and σ^- type. The σ-values result from computation (*i*).[b]

	Substituent	σ	Reaction series 1a	1a′	1b	1b′	1c	1c′	1d	1d′	1e	1e′
1	H	0.000	−0.012	−0.011	0.002	0.001	0.010	0.003	0.014	−0.003	0.009	−0.007
2	*m*-Me	−0.066			−0.017	−0.016	−0.015	−0.022	−0.029	−0.045	−0.005	−0.021
3	*p*-Me	−0.142	0.008	0.009	0.014	0.015	0.016	0.010	0.025	0.011	0.010	−0.005
4	*p*-Et	−0.121			0.001	0.001						
5	*m*-Cl	0.369	0.007		−0.009		−0.018		−0.059		−0.024	
6	*p*-Cl	0.238	0.005	0.005	0.006	0.002	0.012	0.004	0.035	0.013	0.057	0.040
7	*m*-Br	0.370					−0.018		−0.059		−0.025	
8	*p*-Br	0.264	−0.019	−0.020	−0.005	−0.009	0.008	−0.001	0.016	−0.007	0.024	0.007
9	*m*-I	0.324			−0.008				−0.057		−0.026	
10	*p*-I	0.278			−0.003	−0.007	0.001	−0.008	0.036	0.013	0.023	0.005
11	*m*-F	0.334	0.004				−0.009		−0.044		−0.035	
12	*p*-F	0.149	0.007	0.008	−0.006	−0.008	0.000	−0.008	0.016	−0.002	0.022	0.006
13	*m*-OCH_3	0.103					−0.035		−0.019		−0.069	
14	*p*-OCH_3	−0.122	0.003	0.004	0.012	0.013	0.025	0.019	0.041	0.027	0.012	−0.002
15	*m*-NO_2	0.717	−0.008		−0.009		−0.014		−0.007		−0.010	
16	*p*-NO_2	0.815	0.008	0.006	0.010	0.010	0.023	0.012	0.044	0.010	0.007	−0.014
17	*m*-CN	0.626					−0.005	−0.016	0.019	−0.011	0.008	−0.012
18	*p*-CN	0.715					0.017	0.007	0.025	−0.005	0.022	0.004
		s_e	0.0092	0.0102	0.0100	0.0099	0.0167	0.0117	0.0358	0.0179	0.0264	0.0152
		ϱ	0.493	0.496	0.496	0.507	0.535	0.541	0.712	0.733	0.849	0.856
		C_ϱ	0.045	0.066	0.042	0.051	0.056	0.046	0.090	0.050	0.060	0.037

[a] Criterion of goodness of fit, see Ref. 4. $C_\varrho = t_{n-2}$ (0.05) s_ϱ/ϱ. [b] The sigma values derived in case (*ii*) show small differences from case (*i*) due to the change in the data base from which they are calculated. The differences are, however, within 0.002 σ units.

Table 3. Residuals e_{ik} for each reaction of system (*2*), (*i*) with and (*ii*) without the substituents *m*-OCH_3, *m*-halogens, and *m*-NO_2. Notation same as in Table 2.

Substituents		2a	2a′	2b	2b′	2c	2c′	2d	2d′
1	H	0.021	0.008	0.006	−0.001	−0.002	−0.011	0.021	−0.001
2	*m*-Me	0.007	−0.007	0.008	0.002				
3	*p*-Me					0.004	−0.001		
5	*m*-Cl	−0.029		−0.025		−0.040		−0.026	
6	*p*-Cl					0.008	−0.011		
7	*m*-Br	−0.029		−0.005		−0.030		−0.007	
12	*p*-F					0.008	−0.003		
14	*p*-OCH_3					0.026	0.020		
15	*m*-NO_2	−0.012 [a]		−0.025 [a]		−0.019		−0.005	
16	*p*-NO_2	0.020	0.006	0.016	0.001	0.032	0.007	0.008	−0.004
17	*m*-CN	0.012	−0.002	0.009	0.004				
18	*p*-CN	0.009	−0.005	0.015	0.002	0.022	0.000	0.017	0.004
	s_e	0.0195	0.0059	0.0158	0.0024	0.0226	0.0101	0.0158	0.0033
	ϱ	0.382	0.384	0.491	0.500	0.399	0.420	0.481	0.471
	$C\varrho$	0.141	0.057	0.090	0.018	0.125	0.061	0.125	0.149

[a] Calculated σ-values for 3,5-di-NO_2 in reaction 2a; 1.39; 2: 1.27. The primary σ-value is 1.379. The statistical value based on 11 observations is 1.417 (to be published by the present authors). The σ-value for reaction 2a is within the uncertainty of the Hammett equation but the value for reaction 2b is too low. The 3,5-di-NO_2 group thus shows a strong solvent dependence when the alcohol content is increased from 50 % in reaction 2a to 75 % in 2b.

computations were made, denoted by 1a and 1a′ *etc:* (*i*) all substituents included and (*ii*) *meta* substituents with free electron pairs, *i.e. meta* halogens, *m*-OCH_3 and *m*-NO_2 excluded for series of (*1*) and (*2*); *m*-CN showed no anomaly and was not excluded.

RESULTS

The results of the computations are presented in Tables 2 and 3. Reactions 1a and 1b show very good fits in both computations and the deviations are of the normal size for the Hammett equation.[3,4] There is no improvement of the fit in the case 1a′ and 1b′. In reaction 1c, all substituents show good fits except *m*-OCH_3 and there is some improvement of the fit in the case 1c′. Reactions 1d and 1e show big negative residuals for the *meta* halogens and *m*-OCH_3 but the fit is greatly improved in the cases 1d′ and 1e′. The reaction 2c shows the same residual pattern as the reaction 1d and 1e and the fit is improved in case 2c′. In the reactions 2a′, 2b′ and 2d′ the substituents are too few to allow stringent interpretations. The results are, however, included for comparison.

DISCUSSION

The main pattern is best seen in Figs. 1 and 2, where the Hammett plot for the series 1d are made in (Fig. 1) the way corresponding to the analysis of Hoefnagel and (Fig. 2) corresponding to 1d and 1d′ in our analysis. In the plot corresponding to our analysis, the free electron substituents deviate and especially the *meta* halogens (Nos. 5, 7, 9, and 11). As seen in Fig. 1 the Hoefnagel plot relies very much on these substituents, which results in too low ϱ and log K_0 values and, consequently, far too large secondary σ-values for *p*-CN and *p*-NO_2. These abnormal values were interpreted by Hoefnagel *et al.* as due to through-resonance effects.

Since the deviations for the *meta* substituents are absent in the series 1a and 1b and increase through 1c and 1d with increasing amounts of alcohol in the solvent, we suggest that they are due to some solvent related effect. Hence,

we claim that the poor fit for the phenylacetic acids in 50 % EtOH is likely to be a solvent dependent interaction between the *meta* substituents and the reaction site and not due to a through-resonance effect of the *para* substituents.

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Reaction of Esters with Dibromomethyl Methyl Ether

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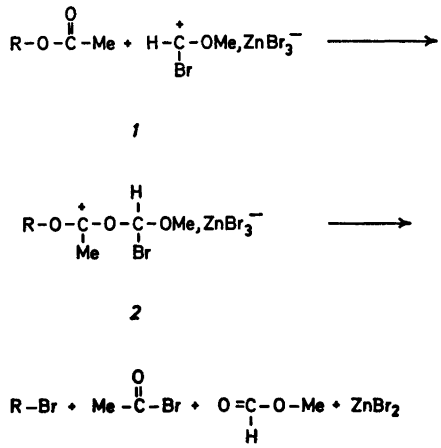
Acetylated monoalcohols react with dibromomethyl methyl ether (DBE) and zinc bromide to give the corresponding alkyl bromides, secondary acetates with inversion of configuration. Both *cis*- and *trans*-1,2-diacetoxycyclohexane react with DBE-$ZnBr_2$, probably *via* an acetoxonium ion, to give *trans*-2-bromocyclohexyl acetate. The latter on further reaction yields *trans* 1,2-dibromocyclohexane. 2-Bromo-3-acetoxytetrahydropyrane was readily converted into 2,3-dibromotetrahydropyrane whereas 3-acetoxytetrahydropyrane did not react. The mechanism of the reaction of acylated carbohydrates with DBE-$ZnBr_2$ is discussed.

Reaction of acylated pentopyranoses or pentofuranoses with dibromomethyl methyl ether (DBE) and zinc bromide leads to formation of 2-bromo-2-deoxy-glycosyl bromides in moderate yield, but with a high degree of stereo- and regio-selectivity.[1,2] Partial mechanisms have been proposed;[1,2] but in order to get a more complete picture of these reactions the behaviour of a number of simple esters towards DBE has now been studied, and the results are described in the present paper.

All reactions of esters with DBE and zinc bromide were carried out in chloroform or deuteriochloroform solutions, the conditions which were also used for the reactions with sugar esters. In the previous work the reaction mixtures were often inhomogeneous, either because the zinc bromide was not completely soluble, or because of formation of complexes between the zinc bromide and the sugar esters. In the present work suitable concentrations were chosen to ensure homogeneous reaction mixtures.

Treatment of the acetylated alcohols shown in Table 1 with DBE and zinc bromide gave the corresponding bromides, methyl formate, and acetyl bromide. The reactions were monitored by NMR spectroscopy and the products were further analysed by GLC. The high rates of reaction of *tert*-butyl and benzyl acetate indicate that these esters react by an S_N1 type of reaction, involving carbonium ion intermediates. The secondary and primary esters do probably not react *via* free carbonium ions since this would lead to rearrangement, and only one product was found in each case. Treatment of 3-β-cholestanyl acetate with DBE and zinc bromide gave a high yield of the thermodynamically unstable 3-α-cholestanyl bromide, the 3-β-bromide not being detectable. Thus secondary esters appear to react with inversion.

The mechanism, as proposed by Szabó *et al.*,[3] probably involves reaction of the ester with the oxocarbonium ion *1* to give an unstable intermediate *2*, which decomposes to the alkyl bromide, acetyl bromide, and methyl formate (Scheme 1).



Scheme 1

Table 1. Conversion of monoacetates to bromides with DBE and zinc bromide in deuteriochloroform.

Acetate	Bromide	Time to complete the reaction
Isobutyl	Isobutyl	10 – 20 days
sec-Butyl	*sec*-Butyl	1 h
Cyclohexyl	Cyclohexyl	10 h
tert-Butyl	*tert*-Butyl	< 2 min
Benzyl	Benzyl	< 2 min

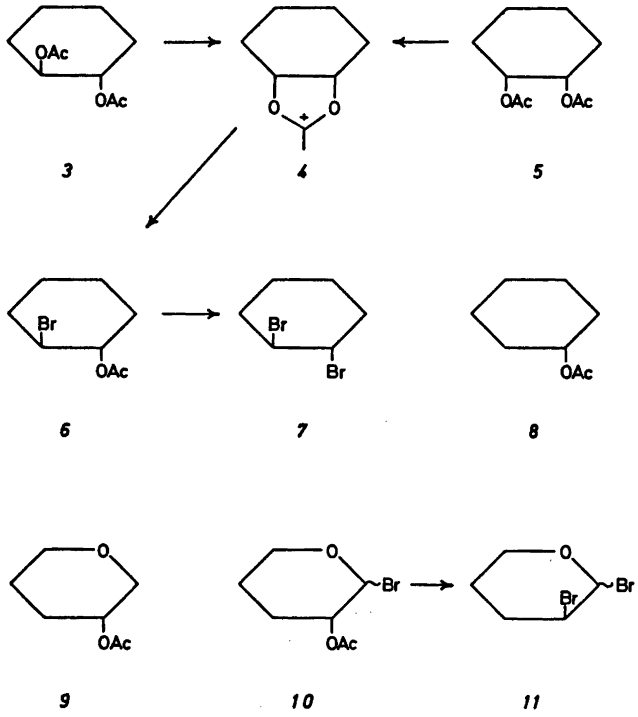
Table 2. Competetive experiments in which a mixture of two esters were treated with DBE-$ZnBr_2$ in deuteriochloroform.

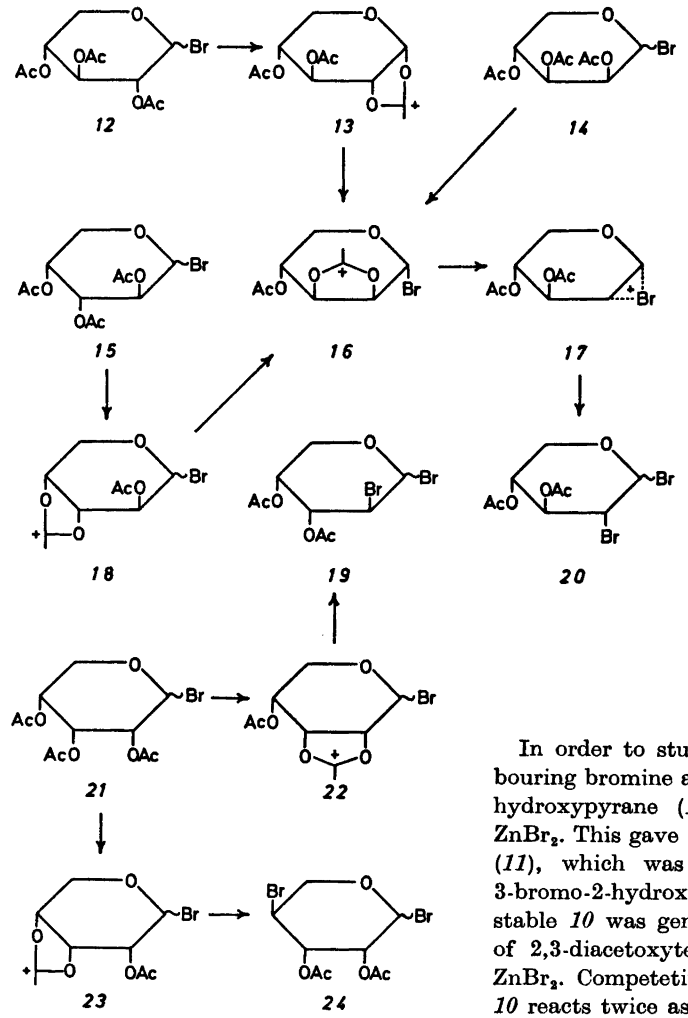
Esters	Reaction time h	% reacted of each ester
3 – *8*	4	3 – 46
5 – 8	4	10 – 57
3 – *5*	48	26 – 50
6 – *8*	2.5	9 – 62
9 – *10*	1 (in CH_3NO_2)	0 – 100
8 – *10*	1	30 – 55
8 – *a*	1.5	40 – 53

[a] Cyclohexyl benzoate.

Reaction of *trans*- or *cis*-1,2-diacetoxycyclohexanes, *3* and *5*, with DBE and zinc bromide gave a mixture of the *trans*-monobromide (*6*) and *trans*-dibromocyclohexane (*7*). Competetive experiments, in which the rate of reaction of cyclohexyl acetate (*8*) was compared with those of *3*, *5* and *6*, gave the results shown in Table 2. It is seen that *3*, *5* and *6* all react slower than cyclohexyl acetate, probably because of the inductive effect of a neighbouring acetoxy or bromo group.[4] The reactions of *3* and *6* must involve a *trans* neighbouring group participation by an acetoxy group or a bromine atom since retention of the configuration is observed. The conversion of *5* to *6*, on the other hand, takes place with inversion which might suggest a direct replacement.

In order to get more information about the influence of neighbouring oxygen on the reaction 3-acetoxytetrahydropyrane (*9*) was treated with DBE-$ZnBr_2$. It was found not to react at all. Thus a β-oxygen has a strongly retarding effect on the reaction of an acetoxy





group, and the fact that *3* and *5* react with a rate not much lower than *8* strongly suggests that neighbouring group assistance is taking place and that the acetoxonium ion *4* is an intermediate in the conversion of both *3* and *5* into *6*.

Thus the reaction of *3* with DBE-$ZnBr_2$ is probably analogous to its reaction with antimony pentachloride to give *4*.[5] The *cis*-neighbouring group assistance in *5* is analogous to its reaction with hydrogen fluoride.[6] The ability of DBE-$ZnBr_2$ to form an acetoxonium ion with both *cis*- and *trans*-diacetates is similar to the effect of trifluoromethane sulfonic acid.[7]

In order to study the influence of a neighbouring bromine atom 3-acetoxy-2-bromotetrahydroxypyrane (*10*) was treated with DBE-$ZnBr_2$. This gave 2,3-dibromotetrahydropyrane (*11*), which was identified by hydrolysis to 3-bromo-2-hydroxy-tetrahydropyrane. The unstable *10* was generated *in situ* by treatment of 2,3-diacetoxytetrahydropyrane with DBE-$ZnBr_2$. Competetive experiments showed that *10* reacts twice as fast as *8* (Table 2). This, in connection with the fact that *9* does not react at a detectable rate, shows that the bromine atom at C1 of *10* must participate in the displacement of the acetoxy group to give *11*. This probably involves the formation of a 2,3-bromonium ion and results in migration of bromine from C1 to C2. Similarly, the conversion of *6* to *7* must proceed *via* a bromonium ion.

From the experiments described above a mechanism for the reaction of sugar esters with DBE and zinc bromide may be proposed. Sugar esters in which the substituent at C2 and C3 are *trans* oriented, such as tri-*O*-acetyl-D-xylopyranosyl bromide (*12*), can react *via* a 1,2-acetoxonium ion (*13*) which rearranges

to a 2,3-ion (*16*) with simultaneous introduction of bromine at C1. Attack of the bromine atom of C1 upon C2 may then yield the 2-bromo-2-deoxy-derivative *20 via* the bromonium ion *17*, as proposed previously.[1] This explains the stereoselectivity of the reaction and the fact that bromine is only introduced at C2. An analogous mechanism can explain the reaction of the corresponding furanoses in which the substituents at C2 and C3 are *trans*-oriented.[2] The compound *20* has *trans*-acetoxy groups at C3 and C4 and it might therefore undergo further substitution similar to that of *3*. This was, however, not observed in *20* or in other sugar derivatives.

Sugar derivatives in which C2 and C3 are *cis*-oriented must react by a different mechanism. The lyxosyl bromide *14* can form the 2,3-ion *16* directly by a mechanism analogous to that by which *5* is converted into *4*. The ion *16* then yields *20* as described above. The ribo- and arabinofuranosyl bromides may react in the same way.[2] Tri-*O*-acetylribopyranosyl bromide (*21*) has C2 and C3 *cis*-oriented and can therefore give a 2,3-acetoxonium ion *22*. On further reaction, analogous to the conversion of *16* to *20*, this would give the 2-bromo-2-deoxyarabinose derivative *19* which was found previously.[1] Alone of all pentose-derivatives investigated *21* gave a 4-bromo-4-deoxy compound (*24*).[1] This probably takes place *via* the 3,4-acetoxonium ion *23*.

Tri-*O*-acetylarabinosyl bromide (*15*) has C2 and C3 *trans*-oriented and could therefore react analogous to *12*. In this case the acetoxonium ion *22* would be formed, and the final product would be the 2-bromo-2-deoxyarabinose derivative *19*. However, *20* and not *19* was the product actually found,[1] and this must mean that the *cis*-3,4-acetoxy groups of *15* react to give an acetoxonium ion (*18*) which rearranges to *16* and subsequently yields *20*.

A number of benzoylated pentosyl bromides have been studied, and it was found that, of the pyranoses, only tri-*O*-benzoylxylopyranosyl bromide gave a 2-bromo-2-deoxy compound with DBE-$ZnBr_2$.[1] Benzoylated ribo- and lyxofuranosyl bromide did not react whereas the corresponding arabino- and xylofuranosyl bromides gave 2-bromo-2-deoxy compounds.[2] This indicates that the benzoylated pentoses in which the reaction would be expected to start from a pair of *cis*-oriented benzoyloxy-groups do not react.

In agreement herewith it was found that *cis*-1,2-dibenzoyloxycyclohexane did not react with DBE-$ZnBr_2$. Cyclohexyl benzoate reacted slightly faster than the acetate *8* to give cyclohexyl bromide and 3-*O*-benzoyl-2-bromotetrahydropyrane readily gave 2,3-dibromo-tetrahydropyrane (*11*).

EXPERIMENTAL

^1H NMR spectra were measured on Varian A60, Varian HA-100, or Bruker HX-90E instruments. For gas chromatography (GLC) was used a Perkin-Elmer F 11 instrument equipped with a column of silicone E 301. Preparative thin layer chromatography (TLC) was done on 1 mm layers of silica gel (Merck PF_{254}).

Dibromomethyl methyl ether (DBE) was prepared according to Gross and Karsch.[3]

Reaction of monoacetates with DBE-$ZnBr_2$ (Table 1)

The ester (0.5 mmol) was dissolved in a mixture of DBE (0.5 ml) and deuteriochloroform (0.5 ml) at room temperature and zinc bromide (50 mg) was added. The mixture was stirred until a homogeneous solution was obtained and NMR spectra were then measured at intervals.

When the reactions were completed methylene chloride was added and the solution was washed with 4 N hydrochloric acid and with aqueous sodium hydrogen carbonate and dried. The methylene chloride solution was then subjected to GLC to identify the products. The identifications were confirmed by addition of authentic compounds.

Isobutyl acetate reacted very slowly and the reaction was interrupted after 48 h. GLC showed that isobutyl acetate and isobutyl bromide were present in a ratio of 83:17 at this stage.

The other reactions shown in Table 1 were allowed to continue until no ester could be detected from the NMR spectra. *tert*-Butyl acetate was completely converted into *tert*-butyl bromide within 2 min after the addition of $ZnBr_2$. The NMR spectrum showed that one equivalent of methyl formate and acetyl bromide were formed at the same time. *tert*-Butyl acetate reacted with DBE in the absence of $ZnBr_2$, the reaction being completed within 1.5 h. Benzyl acetate also reacted rapidly with DBE and $ZnBr_2$, but no reaction took place in the absence of $ZnBr_2$.

3-α-Cholestanyl bromide. 3-β-Cholestanyl acetate (990 mg) in chloroform (5 ml) was treated with DBE (2.0 ml) and $ZnBr_2$ (260 mg) for 5 days at room temperature. Work up as described above gave a product which was purified by preparative TLC with pentane as eluent to give 781 mg (73 %) of 3-α-cholestanyl bromide after recrystallization from acetone, m.p. 96–97 °C, $[\alpha]_D^{20}$ +28.4° (*c* 2, $CHCl_3$). An additional recrystallization gave a product with m.p. 101–102 °C, $[\alpha]_D^{20}$ +27.9° (*c* 2.5, $CHCl_3$) (reported [9] m.p. 101–102 °C, $[\alpha]_D$ +26°). No β-bromide was found.

Reaction of diacetoxycyclohexanes with DBE-$ZnBr_2$

cis-1,2-Diacetoxycyclohexane (512 mg) in chloroform (5 ml) was treated with DBE (2 ml) and $ZnBr_2$ (146 mg) for 48 h at room temperature. Work up as described above gave a product which was separated into 3 fractions by preparative TLC using ether-pentane (1:3) as eluent. The first fraction contained 140 mg (28 %) of *trans*-1,2-dibromocyclohexane (*7*), the second fraction gave 124 mg (27 %) of *trans*-1-acetoxy-2-bromocyclohexane (*6*), and the third fraction 126 mg (25 %) unreacted *5*. The products were identified by comparing their NMR and IR spectra with those of authentic samples.

When the reaction was allowed to proceed for 5 days 61 % of *7*, 5.5 % of *6*, and 4.5 % of unreacted *5* was obtained.

trans-*1,2-Diacetoxycyclohexane* (*3*) when mixed with DBE and $ZnBr_2$ in chloroform gave a precipitate and therefore reacted slowly. After 15 days the mixture was worked up and analysed as described above. It gave 6 % of *7*, 9.7 % of *6*, and 26 % of *3*.

Competetive experiments (Table 2). 1.0 mmol of each of the two acetates were dissolved in deuteriochloroform (1.0 ml) and DBE (1.0 ml) and $ZnBr_2$ (100 mg) were added. The mixtures all became homogeneous when stirred for 30 min. The reactions were followed by NMR spectroscopy and when a suitable amount had reacted the mixtures were worked up as described above. The resulting methylene chloride solutions were analysed by GLC using co-injection of authentic compounds for identifications.

3-Acetoxy-2-bromotetrahydropyrane (*10*) was prepared *in situ*. To a solution of 2,3-diacetoxytetrahydropyrane [10] (515 mg) in chloroform (2 ml) was added DBE (1 ml) and $ZnBr_2$ (165 mg). An NMR spectrum measured after 5 min showed that only *10* was present as a 1:2 *cis–trans* mixture.

After 4 h at room temperature an NMR spectrum showed that *10* was completely converted in 2,3-dibromotetrahydropyrane (*11*). The spectrum was identical with that of a sample prepared by addition of bromine to 3,4-dihydro-2*H*-pyrane.[11]

The reaction mixture was evaporated. The residue in acetone (20 ml) was stirred for 2 h with water (2 ml) and silver carbonate (1 g). Filtration and evaporation gave a residue which was dissolved in dichloromethane and dried. Evaporation gave a product (360 mg) which was purified by preparative TLC (ether-pentane 1:1) to give 215 mg (47 %) of 3-bromo-2-hydroxytetrahydropyrane, which was recrystallized from ether-pentane, m.p. 77–80 °C (reported [11] m.p. 79–80 °C). An NMR spectrum was identical with that of an authentic sample.[11]

Reaction of a mixture of 9 *and* 10 *with DBE-$ZnBr_2$.* 2,3-Diacetoxytetrahydropyrane (201 mg, 1.0 mmol) and *9* (142 mg, 1.0 mmol) were dissolved in deuteriochloroform (1 ml); DBE (1 ml) and $ZnBr_2$ (106 mg) were then added. After 1 h at room temperature an NMR spectrum showed that all the diacetoxy compound was converted into *10*. Since the mixture was inhomogeneous nitromethane (1 ml) was added. After 1 h an NMR spectrum showed that *10* was completely converted in *11*, whereas *9* was unaffected. The mixture was worked up as described above and the products were further identified through GLC.

Reaction of a mixture of 8 *and* 10 *with DBE and $ZnBr_2$.* Cyclohexyl acetate (*8*) (138 mg, 1.0 mmol) and diacetoxytetrahydropyrane (203 mg, 1.0 mmol) in deuteriochloroform (1 ml) were mixed with DBE (1 ml) and $ZnBr_2$ (98 mg). After 1 h the mixture was diluted with dichloromethane and washed. GLC and NMR spectra showed that 30 % of *8* was converted into cyclohexyl bromide and 55 % of the diacetoxy compound was converted into *11*.

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Short Communications

(—)-Decylcitric Acid and (+)-Isocitric Acid as Metabolites from *Penicillium spiculisporum* — a Correction

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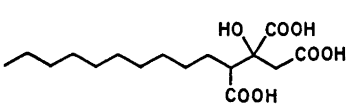
The isolation of two diastereomeric metabolites, (+)-decylcitric acid and (−)-decylcitric acid, from a strain of *Penicillium spiculisporum* (ATCC 24792) was reported in 1968.[1] We here report that of these only (−)-decylcitric acid is a metabolite from the fungus and that the substance considered to be (+)-decylcitric acid is a mixture of (−)-decylcitric acid (*1*) and (+)-isocitric acid (*2*).



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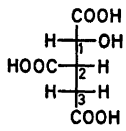

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In the previous work[1] two spots were obtained on TLC which were ascribed to (+)-decylcitric acid ($R_F = 0.25$) and (−)-decylcitric acid ($R_F = 0.44$). Working on the absolute configurations of alkylcitric acids we investigated a product available in a small amount from the previous work. This product showed $[\alpha]_{578}^{22} + 1.9°$ (*c* 1.8, acetone), compared with $[\alpha]_{578}^{20} + 2.4°$ reported for the assumed (+)-decylcitric acid. Esterification with methanol and conc. sulfuric acid gave a product which contained (NMR, GLC-MS) trimethyl isocitrate and trimethyl decylcitrate as major components in approximately equimolar amounts and dimethyl isocitrate lactone in a few mol per cent.

The separation of decylcitric and isocitric acids is difficult to achieve by fractional crystallisation but is easily performed by a partition of a mixture of the methyl esters between water and light petroleum. Using this technique it was found that the crude, acidic fermentation product contained the two acids in approximately equimolar amounts. Hydrolysis of the trimethyl decylcitrate yielded only (−)-decylcitric acid. The spot with the R_F-value 0.25 previously ascribed to (+)-decylcitric acid should thus be ascribed to isocitric acid. Authentic (±)-isocitric acid shows this mobility. The absolute configuration of the trimethyl isocitrate was determined by its conversion to (−)-dimethyl isocitrate lactone,[2,3] a crystalline compound derived from (+)-isocitric acid (*2*) of known absolute configuration (1*R*, 2*S*).[4]

The (1*S*, 2*S*) diastereomer of *2*, but not *2* itself, has previously been found as a metabolite from *Penicillium* species.[5] The (1*R*, 2*S*) isomer *2* which has been isolated from many plants is the one formed in the citric acid cycle.[6]

Experimental. Melting points are corrected. Optical rotations were measured on a Perkin-Elmer 141 polarimeter and NMR spectra on a Varian XL-100 instrument. GLC operations were carried out using a Perkin-Elmer 900 chromatograph equipped with a JXR column (3 % on Gas-Chrom Q, 100 − 120 mesh, 0.2 × 180 cm). For the simultaneous analysis of trimethyl decylcitrate and esters of isocitric acid and its lactone a temperature programme was used: initial temp. 100 °C, heating 10 °C/min to 210 °C.

Separation of the fermentation products. The crude fermentation product[1] (5.0 g) was treated (reflux, 4 days) with methanol (700 ml) and conc. sulfuric acid (10 ml). Sodium hydrogen carbonate solution was added under ice-cooling until pH 5 − 6 was reached. The methanol was evaporated and the resulting two-layered system (100 ml) was extracted five times with light petroleum and then ten times with chloroform-ethanol (3:2). The combined petroleum layers were washed once with water and then dried (Na_2SO_4). Evaporation of the solvent followed by drying in a vacuum desiccator left trimethyl decylcitrate (3.6 g), at least 95 % pure (NMR, GLC). ^{1}H NMR, $\delta(CDCl_3)$: 3.87 (s, 1 H), 3.79 (s, 3 H), 3.69 (s, 3 H), 3.65 (s, 3 H), 3.17, 3.01, 2.76 and 2.60 (AB-spectrum, 2 H), 2.80 − 2.56 (1 H, probably four signals), 2.00 − 0.70 (21 H). The combined chloroform-ethanol layers were dried (Na_2SO_4) and then concentrated. Removal of

inorganic salts by extraction of the residue twice with chloroform followed by evaporation of the solvent left an oil (1.7 g), which to over 95 % extent consisted of a mixture of trimethyl isocitrate and dimethyl isocitrate lactone in the approximate ratio 10:1 (NMR).

Trimethyl isocitrate: ^{1}H NMR, δ($CDCl_3$): 4.39 (d, 1 H, J = 3 Hz), 3.9 – 3.3 (11 H), 3.05 – 2.52 (2 H, AB part of ABX spectrum, J_{AB} = 17 Hz, J_{AX} = 7 Hz, J_{BX} = 8 Hz).

(−)-Decylcitric acid. The ester obtained in the extraction with light petroleum was hydrolysed with sodium hydroxide solution (2 M, reflux overnight). After washing with ether and acidification, the decylcitric acid was extracted with ether. The ether solution was dried (Na_2SO_4) and concentrated, and the residue was recrystallised first from water and then from acetone-light petroleum affording (−)-decylcitric acid, m.p. 135 – 139 °C, $[\alpha]_{578}^{21}$ −21° (*c* 1.7, acetone). Lit.[1] value: $[\alpha]_{578}^{20}$ −10.9° (*c* 1.8, acetone).

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Tobacco Chemistry 37. The Absolute Configuration of Prenylsolanone, (9*S*)-6,12-Dimethyl-9-isopropyltrideca-5*E*,10*E*,12-trien-2-one, a Nor-thunberganoid of *Nicotiana tabacum* L.

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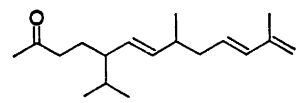
Recent studies have revealed that both the tobacco diterpenoids of the thunbergane type and several of the tobacco volatiles which are structurally reminiscent of the thunberganoids, possess the same absolute configuration, *S*, at the carbon atom carrying the isopropyl group thus strengthening the hypothesis that such diterpenoids act as precursors of the smaller molecules.[1–4] In the present communication we wish to report the chirality of another tobacco nor-thunberganoid, prenylsolanone.



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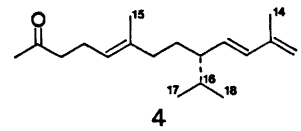

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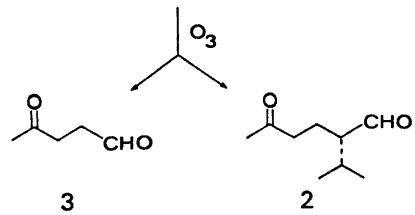

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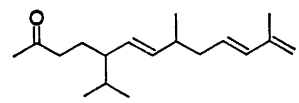

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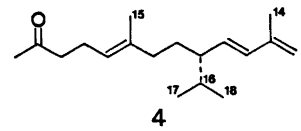

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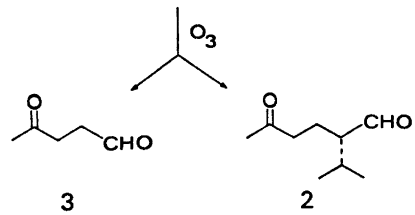

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Table 1. ^{13}C chemical shifts and lanthanide-induced shifts (LIS) of prenylsolanone (*4*).

Carbon	Chemical shift [a]	LIS [b]
1	29.87	42.3
2	208.65	100.0
3	43.80	39.7
4	22.54	25.4
5	122.52	12.9
6	136.67	7.1
7	37.74	3.1
8	30.71	1.9
9	49.18	0.9
10	133.99	0.6
11	132.88	0.2
12	142.16	0
13	114.11	0.2
14	18.82	0.1
15	16.02	5.0
16	32.15	0.3
17	20.82	0.2
18	19.10	0.2

[a] δ-values in ppm downfield from TMS. [b] Relative shifts induced by $Yb(dpm)_3$. The effect [9] of "complex-formation shift" was not evaluated.

with the assumed constitution. Similarly, the ^{13}C NMR spectrum revealed two non-protonated olefinic carbons and the ^1H NMR spectrum showed five olefinic protons and two vinylic methyl groups (δ 1.83 and 1.59). These NMR data suggested that the structure tentatively proposed by Devreux and Beaulieu,[5] 8,12-dimethyl-5-isopropyltrideca-6,10,12-trien-2-one (*1*) should be revised to 6,12-dimethyl-9-isopropyltrideca-5*E*,10*E*,12-trien-2-one (*4*) which also complies with the ozonolysis products. The ^{13}C chemical shifts of this compound (*4*) are given in Table 1 and the assignments, made by comparison of these data and those of structurally related compounds [3,7] were confirmed with the aid of $Yb(dpm)_3$-induced shifts. The revised structure *4* is the same as that assigned to prenylsolanone which Demole and Enggist [6] very recently reported as a constituent of Burley tobacco flavour. Direct comparison of our sample with synthetic prenylsolanone kindly provided by Dr. Demole confirmed the identity.

The present ozonolytic degradation to (2*S*)-5-oxo-2-isopropylhexanal (*2*) established the absolute configuration of prenylsolanone (*2*) as *S* in agreement with the chirality of other tobacco constituents assumed to be northunberganoids.[1–4] The optical activity of the ketoaldehyde *2* was somewhat lower than that previously reported [2] for this compound implying that the present prenylsolanone was partially racemic (enantiomeric ratio *ca.* 70:30), or that partial racemisation of the ketoaldehyde *2* had occurred during work-up. The present ^{13}C NMR results confirmed the *E*-configuration previously assigned to the 5,6-double bond ($\delta_{C(15)} = 16.02$; $\Delta\delta_{C(15)} > \Delta\delta_{C(7)}$).[8]

Experimental. IR spectra, rotations and mass spectra were recorded on Digilab FTS-14, Perkin-Elmer 141 and LKB 2091 instruments, respectively. Proton noise decoupled and single frequency off-resonance decoupled ^{13}C NMR and ^1H NMR spectra were obtained in $CDCl_3$ solutions in FT-mode on a Varian XL-100-12 spectrometer equipped with S-124 FT/disk accessories and controlled by a Varian 620 L computer. GC–MS was performed on a Varian 1700 chromatograph attached to the LKB 2091 instrument using glass capillary columns (5 m × 0.25 mm) coated with Emulphor. The spectral data of the title compound were indistinguishable from those reported by Demole and Enggist [6] for prenylsolanone. ^{13}C NMR data: see Table 1. $[\alpha]^{20}$ +10.8° (589 nm), +11.5° (578), +13.6° (546), +27.6° (436), +53.1° (365) (*c* 1.1; $CHCl_3$). The present compound did not separate from synthetic prenylsolanone when co-injected on a glass capillary column. Ozonolysis of prenylsolanone was performed as outlined previously [3] furnishing 4-oxopentanal (*3*) and (2*S*)-5-oxo-2-isopropylhexanal (*2*) possessing spectral data identical with those previously published.[3] The optical activity of the latter ketoaldehyde (*2*) was $[\alpha]^{20}$ +17.4° (589 nm), +17.9° (578), +22.0° (546), +60.5° (436), +184.1° (365) (*c* 0.39; $CHCl_3$); lit.[2] $[\alpha]^{20}$ +47.8° (589 nm), +50.7° (578), +61.4° (546), +146.6° (436), +400.8° (365) (*c* 0.73; $CHCl_3$).

Acknowledgements. The authors are indebted to Miss Ann-Marie Eklund for skilful technical assistance and Dr. Edouard Demole, Firmenich & Cie., Geneva, for a generous sample of prenylsolanone.

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A New Method for the Preparation of Jack-bean Urease Involving Covalent Chromatography

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Urease (EC 3.5.1.5) can be prepared from jack-bean meal by a simple extraction crystallization procedure devised by Sumner.[1] The method, which does not reproducibly give urease preparations of high specific activity and homogeneity, has been improved by a number of additional separation steps.[2,3] The extended procedures, however, tend to be rather laborious and time consuming.

Recently we have developed a method for the immobilization of urease by thiol–disulfide exchange.[4] An agarose-2-pyridyl-disulfide gel was used as activated solid support. Since the immobilization of partially purified urease was followed by a 6–10-fold purification and since the immobilization is reversible, it was proposed that the technique could be used for preparation of urease. The same technique, known as covalent chromatography by thiol-disulfide exchange, has recently been used to prepare papain from papaya latex and mercaptalbumin from bovine serum albumin.[5,6]

In this communication a simple 3-step method for the preparation of highly active urease from jack-bean meal is described which involves an ethanol extraction with subsequent covalent chromatography followed by a gel filtration step.

The covalent chromatography procedure requires consecutive thiol–disulfide interchange reactions. Active urease, containing reactive thiol groups, is covalently bonded to the chromatographic material (agarose-2-pyridyl disulfide) *via* disulfide bridges and subsequently removed by a low molecular weight thiol compound (see Fig. 1a–b). Previous work with thiol–disulfide exchange immobilization of urease has been carried out using a gel containing 60 μmol SH groups per g of dried gel, 70 % of which could be activated for thiol-disulfide exchange by reaction with 2,2′-dipyridyl disulfide.[4]

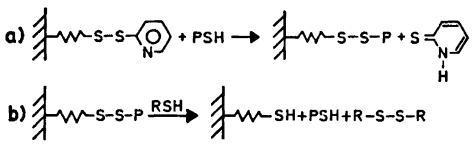

Fig. 1. Reaction scheme for covalent chromatography of jack-bean urease (PSH). RSH low molecular weight thiol such as DTT.

An attempt to increase the number of 2-pyridyl disulfide structures, and thus the immobilization capacity of the gel, by increasing its thiol content[7] was unsuccessful, since a 10-fold increase in gel bound –SH groups failed to increase the degree of activation.[8] The proposed explanation for this behaviour is the formation of disulfide bridges between neighbouring gel bound –SH groups by a thiol–disulfide exchange reaction involving newly formed 2-pyridyl disulfide residues and still unactivated thiol groups.[9] The effect is more pronounced with gels of high thiol concentration (> 100 μmol of SH-groups/g dried gel), since the thiol groups are then closely situated in space. The dipyridyl disulfide activation normally takes place in aqueous solution where the reagent is sparingly soluble (~1.5 mM).[5] An increased concentration of the activating agent, necessitating work in less than 100 % aqueous solution, should then diminish the mentioned undesirable side reaction. Indeed we have been able to show that the activation of highly substituted SH containing gel (600 μmol SH groups/g dried gel) in 60 % aqueous acetone, where the dipyridyl disulfide reagent is soluble enough to permit work at a concentration of 0.2 M, results in the conversion of *ca.* 67 % of the thiol groups into the active structure. This gives an agarose-2-pyridyl disulfide derivative containing about 400 μmol of 2-pyridyl disulfide structures/g dried derivative.

The increase in the amount of 2-pyridyl disulfide structures is particularly useful in work with raw extracts. Since they have undergone no selective treatment, they are bound to contain a sizeable amount of thiol containing compounds other than urease, leading to a competition with the enzyme for the reactive disulfide structures.

The release of gel-bound material is accomplished through reduction with dithiothreitol (Fig. 1b). This reaction, however, liberates not only thiol containing material of biological origin. Remaining active structures are simultaneously eliminated in the form of 2-thiopyridone.

The released material was passed through a column of Sephadex G-25 for elimination of low molecular weight material such as dithiothreitol and 2-thiopyridone. This step was introduced merely as a check on the performance of the covalent chromatography and should be omitted when the method is used preparatively.

Subsequent gel filtration on Sepharose 6B of void material from the previous step showed

that it contained about 50 % of high molecular weight material of insignificant urease activity. After removal of this, about 16 mg of urease with a specific activity of 2515 units/mg protein was obtained. This activity is about 50 % higher than the value reported previously for urease extensively purified by conventional techniques.[2] The overall efficiency of the preparation procedure is evident from Table 1, which indicates a 280-fold purification of the enzyme in the two steps following ethanol extraction of the jack bean meal. The capacity of the agarose-2-pyridyl disulfide derivative to bind urease active material was 5.1 mg/ml gel.

The pure urease preparation seemed to be quite stable, since no activity loss was detected when it was stored dissolved in 0.05 M Tris-HCl buffer containing 0.1 M KCl and 1 mM EDTA pH 7.2 at +4 °C for 3 weeks. To avoid aggregation and inactivation phenomena that might occur under longer periods of time, the active urease might be stored in immobilized form on the covalent chromatography gel. For some purposes it might even be preferable to use this immobilized conjugate which also shows urease activity.[4] In this form the urease should also be useful for studying its subunit organization which has not yet been fully elucidated despite considerable investigation.[10]

Materials and methods. Jack bean meal and dithiothreitol were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Sepharose 2B, Sepharose 6B and Sephadex G-25 were the products of Pharmacia Fine Chemicals Co., Uppsala, Sweden. 2,2′-Dipyridyl disulfide (2-PDS) was obtained from Aldrich-Europe (B-2340 Beerse, Belgium) and Nessler's reagent A (potassium tetraiodomercurate(II) solution) and Nessler's reagent B (sodium hydroxide solution) were obtained from E. Merck AG (Darmstadt, Germany).

Preparation of chromatographic material. A. Thiolation of agarose (Sepharose 2B) was performed according to Axén *et al.*[7] The thiolated agarose contained ~600 μmol of SH/g dry conjugate as determined by its reaction with 2,2′-dipyridyl disulfide.[5]

B. Activation of thiol-agarose. 25 g of freshly thiolated agarose (25 g of sedimented gel corresponds to 500 mg dry derivative) was washed with 0.025 M $NaHCO_3$ containing 60 % acetone and 1 mM EDTA and was then suspended in 20 ml of the same solution. The suspension was added to a solution of 2,2′-dipyridyl disulfide (2.6 g) in 20 ml 60 % acetone containing 0.015 % EDTA. The mixture was allowed to react under stirring for 1 h at +23 °C. The activated gel was then carefully washed free of excess reagent with 60 % acetone and 1 mM EDTA. The activated thiol-agarose contained 400 μmol of reactive disulfide structures per g dry conjugate. This figure which states the theoretical capacity of the chromatographic material to bind thiols, was determined by estimating the amount of 2-thiopyridone released as a result of reducing the activated gel with dithiothreitol.[5] The agarose-2-pyridyl disulfide derivative was stored as a suspension in 1 mM EDTA at +4 °C.

Preparation of jack bean meal extract. Jack-bean meal, 60 g, was mixed with 300 ml 0.05 M Tris-HCl buffer containing 36 % ethanol, 0.1 M KCl and 1 mM EDTA pH 7.2 (final pH was 6.5). The mixture was stirred for 5 min at +28 °C and was subsequently filtered on a Büchner funnel. The slightly turbid filtrate was then centrifuged (5000 *g*, 20 min). The supernatant (about 210 ml) was diluted to 300 ml by 0.05 M Tris-HCl buffer pH 7.2

Table 1. Efficiency of purification of urease from 50 g of jack-bean meal.

Procedure	Total activity [a] recovered (units)	Amount of dry weight material (mg)	Specific activity [a] (units/mg dry weight material)	Activity yield (%)
Ethanol extraction	56 100	6250 [b]	9	100
Covalent chromatography + gel filtration on G-25	48 000	32 [c]	1500	86
Gel filtration on Sepharose 6B	40 240	16 [c]	2515	72

[a] The ureolytic activity was determined essentially as described in Ref. 4. One unit is the amount of enzyme that liberates 1 μmol of NH_3 from urea at 25 °C in 1 min. [b] Calculated by dry weight determination. [c] Calculated from A_{280} using A_{280} (0.1 %) = 0.589 assuming that essentially all of the material is urease or polymers of urease.

(same as above) and the pH was adjusted to 7.2 by addition of 0.5 M NaOH. The final extract thus contained 25 % ethanol.

Covalent chromatography. A column with a total volume of 6.3 ml (1 × 8 cm) was prepared from the agarose-2-pyridyl disulfide gel. The column was equilibrated with 0.05 M Tris-HCl buffer pH 7.2 containing 0.1 M KCl and 1 mM EDTA. Jack bean meal extract (250 ml) was passed through the column at a flow rate of 20 ml/h. Ultraviolet absorption at 280 nm and ureolytic activity were determined in fractions of the effluent. Most of the UV-absorbing material passed through the column unretained. In the first 150 ml of eluate no urease activity was detected. A small activity that gradually increased to that of the sample applied was then observed within the next 100 ml of eluate. The column was then washed with Tris-HCl buffer (same as above) until A_{280} in the eluate was less than 0.04. Covalently bonded material was detached from the chromatographic material with 20 mM dithiothreitol (20 ml) dissolved in 0.05 M Tris-HCl buffer pH 8.0 containing 0.1 M KCl and 1 mM EDTA.

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Biosynthesis of a Quinol Glucoside in *Cornus*

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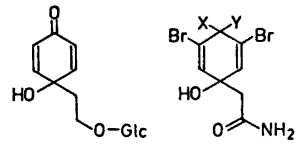
The glucoside *1*, first isolated from *Cornus foemina* Mill.,[1] but subsequently recognized as a component of probably all species of the *Cornus* subgenera Kraniopsis and Mesomora,[2] has no known obvious structural relative among constituents of higher plants, though the parent 4-alkyl-4-hydroxy-cyclohexa-2,5-dienone (quinol) system, or its formal addition or rearrangement derivatives, has been repeatedly encountered in natural products, such as *2*,[3] *3*,[4] *4*,[5] and *5*,[6] all from marine sponges of the genus *Verongia*; *6* (thelepin) from a marine worm;[7] and botrallin *7* of fungal origin.[8] An oxidative *in vivo* derivation of *1* from tyrosol *8*, possibly *via* salidroside *9*, a phenolic glucoside encountered within the families Crassulaceae, Ericaceae, Oleaceae, and Salicaceae, but also accompanying *1* in *C. foemina*[1] and other *Cornus* species,[2] appeared likely and has now been confirmed. We report the results.



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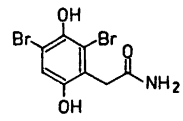

5

The potential precursors, L-tyrosine, tyrosol *8*, and salidroside *9*, labelled with ^{14}C or ^{3}H, were administered to young shoots of *C. stolonifera* Michx. After a metabolic period of 44 h, the glucosides *9* and *1* were isolated and purified by chromatography before their total radioactivities were determined. The results are presented in Table 1.

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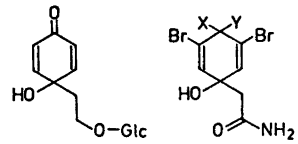

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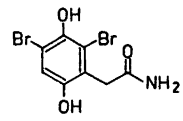

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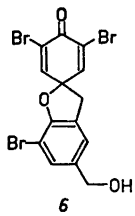

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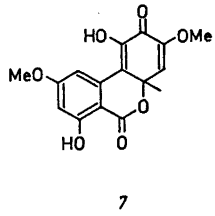

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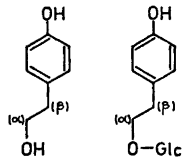

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9

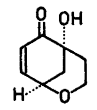
It appears that tyrosol *8* and salidroside *9* are both efficiently converted into *1*, whereas L-tyrosine and glucose, not surprisingly, act as far inferior precursors. Though unproven, the observed incorporation of L-tyrosine may conceivably proceed through *p*-hydroxyphenylacetaldoxime and tyrosol, an established pathway in certain dicotyledonous angiosperms.[9] [α,β-^{3}H][*glucose-U*-^{14}C]Salidroside *9* was administered to the plant in order to learn to what extent incorporation proceeded in a direct step, *i.e.* without rupture of the glucosidic linkage. Even when its corporation was accompanied by only a minor change in the ^{3}H:^{14}C ratio (Table 1), a moderate *in vivo* exchange of glucose in *9* was noticed in recovery experiments. The simultaneous operation of alternative, minor pathways, such as oxidation of tyrosol to the aglucone of *1* (formula iii in the footnote), followed by glucosylation of the latter, cannot be dismissed on the basis of the present results.

The natural distribution of the glucoside *1* is unknown. Apart from the two formerly specified subgenera within *Cornus*, *Forsythia* species [10] and *Digitalis purpurea* L.[10,11]* are now to be counted as established sources of *1*. The facile conversions of the tetraacetate of *1* into homogentisyl or resorcinol derivatives,[1] may conceivably have their enzymic counterparts in Nature.

The detailed character of the enzymic oxidation of *9* to *1* is unknown. Singlet oxygen participation [13] and the intermediacy of arene oxides [5] are both conceivable reactions among which the present results, however, cannot distinguish.

Experimental. Plant material and feeding technique. The experiments were performed in the last week of April on small spring shoots of *C. stolonifera* Michx. The radioactive com-

* A cyclohexanone derivative, isolated from the growing tips of *D. purpurea* and formulated as (i) (or its mirror image) possesses properties and spectroscopical data [12] suggesting its identity with (ii) [11,13] that arises from intramolecular cyclization of (iii), liberated on enzymic hydrolysis of *1* in *Cornus* extracts.[1,11]



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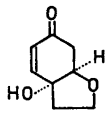

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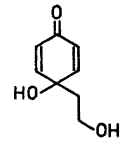

(iii)

Table 1. Absolute incorporations of ^{14}C- and ^{3}H-labelled precursors into salidroside *9* and the quinol glucoside *1* in *Cornus stolonifera* Michx.

Exp. No.	Compound administered	Total activity administered (mμCi)	Total activity isolated in *9* (mμCi)	% Incorporation into salidroside *9*	Total activity isolated in *1* (mμCi)	% Incorporation into the quinol glucoside *1*
1	D-[*U*-^{14}C] Glucose	4860	12	0.25	6.2	0.13
2a	L-[α, β-^{3}H] Tyrosine	4850	2.3	0.05	8.1	0.17
2b	L-[α, β-^{3}H] Tyrosine	4830	2.6	0.05	7.1	0.15
3a	[α, β-^{3}H] Tyrosol, *8*	2760	730	26.4	430	15.7
3b	[α, β-^{3}H] Tyrosol, *8*	2730	560	20.5	480	17.6
4a	[α, β-^{3}H] Salidroside, *9* + [*glucose-U*-^{14}C] Salidroside, *9*, (^{3}H:^{14}C = 5.6)[a]	3629 653			784[b] 116[b]	21.6 17.8
4b	[α, β-^{3}H] Salidroside, *9* + [*glucose-U*-^{14}C] Salidroside, *9* (^{3}H: ^{14}C = 5.6)[a]	3799 683			691[c] 102[c]	18.2 15.0

[a] The ^{3}H: ^{14}C-ratio was 6.0 after correction for a radioactive impurity in the salidroside administered. [b] ^{3}H: ^{14}C = 6.7. [c] ^{3}H: ^{14}C = 6.7.

pounds were administered to the leaves through the petioles. The metabolic period was 44 h.

Radioactive compounds. D-[*U*-^{14}C] *Glucose* and L-[α,β-^{3}*H*]*tyrosine* were obtained from Amersham Radiochemical Centre, England. [α,β-^{3}*H*]*Tyrosol 8* was synthesized by a modification of the method of Ehrlich:[14] to a solution of L-tyrosine (6 mg) and sucrose (0.5 g) in water (5 ml) were added 220 μCi (1 μCi/μl) of L-[α,β-^{3}H]tyrosine and baker's yeast (250 mg). At the end of 5 days at 22 °C, carrier tyrosol (14.4 mg) was added. After centrifugation, the supernatant, combined with the washings, was adjusted to pH 7 with solid $NaHCO_3$, and continuously extracted with ether. After drying and evaporation, the ether solution afforded a crystalline residue which was purified by chromatography on SiO_2-plates ($CHCl_3$:MeOH, 5:1) to give homogeneous [α,β-^{3}H]tyrosol (18.9 mg). [α,β-^{3}*H*]*Salidroside 9* was prepared, essentially as reported,[15] by reaction of the total amount of ^{3}H-tyrosol described above with tetra-*O*-acetyl-α-D-glucopyranosyl bromide to give a crude product, separated on SiO_2-plates ($CHCl_3$:MeOH, 95:5) into [α,β-^{3}H]-2-[4-hydroxyphenyl]ethyl tetra-*O*-acetyl-β-D-glucopyranoside (49 mg) and [α,β-^{3}H]tyrosol, the latter isolated after addition of carrier (37 mg) and possessing a total activity of 30.1 μCi. The tetraacetate was converted into salidroside *9* on standing in MeOH (8 ml), saturated with NH_3 at 0 °C, for 17 h at 20 °C. The product was purified by chromatography on SiO_2-plates ($CHCl_3$:MeOH, 4:1) to give a homogeneous product (20 mg) with a total activity of 15.3 μCi. [glucose-U-^{14}*C*]*Salidroside* (55 mg, total activity 23 μCi) was prepared similarly, by deacetylation of the tetraacetate (121 mg), resulting from the reaction between tyrosol (44 mg) and tetra-*O*-acetyl-α-D-[*U*-^{14}C]glucopyranosyl bromide, the latter produced by dissolving D-glucose (57 mg), together with the residue from 225 μl (55.1 μCi) of a D-[*U*-^{14}C]glucose solution, in pyridine (2 ml), to which Ac_2O (0.5 ml) was added. After 1 h at 100 °C, H_2O (2 ml) was added. After 15 min, the solution was extracted with $CHCl_3$ (4 × 5 ml) and the organic phase was washed with H_2SO_4, $NaHCO_3$, and H_2O. The remaining syrup was dissolved in $CHCl_3$ (5 ml), containing HBr/AcOH (30 % v/v) (1.3 ml), and kept for 2 h at 20 °C. Additional $CHCl_3$ was added; the organic phase, after washing and drying, gave a syrup (159 mg) used in the reaction described above.

Isolation of the quinol glucoside 1. The plant material (5 – 10 g) was homogenized in EtOH and the suspension filtered. The combined filtrate and washings were taken to dryness and the residue was dissolved in H_2O; the aqueous phase was extracted twice with ether, and concentrated *in vacuo*. After passage through a column of neutral Al_2O_3, and thorough washing of the column, the solution was taken to dryness. The residue was taken up in MeOH and the solution evaporated after the addition of silica gel (3 g). The powder was placed on top of a silica gel column, packed in acetone, and the column was eluted with acetone; the fractions containing *1* and *9* were combined and evaporated to dryness. The residue was purified by preparative chromatography on SiO_2-plates, first with $CHCl_3$:MeOH (4:1) and then with BuOH:MeOH:H_2O (7:1:3) as the mobile phase.

Determination of radioactivity. All measurements were made by liquid scintillation counting on a Packard Tricarb scintillation spectrometer model 3320. Samples were dissolved in 1 ml H_2O (the precursors) or MeOH (the glucosides). The solutions were mixed with 10 ml scintillation solvent.[16] Counting efficiency and ^{14}C-overlap were determined by counting ^{14}C- and ^{3}H-labelled hexadecane standards under the same circumstances.

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Interaction between Dimethyl Sulfoxide and the Anomeric Proton in Anomeric Glycopyranosides

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Chromatography on dimethyl sulfoxide impregnated papers was devised in the late 1950's. It provided a means for separating lipophilic compounds not amenable to the usual paper chromatographic technique using water-saturated cellulose as the stationary phase. Preparative separations using dimethyl sulfoxide as the stationary phase on impregnated silica gel were also described.[1,2] These preparative separations were often better than the corresponding ones on paper. Similar separations can also, most conveniently, be carried out on impregnated silica gel thin-layer plates. Separations of moderately polar compounds such as aromatic amines, phenols, aldose and alditol acetates, aldose methyl ethers and acetylated methyl glycosides were readily accomplished. With the advent of thin-layer chromatography and of gas-liquid chromatography, the technique of chromatographic separation on dimethyl sulfoxide as stationary phase fell into disuse. It does, however, remain as an excellent method for the separation of anomeric glycoside derivatives. Anomers are frequently separated much better than are positional and other stereoisomers, those with axial aglycons in the more stable conformation, generally the α-forms, having the higher mobility. These observations have interested us for some time and is the subject matter of this communication.

A current rationale of the anomeric effect is represented in Fig. 1.[3,4] In glycosides with axial aglycones *1*, the antibonding orbital of bond *a* is stabilized by the *trans*-periplanar free electron pair *a'* at O-5. In the same manner, the antibonding orbital of the C-1 – O-5 bond *b* is stabilized by the *trans*-periplanar free electron pair *b'* at O-1. By contrast in

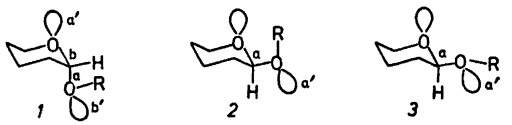

Fig. 1. Orbital representations of the anomeric and *exo*-anomeric effects. For the sake of clarity, only the participating electron pair on each oxygen atom is represented. The argument is independent of the state of hybridization at the oxygen atoms (sp^2 or sp^3).

glycosides with equatorial aglycons *2* and *3* only one such orbital interaction is possible, that of the C-1 – O-5 bond *a* with the free electron pair *a'*. In the absence of other effects the β-D-anomer (*2*, *3*) should be the less stable of the two anomers, with a lower electron density at C-1 and, hence at H-1 than that for the α-D-anomer *1*.

Cations are solvated by dimethyl sulfoxide. Since H-1 of β-D-anomers has a lower electron density than H-1 of α-D-anomers the former should interact more strongly with dimethyl sulfoxide when this is employed for example as the stationary phase in chromatography.

This possibility has now been examined by NMR, for the fully acetylated anomeric methyl hexopyranosides with the D-*galacto*-, D-*gluco*- and D-*manno*-configurations. Spectra were recorded for each glycoside in deuteriochloroform containing increasing concentrations of hexadeuteriodimethyl sulfoxide (from 0 to 100 %). In the spectra obtained for the α-D-glycosides, upfield shifts in δ values for all individual protons except one, including acetoxyl and methoxyl protons, were recorded. The magnitude of this shift for individual protons varied from 0.40 to 0.21 on changing the solvent from chloroform to dimethyl sulfoxide. The exception was the δ value for H-1 of the α-mannoside for which maximum down-field shift of 0.04 ppm was observed. By contrast, the δ values recorded for H-1, H-3 and H-5 in the β-D-glycosides were shifted down-field; upfield shifts were recorded for other protons. The maximum down-field shift for H-3 was only 0.05 ppm, but those for H-1 and H-5 for the three β-D-glycosides ranged from 0.18 to 0.28 ppm on changing the solvent from chloroform to dimethyl sulfoxide (Table 1). We interpret this as being due to interaction of H-1 of the β-D-glycosides with the partially negative oxygen atom in dimethyl sulfoxide and the down-field shifts of H-1, H-5 and, to a much lesser degree H-3, as being due to the anisotropy of the S=O bond.[5] The stronger interaction between the β-D-glycosides and dimethyl sulfoxide correlates well with the differences in chromatographic mobility between α-D- and β-D-glycosides using dimethyl sulfoxide as the stationary phase.

Experimental. The spectra of the six methyl hexopyranosides (~25 mg in 0.50 ml $CDCl_3$ containing ~0.1 % tetramethylsilane as internal standard) were recorded using a Varian XL 100 spectrometer. $(CD_3)_2SO$ was added incrementally between recording the spectra. Values of shift gradients thus determined are recorded in Table 1.

NMR assignments. Since significant down-field shifts were obtained for the β-D-anomers and not for the corresponding α-D-anomers, assignments for the former only will be described here.

Methyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside. Solvent, $CDCl_3$. Decoupling irradiation

Table 1. Variations with solvent composition of δ for H-1, H-3 and H-5 of three methyl 2,3,4,6-tetra-*O*-acetyl-β-D-hexopyranosides.

	$CDCl_3$	$CDCl_3/(CD_3)_2SO$ 5:2	$CDCl_3/(CD_3)_2SO$ 1:1	$(CD_3)_2SO$
galacto configuration				
H-1	4.39	4.48	4.54	4.59
H-3	4.99	—	—	5.04
H-5	3.91	—	—	4.09
gluco configuration				
H-1	4.43	4.52	4.59	4.68
H-3	5.21	5.23	5.24	5.26
H-5	3.70	3.82	(3.89)[a]	(3.98)
manno configuration				
H-1	4.56	4.72	4.78	4.84
H-3	5.04	—	—	(5.09)
H-5	3.66	—	—	3.86

[a] Values in parentheses are less accurate due to overlapping signals or second-order effects and those not given could not be determined, for the same reasons.

at the centre of frequency of the presumed H-5 signal (identified from its chemical shift and spin coupling pattern) caused a simplification of the H-6 and H-6′ signals and a sharpening of the H-4 broad doublet. Irradiation at δ 5.18 (H-2) caused the presumed H-1 signal to collapse into a singlet. The converse collapse of the presumed H-2 triplet into a doublet when irradiating at the presumed H-1 frequency was also observed. Irradiation at the H-4 frequency caused collapse of the H-3 signal (dd) into the expected doublet. The following assignments were therefore made: δ values: H-1 4.39, H-2 5.18, H-3 4.99, H-4 5.39, H-5 3.91, H-6 and H-6′ 4.10-4.22. First-order coupling constants: $J_{1,2}$ 8 Hz, $J_{2,3}$ 10 Hz, $J_{3,4}$ 4 Hz, $J_{4,5} < 1$ Hz.

*Methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside.* Solvent $CDCl_3/(CD_3)_2SO$ 5:1. Using decoupling techniques similar to those described above, the following assignments were made: δ values: H-1 4.51, H-2 4.89, H-3 5.23, H-4 5.00, H-5 3.80, H-6 4.08, H-6′ 4.28. First-order coupling constants: $J_{1,2}$ 8 Hz, $J_{2,3}$ 8 Hz, $J_{3,4}$ 8 Hz, $J_{4,5}$ 8 Hz, $J_{5,6}$ 3 Hz, $J_{5,6'}$ 5 Hz, $J_{6,6'}$ 12 Hz.

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Studies on the Kolbe Electrolysis. XI.* Racemization of Optically Active *sec*-Butyl Radicals in a Mixed Coupling Reaction

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In the preceding article of this series,[1] the mixed Kolbe coupling between radicals from ethyl hydrogen (+)-ethylmethylmalonate and isovaleric acid was found to give ethyl ethylisobutylmethylacetate which was racemic to an extent of at least 99.98%. From the theoretical point of view, one possible objection against this system is that the radical center of the optically active radical is strongly conjugated with an ethoxycarbonyl group, thus (1) providing a strong driving force for the radical to attain a planar structure and (2) causing a weakening of any chemisorption bond between the radical and the electrode surface (platinum). Both factors would de-

* Part X. See Ref. 1.

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Studies on the Kolbe Electrolysis. XI.* Racemization of Optically Active *sec*-Butyl Radicals in a Mixed Coupling Reaction

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crease any tendency for the formation of a product with at least partially retained configuration, postulated to be the outcome of a mechanism involving a strong chemisorptive interaction between the initially formed radical and the electrode.[2,3]

The mixed coupling reaction between optically active *sec*-butyl radicals from (+)-2-methylbutanoic acid and methoxycarbonylmethyl radicals from methyl hydrogen malonate (eqn. 1) is not marred with the problem indicated above. We now report that methyl 3-methylpentanoate formed in this reaction

$$\begin{matrix} CH_3CH_2CH(CH_3)COO^- \\ CH_3OCOCH_2COO^- \end{matrix} \xrightarrow[\text{Pt anode}]{-2e^-}$$

$$CH_3CH_2CH(CH_3)CH_2COOCH_3 \qquad (1)$$

is completely racemized, in spite of the fact that the optically active radical is of purely hydrocarbon type.

Optically pure (+)-2-methylbutanoic acid was prepared by oxidation of (−)-2-methyl-1-butanol in "purple benzene".[4] It was then electrolyzed together with methyl hydrogen malonate in a 3:1 ratio (several test runs with inactive material showed that the formation of methyl 3-methylpentanoate was suppressed when the reaction was run with an excess of monomethyl malonate) in methanolic solution, the acid mixture being neutralized by potassium hydroxide to an extent of *ca.* 3%. Methyl 3-methylpentanoate was isolated from the product mixture by preparative GLC, $[\alpha]_{578}^{25}$ being $0.000 \pm 0.002°$. An ORD curve in ethanol indicated no rotation down to 220 nm. Optically pure methyl 3-methylpentanoate has $[\alpha]_{589}^{25} = \pm 7.57°$ (neat).[5] Thus, complete racemization had taken place during the coupling process, and no conclusion regarding the possible intervention of chemisorbed radicals can be drawn from this experiment. As stressed before,[1] only the positive establishment of a retention mechanism would be conclusive in this respect.

Experimental. (+)-2-Methylbutanoic acid. Potassium permanganate (144 g, 0.90 mol), water (1250 ml), benzene (300 ml), tetrabutylammonium bromide (15 g, 0.047 mol) and (−)-2-methyl-1-butanol was stirred vigorously for 24 h at room temperature. Then sodium pyrosulfite and sulfuric acid was added in small portions (the flask being immersed in an ice-bath) until a clear solution was obtained. The benzene layer was separated and the acidic aqueous phase extracted with three portions of benzene. The combined benzene extracts were evaporated and the residual oil dissolved in aqueous sodium carbonate. This solution was extracted with two portions of ether to remove neutral components. Acidification, ether extraction, and distillation gave (+)-2-methylbutanoic acid, b.p. 76–78 °C/15 mmHg (10.5 g, 34%), $[\alpha]_{578}^{25} = 18.2°$ (neat; lit.[6] $[\alpha]_{589}^{27} = 17.9°$).

Mixed coupling of (+)-2-methylbutanoic acid and methyl hydrogen malonate. This reaction was carried out as described previously [7,8] with (+)-2-methylbutanoic acid (18.6 g, 0.182 mol), methyl hydrogen malonate (7.2 g, 0.061 mol), methanol (100 ml), and potassium hydroxide (0.45 g, 0.008 mol). After electrolysis at 1.0 A for 10.5 h, water (400 ml) was added and the mixture extracted with pentane (2 × 200 ml). After drying with magnesium sulfate, the pentane was distilled off through a Vigreux column. The residual oil (6.0 g) was subjected to preparative GLC (6 m × 10 mm 20 % SE-30 on Chromosorb W), pure methyl 3-methylpentanoate being collected in low yield (0.327 g, 4 %). This sample showed $[\alpha]_{578}^{25} = 0.000 \pm 0.002°$ (*c* 12.8, methanol).

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The Photocycloaddition of Cyclohexene to Carbostyrils

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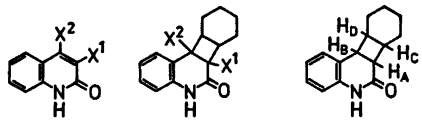
Photocycloaddition of cyclohexene to carbostyril, and to carbostyrils with methyl groups in the 3- and 4-position, has been investigated. The reaction was shown to proceed from the lowest triplet state of the carbostyrils. In the case of 4-methyl- and 3,4-dimethylcarbostyril the addition was stereospecific, and the unsubstituted carbostyril gave predominantly *cis*-product, whereas 3-methylcarbostyril gave a mixture of products. The predominant products in the two former cases were shown to be *cis* by NMR spectroscopy.



I II IIa

a. $X^1 = X^2 = H$
b. $X^1 = CH_3$ $X^2 = H$
c. $X^1 = H$ $X^2 = CH_3$
d. $X^1 = X^2 = CH_3$

We have previously demonstrated that unsubstituted carbostyrils readily dimerize to give cyclobutane dimers. [1–3] The unsubstituted carbostyril dimer was shown to be *trans-head-head* by chemical means, and the same type of stereochemistry was found for a series of carbostyril dimers by NMR spectroscopy.[3] From these and other studies [4] it appeared that substituents in the 3- and/or 4-position of carbostyrils hindered the dimerization reaction.

The carbostyril dimerization was later shown to proceed from a triplet excited state, presumably *via* a triplet excimer,[5] and it was found that unsubstituted carbostyril underwent photoaddition to a series of alkenes.[6]

In this paper we present results which demonstrate that the triplet state of the carbostyrils is responsible for the addition to cyclohexene, and that cycloaddition, as opposed to photodimerization, also proceeds with carbostyrils substituted in the 3- or the 4-position.

Irradiation of compounds Ia–d in methanol containing *ca.* 10 % of cyclohexene gives products IIa–d in good yields. The structures of the products were assigned on the basis of UV, IR, and NMR spectroscopy, as well as elemental analysis. The stereochemistry of the cyclobutane ring was elucidated by means of NMR spectroscopy.[3]

The 220 MHz NMR spectrum of IIa shows two triplets δ 3.89 (A) and 3.41 (B). The protons H_C and H_D give rise to two multiplets, which are assigned on the basis of a decoupling experiment; δ 2.80 (C) and 3.02 (D). The coupling constants were all equal, *i.e.* $J_{AC} = J_{BD} = J_{AB} = 9.2$ Hz. On the basis of the values of the coupling constants we conclude that the compound has the *cis* configuration.[3] It was not possible to obtain a sharp melting point for the compound due to the presence of a small amount of impurity, presumably *trans* isomer (< 5 % estimated by NMR spectroscopy). It should be noted that Evanega and Fabiny [6] obtained a 1:3 mixture of the *cis* and *trans* isomers from the photocycloaddition of cyclopentene to carbostyril.

No 220 MHz NMR spectrum was obtained for the product from the carbostyril Ib. The reaction mixture resisted all attempts at crystallization. Thin layer chromatography revealed that the reaction mixture was not homogeneous. It consisted of at least six products, the major one being isolated by

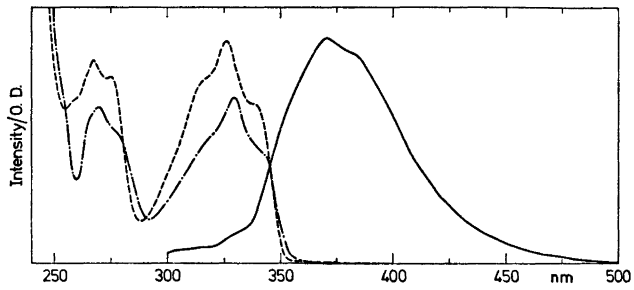


Fig. 1. UV absorption (dashed line), corrected fluorescence (full line), and corrected fluorescence excitation of 4-methylcarbostyril in absolute ethanol at room temperature. Intensity is in arbitrary units.

means of preparative layer chromatography. It is believed to be *cis* IIb.

In the 220 MHz NMR spectrum of IIc, the H_A proton gives rise to a doublet, δ 2.98 (A), with a coupling constant, $J_{AC} = 9.0$ Hz, indicating, that this compound also has the *cis* configuration. No *trans* isomer was present.

For compound IId it was not possible to elucidate the stereochemistry by means of proton NMR spectroscopy.

From these results we conclude, that whereas substituents in the 3- and 4-position hinder photodimerization, presumably by steric hindrance, they do not hinder photocycloaddition.

The mechanistic studies were performed on 4-methylcarbostyril in order to avoid photodimerization.

The first excited singlet state of 4-methylcarbostyril was characterized by means of spectroscopic methods. The UV spectrum of 4-methylcarbostyril was recorded at room temperature in various solvents. No noticeable solvochromism was observed. Neither did we observe any change in vibrational fine structure by increasing the solvent polarity. In Fig. 1 the UV spectrum of 4-methylcarbostyril in absolute ethanol at room temperature is shown. For the maximum at 325 nm, log ε = 2.80. Fig. 1 also shows the corrected fluorescence spectrum and the corrected fluorescence excitation spectrum of 4-methylcarbostyril in absolute ethanol at room temperature. This is in good agreement with the absorption spectrum. The excitation spectrum was corrected according to Parker [7] with Rhodamin B as quantum counter.

The total corrected emission spectrum of 4-methylcarbostyril was recorded in EPA† at 77 K (Fig. 2). From the 0 – 0 transition the energy of the first excited singlet state S_1 (82.7 kcal mol^{-1}) and of the lowest triplet state T_1 (66.4 kcal mol^{-1}) were determined. The energy gap between the singlet and the triplet state is 16.3 kcal mol^{-1} = 5700 cm^{-1}.

The phosphorescence lifetime of 4-methylcarbostyril at 77 K was determined to be 1.04 s in EPA and 1.20 s in absolute ethanol.

On the basis of these results we conclude that the first excited singlet of 4-methylcarbostyril is (π, π^*) in character.[8]

In order to determine the multiplicity of the excited state, from which the photocycloaddition proceeds, the influence of piperylene (triplet energy 57 kcal mol^{-1}) on the quantum yield of 4-methylcarbostyril disappearance

† Diethyl ether-isopentane-ethanol, 5:5:2.

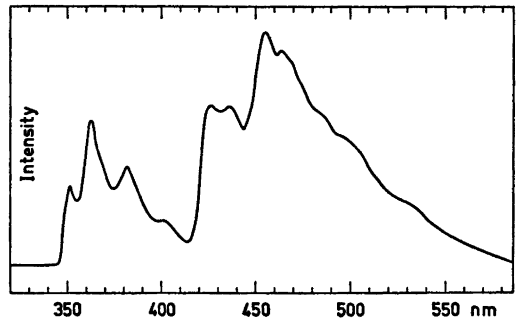


Fig. 2. Corrected total emission of 4-methylcarbostyril in EPA at 77 K. Intensity is in arbitrary units.

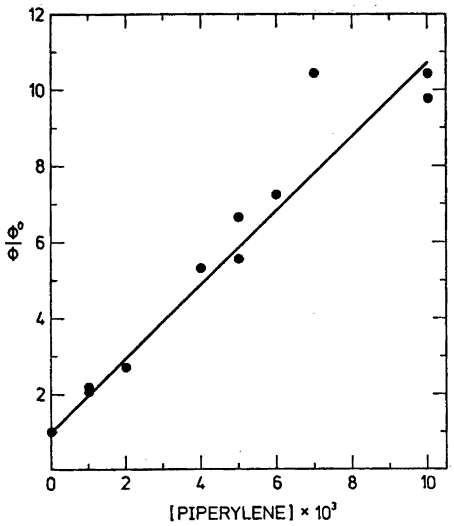


Fig. 3. Stern-Volmer plot of 4-methylcarbostyril disappearance with piperylene as triplet quencher; degassed methanol at room temperature. Starting concentrations of 4-methylcarbostyril and cyclohexene were 5.2×10^{-4} mol/l and 2×10^{-3} mol/l, respectively.

was investigated. Fig. 3 shows the plot of the relative quantum yield of 4-methylcarbostyril disappearance (Φ^0/Φ) against piperylene concentration. Φ^0 and Φ are the quantum yields of 4-methylcarbostyril disappearance in the absence and presence of piperylene. The plot follows the Stern-Volmer relation

$$\Phi^0/\Phi = 1 + k[Q]$$

We have also investigated the influence of cyclohexene on the fluorescence quantum yield of 4-methylcarbostyril. The corrected fluorescence spectrum of 4-methylcarbostyril in absolute ethanol with various concentrations of cyclohexene at room temperature was recorded (0 to 5×10^{-3} mol/l). No variation in the fluorescence quantum yield was observed.

Thus it is concluded that the photocycloaddition proceeds from the lowest triplet state of 4-methylcarbostyril.

EXPERIMENTAL

The carbostyrils used were prepared by UV irradiation of the quinoline *N*-oxides in ethanol [4] and purified by several recrystallizations from ethanol. Reagent grade cyclohexene (BDH) was purified by passage through a column packed with neutral active aluminium oxide. In the preparative experiments reagent grade methanol was used. In the mechanistic experiments methanol (*pro analysi*, Merck) was used. Absolute ethanol was purified by means of activated carbon.

Microanalysis were carried out in the microanalysis department of this laboratory by Mr. Preben Hansen and his staff.

Melting points (uncorrected) were determined on a Reichert melting point microscope.

Infrared spectra were recorded on a Perkin Elmer 337 Grating Infrared Spectrophotometer.

Ultraviolet spectra were recorded on a Unicam SP 800 A spectrophotometer.

Nuclear magnetic resonance spectra were recorded on a Varian A-60 A NMR Spectrometer, a Bruker 90 MHz NMR spectrometer, or a Varian 220 MHz NMR spectrometer.

Emission spectra were recorded on a Hitachi-Perkin Elmer Fluorescence Spectrophotometer, MPF-3, with the phosphorescence accessory.

Thin layer chromatograms (TLC) were undertaken on 8×10 cm plates with a 0.25 mm layer of aluminium oxide ($PF_{254+366}$ Merck) using a 9:1 mixture of benzene and 2-propanol and visualized with UV light.

The preparative irradiations were performed through a Pyrex filter. The light source was either an "Original Hanau" Q-700 lamp or the 300 nm lamps of a Rayonet reactor (Type RS). The irradiations were monitored by TLC or by UV spectroscopy, and continued until no more starting material could be detected.

Photocycloaddition of cyclohexene to carbostyril (Ia). Carbostyril (1.00 g) was dissolved in 500 ml ethanol containing 50 ml cyclohexene and irradiated in a Rayonet reactor. Evaporation of the solvent gave a yellow oil, which after treatment with petroleum ether gave colorless crystals (1.47 g; 94 %), m.p. 173–188 °C. Recrystallization from benzene-petroleum ether several times raised the m.p. to 180–194 °C. (Found: C 79.25; H 7.55; N 6.16. Calc. for $C_{15}H_{17}NO$: C 79.15; H 7.32; N 6.13). IR 1670 cm^{-1}. UV (EtOH): 258 nm, log $\varepsilon = 2.92$. NMR (220 MHz): δ 0.91–2.04 (m, 8 H); 2.80 (m, 1 H); 3.02 (m, 1 H); 3.41 (t, 1 H); 3.89 (t, 1 H); 6.77–7.27 (m, 4 H).

Photocycloaddition of cyclohexene to 3-methylcarbostyril (Ib). 3-Methylcarbostyril (0.50 g) was dissolved in 225 ml methanol containing 25 ml cyclohexene and irradiated in a Rayonet reactor. Evaporation of the solvent gave a yellow oil (3.59 g), which could not be brought to crystallization. A portion of this oil (1.04 g) was purified by preparative layer chromatography on silica gel using benzene–2-propanol (9:1 by volume) as the eluent, with two developments. The major fraction was isolated and extracted with methanol using

a Soxhlet apparatus. Evaporation gave a semi-crystalline mass (280 mg). IR: 1670 cm^{-1}. UV (EtOH): 258 nm. NMR (60 MHz): δ 0.7–2.9 (m, 10 H); 1.52 (s, 3 H); 3.1–3.6 (m, 1 H); 6.7–7.2 (m, 4 H); 9.7 (s, 1 H). Obviously the cyclohexene had undergone some degree of polymerization as well, but no attempt was made to examine this.

Photocycloaddition of cyclohexene to 4-methylcarbostyril (Ic). 4-Methylcarbostyril (3.00 g) was dissolved in 1000 ml methanol containing 100 ml cyclohexene and irradiated with an "Original Hanau" Q-700 lamp contained in a Pyrex immersion well. After 9 h irradiation no more starting material could be detected by TLC. Evaporation gave colorless crystals (4.70 g ~ 100 %), m.p. 212–226 °C. Recrystallization from methanol several times gave needles, m.p. 238–239 °C. (Found: C 79.71; H 7.46; N 5.88. Calc. for $C_{16}H_{15}NO$: C 79.62; H 7.93; N 5.81). IR: 1670 cm^{-1}. UV (EtOH): 256 nm, log $\varepsilon = 2.91$. NMR (220 MHz): δ 1.18–1.86 (m, 8 H); 1.63 (s, 3 H); 1.86–2.12 (m, 1 H); 2.32–2.54 (m, 1 H); 2.98 (d, 1 H); 6.80–7.27 (m, 4 H).

Photocycloaddition of cyclohexene to 3,4-dimethylcarbostyril (Id). 3,4-Dimethylcarbostyril (3.00 g) was dissolved in 1000 ml methanol containing 100 ml cyclohexene and irradiated as above. After 10 h irradiation no more starting material could be detected by TLC. Evaporation gave a yellow oil, which after treatment with ether crystallized (2.79 g ~ 63 %), m.p. 162–198 °C. Recrystallization several times from methanol–water gave colorless crystals, m.p. 206–207 °C. (Found: C 79.75; H 8.09; N 5.32. Calc. for $C_{17}H_{21}NO$: C 79.84; H 8.31; N 5.32). IR: 1670 cm^{-1}. UV (EtOH): 256 nm, log $\varepsilon = 3.03$. NMR (220 MHz): δ 1.14–1.59 (m, 8 H); 1.30 (s, 3 H); 1.48 (s, 3 H); 1.64–2.22 (m, 2 H); 6.80–7.27 (m, 4 H).

The Stern-Volmer plot. Samples (10 ml) containing 5.20×10^{-4} mol/l 4-methylcarbostyril and 2.0×10^{-2} mol/l cyclohexene in methanol, and various concentrations of piperylene ranging from 0 to 1×10^{-2} mol/l were prepared. The piperylene used was the 90 % technical product from Aldrich, which is a mixture of the isomers with cyclopentene as an impurity. The cyclopentene was assumed to have no significant influence on the results.

The samples were placed in Pyrex test tubes fitted with glass stoppers and provided with magnet stirrers. Degassing was performed by purging the solutions with argon before closing the tubes. The tubes were placed on a carousel and irradiated (magnet stirring) at room temperature with the Q-700 lamp. The irradiation was continued until about 40 % conversion in the absence of piperylene. The disappearance of 4-methylcarbostyril was measured spectrophotometrically at 325 nm after dilution by a factor of 10.

Acknowledgement. We wish to thank Dr. Herman Ziffer for the 220 MHz NMR spectra and for a very helpful discussion.

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The Crystal and Molecular Structure of (—)-Phenylephrine

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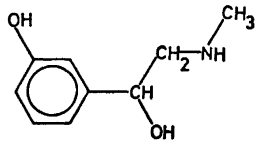
The crystal structure of (−)-phenylephrine has been determined by X-ray methods, using 929 observed reflections collected by counter diffractometer techniques. The crystals are monoclinic; space group $P2_1$ with $a = 7.833(3)$ Å, $b = 6.572(3)$ Å, $c = 8.658(4)$ Å, and $\beta = 99.09(1)°$. Least-squares refinements yielded a conventional R-factor of 0.040. Standard deviations in bond lengths are 0.003 − 0.004 Å and in bond angles 0.2 − 0.3°. The phenylephrine molecules were found to exist as zwitterions in the crystals, formed by a proton transfer from the phenolic hydroxyl group to the nitrogen atom. The ions are connected by hydrogen bonds to build double molecular layers parallel to (100).

The structure investigation of (−)-phenylephrine forms part of a research project on the free base form of sympathomimetic amines. So far, crystal structure determinations of (−)-adrenaline [1] and (−)-noradrenaline [2] have been reported.

Generally, maximal adrenergic activity of sympathomimetic amines depends on the presence of hydroxyl groups in *p*- and *m*-positions on the benzene ring. The absence of one or both of these groups results in a reduction in adrenergic potency, and especially β-activity is reduced. This is clearly seen in the different pharmacological activity of phenylephrine and adrenaline. Phenylephrine differs chemically from adrenaline only by lacking the *para* hydroxyl group, and is thus in contrast to adrenaline a weak β-adrenergic agonist.

It is assumed that a functional group in *m*-position on the phenyl ring is a requirement for β-adrenergic activity in particular.[3] The nature of this group is critical, but its chemical role in stimulating the receptor is not established. The crystal structures of (−)-adrenaline [1] and (−)-noradrenaline [2] revealed a zwitterionic state of the amines, formed by a proton transfer from the *meta* hydroxyl group to the nitrogen atom. In view of the central role this *meta* substituent is assumed to play in β-receptor stimulation, it seems of importance to obtain more information about the zwitterionic character of this class of drugs.



EXPERIMENTAL

Commercially obtained (−)-phenylephrine hydrochloride was dissolved in a 1 M ammonia solution. By slow diffusion of ethanol into the solution, thin needle-formed crystals appeared. The low water solubility of these crystals indicated the formation of the free base form of the compound.

The crystals are monoclinic; systematically absence of reflections $0k0$ with k odd determined the space group to be $P2_1$ since the phenylephrine molecule is optically active. Unit cell parameters were determined from measurements on a manual Picker diffractometer with CuK-radiation. Three-dimensional intensity data were obtained from a crystal of dimensions $0.48 \times 0.16 \times 0.05$ mm³ on an automatic Picker diffractometer with crystal monochromated Mo$K\alpha$-radiation. Intensities of 1030 reflections with $2\theta < 60°$ were measured using the $\omega - 2\theta$ scan technique. The scan speed was 1° min^{-1}, and scan range 2°. 929 reflections with net intensities larger than $2.5\sigma(I)$ were considered to be observed and were used in the structure determination. Lorentz and polarization corrections were applied to the intensity data.

Atomic form factors used were those of Doyle and Turner [4] for oxygen, nitrogen, and

carbon atoms, and of Stewart *et al.*[5] for hydrogen atoms.

The computer programs employed during the present study are described in Ref. 6.

CRYSTAL DATA

Phenylephrine, $C_9H_{13}O_2N$
Space group $P2_1$, monoclinic
$a = 7.833(3)$ Å; $b = 6.572(3)$ Å; $c = 8.658(4)$ Å; $\beta = 99.09(1)°$
$V = 440.1$ Å^3; $M = 167.23$; $F(000) = 180$; $Z = 2$; $D_{calc} = 1.262$ g cm^{-3}

STRUCTURE DETERMINATION

Unit cell dimensions of the phenylephrine crystals correspond closely to those of adrenaline;[1] both compounds crystallize with space group $P2_1$. Coordinates of nitrogen and carbon atoms from the adrenaline study were therefore selected as trial structure in the calculation of a Fourier map, in which all non-hydrogen atoms could be located. Subsequent least-squares refinement, with anisotropic thermal parameters introduced, yielded an R-factor of 0.08. Positional parameters for hydrogen atoms bonded to carbon atoms were calculated. The remaining three hydrogen atoms were located in a difference Fourier map in positions indicating a zwitterionic structure of the molecule. Full-matrix least-squares refinements including positional and isotropic thermal parameters of all hydrogen atoms converged with a conventional R-factor of 0.040 and a weighted R-factor of 0.029. A final difference Fourier map showed electron density fluctuations in the range ± 0.17 e Å^{-3}.

An analysis of the molecular thermal motions in terms of a rigid body model indicated that the phenol part of the molecule could be regarded as a rigid unit, as the r.m.s. discrepancy between atomic vibration components calculated from this analysis and those obtained from the structure determination was 0.0028. The bond lengths in the phenol part of the molecule were accordingly corrected for librational effects.

The final parameters for non-hydrogen atoms are listed in Table 1 and for hydrogen atoms in Table 2. Bond lengths and angles are given in Table 3. Thermal ellipsoids drawn at the

Table 1. Fractional atomic coordinates and thermal parameters with estimated standard deviations for non-hydrogen atoms ($\times 10^5$). 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_{13}	B_{23}
O1	95052(26)	31376(46)	83278(19)	1807(45)	1590(53)	624(24)	− 495(85)	332(55)	− 19(60)
O2	81903(32)	11076(0)	14640(21)	2224(55)	1686(57)	712(27)	909(108)	7(59)	− 320(72)
N	81952(34)	52753(51)	5386(25)	1500(54)	1282(60)	588(29)	216(103)	213(66)	55(72)
C1	80430(35)	20701(53)	41821(28)	1119(54)	1150(68)	662(34)	493(109)	278(71)	267(83)
C2	89674(37)	28737(58)	55353(31)	1194(55)	1218(69)	812(37)	− 343(116)	622(75)	95(86)
C3	85446(35)	24455(52)	70186(29)	1336(56)	1137(75)	766(36)	227(114)	617(75)	133(84)
C4	71128(39)	12020(68)	70672(31)	1763(66)	1793(83)	853(40)	− 330(143)	976(86)	641(110)
C5	61931(43)	3918(63)	57094(39)	1329(65)	2030(93)	1486(51)	− 1072(142)	724(94)	90(125)
C6	66600(40)	7958(58)	42642(32)	1297(57)	1718(90)	916(42)	− 457(132)	− 13(82)	− 36(98)
C7	85663(37)	26496(56)	26213(28)	1146(57)	1642(82)	609(33)	433(119)	343(71)	112(85)
C8	75443(45)	45003(60)	19535(32)	1331(64)	1398(72)	776(36)	120(121)	617(82)	64(91)
C9	68903(57)	65157(72)	− 4803(43)	2047(88)	2314(109)	1117(48)	982(178)	− 200(112)	652(132)

Table 2. Fractional atomic coordinates ($\times 10^4$) and isotropic thermal parameters with estimated standard deviations for hydrogen atoms.

Atom	*x*	*y*	*z*	*B*
HC2	9885(35)	3727(39)	5482(26)	2.3(6)
HC4	6779(34)	891(49)	8027(31)	3.5(6)
HC5	5319(38)	−484(49)	5746(27)	2.9(7)
HC6	5957(35)	203(45)	3356(30)	3.4(7)
HC7	9935(36)	2960(53)	2722(28)	3.5(6)
H1C8	7596(33)	5716(50)	2720(28)	3.1(6)
H2C8	6291(35)	4078(42)	1676(29)	2.2(6)
HO2	8977(43)	258(53)	1710(36)	4.1(9)
H1N	9282(39)	6114(62)	885(33)	5.4(8)
H2N	8481(38)	4211(48)	−91(32)	3.5(7)
H1C9	7425(44)	6934(57)	−1437(42)	7.1(10)
H2C9	6546(41)	7516(60)	102(38)	4.9(9)
H3C9	5880(49)	5643(70)	−810(38)	7.6(12)

Table 3. Bond lengths (Å) and angles (°) with estimated standard deviations in parantheses.

Bond length		Corrected	Bond angle	
C1–C2	1.382(4)	1.385	C2–C1–C6	119.9(3)
C2–C3	1.404(3)	1.407	C1–C2–C3	122.1(3)
C3–C4	1.393(4)	1.400	C2–C3–C4	116.9(3)
C4–C5	1.385(4)	1.388	C3–C4–C5	120.9(3)
C5–C6	1.383(4)	1.386	C4–C5–C6	121.2(3)
C1–C6	1.380(4)	1.386	C5–C6–C1	119.0(3)
C1–C7	1.521(3)	1.524	O1–C3–C4	121.5(2)
C3–O1	1.338(3)	1.341	O1–C3–C2	121.7(3)
C7–O2	1.423(3)		C2–C1–C7	118.6(3)
C7–C8	1.519(4)		C6–C1–C7	121.4(2)
C8–N	1.489(3)		C1–C7–O2	113.1(2)
N–C9	1.484(4)		O2–C7–C8	105.3(2)
C2–HC2	0.92(3)		C1–C7–C8	109.8(2)
C4–HC4	0.93(3)		C7–C8–N	111.0(2)
C5–HC5	0.90(3)		C8–N–C9	112.5(3)
C6–HC6	0.97(3)			
C7–HC7	1.08(3)			
C8–H1C8	1.04(3)			
C8–H2C8	1.01(3)			
C9–H1C9	1.02(3)			
C9–H2C9	0.90(3)			
C9–H3C9	0.98(3)			
N–H1N	1.02(3)			
N–H2N	0.94(3)			
O2–HO2	0.83(3)			

50 % probability level are illustrated in Fig. 1, which also presents the numbering of the atoms and bond lengths (corrected).

The structure factor list may be obtained from this institute upon request.

DISCUSSION

The present study demonstrates a zwitterionic structure of the phenylephrine molecule corresponding to that observed for adrenaline [1] and noradrenaline.[2] The zwitterionic character

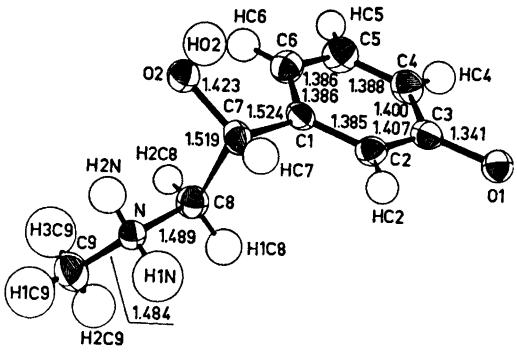


Fig. 1. Bond lengths and ellipsoids of thermal motion. (The drawing was prepared using the computer program ORTEP [13]).

of these molecules is produced by a proton transfer from a *meta* phenolic hydroxyl group to the nitrogen atom. This indicates a stronger acidic character of the *meta* hydroxyl group compared with the protonated amino group, and also a stronger acidity of the *meta* hydroxyl relative to the *para* hydroxyl. Even though the phenolic dissociation constant is reported to be less for monophenolic phenylethylamines than for catecholamines,[7] there is also observed a deprotonation of the *meta* hydroxyl group in the phenylephrine molecule. The bond lengths associated with the zwitterionic character of phenylephrine, C–N$^+$ (1.489 and 1.484 Å), N$^+$–H (0.94 and 1.02 Å), and C–O$^-$ (1.341 Å), are within the accuracies of the measurements identical to those of the free bases formerly investigated.[1,2] The remaining bond lengths of the molecule are in agreement with corresponding values found in both the free bases and salts [8–11] of sympathomimetic amines.

The benzene ring is planar, the deviations of the ring carbon atoms from a least-squares plane defined by these atoms being in the

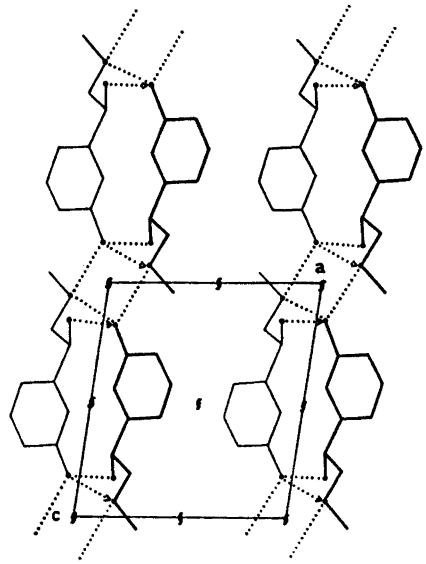


Fig. 2. The structure as viewed down the *b*-axis.

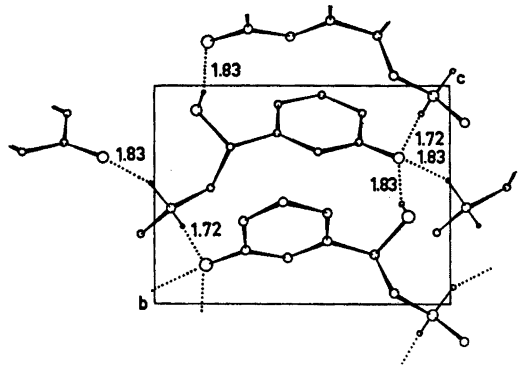


Fig. 3. The structure as viewed down the *a*-axis with hydrogen bonds (Å) indicated.

Table 4. Hydrogen bonded interactions X–H . . . Y.

X	Y	X . . Y(Å)	H . . Y (Å)	X–H . . . Y (°)
N	O1($x,y,z-1$)	2.705	1.83	153.7
N	O1($-x+2, y+\frac{1}{2}, -z+1$)	2.681	1.72	157.3
O2	O1($-x+2, y-\frac{1}{2}, -z+1$)	2.645	1.83	163.4

range 0.005 – 0.012 Å. The exocyclic atoms O1 and C7 are situated 0.090 and −0.056 Å, respectively, out of the ring plane. The external C – C – O^- angles are identical, in contrast to the situation in the zwitterionic form of the related catechol derivatives.[1,2] For the latter compounds an increase of the C2 – C3 – O^- angle is observed, indicative of an attraction between the rather close phenolic hydrogen atom and the charged oxygen atom.

The conformation of the phenylephrine molecule is in conformity with that usually found for this class of compounds in the crystals.[1,2,11]. The ethylamine side chain is fully extended and approximately perpendicular to the ring system; the dihedral angle C1 – C7 – C8 – N is 172.2° and C6 – C1 – C7 – C8 is 87.0°. The hydroxyl group of the side chain is situated in a *trans* position relative to the oxygen atom on the benzene ring. The dihedral angle C6 – C1 – C7 – O2 is −30.3°. The valence angles in the side chain are close to the expected tetrahedral value.

The crystal structure is characterized by chains of zwitterions, which are connected through hydrogen bonds to build double molecular layers parallel to (100). The only interactions between these layers are van der Waals contacts between benzene rings of magnitude 3.8 Å. This molecular arrangement, which is illustrated in Fig. 2, corresponds closely to that observed for adrenaline [1] and noradrenaline [2] in the crystals. The charged oxygen atom in all of the free bases mentioned is engaged in three fairly short intermolecular hydrogen bonds, which are arranged in infinite spirals about screw axes. Hydrogen bond distances and angles are given in Table 4 and also indicated in Fig. 3. The intramolecular contact between the nitrogen atom and hydroxyl oxygen atom of the side chain is 2.853 Å. This interaction is not representing a hydrogen bond, as all of the amino and hyroxyl hydrogen atoms are engaged in intermolecular hydrogen bonds, and furthermore their positions are not favourable for the formation of such a bond. This conclusion has also been reached in several other crystal structure determinations of sympathomimetic amines,[1,2,8,9] in which a short intramolecular contact between the amino and hydroxyl groups has been observed.

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Indolizine Derivatives. V.* The Perkin Reaction of 2-Pyridinecarbaldehyde. Disproportionation of 3-(2-Pyridyl)acrylic Acid

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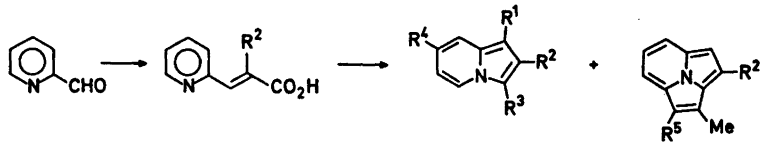
The Perkin reaction of 2-pyridinecarbaldehyde with acetic anhydride/potassium acetate, propionic anhydride/potassium propionate, or acetic anhydride/potassium acetate in the presence of phenylacetic acid gives indolizines and pyrrolo[2,1,5-*cd*]indolizines *via* disproportionation of the normal Perkin reaction products. The mechanisms of these and related reactions are discussed.

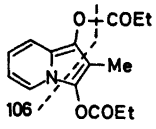
The anticipated 3-(2-pyridyl)acrylic acids (*2*) cannot be isolated from the Perkin reaction of 2-pyridinecarbaldehyde (*1*); instead, indolizine derivatives are formed *via* the normal Perkin reaction products, 3-(2-pyridyl)acrylic anhydrides, as was briefly reported recently.[1] Thus, with acetic anhydride/potassium acetate, compound *1* afforded the indolizines *3a* and *3b* [2] and the pyrrolo[2,1,5-*cd*]indolizine *4a*;[1] with propionic anhydride/potassium propionate the analogous indolizines *3c* and *3d*,[3,1] further, *3h*, *3i* and *3j* and the pyridylindolizine *3e*;[1] and with acetic anhydride/potassium acetate in the presence of phenylacetic acid *3f* and *3g*.[3,4,1] In the light of further investigations, a possible disproportionation mechanism of 3-(2-pyridyl)acrylic acids (*2*) under the conditions of the Perkin reaction leading to the indolizine derivatives *3* and *4* is discussed. The structures of the new indolizines were unambiguously established from their analyses and spectral

* Part IV; Ref. 10.

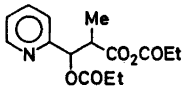


1 *2* *3* *4*

No.	R^2	R^5	No.	R^1	R^2	R^3	R^4
2a	H	–	*3a*	H	H	OAc	H
2b	Me	–	*3b*	H	H	Ac	H
2c	Ph	–	*3c*	H	Me	OCOEt	H
			3d	H	Me	COEt	H
			3e	COEt	Me	2-Pyridyl	H
4a	H	Ac	*3f*	H	Ph	OAc	H
4b	H	COOEt	*3g*	H	Ph	Ac	H
4c	Me	Ac	*3h*	OCOEt	Me	OCOEt	H
			3i	MeCH(OCOEt)	Me	COEt	OCOEt
			3j	H	Me	2-Pyridyl	H
			3k	OCOEt	Me	COEt	MeCH(OCOEt)
			3l	H	Me	Ac	H



3h



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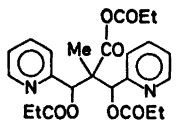

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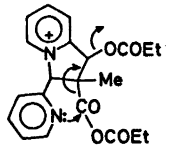

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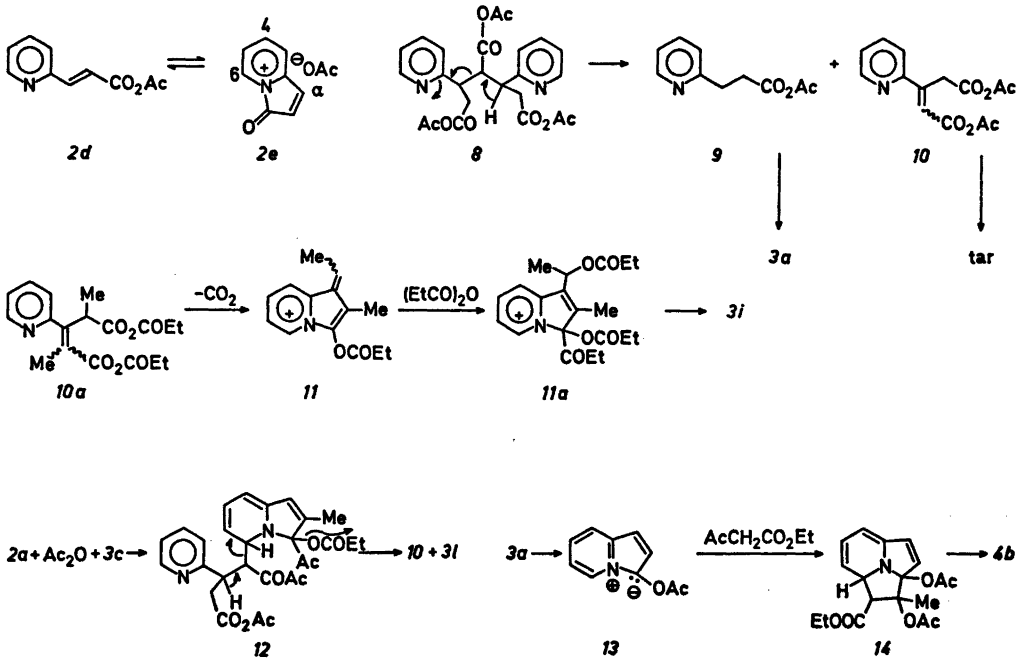
data (UV, IR, NMR, MS)[3,5–7] except *3i*, for which the alternative structure *3k* is possible as well. The structure *3i* is preferred over *3k*, however, because the mass spectrum showed no significant peaks characteristic of the known 1-indolizinyl acylates,[7] for example, the $m/e=$ 106 peak of *3h*.

Formation of indolizines

No change in the oxidation level. The indolizine *3h* is formed if cyclocondensation takes place before the elimination of propionic acid from the acylated aldol *5*. The indolizines *3e* and *3j* can originate from either the dimeric aldol *6*[8] or the cycloaddition product *7*, both routes involving decarboxylation. The indolizine *3j* is readily acylated to give *3e*.

Reduced products. 2-Pyridinecarbaldehyde (*1*) gives with acetic anhydride/potassium acetate the normal Perkin reaction product *2d*, which is susceptible to nucleophilic attack by the enolate anion of acetic anhydride, affording the dimeric addition product *8*.* The latter is cleaved into reduced (*9*) and oxidized (*10*) moieties. The reduced part *9* then cyclizes to the indolizine *3a*. This is in accordance with the facts that 3-(2-pyridyl)acrylic acid (*2a*) (prepared by the Doebner reaction [9]) and 3-(2-pyridyl)propanoic acid also give *3a* when treated with acetic anhydride/potassium acetate.[10]

* The positions 4 and 6 of the pyridine are susceptible to nucleophilic addition as well. Besides, *2d* might also react through its cyclic form *2e*.



Oxidized products. The oxidized part *10* is apparently not able to give any definite molecules, thereby accounting for the complete absence of simple oxidized species in the product mixture; a considerable amount of tar is formed in the Perkin reaction of *1*. In the propionate case, after addition and cleavage steps, the oxidized part *10a* cyclizes to the pyridinium compound *11*. This step involves decarboxylation. The compound *11* then rearranges into the indolizine *3i* through a net addition of propionic anhydride.

Doubly reduced products. Because *2a* gave with acetic anhydride/potassium acetate the same products as *1*, particularly *3b*, it is assumed that also the other doubly reduced products, *3d* and *3g* were formed through the corresponding 3-(2-pyridyl)acrylic anhydrides. They are definitely not formed *via* disproportionation of the corresponding 3-indolizinyl acylates alone. Interestingly, treatment of the indolizine *3c* with acetic anhydride/potassium acetate in the presence of *2a* produced traces of the indolizinylethanone *3l* suggesting that *3l* can be formed through the sequence shown above.

Formation of pyrrolo[2,1,5-*cd*]-indolizines

The indolizine *3a* cyclizes with acetic anhydride/potassium acetate to *4a* and the reaction of *3a* in the presence of ethyl acetoacetate gives *4b*,[1] *via* nucleophilic attack by the anions derived from 2,4-pentanedione (from the self-condensation of acetic anhydride[11]) and ethyl acetoacetate, respectively, on C-5 of the zwitterion *13*. In the propionate case or in the presence of phenylacetic acid the formation of the third ring is prevented owing to the methyl and phenyl substituents, or 3-indolizinyl acylates are attacked at positions other than C-5. When *3c* was treated with acetic anhydride/potassium acetate *4c* was obtained.

An amazing feature of 3-indolizinyl acylates is that they are not acylated at C-1, although 3-alkylindolizines, for example, are easily acylated at this position.[12] The preferred reaction at C-3 (and at C-5) probably explains the absence of 1-acyl derivatives of the compounds *3*.

EXPERIMENTAL

Acid anhydrides contained less than 3 % of the free acid. Potassium acetate and potassium propionate were dried at 110 °C before use. Product mixtures were worked up by hydrolyzing the excess of acid anhydrides with water, extracting into ether and drying on sodium sulfate. Woelm silica was used for dry-column chromatography, and benzene, containing increasing amounts of methylene chloride, was used as eluent. Thin-layer chromatography was carried out using Merck silica gel $HF_{254+366}$ with benzene containing 2 – 5 % of methanol. The solid products were recrystallized from light petroleum (b.p. 40 – 60 °C) if not stated otherwise. Melting points are uncorrected. Elemental analyses were performed by Mrs. A. M. Horko. UV spectra were obtained for solutions in ethanol, IR spectra were obtained for KBr-tablets or liquid films. NMR spectra were measured for solutions in $CDCl_3$ or CCl_4 at 60 MHz. Mass spectra were recorded at 70 eV through the cooperation of Mr. P. Karvonen.

The Perkin reactions of 2-pyridinecarbaldehyde

With Ac_2O/KOAc. 2-Pyridinecarbaldehyde (10.7 g, 0.10 mol), Ac_2O (50 g, 0.5 mol) and KOAc (25 g, 0.25 mol) were refluxed for 0.5 h. After work-up and chromatography the following four compounds were obtained: *3-Indolizinyl acetate*, (*3a*), yield 0.7 g (4 %), m.p. 20 °C. (Found: C 68.85; H 5.10; N 7.75. Calc. for $C_{10}H_9NO_2$: C 68.55; H 5.20; N 8.00). IR, ν_{max} 1775 (s), 1750 (s). ^{1}H NMR: δ 7.52 (1 H, br d, J 7 Hz), 7.21 (1 H, br d, J 9), 6.6 – 6.2 (2 H, m), 6.52 (1 H, d, J 4.3), 6.28 (1 H, d, J 4.3), 2.18 (3 H, s). MS, *m/e* (%): 175 (M^+). *1-(3-Indolizinyl)ethanone*, (*3b*),[2] yield 0.95 g (6 %), m.p. 32 °C. ^{1}H NMR: δ 9.87 (1 H, br d 7), 7.40 (1 H, br d 9), 7.32 (1 H, d 4.7), 7.2 – 6.55 (2 H, m), 6.35 (1 H, br d, J 4.7), 2.46 (3 H, s). MS, *m/e* (%): 159 (M^+, 64), 145 (9), 144 (100), 116 (59), 89 (29), 43 (41). *1-(2-Methyl-1-pyrrolo[2,1,5-cd]-indolizinyl)ethanone*, (*4a*), yield 0.79 g, (4 %), m.p. 79 °C. (Found: C 79.05; H 5.80; N 6.75. Calc. for $C_{13}H_{11}NO$: C 79.15; H 5.60; N 7.10). UV, λ_{max} (log ε): 411 (3.89), 403 (3.86), 395 (sh, 3.77), 313 (3.78), 277 (sh, 3.80), 2.57 (4.23), 240 (4.05). IR, ν_{max}: 1645 (s), 1640 (s), 1630 (s). ^{1}H NMR: δ 8.21 (1 H, dd, J 5.2 and 2.8), 7.90 – 7.70 (2 H, m), 7.60 (1 H, d, J 4.4), 7.24 (1 H, d, J 4.4), 2.95 (3 H, s), 2.72 (3 H, s). MS, *m/e* (%): 197 (M^+, 14), 183 (9), 182 (100), 155 (22), 154 (33), 153 (11). *3-Acetyl-2,6-dimethyl-4H-pyran-4-one*,[11] yield 2.4 g, m.p. 57 °C. When the reaction time was reduced to 10 min, *3a*, 2.6 g (15 %) and *3b*, 1.3 g (8 %), but none of *4a*, were obtained, while *3a* was absent from the product mixture after periods longer than 1 h.

With $(EtCO)_2O$/KOCOEt. 2-Pyridinecarbaldehyde (21.4 g, 0.2 mol) was added to the hot mixture of $(EtCO)_2O$ (130 g, 1.0 mol) and KOCOEt (55 g, 0.5 mol) at once and boiled for 0.5 h. Work-up and chromatography gave: *2-Methyl-3-indolizinyl propionate, (3c)*, yield 4.0 g (10 %), m.p. 33 °C. (Found: C 70.65; H 6.20; N 6.75. Calc. for $C_{12}H_{13}NO_2$: C 70.90; H 6.45; N 6.90). UV, λ_{max} (log ε): 3.72 (sh, 2.94), 353 (sh, 3.30), 344 (3.33), 292 (3.49), 281 (3.51), 272 (3.48), 243 (sh, 4.31), 232 (4.44). IR, ν_{max}: 1765 (s). ^{1}H NMR: δ 7.21 (1 H, br d, J 7), 7.08 (1 H, br d, J 9), 6.07 (1 H, br s), 2.49 (2 H, q, J 7.5), 2.12 (3 H, dd, J 0.4 and 0.1), 1.18 (3 H, t, J 7.5). *1-(2-Methyl-3-indolizinyl)-1-propanone, (3d)*,[3] yield 2.3 g (6 %), m.p. 48 °C. ^{1}H NMR identical with that given in Ref. 3, particularly, H-5 at δ 9.92 (br d, J 7). *2-Methyl-3-(2-pyridyl)-indolizine, (3j)*, yield 0.2 g (1 %), as liquid. (Found C 80.75; H 5.60; N 13.45. Calc. for $C_{14}H_{12}N_2$: C 80.75; H 5.80; N 13.45). ^{1}H NMR: δ 9.26 (1 H, br d, J 7), 8.53 (1 H, br d, J 5), 7.9 – 6.15 (6 H, m), 6.20 (1 H, br s), 2.48 (3 H, s). *2-Methyl-1,3-indolizinediyl dipropionate, (3h)*, yield 1.6 g (3 %), m.p. 104 °C. (Found: C 65.80; H 6.00; N 5.20. Calc. for $C_{15}H_{17}NO_4$: C 65.45; H 6.20; N 5.10). UV similar to that of *3c*. IR, ν_{max}: 1765 (s), 1745 (s), 1740 (s). MS, *m/e* (%): 275 (M^+, 10), 219 (16), 190 (10), 163 (49), 162 (100), 106 (19). *1-[2-Methyl-7-propionyloxy-1-(1-propionyloxy-1-ethyl)-3-indolizinyl]-1-propanone, (3i)*, yield 1.4 g (2 %), m.p. 99 °C. (Found: C 66.85; H 6.95; N 3.95. Calc. for $C_{20}H_{25}NO_5$: C 66.85; H 7.00; N 3.90). UV, λ_{max} (log ε): 408 (sh, 2.69), 360 (4.00), 349 (sh, 3.94), 267 (4.21), 263 (sh, 4.18), 253 (sh, 3.99), 244 (sh, 3.93), 228 (4.14). IR, ν_{max} 1745 (s), 1730 (s). ^{1}H NMR: δ 9.92 (1 H, br d 7), 7.01 (1 H, br s), 6.71 (1 H, br d 7), 5.76 (1 H, q 6), 2.82 (2 H, q 7), 2.67 (2 H, q 7), 2.35 (3 H, s), 2.33 (2 H, q 7), 1.56 (3 H, d 6), 1.34 (3 H, t 7), 1.22 (3 H, t 7), 1.00 (3 H, t 7). MS, *m/e* (%): 359 (M^+, 16), 304 (22), 303 (100), 302 (45), 274 (12), 231 (14), 230 (46), 229 (32), 228 (22), 203 (15), 202 (20), 174 (19), 118 (9), 104 (20). *1-[2-Methyl-3-(2-pyridyl)-1-indolizinyl]-1-propanone, (3e)*, yield 7.0 g (27 %), m.p. (EtOH) 147 °C. (Found: C 77.45; H 5.95; N 10.55. Calc. for $C_{17}H_{16}N_2O$: C 77.25; H 6.10; N 10.60). UV, λ_{max} (log ε): 370 (sh, 3.79), 352 (sh, 4.01), 338 (4.06), 325 (4.04), 270 (4.15), 265 (sh, 4.09), 231 (4.20). IR, ν_{max}: 1620 (s), 1610 (s). ^{1}H NMR: δ 8.75 (1 H, br d 5), 8.69 (1 H, br d 7), 8.36 (1 H, br d 9), 2.60 (3 H, s), +EtCO. MS, *m/e* (%): 264 (M^+, 37), 246 (8), 236 (19), 235 (100).

With Ac_2O/KOAc in the presence of phenylacetic acid. 2-Pyridinecarbaldehyde (10.7 g, 0.10 mol), Ac_2O (50 g, 0.5 mol), KOAc (25 g, 0.25 mol) and phenylacetic acid (13.6 g, 0.10 mol) were boiled for 0.5 h. Work-up and chromatography gave: *2-Phenyl-3-indolizinyl acetate, (3f)*, yield 6.8 g (27 %), m.p. (MeOH) 118 °C. (Found: C 76.25; H 5.25; N 5.35. Calc. for $C_{16}H_{13}NO_2$: C 76.45; H 5.20; N 5.55). UV, λ_{max} (log ε): 384 (sh, 3.54), 365 (3.65), 352 (3.69), 307 (sh, 3.62), 294 (sh, 3.88), 284 (sh, 3.99), 252 (4.68). IR, ν_{max}: 1770 (s), 1765 (s). ^{1}H NMR: δ 7.80 – 7.20 (7 H, m), 6.80 – 6.45 (2 H, m), 6.64 (1 H, s), 2.39 (3 H, s). MS, *m/e* (%): 251 (M^+, 9), 210 (16), 209 (100), 208 (65), 181 (13), 180 (76). *1-(2-Phenyl-3-indolizinyl)ethanone, (3g)*,[3,4] yield 1.2 g, (5 %), m.p. 65 °C. ^{1}H NMR: δ 10.02 (1 H, br d, J 7), 7.36 (5 H, s), 6.36 (1 H, s), 1.95 (3 H, s).

Disproportionation of 3-(2-pyridyl)acrylic acid (2a). 2a[9] (7.5 g, 0.05 mol), Ac_2O (50 g, 0.5 mol), KOAc (25 g, 0.25 mol) were refluxed for 0.5 h producing *3a, 3b* and *4a* in 15, 9 and 4 % yields, resp. In neat Ac_2O *2a* gave 90 % of acetic 3-(2-pyridyl)acrylic anhydride, *(2d)*, m.p. 68 °C. (Found: C 62.60; H 4.50; N 7.65. Calc. for $C_{10}H_9NO_3$: C 62.80; H 4.75; N 7.65). IR, ν_{max}: 1790 (s), 1725 (s), 1625 (s). MS, *m/e* (%): 191 (M^+, –) 148 (57), 132 (100).

Cyclocondensation of 3-(2-pyridyl)propanoic acid with Ac_2O/KOAc. 2a[9] (14.9 g, 0.10 mol), 10 %-Pd/C (0.5 g) and ethanol (150 ml) were shaken under hydrogen (1 atm) at room temperature for 12 h. After filtration, evaporation and recrystallization from chloroform 11.2 g (74 %) of *3-(2-pyridyl)propanoic acid*[13] was collected. This saturated acid (1.5 g, 0.01 mol) was heated with Ac_2O (10 ml) and KOAc (5 g) at 100 °C for 15 min to produce *3a*, yield 0.77 g (44 %), but none of *3b*.

Cyclization of 3-indolizinyl acylates with Ac_2O/KOAc

3-Indolizinyl acetate (3a) with Ac_2O/KOAc. 3a (3.5 g, 0.020 mol) was added to the hot mixture of 20 ml Ac_2O and 10 g of KOAc and boiled for 20 min. Work-up and chromatography gave *4a*, yield 0.44 g, (11 %).

3-Indolizinyl acetate (3a) with Ac_2O/KOAc in the presence of ethyl acetoacetate. 3a (3.5 g, 0.020 mol), Ac_2O (20 ml), KOAc (10 g) and ethyl acetoacetate (2.6 g, 0.020 mol) gave *ethyl 2-methyl-1-pyrrolo[2,1,5-cd]indolizine-carboxylate (4b)*, yield 0.95 g, (21 %), m.p. 64 °C. (Found: C 73.90; H 5.85; N 6.00. Calc. for $C_{14}H_{13}NO_2$: C 74.00; H 5.75; N 6.15). UV similar to that of *4a*. IR, ν_{max}: 1690 (s), 1685 (s). ^{1}H NMR similar to that of *4a*, COOEt instead of $COCH_3$. MS, *m/e* (%): 227 (M^+, 69), 199 (22), 198 (23), 182 (100), 155 (40), 154 (52), 153 (34), 73 (37). Starting from *2a* afforded similarly *4b* in 12 % yield.

2-Methyl-3-indolizinyl propionate (3c) with Ac_2O/KOAc. 3c (2.0 g, 0.010 mol) was boiled with Ac_2O (10 ml) and KOAc (5 g) for 15 min. After work-up and chromatography *1-(2,3-dimethyl-1-pyrrolo[2,1,5-cd]indolizinyl)ethanone (4c)* was obtained, yield 0.34 g (16 %), m.p. 70 °C. (Found: C 79.35; H 6.30; N 6.75. Calc. for $C_{14}H_{13}NO$: C 79.60; H 6.20; N 6.65). UV and IR similar to those of *4a*. ^{1}H NMR: δ 2.77

(3 H, d, *J* 1) instead of *δ* 7.24 (1 H, d, *J* 4.4) in *4a*. MS, *m/e* (%): 211 (M^+, 55), 197 (15), 196 (100), 168 (11), 167 (28).

2-Methyl-3-indolizinyl propionate (3c) with Ac_2O/KOAc in the presence of 2a. *3c* (2.0 g, 0.010 mol) and *2a* (1.5 g, 0.010 mol) were treated with Ac_2O (20 ml) and KOAc (10 g). After work-up *ca.* 15 mg of *1-(2-methyl-3-indolizinyl)ethanone (3l)*[3] was collected by means of preparative thin-layer chromatography, m.p. 81 °C. ^1H NMR identical with that given in Ref. 3, particularly, H-5 at *δ* 9.93 (br, d, *J* 7). The product mixture contained several other indolizines, such as *3a*, *3b* and *4a*.

All attempts to cyclize *3a* with $(EtCO)_2O$/KOCOEt failed; *3a* disappeared during prolonged heating.

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Ion Pair Extraction in Preparative Organic Chemistry. X. Kinetic Evidence for the Ion Pair as the Nucleophile in Alkylation of Acetylacetone and Related Compounds

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The kinetics of the methylation of some beta-diketones with methyl iodide or dimethyl sulfate has been studied using the 'extractive alkylation' method. It was found that both the anion of the beta-diketone and its ion pair with tetrabutylammonium can function as the nucleophile. Due to the low degree of dissociation of the ion pair in methylene chloride, the ion pair is the dominating nucleophile. Under preparative conditions, it can be regarded as the only nucleophile.

In 1953 Brändström[1] presented a theory for the carbon alkylation of beta-dicarbonyl compounds. According to this theory it is, in modern nomenclature, the chelated ion pair that is the nucleophile in the carbon alkylation reaction. Since then several investigations have been performed which strongly support this theory. However, it still seems to be a controversial question.[2]

The extractive alkylation process recently introduced by Brändström and Junggren,[3] however, provides an excellent opportunity to explore the mechanism of the alkylation reaction. The fundamental points in this reaction are:

1. The reaction between the quaternary ammonium compound of the diketone and the alkylation agent takes place in a two layer suspension where the velocity of the interfacial transport, by proper stirring, can be made very rapid compared to the alkylation reaction.

2. The concentrations (and activities) of the mesomeric anion and the quaternary cation in the aqueous layer can be readily controlled and measured.

3. By means of simple distribution experiments it is possible to measure, calculate, and predict the concentration of all species of interest in connection with the alkylation.

4. Reaction kinetics can be readily performed at different concentrations of these species and the velocity of the reaction can be correlated to the concentration of the different nucleophiles.

LIST OF SYMBOLS

w and s = ml of *w*ater or *s*olvent layer

L'	= mmol of NaOH added
L	= mmol of NaOH added corrected for the content of OH^- and H_3O^+
A	= total quantity of HA in mmol
Q	= total quantity of Q in mmol
R	= total quantity of RI in mmol
HA	= symbol for weak acid
RI	= symbol for alkylation agent
Q^+	= symbol for tetrabutylammonium
RA	= symbol for alkylated product
$[\ ]_w$	= concentration in aqueous layer, M
$[\ ]_s$	= concentration in organic layer, M
C_{QA}	= total concentration of QA in the solvent, M
K_D	= distribution constant of HA
k'_{HA}	= apparent dissociation constant of HA in water
k^*_{HA}	= apparent dissociation constant of HA defined by eqn. 8
E_{QA}	= extraction constant of QA

E^*_{QA} = apparent extraction constant of QA defined by eqn. 16
E'_{QA} = extraction constant of QA obtained from eqn. 34
L^0, w^0, s^0, C^0_{QA}, $[\ ]^0_w$, $[\ ]^0_s$ = values read before RI was added
t_0 = starting time used in calculation
$[RA]_0$ = value of $[RA]_s$ at time $t=t_0$
k_{obs} = observed second order rate constant, dm³/mol min
k_A = second order rate constant for the alkylation of the anion, dm³/mol min
k_{QA} = second order rate constant for the alkylation of the ion pair, dm³/mol min
K_{diss} = thermodynamic dissociation constant of the ion pair
γ_s = mean activity coefficient in the solvent
θ = integral defined by eqn. 31
a_0, a_1, a_2 and a_3 = parameters obtained by the least square fitting of eqn. 33
S.D.(E) S.D.(pH) and S.D.(k) = standard deviations of the quantities E, pH and k obtained

METHOD

The reaction studied is of the type

$$HA_s + OH^-_w \rightleftharpoons A^-_w + H_2O$$
$$A^-_w + Q^+_w \rightleftharpoons QA_s$$
$$QA_s + RI_s \rightleftharpoons QI_s + RA_s$$

where the subscripts s and w indicate that the ion or molecule is present in the solvent or the water layer. It was followed by registrating the quantity of sodium hydroxide added to keep the pH constant during the reaction.

CALCULATIONS

The distribution of HA follows the equations

$$HA_s \rightleftharpoons HA_w \qquad [HA]_s = K_D[HA]_w \qquad (1)$$

$$HA_w + H_2O \rightleftharpoons A^-_w + H_3O^+ \qquad [A^-]_w a_{H_3O^+} = k'_{HA}[HA]_w \qquad (2)$$

The total quantity L' of NaOH added is

$$L' = w([A^-]_w + [OH^-]_w - [H_3O^+]_w) \qquad (3)$$

$[OH^-]_w$ and $[H_3O^+]_w$ can be calculated from the pH reading, or better, be determined by blank experiments. From this we can calculate the quantity L

$$L = L' - w([OH^-]_w - [H_3O^+]_w) \qquad (4)$$

we thus have

$$L = w[A^-]_w \qquad (5)$$

From the total quantity A of HA we can deduct

$$A = s[HA]_s + w[HA]_w + w[A^-]_w \qquad (6)$$

From eqns. 1, 5, and 6 we obtain

$$[HA]_w = (A - L)/(sK_D + w) \qquad (7)$$

Introducing the values for $[A^-]_w$ and $[HA]_w$ into the dissociation equilibrium we obtain

$$k^*_{HA} = k'_{HA}/(K_D + w/s) = sLa_{H_3O^+}/w(A - L) \qquad (8)$$

From this we can calculate the apparent dissociation constant k^*_{HA} used in all calculations in the following.

The extraction of QA follows the following equations:

$$Q^+_w + A^-_w \rightleftharpoons QA_s \qquad [QA]_s = E_{QA}[Q^+]_w[A^-]_w \qquad (9)$$

Using C_{QA} for the concentration of all compounds in the organic layer containing Q and A in the molar portions one to one and supposing that no other combinations of Q and A are formed we obtain for the total quantities L, A and Q

$$L = w[A^-]_w + sC_{QA} \qquad (10)$$

$$A = w[A^-]_w + sC_{QA} + w[HA]_w + s[HA]_s \qquad (11)$$

$$Q = w[Q^+]_w + sC_{QA} \qquad (12)$$

A combination of 10 and 12 gives

$$[Q^+]_w = (Q - L)/w + [A^-]_w \qquad (13)$$

A combination of 1, 10, and 11 gives

$$[HA]_w = (A - L)/(sK_D + w) \qquad (14)$$
$$[HA]_s = (A - L)K_D/(sK_D + w]$$

A combination of 2, 8, and 14 gives

$$[A^-]_w = (A - L)k^*_{HA}/s\, a_{H_3O^+} \qquad (15)$$

The eqns. 15, 13, and 10 enable us to calculate $[A^-]_w$, $[Q^+]_w$ and C_{QA}. From these we can calculate an apparent extraction constant E^*_{QA} defined by

$$E^*_{QA} = C_{QA}/[Q^+]_w[A^-]_w \quad (16)$$

The extraction equilibrium during the alkylation can be calculated in the following way. Assume that no side reactions occur such as hydrolysis of the alkylation agent or dialkylation. The following equations are now valid.

$$L = w[A^-]_w + sC_{QA} + s[RA]_s \quad (17)$$

$$A = w[A^-]_w + sC_{QA} + s[RA]_s + w[HA]_w + s[HA]_s \quad (18)$$

$$Q = w[Q^+]_w + sC_{QA} + s[QI]_s \quad (19)$$

$$R = s[RI]_s + s[RA]_s \quad (20)$$

$$[RA]_s = [QI]_s \quad (21)$$

In the same way as before we obtain

$$[Q^+]_w = (Q-L)/w + [A^-]_w \quad (22)$$

$$[A^-]_w = (A-L)\, k^*_{HA}/sa_{H_3O^+} \quad (23)$$

A combination of 16 and 17 gives

$$s[RA]_s = L - w[A^-]_w - sE^*_{QA}[Q^+]_w[A^-]_w \quad (24)$$

From 20 we obtain

$$[RI]_s = R/s - [RA]_s \quad (25)$$

The eqns. 17 and 22 – 25 thus provide a possibility of calculating all concentrations of interest in the kinetic measurements if the values of E^*_{AQ} and k^*_{HA} are known. It is, however, not necessary to introduce E^*_{QA} at this point in the calculations since it is readily eliminated in the following way: If the readings at equilibrium prior to the addition of RI are marked with the superscript ^{0}, we obtain, since $[RA]^0_s = 0$

$$C^0_{AQ} = (L^0 - w^0[A^-]^0_w)/s_0 = E^*_{QA}[Q^+]^0_w[A^-]^0_w \quad (26)$$

This is solved for E^*_{QA} which is constant throughout the experiment and therefore can be introduced into eqn. 24 giving

$$s[RA]_s = L - w[A^-]_w - s[Q^+]_w[A^-]_w - (L^0 - w^0[A^-]^0_w)/(s_0[Q^+]^0_w[A^-]^0_w) \quad (27)$$

THE CALCULATION OF THE RATE CONSTANT

During each run the following rate equation has been found to be valid

$$d[RA]_s/dt = k_{obs}C_{QA}[RI]_s \quad (28)$$

This is integrated between the limits t and t_0 giving

$$[RA]_s - [RA]_0 = k_{obs}\int_{t_0}^{t} C_{QA}[RI]_s dt \quad (29)$$

Combining this with 23 and 16 gives

$$[RA]_s - [RA]_0 = (k_{obs}\, E^*_{QA}k^*_{HA}/a_{H_3O^+}) \int_{t_0}^{t} (A-L)\,[Q^+]_w[RI]_s dt/s \quad (30)$$

In this equation the integral

$$\theta = \int_{t_0}^{t} (A-L)\,[Q^+]_w[RI]_s\, dt/s \quad (31)$$

is readily calculated by numerical integration. In fact, the simple trapetzoid formula is quite satisfactory. A plot of $[RA]_s$ against θ should give a straight line with the slope a_1

$$a_1 = k_{obs}\, E^*_{QA}k^*_{HA}/a_{H_3O^+} \quad (32)$$

from which k_{obs} can be calculated.

In some cases when the reaction was allowed to proceed to more than 20 % conversion, the lines were slightly curved, especially when disubstitution was possible. It was, however, never difficult to obtain the slope at the time $t=0$ which is that of primary interest.

Due to the rather extensive amount of numeric calculations involved in the determination of each velocity constant, a computer program was constructed. In order to calculate the value for the slope of the plot of $[RA]_s$ against θ, the curve was fitted with a polynom of the type

$$[RA]_s = a_0 + a_1\theta + a_2\theta^2 + a_3\theta^3 \quad (33)$$

The parameters a_0, a_1, a_2, and a_3 were determined by the method of least square, minimizing the error in $[RA]_s$. In all runs the fit was excellent. The slope at $t=t_0$ is equal to a_1 which is obtained accurately in this way.

In order to cover as wide a concentration range as possible, it became necessary to study both rapid and slow reactions. The slow reactions, down to 2 % conversion in 24 h, presented no problems other than trivial mechanical ones such as performing very rapid stirring in a closed system for a long period.

The rapid reactions (20 % conversion in less than 10 min) raised two additional complications, however. The first one was that the velocity was sometimes controlled by the rate of the interfacial transport. When this occurred, the needle of the pH meter showed small and rapid fluctuations in time with additions of the base. In the cases where this could not be avoided by increasing the rate of stirring, the experiment was rejected. The second complication arose from the construction of the instrument. In the most rapid reactions the instrument could not keep the pH at the same level as that at equilibrium. This was seen by a slight S shape of the plot of L' against t presented by the instrument. A pH was also read on the instrument that was slightly lower than that at equilibrium. This problem was handled in the following way.

The time $t=0$ for the addition of the alkylation agent was marked on the t axis of the plot of L' against t presented by the instrument. The first part of the curve, before the pH had been stabilized, was replaced by a line obtained by back extrapolating the curve to $t=0$. The calculation was then performed in the usual way.

RESULTS AND DISCUSSIONS

The dissociation constant of acetylacetone in 0.5 M Na_2SO_4 was determined in the usual way giving $pk'_{HA}=8.81$. The determination of the apparent dissociation constant k^*_{HA} in the two layer system, methylene chloride and 0.5 M Na_2SO_4, presented no problem. Five titration curves with the starting concentration of HA ranging from 0.1 to 0.5 M gave $pk^*_{HA}=10.42$. S.D.$(pk)=0.016$.

The determinations of the apparent extraction constants E^*_{QA} were performed in rather concentrated solutions. The transport of matter from one layer to the other thus resulted in a slight change in the volumes s and w. Control experiments gave the result that, within the experimental errors, the change in volume expressed in ml was identical to the weight of the transported matter in grams. s and w were therefore corrected in accordance with this.

A typical run for the determination of the apparent extraction constant E^*_{QA} of tetrabutyl ammonium acetylacetonate is given in Table 1 and the results of 5 such runs are given in Table 2.

The constants obtained can be summarized by the formula

$$E'_{QA}=5.43+0.128A \qquad (34)$$

The small but highly significant variation of E'_{QA} with A is best interpreted as a medium effect. A solution containing HA or QA in methylene chloride is a better extraction solvent for QA than pure methylene chloride. Every attempt to correlate the variation of E^*_{QA} with the formation of species such as $(QA)_2$ and QAHA gave, as a result, such low formation constants of these species that their physical significance is highly questionable.[4] There is thus no reason to introduce species other than the ion pair AQ (and the free anion A^-) in the kinetical discussions.

Table 1. Determination of the apparent extraction constant E^*_{QA} of tetrabutylammonium acetylacetonate. $A=2.5$, $Q=12.5$, NaOH = 5.095, $s_0=25$, $w_0=25$, $t=25$ °C.

ml	L	pH	w	s	$[A^-]_w$	$[Q^+]_w$	C_{QA}	E^*_{QA}[a]
0.05	0.255	8.92	25.02	25.03	0.00284	0.493	0.00734	5.24
0.10	0.510	9.28	25.03	25.07	0.00579	0.484	0.01457	5.20
0.15	0.764	9.50	25.04	25.11	0.00839	0.477	0.02206	5.51
0.20	1.019	9.70	25.06	25.14	0.01135	0.470	0.02922	5.48
0.25	1.269	9.86	25.06	25.19	0.01366	0.461	0.03678	5.84
0.30	1.524	10.04	25.07	25.23	0.01642	0.454	0.04408	5.91
0.35	1.773	10.23	25.08	25.26	0.01897	0.447	0.05136	6.06
0.40	2.023	10.48	25.10	25.30	0.02216	0.439	0.05797	5.96

[a] $E^*_{QA}=5.65$, S.D. $(E)=0.31$.

Table 2. The apparent extraction constant E^*_{QA} of tetrabutylammonium acetylacetonate. $E'_{QA} = 5.43 + 0.1284A$; $Q = 12.5$.

A	ml	pH	E^*_{QA}	Mean	S.D.(E)	E'_{QA}
2.5	0.05	8.92	5.24			
2.5	0.10	9.28	5.20			
2.5	0.15	9.50	5.51			
2.5	0.20	9.70	5.48			
2.5	0.25	9.86	5.84			
2.5	0.30	10.04	5.91			
2.5	0.35	10.23	6.06			
2.5	0.40	10.48	5.96	5.65	0.31	5.75
5.0	0.10	8.88	5.99			
5.0	0.20	9.24	6.03			
5.0	0.30	9.48	6.11			
5.0	0.40	9.69	5.96			
5.0	0.50	9.87	6.05			
5.0	0.60	10.05	6.23			
5.0	0.70	10.25	6.33			
5.0	0.80	10.50	6.42	6.14	0.16	6.07
7.5	0.15	8.85	6.69			
7.5	0.30	9.22	6.60			
7.5	0.45	9.48	6.27			
7.5	0.60	9.69	6.31			
7.5	0.75	9.88	6.32			
7.5	0.90	10.07	6.41			
7.5	1.05	10.28	6.44			
7.5	1.20	10.52	6.92	6.50	0.21	6.39
10.0	0.20	8.85	6.67			
10.0	0.40	9.22	6.78			
10.0	0.60	9.48	6.64			
10.0	0.80	9.69	6.69			
10.0	1.00	9.89	6.62			
10.0	1.20	10.09	6.60			
10.0	1.40	10.29	7.05	6.73	0.14	6.71
12.5	0.25	8.83	7.30			
12.5	0.50	9.22	7.00			
12.5	0.75	9.49	6.74			
12.5	1.00	9.70	6.93			
12.5	1.25	9.90	6.97			
12.5	1.50	10.11	6.85			
12.5	1.75	10.33	6.93			
12.5	2.00	10.60	6.95	6.96	0.19	7.04

In the following E'_{QA} obtained from eqn. 34 is used instead of E^*_{QA} in eqn. 32 in the calculation of k_{obs}.

Some remarks must be made, however, concerning the use of eqn. 34. It has been obtained from measurements in the concentration range 0.007 – 0.25 M in $[QA]_s$. In this concentration range the dissociation of $[QA]_s$ into Q^+_s and A^-_s in the organic layer can be expected to be very low and its influence on E^*_{QA} can be neglected. From the discussion above, it is obvious that eqn. 34 is obtained in the concentration region where $C_{QA} = [QA]_s$ and E'_{QA} thus connects the concentrations in both layers by the equation

$$[QA]_s = E'_{QA}[Q^+]_w[A^-]_w \qquad (35)$$

When E'_{QA} is used instead of E^*_{QA} to calculate k_{obs} by eqn. 32, it follows from the deductions of eqn. 30 that C_{QA} in eqn. 28

has to be replaced by $[QA]_s$. These are, however, the only points where the use of E'_{QA} affects the calculations.

In this connection, it should be pointed out that there seems to be no method available at present to determine E^*_{QA} in the concentration range where the dissociation is of importance. The present method is too inaccurate in this concentration range and the reactivity of QA towards methylene chloride is too high to allow accurate measurements by other, more time consuming methods.

The kinetic measurements of the methylation of acetylacetone with methyl iodine in the presence of tetrabutylammonium ions have been performed at different pH values (5.5 – 9.0), different initial concentration of acetylacetone (0.04 – 0.5 M) and of tetrabutylammonium ions (0.1 – 0.5 M). A typical run is given in Table 3 and the results of 41 runs of this type are given in Table 4.

The k_{obs} values from different runs, but under the same conditions may differ by up to 10 % from the mean value. This can be explained by considering the difficulties in determining the pH values in the different runs. From the calculation of k^*_{HA}, we can see that the standard deviation in the pk determination is about 0.016 pk unit. This corresponds to a standard deviation in the pH readings of 0.016 pH units and in k_{obs} of about 3.7 %. This is in good agreement with mean standard deviation in k_{obs} of 4.2 % found by experiment.

From Table 4, we can see that k_{obs} is fairly constant (within a factor of 2) in all experiments in spite of the fact that C_{QA} is varied within a factor of 3000. This is in good agreement with the assumption that the ion pair QA is the dominating nucleophile. (In the calculations it was assumed that the velocity is proportional to C_{QA} and from the equilibrium studies it was demonstrated that $C_{QA} \simeq [QA]_s$).

A careful inspection of the kinetic runs made at pH 9 (Run 17 – 41) reveals that k_{obs} is independent of the value of Q and almost independent of the value of A. The small decrease in k_{obs} with an increasing $[HA]_s$ is probably a medium effect. Thus an increase in $[HA]_s$ slightly increases the extraction and at the same time makes the ion pair less reactive toward methyl iodide.

Table 3. Methylation of acetylacetone with methyl iodide at pH 8.98. $A = 7.5$, $Q = 2.5$, $R = 16.07$, $s = s^0 = 26.0$, $w = 25.0$, $L^0 = 0.492$.

$t - t_0$ min	L	$\theta \times 10^2$	$[RA]_{calc} \times 10^2$ [a]	$[RA]_{found} \times 10^2$
0	0.515	0	0.103	0.105
0.25	0.542	0.359	0.224	0.223
0.50	0.568	0.712	0.346	0.344
0.75	0.595	1.057	0.465	0.464
1.00	0.622	1.396	0.585	0.587
1.25	0.648	1.729	0.705	0.704
1.50	0.674	2.055	0.823	0.822
1.75	0.700	2.374	0.940	0.938
2.00	0.726	2.688	1.055	1.055
2.25	0.752	2.995	1.171	1.073
2.50	0.777	3.296	1.286	1.288
2.75	0.802	3.591	1.398	1.401
3.00	0.827	3.880	1.511	1.509
3.25	0.851	4.164	1.622	1.619
3.50	0.876	4.442	1.732	1.730
3.75	0.900	4.715	1.840	1.840
4.00	0.925	4.982	1.949	1.950
4.25	0.948	5.244	2.055	2.054
4.50	0.973	5.501	2.160	2.161

[a] $a_0 = 0.001031$; $a_1 = 0.3352$; $a_2 = 0.7232$; $a_3 = -0.3409$; $[RA]_{calc} = a_0 + a_1\theta + a_2\theta^2 + a_3\theta^3$.

Table 4. Methylation of acetylacetone with methyl iodide.

pH	A	Q	$a_{H_3O^+}/k^*_{HA}$	E'_{QA}	$1000a_1$	k_{obs}	Mean	S.D.(k)	Exp. No.
5.50	12.5	2.5	83180	7.04	0.2277	2.69	2.69		1
6.00	12.5	2.5	26300	7.04	0.5659	2.11	2.11		2
6.50	12.5	2.5	8318	7.04	1.618	1.91	1.91		3
7.00	12.5	2.5	2630	7.04	4.493	1.68			4
7.00	12.5	2.5	2630	7.04	4.579	1.71	1.70	0.02	5
7.50	12.5	2.5	831.8	7.04	13.59	1.61			6
7.50	12.5	2.5	831.8	7.04	13.58	1.61			7
7.50	12.5	2.5	831.8	7.04	14.60	1.73			8
7.50	12.5	2.5	831.8	7.04	13.25	1.57	1.63	0.07	9
8.00	12.5	2.5	263	7.04	40.45	1.51			10
8.00	12.5	2.5	263	7.04	34.59	1.29			11
8.00	12.5	2.5	263	7.04	38.43	1.44			12
8.00	12.5	2.5	263	7.04	41.95	1.57	1.45	0.12	13
8.48	12.5	2.5	87.1	7.04	100.6	1.24			14
8.48	12.5	2.5	87.1	7.04	113.6	1.41			15
8.50	12.5	2.5	83.2	7.04	135.2	1.60	1.42	0.18	16
9.00	1.0	2.5	26.3	5.56	337.9	1.60	1.60		17
9.00	1.5	2.5	26.3	5.62	337.1	1.58	1.58		18
9.00	2.5	2.5	26.3	5.75	342.7	1.57			19
9.00	2.5	2.5	26.3	5.75	323.6	1.48	1.53	0.06	20
8.99	5.0	2.5	26.92	6.07	346.5	1.54			21
9.00	5.0	2.5	26.30	6.07	312.4	1.35	1.45	0.13	22
8.98	7.5	2.5	27.54	6.39	335.2	1.44			23
9.00	7.5	2.5	26.30	6.39	331.6	1.36	1.40	0.06	24
8.98	10.0	2.5	27.54	6.71	345.4	1.42			25
9.00	10.0	2.5	26.30	6.71	340.4	1.33	1.38	0.06	26
8.97	12.5	2.5	28.18	7.04	333.4	1.33			27
9.00	12.5	2.5	26.30	7.04	369.1	1.38			28
8.99	12.5	2.5	26.92	7.04	371.2	1.42			29
8.96	12.5	2.5	28.84	7.04	349.0	1.43	1.39	0.05	30
9.00	2.5	5.0	26.30	5.75	303.2	1.39			31
9.00	2.5	5.0	26.30	5.75	319.9	1.46	1.43	0.05	32
8.97	2.5	7.5	28.18	5.75	291.0	1.43			33
8.98	2.5	7.5	27.54	5.75	303.8	1.46			34
9.00	2.5	7.5	26.30	5.75	304.5	1.39	1.43	0.04	35
8.97	2.5	10.0	28.18	5.75	273.8	1.34			36
8.97	2.5	10.0	28.18	5.75	278.1	1.36			37
9.00	2.5	10.0	26.30	5.75	345.0	1.56	1.42	0.12	38
8.96	2.5	12.5	28.84	5.75	295.1	1.48			39
8.96	2.5	12.5	28.84	5.75	274.8	1.38			40
9.00	2.5	12.5	26.30	5.75	328.6	1.50	1.45	0.06	41

Still more interesting results were obtained by varying the pH (Run 1 – 16 and 27 – 30). We can observe a small but statistically highly significant increase in k_{obs} with a decrease in pH. The greatest increase in k_{obs} for a given decrease in pH can be observed in the solutions with the lowest concentration C_{QA}. In these solutions we can expect the most extensive dissociation of the ion pair. It is therefore natural to consider the possibility that both the ion pair and the free anion can function as the nucleophile. If we assume that both the anion and the ion pair react with RI by second order kinetics we obtain the total reactions rate

$$d[RA]_s/dt = (k_A[A^-]_s + k_{QA}[QA]_s)\,[RI]_s \quad (36)$$

From the discussion given in connection with the use of eqn. 34 it follows that this should be equal to that obtained from the modified eqn. 28

$$d[RA]_s/dt = k_{obs}[QA]_s\,[RI]_s \quad (37)$$

we thus obtain

$$k_{obs} = k_{QA} + k_A[A^-]_s/[QA]_s \quad (38)$$

In the organic layer we have at $t = 0$, $[Q^+]_s = [A^-]_s$ and the dissociation equilibrium in the organic layer gives

$$(\gamma_s)^2[A^-]^2_s = K_{diss}[QA]_s \quad (39)$$

where γ_s is the mean activity coefficient of the ions in the solvent and K_{diss} the dissociation constant of the ion pair. From 38 and 39 we obtain.

$$k_{obs} = k_{QA} + k_A(K_{diss}/[QA]_s)^{1/2}/\gamma_s \quad (40)$$

A plot of k_{obs} against $1/\gamma_s[QA]_s^{1/2}$ should thus give a straight line with the intercept k_{QA} and the slope $k_A(K_{diss})^{1/2}$. In the present investigation the concentration $[QA]_s$ is in the range $10^{-2} - 10^{-5.4}$ and the dissociation constant K_{diss} can be estimated to be not much higher than 10^{-5}. In this case γ_s will be close to unity and a good approximation of its value can be obtained by the formula of Marshall and Grünwald [5] using $K_{diss} = 10^{-5}$ as an approximative value of this constant. The value of $[QA]_s$ is obtained from 35 (9 with $E'_{QA} = E_{QA}$).

The results of a least square calculation according to eqn. 40 are given in Table 5. We can see that k_{obs} is very close to k_{calc} which means that the fit of eqn. 40 is very good. In fact, the difference $k_{obs} - k_{calc}$ is of the same magnitude as the standard deviation of the k_{obs} values used in the calculation. The results obtained thus strongly support the assumption that both the anion and the ion pair are nucleophiles in the methylation of acetylacetone with methyl iodide by the process "extractive alkylation". Under preparative conditions, the reaction is almost exclusively an alkylation of the ion pair.

Eqn. 40 gives k_{QA} directly. For the calculation of k_A a knowledge of K_{diss} is necessary. This is not available by experiments. From the magnitude of the dissociation constants of other tetrabutylammonium salts,[11] we can make a rough guess or estimate $K_{diss} \simeq 10^{-5}$. Using this value we can see that k_A is of the same magnitude as k_{QA}. Due to the low degree of dissociation, the dominating reaction, however, will be that of the ion pair.

This result is in direct contrast to the results obtained by Kurts *et al.*[2] for the alkylation of alkali salts of ethyl acetoacetate with ethyl tosylate in hexamethylphosphortriamide in which no ion pair reactivity could be detected. This difference might be explained by the differences in size of the cation [9] and by differences in the solvation of the cation part of the ion pair.[10] An increase in the distance between the two charged centres can be expected to increase the reactivity of the anion part of the ion pair. The same effect can also be expected from a better solvation of the cation part of the ion pair.

The methylation of acetylacetone with dimethyl sulfate, the methylation of methylacetylacetone with methyl iodide, and the methylation of benzoylacetone with methyl iodide were also studied.

Table 5. Methylation of acetylacetone with methyl iodide. Summary of results. $A = 12.5$, $Q = 2.5$, $R = 16.07$, 25.0 °C.

pH	$[OA]_s \times 10^3$	γ_s	$1/\gamma_s[QA]_s^{1/2}$	k_{obs}	k_{calc} [a]	% ion pair reaction
5.50	0.00423	0.99	491	2.69	2.70	53
6.00	0.0134	0.98	279	2.11	2.15	68
6.50	0.0418	0.98	158	1.91	1.83	78
7.00	0.131	0.97	90	1.70	1.65	86
7.50	0.414	0.96	51	1.63	1.55	92
8.00	1.28	0.95	29	1.45	1.50	95
8.50	3.68	0.93	18	1.42	1.47	97
9.00	9.99	0.91	11	1.39	1.44	99

[a] $k_{QA} = 1.42$, S.D. $= 0.03$; $k_A(K_{diss})^{1/2} = 2.62 \times 10^{-3}$; S.D. $= 0.13 \times 10^{-3}$; $k_{calc} = k_{QA} + k_A(K_{diss}/[QA]_s)^{1/2}/\gamma_s$.

Table 6. Methylation of acetylacetone with dimethyl sulfate. $A = 12.5$, $Q = 2.5$, $R = 10.58$, 25.0 °C, $E'_{QA} = 7.04$.

pH	$a_{H_3O^+}/k^*_{HA}$	γ_s	a_1	$[QA]_s$	k_{obs}	k_{calc}[a]
7.00	2630	0.97	0.00847	1.31×10^{-4}	3.16	3.22
7.50	831.8	0.96	0.02392	4.14×10^{-4}	2.83	2.69
8.00	263	0.95	0.06401	1.28×10^{-3}	2.39	2.40
8.47	89.1	0.93	0.1691	3.68×10^{-3}	2.14	2.25
9.00	26.3	0.91	0.5882	9.99×10^{-3}	2.20	2.16

[a] $k_{QA} = 2.01$; S.D. $= 0.08$; $k_A(K_{diss})^{1/2} = 13.4 \times 10^{-3}$; S.D. $= 1.7 \times 10^{-3}$; $k_{calc} = k_{QA} + k_A(K_{diss}/[OA]_s)^{1/2}/\gamma_s$.

Table 7. Determination of the apparent extraction constant E^*_{QA} of tetrabutylammonium methylacetylacetonate. $A = 2.5$, $Q = 12.5$, NaOH $= 1.017$, $s = 25$, $pk^*_{HA} = 12.41$, $pk'_{HA} = 10.76$.

ml	L	pH	w	$[A^-]_w$	$[Q^+]_w$	C_{QA}	E^*_{QA}	E'_{QA}[a]
0.50	0.361	10.97	25.50	0.00310	0.479	0.01133	7.61	7.69
0.75	0.591	11.23	25.75	0.00504	0.468	0.01860	7.88	7.85
1.00	0.823	11.43	26.00	0.00702	0.456	0.02590	8.08	8.01
1.25	1.048	11.60	26.25	0.00900	0.445	0.03293	8.23	8.16
1.50	1.264	11.76	26.50	0.01107	0.435	0.03949	8.20	8.31
1.75	1.463	11.90	26.75	0.01282	0.425	0.04570	8.39	8.44
2.00	1.655	12.03	27.00	0.01409	0.416	0.05211	8.89	8.59
2.25	1.798	12.17	27.25	0.01616	0.409	0.05576	8.44	8.67

[a] $E'_{QA} = 7.44 + 22.0\ C_{QA}$

Table 8. Methylation of methylacetylacetone with methyl iodide. $A = 2.5$, $R = 16.07$, $pk^*_{HA} = 12.41$, 25.0 °C.

pH	Q	$a_{H_3O^+}/k^*_{HA}$	E'_{QA}	$a_1 \times 10^3$	$[QA]_s \times 10^5$	γ_s	$1/\gamma_s[QA]_s^{1/2}$	k_{obs}	k_{calc}[a]
8.50	2.5	8130	7.44	1.003	0.909	0.99	335	1.10	1.09
8.50	12.5	8130	7.44	0.958	4.55	0.98	151	1.05	0.97
9.00	2.5	2570	7.44	2.707	2.82	0.98	192	0.94	0.99
9.50	2.5	813	7.44	8.055	8.84	0.97	110	0.88	0.94
10.00	2.5	257	7.44	26.11	27.2	0.96	63	0.91	0.91
10.00	12.5	257	7.47	26.58	135	0.95	29	0.91	0.89

[a] $k_{QA} = 0.87$, S.D. $= 0.03$; $k_A(K_{diss})^{1/2} = 0.65 \times 10^{-3}$, S.D. $= 0.15 \times 10^{-3}$; $k_{calc} = k_{QA} + k_A(K_{diss}/[QA]_s)^{1/2}/\gamma_s$.

The apparent extraction constants E^*_{QA} for the tetrabutylammonium salts of methylacetylacetone and benzoylacetone were not investigated as carefully as that for acetylacetone. Even in these cases E^*_{QA} showed a slight variation with the composition of the organic layer, but the variation was too low to be interpreted as the formation of any other species than the ion pair and the anion. For interpolation purposes the formulas given for E'_{QA} at the bottom of Tables 7 and 9 were used. They fit the experimental values very nicely, but should not be used for any other purpose than interpolations.

In the methylation with dimethyl sulfate a slight complication occurs. Besides the main reaction there is a slow pH independent hydrolysis of dimethyl sulfate. This is readily cor-

Table 9. Determination of the apparent extraction constant E^*_{QA} of tetrabutylammonium benzoyl acetonate. $A = 2.5$. $Q = 12.5$, NaOH = 1.017, $s = 25$, $pk^*_{HA} = 12.37$.

ml	L	pH	w	$[A^-]_w \times 10^5$	$[Q^+]_w$	C_{QA}	E^*_{QA}	E'_{QA}[a]
0.25	0.2441	8.82	25.25	2.54	0.485	0.00974	790	775
0.50	0.4780	9.15	25.50	4.87	0.471	0.01907	831	822
0.75	0.7160	9.38	25.75	7.30	0.458	0.02856	854	869
1.00	0.9580	9.55	26.00	9.34	0.444	0.03822	922	916
1.25	1.1985	9.72	26.25	11.65	0.431	0.04782	953	964
1.50	1.4396	9.88	26.50	13.73	0.418	0.05744	1002	1012
1.75	1.6796	10.06	26.75	16.07	0.405	0.06701	1030	1059
2.00	1.9211	10.25	27.00	17.57	0.392	0.07665	1113	1107
2.25	2.1601	10.52	27.25	19.21	0.380	0.08619	1182	1154

[a] $E'_{QA} = 727 + 4953\ C_{QA}$.

Table 10. Methylation of benzoylacetone with methyl iodide. $A = 2.5$, $Q = 2.5$, $R = 16.07$, $pk^*_{HA} = 12.37$; 25.0 °C.

pH	$a_{H_3O^+}/k^*_{HA}$	E'_{QA}	a_1	$[QA]_s \times 10^4$	γ_s	$1/\gamma_s[QA]_s^{1/2}$	k_{obs}	k_{calc}[a]
8.00	23440	728	0.0341	3.08	0.96	59.4	1.098	1.097
8.50	7413	732	0.1027	9.52	0.95	34.1	1.040	1.049
9.00	2344	742	0.3296	29.66	0.93	19.7	1.041	1.022
9.50	741	769	1.034	85.44	0.91	11.9	0.997	1.008

[a] $k_{QA} = 0.985$, S.D. = 0.017; $k_A(K_{diss})^{1/2} = 1.88 \times 10^{-3}$, S.D. $= 0.46 \times 10^{-3}$; $k_{calc} = k_{OX} + k_A(K_{diss}/[QA])^{1/2}/\gamma_s$.

rected for, however. In the other reactions no complication occurred. The results are given in Tables 6–10.

All these reactions behaved in the same way, indicating that the ion pair is the dominating nucleophile, but some alkylation of the free ion also occurs. The results are summarized in Table 11. It is interesting to note that both the carbon alkylation with methyl iodide and the oxygen alkylation with dimethyl sulfate follow the same type of kinetics.

From the rather uncertain $k_A(K_{diss})^{1/2}$ values it can be concluded that the reaction of the anion seems to be somewhat more important for the oxygen alkylation with dimethyl sulfate than for the carbon alkylation with methyl iodide.

The interesting question as to whether the oxygen and carbon alkylation are using the same nucleophile or not, was also studied in another way. The isopropylation of the tetrabutylammonium salt of acetylacetone with isopropyl iodide in methylene chloride gives almost equal quantities of *C*- and *O*-alkylation. Assume that the free anion takes part in the oxygen alkylation to a different extent than in the carbon alkylation and that the reaction with the anion is of significant importance for the formation of at least one of the products. A change in the degree of dissociation should then influence the product composition. Due to the common ion effect, the addition of QI to the mixture should depress the dissociation of QA. The addition of a large amount of QI to the isopropylation reaction solution had no effect on the product composition.

The anion, therefore, is of the same, or of very little importance for both the carbon and oxygen alkylation in this case.

EXPERIMENTAL

The solution of tetrabutylammonium sulfate was prepared by careful neutralisation of pure tetrabutylammonium hydrogen sulfate with carbonate free sodium hydroxide. The absence

Table 11. The alkylation of tetrabutylammonium salts of some β-diketones. Summary.

β-Diketone [a]	Alkylating agent	k_{QA}	S.D.	$k_A(K_{diss})^{1/2} \times 10^3$	S.D. $\times 10^3$	Type of alkylation
I	CH_3I	1.42	0.03	2.62	0.13	C
I	$(CH_3)_2SO_4$	2.01	0.08	13.4	1.7	O
II	CH_3I	0.87	0.03	0.65	0.15	C
III	CH_3I	0.99	0.02	1.88	0.46	C

[a] I = $CH_3COCH_2COCH_3$; II = $CH_3COCHCOCH_3$ (CH_3 on the central C); III = $C_6H_5COCH_2COCH_3$.

of buffer capacity in the pH range 5 – 9 demonstrated that it was free from tributylammonium hydrogen sulfate.

The kinetic measurements were performed in the following way. 25 ml of a solution of the compound HA in methylene chloride was added to 25 ml of an aqueous solution containing tetrabutylammonium sulphate Q_2SO_4 and Na_2SO_4 to give an ionic strength corresponding to 0.5 M Na_2SO_4. The flask with this mixture was placed in a water thermostat at 25.0 °C and fitted with a very efficient Teflon stirrer that vigorously mixed the two layers. A glass and a calomel elextrode, as well as a capillary tube for the addition of aqueous NaOH from a syringe, were introduced and connected to an instrument (Radiometer Titrator TTTl with Titrigraph SBR2/SBU2) which registers the quantity of NaOH needed to keep the pH constant. A pH value and a proper paper speed were chosen and the experiment started. When equilibrium was reached, 1.000 ml of methyl iodide, or dimethyl sulfate, was rapidly injected. The reaction was followed by registering the volume L' of NaOH added to keep the pH constant.

The distribution measurements were performed with the same type of equipment, but the instrument was now used as a pH meter. Portions of sodium hydroxide corresponding to about 10 % of the total quantity of HA present were introduced and the pH was measured after each addition. When Q^+ was present, it was usually not necessary to stop the stirrer in order to get a stabile reading, in the other cases, the layers were allowed to separate and the pH was read in the aqueous layer.

Methylacetylacetone was prepared by the method of Brändström,[6] comp. Ref. 7. Acetylacetone was removed from the product by dissolving it in 10 parts of ether and extracting it with small portions of 1 M sodium hydroxide, each corresponding to about 5 % of the total content of diketone. The pH of each aqueous extract was measured. The extraction was continued until the acetylacetone content could be calculated to be about 0.1 %. The apparent dissociation constants of acetylacetone and methylacetone are 9.6 and 11.6, respectively, in this two layer system. (The pH of the two last portions were 11.5 and 11.7, respectively).

The isopropylation of the tetrabutylammonium acetylacetonate was performed in methylene chloride by the method of Brändström and Junggren.[8]

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Bacterial Carotenoids. XLVIII.* C_{50}-Carotenoids. 16.* Synthesis and Chiroptical Properties of the Model Compounds (2*R*, 6*R*, 2′*R*, 6′*R*)-2,2′-Dimethyl-ε,ε-carotene and (2*R*, 6*S*, 2′*R*, 6′*S*)-2,2′-Dimethyl-ε,ε-carotene

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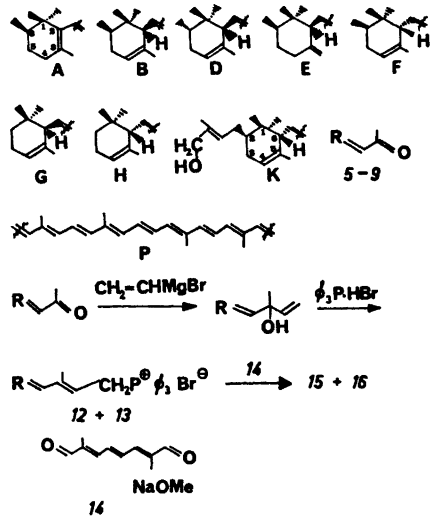
(2*R*,6*S*,2′*R*,6′*S*)-2,2′-Dimethyl-ε,ε-carotene (*15*) and (2*R*,6*R*,2′*R*,6′*R*)-2,2′-dimethyl-ε,ε-carotene (*16*) were synthesized from the corresponding optically active irones (*6*,*9*). ^{1}H NMR and CD properties of the optically active C_{42}-carotenes and synthetic intermediates are reported. The absolute configuration 2*R*,6*R*,2′*R*,6′*R* of the bacterial C_{50}-carotenoid decaprenoxanthin (*3a*) is based on these results.

Recently [1] we reported the synthesis and CD properties of (2*R*,2′*R*)-2,2′-dimethyl-β,β-carotene (*1*) from which the absolute configuration of *C.p.* 450 [2] = (2*R*,2′*R*)-2-(4-hydroxy-3-hydroxymethyl-2-butenyl)-2′-(3-methyl-2-butenyl)-β,β-carotene (*2*, with opposite configuration at C-2,2′ relative to *1*) was established, Scheme 1. The synthesis was based on the isolation of optically active β-irone (*5*) from Iris oil.[3] Optically active α-irones (*6* and *7*) of known absolute configuration are also present in Iris oil.[3]

An obvious extention to our earlier work was the synthesis of optically active 2-methyl substituted ε,ε-carotenes in order to illuminate the absolute configurations of C_{50}-carotenoids containing ε-ring systems,[4,5] particularly of decaprenoxanthin (*3*).[6,7] Simpler, appropriate models for CD-comparison already available were the enantiomeric ε,ε-carotenes (*4a*,*b*).[8–10]

Optically inactive 2,2′-dimethyl-ε,ε-carotene has previously been synthesized by a different approach [11] than that reported below.



Scheme 1. *1*, A–P–A; *3*, K–P–K (chirality unknown); *3a*, K–P–K; *4a*, G–P–G; *4b*, H–P–H; *5*, R=A; 6, R=B; *7*, R=D; *8*, R=E; *9*, R=F; *12*, R=B; *13*, R=F; *15*, B–P–B; *16*, F–P–F.

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RESULTS AND DISCUSSION

The irones *5*, *6*, *7* and *8* are constitutents of natural Iris oil.[3] (2*S*,6*R*)-*cis*-α-Irone (*6*, numbering and nomenclature of the irones follow those used by Rautenstrauch and Ohloff,[3] *cf.* A, and differ from those of the carotenoids,[12] *cf.* K) was isolated by direct preparative GLC of Iris oil. The C-2 epimeric (2*R*,6*R*)-*trans*-α-irone (*9*) was isolated by preparative GLC of a base isomerized mixture of natural irones. The optical purity of *9* used in the synthesis of (2*R*,6*R*,2′*R*,6′*R*)-2,2′-dimethyl-ε,ε-carotene was diluted to some extent by the presence of a small amount of its enantiomer *7*.

The route utilized for the synthesis of optically active dimethyl-ε,ε-carotenes is illustrated in Scheme 1. In reactions known to retain stereochemical integrity at position C-2 of ionones,[13] *6* and *9* were successively treated with excess vinyl magnesium bromide and triphenyl phosphonium bromide to yield the phosphonium salts *12* and *13*. CD curves of the intermediate vinyl alcohols *10* and *11* are given in Fig. 1 and have the same sign as the irone precursors *6* and *9*, respectively. Reaction of the ylids of the phosphonium salts *12* and *13*, generated in the presence of sodium methoxide, with the dial *14* gave (2*R*,6*S*,2′*R*,6′*S*)-2,2′-dimethyl-ε,ε-carotene (*15*) and (2*R*,6*R*,2′*R*,6′*R*)-2,2′-dimethyl-ε,ε-carotene (*16*), respectively.

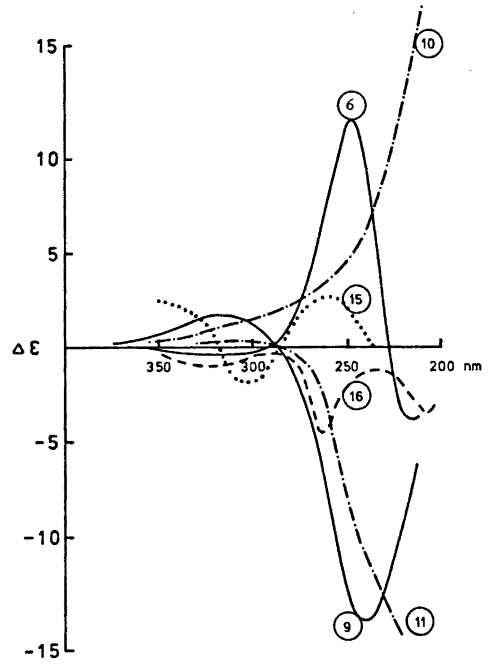

Fig. 1. CD spectra of (2*S*,6*R*)-cis-α-irone (*6*), (2*R*,6*R*)-*trans*-α-irone (*9*), (2*S*,6*R*)-*cis*-vinyl-α-ironol (*10*), (2*R*,6*R*)-*trans*-vinyl-α-ironol (*11*), (2*R*,6*S*,2′*R*,6′*S*)-2,2′-dimethyl-ε,ε-carotene (*15*), and (2*R*,6*R*,2′*R*,6′*R*)-2,2′-dimethyl-ε,ε-carotene (*16*) in EPA solution.

Table 1. Diagnostically useful ^{1}H NMR signals (δ values) in the 2,6-*cis* (*6*, *10*, *15*) and 2,6-*trans* (*9*, *11*, *16*) series (carotenoid numbering used).

Compound	Methyl at C-2,2′	16,17,16′,17′-*gem* dimethyl		*Δ*
2,6-*cis*				
6	0.87	0.72	0.87	0.15
10	0.85	0.63	0.85	0.22
15	0.87	0.68	0.87	0.19
2,6-*trans*				
9	0.85	0.83	0.87	0.04
11	0.82	0.77	0.81	0.04
16	0.82	0.81	0.82	0.01

The dimethyl carotenes *15* and *16* had electronic spectra identical with that of ε,ε-carotene.[8,14] Mass spectra of the here synthesized optically active dimethyl-carotenes *15* and *16* were identical and consistent with the fragmentation pattern reported for racemic 2,2′-dimethyl-ε,ε-carotene.[14,15] The ^{1}H NMR signals of *15* and *16* are given in the Experimental part. Signals assigned to the *gem* dimethyl and adjacent methyl groups of the irones *6* and *9*, vinyl-ironols *10* and *11* and the synthesized C_{42}-carotenes *15* and *16* are compared in Table 1. There is a clear distinction between the chemical shifts of the *gem* dimethyl groups associated with the 2,6-*cis* and 2,6-*trans* isomers which are consistent with the starting irones, intermediate vinyl-ironols and product carotenes within each isomeric group. Mention should be made that the ^{1}H NMR signals reported by Vetter *et al.*[14] for synthetic, racemic [16] 2,2′-dimethyl-ε,ε-carotene correlate with the 2,6-*trans*-isomer.

The chromatographic behaviour of the dimethyl-carotenes *15* and *16* were very similar on alumina paper and alumina plates. Both *15* and *16* were less strongly adsorbed than

synthetic ε,ε-carotene, β,ε-carotene, and β,β-carotene.

CD spectra of *15* and *16* are included in Fig. 1.

In a separate report [17] comparison of the present ^{1}H NMR results (Table 1) with data for decaprenoxanthin (*3*) [6] has led to 2,6-*cis* (2′,6′-*cis*) assignment for decaprenoxanthin. Furthermore comparison of CD data for the synthetic dimethyl-carotenes *15* and *16* (Fig. 1), decaprenoxanthin (*3*),[18] and synthetic 6*S*,6′*S*-ε,ε-carotene (*4b*) [10] has revealed that the chiral center at C-2(C-2′) does not contribute significantly to the CD of *15*, *16* and *3*. This is consistent with previous findings by Rautenstrauch and Ohloff in the α-ionone/α-irone series.[3] Consequently decaprenoxanthin has been assigned the 2*R*,6*R*,2′*R*,6′*R*-configuration *3a*,[17] Scheme 1.

Whereas the sign of the Cotton effect of *15* below 280 nm is as predicted, the lower magnitude could partly be due to some presence of its enantiomer derived from *7*. Further differences in shape and magnitude relative to *16* could be sought in a conformational preference of quasiaxial polyene chain,[19] in which case an axial methyl group at C-2 of *15* should destabilize the preferred conformation and could alter the CD. However, it seems questionable (*cf.* conformations discussed in Ref. 17) that this finding for enones of the α-irone/α-ionone series [19] may be extended to the carotenes.

EXPERIMENTAL

Materials and methods. All solvents were of analytical grade or distilled before use. Separation and purification of synthetic carotenes were accomplished on alumina plates (1 mm) developed with petroleum ether-ethyl ether mixtures. Comparative TLC was on Merck HF_{254} pre-coated alumina plates (0.2 mm).

Instruments used were as specified elsewhere.[20] 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.

The composition of Iris oil before and after base isomerization has been reported;[1,3] GLC separation of the irones has also been described.[1,3]

(+)-(2S,6R)-cis-α-*Irone (6).* (*6*) isolated from natural Iris oil by preparative GLC had the following physical characteristics: $[\alpha]_D^{20}$ (EPA) 589 nm = 9°, 578 nm = 10°, 546 nm = 11°, 436 nm = 23° and 365 nm = 43°; λ_{max} 225 nm (EPA), 228 nm (MeOH); IR(liq.) 3015 (m), 2980, 2935, 2880, 1695, 1675, 1620, 1450, 1365, 1255 (s), 1210, 1170, 1065, 1000, 985 (m), 910, 885, 795 and 740 (w) cm^{-1}; δ ($CDCl_3$) 0.72, 0.87 (6 H, *gem.* dimethyl), 0.87 d (3 H, J = 5 Hz, >CH−CH_3), 1.54 (3 H, =C−CH_3), 1.9−1.7 (3 H, −CH_2 and $CH_3CH<$), 2.25 O=C−CH_3), 2.53 d (1 H, J = 10.5 Hz, >CH−C=C), 5.39 (1 H, broad, −CH=C−CH_3), 6.08 d (1 H, J = 15 Hz, =CH−C=O) and 6.27 q (1 H, J_1 = 16 Hz, J_2 = 10 Hz, CH=CH−C=O; *m/e* 206 (M), 136 (M−70, 121 (M−85) and 93 (M−113).

(+)-(2S,6R)-cis-*Vinyl-α-ironol (10).* Vinyl magnesium bromide (5 g) in tetrahydrofuran (THF, 30 ml) was added to a stirred solution of *4* (544 mg) in THF (5 ml). After 1.5 h at 25 °C saturated NH_4Cl (5 ml) was added and the mixture extracted with ether. The organic layer was dried over Na_2SO_4, concentrated under reduced pressure and chromatographed on a silica gel column developed with benzene-$CHCl_3$ mixtures to give *8* (625 mg, quantitative yield). Product *10* had $[\alpha]_D^{20}$ (EPA) 589 nm = 7°, 578 nm = 8°, 546 nm = 9°, 436 nm = 17° and 365 nm = 33°; IR (liq.) 3400, 2980, 2900, 2800 (s), 1455, 1375 (w), 1100, 1060, 1000, 925, 890 (m) and 810 (w) cm^{-1}; δ ($CDCl_3$) 0.63, 0.85 (6 H, *gem.* dimethyl), 0.85 d (3 H, J = *ca.* 5 Hz, >CH−CH_3), 1.54 (3 H, =C−CH_3), 1.38 (3 H, CH_3−C−OH), 1.9−1.5 (3 H, complex, −CH_2, CH_3−CH<), 2.1−2.2 (1 H, broad >CH−C=C<), 3.65 (1 H, broad, OH), 5.03 q (1 H, J_1 = 10.5 Hz, J_2 = 1.5 Hz, *cis* CH=CH_2), 5.22 q (1 H, J_1 = 16.5 Hz, J_2 = 1.5 Hz, *trans* CH=CH_2), *ca.* 5.37 (1 H, CH=C−CH_3), *ca.* 5.50 (1 H, −CH=CH_2), 5.58 (1 H, =CH−COH) and 6.02 q (1 H, J_1 = 17.5 Hz, J_2 = 10.5 Hz, CH=CH−COH); *m/e* 234 (M), 216 (M−18) and 263 (M−71).

(−)-(2R,6R)-trans-α-*Irone (9).* *9* isolated from a base isomerized mixture of irones as described earlier [1,3] had the following properties: $[\alpha]_D^{20}$ (EPA) 589 nm = −421°, 578 nm = −440°, 546 nm = −506°, 436 nm = −898°; λ_{max} 225 nm (EPA), 228 nm (MeOH); IR (liq.) 3035 (w), 2960, 2935, 2910, 2880 (s), 2835 (m), 1697, 1675, 1620 (s), 1450, 1430, 1390 (m), 1365, 1255 (s), 1200, 1170, 1140, 1120, 1080, 1050 (w), 995, 980 (m) 910 and 810 (w) cm^{-1}; δ ($CDCl_3$) 0.83, 0.87 (6 H, *gem.* dimethyl), *ca.* 0.85 d (3 H, J = 5 Hz, >CH−CH_3), 1.58 (3 H, =C−CH_3), 2.1−1.7 (3 H, complex, −CH_2, CH_3CH<), 2.22 (3 H, O=C−CH_3), 2.31 d (1 H, J = *ca.* 8 Hz, >CH−C=C), 5.47 (1 H, broad, CH=C−CH_3), 6.08 d (1 H, J = 16 Hz, =CH−C=O), and 6.70 q (1 H, J_1 = 16 Hz, J_2 = 9 Hz, CH=CH−C=O); *m/e* 206 (M), 136 (M−70), 121 (M−85) and 93 (M−113).

(−)-(2R,6R)-trans-*Vinyl-α-ironol (11).* (*11*) was prepared from *9* (636 mg) and vinyl magnesium bromide by the procedure earlier described for the preparation of *10*;[3] yield 715 mg (quantitative); $[\alpha]_D^{20}$ (EPA) 589 nm =

$-48°$, 578 nm $= -50°$, 546 nm $= -57°$, 436 nm $= -103°$; IR (liq.) 3400, 2980, 2900, 2800 (s), 1455, 1375 (w), 1100, 1050, 890 (m) and 810 (w) cm^{-1}; δ ($CDCl_3$) 0.77, 0.81 (6 H, *gem.* dimethyl), *ca.* 0.82 d (3 H, $J = ca.$ 5 Hz, $>CH-CH_3$), 1.58 (3 H, $=C-CH_3$), 1.35 (3 H, CH_3-C-OH), 1.3–1.8 (3 H, complex, $-CH_2$, $CH_3-CH<$), 2.1 (1 H, broad $>CH-C=C$), 3.65 (1 H, broad, $-OH$), 5.00 (q, 1 H, $J_1 = 10$ Hz, $J_2 = 2$ Hz, *cis* $CH=CH_2$), 5.18 (q, 1 H, $J_1 = 18$ Hz, $J_2 = 1.5$ Hz, *trans* $CH=CH_2$), *ca.* 5.37 (1 H, $CH=C-CH_3$), *ca.* 5.49 (1 H, $CH=CH_2$), 5.56 (1 H, $=CH-COH$) and 5.97 (q, 1 H, $J_1 = 17.5$ Hz, $J_2 = 10.5$ Hz, $CH=CH-COH$; *m/e* 234 (M), 216 (M – 18) and 263 (M – 71).

(2S,6R)-cis-α-Ironylidene-ethyltriphenylphosphonium bromide (12). A solution of *10* (0.6 g) and triphenylphosphonium bromide (1.1 g) in dry MeOH (15 ml) was stirred at 20 °C for 48 h under nitrogen. The solvent was removed under reduced pressure, the oily residue dissolved in a minimum amount of acetone and chromatographed on a silica gel column. Elution with acetone removed unreacted *10* and triphenylphosphine. *12* was eluted with MeOH and obtained as an amorphous powder after solvent evaporation. Yield of *12* was 1.02 g (62 %).

(2R,6R)-trans-α-Ironylidene-ethyltriphenylphosphonium bromide (13). *13* was prepared from *11* (0.56 g) according to the procedure described for *12*; yield 0.91 g (70 %).

(2R,6S,2'R,6'S)-2,2'-Dimethyl-ε,ε-carotene (15). NaOMe (0.6 M) was slowly added to a stirred solution of *12* (250 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-triene-1,8-dial (*14*, 15 mg, prepared from the acetylenic analogue [21]) in CH_2Cl_2 (1 ml) was added and the mixture stirred at room temperature. Progress of the reaction was monitored by TLC and additional phosphorane, generated externally, was added until *14* was completely consumed. Water was added, the crude product extracted into ether, concentrated under vacuum and chromatographed on alumina plates. Yield of *15* (*cis-trans*-mixture) was 25.2 mg (47 % based on dial *14*). All *trans-15* was obtained after rechromatography on alumina plates followed by crystallization from MeOH-$CHCl_3$ solution. *15* melted at 197 °C; λ_{max} (petroleum ether) 268, (396), 417, 440 [E(1 %, 1 cm) = 2600; $\varepsilon = 147\,000$ compared to 151 700 reported for racemic 2,2'dimethyl-ε,ε-carotene [11], 471 [E(1 %, 1 cm) = 2680; $\varepsilon = 149\,000$ compared to 152 300 [11]], (acetone) 416, (426), 441 [E(1 %, 1 cm) = 2520], and 472 nm [E(1 %, 1 cm) = 2510]; IR (KBr) 3030 (w), 2980, 2920, 2880 (s), 1730, 1440 (w), 1390, 1365 (m), 1210, 1120, 1060, 1030, 1010 (w), 965 (s), 910, 890, 860, 835, 820 and 800 cm^{-1} (w); δ ($CDCl_3$) 0.68, 0.87 (6 H + 6 H, *gem.* dimethyl), 0.87 (6 H, d, $J = 5$ Hz, CH_3 at C-2,2'), 1.53 (6 H, CH_3-18,18'), 1.93 (6 H, CH_3-19,19'), 1.97 (6 H, CH_3-20,20'), *ca.* 2.4 (2 H, H-6,6'), *ca.* 5.5 (2 H, H-4,4'), 5.58 (2 H, dd, $J_1 = 16$ Hz, $J_2 = 7$ Hz, H-7,7') and 6.0–7.0 (in-chain olefinic H); *m/e* 364 (M), 494 (M – 70), 472 (M – 92), 458 (M – 106), 406 (M – 158) and 402 (M – 70 – 92); CD spectrum Fig. 1; $R_F = 0.42$ on alumina plates developed with petroleum ether-ether (97:3) compared to 0.38 for ε,ε-carotene, 0.32 for β,ε-carotene, and 0.25 for β,β-carotene; on Schleicher and Schüll No. 288 alumina paper *15* had $R_F = 0.50$ when developed in petroleum ether.

(2R,6R,2'R,6'R)-2,2'Dimethyl-ε,ε-carotene (16). *16* was prepared from *13* and *14* (15 mg), isolated, purified and crystallized by using the procedures described for the preparation of *15*; yield 15.75 mg (29.3 %). The electronic, IR and mass spectra could not be distinguished from those of *15*; δ ($CDCl_3$), 0.81 (12 H, *gem.* dimethyl), 0.82 (6 H, d, $J = 5$ Hz, CH_3 at C-2,2'), 1.59 (6 H, CH_3-18,18'), 1.81 (6 H, CH_3-19,19'), 1.97 (6 H, CH_3-20,20'), 2.18 (d, $J = 9$ Hz, C-6,6'), *ca.* 5.4 (2 H, C-4,4'), 5.52 (2 H, dd, $J_1 = 16$ Hz, $J_2 = 7$ Hz, H-7,7') and 6.0–7.0 (in-chain olefinic H). Crystalline all-*trans-16* melted at 222–223 °C. The CD spectrum is reproduced in Fig. 1. On Schleicher and Schüll No. 288 paper *16* had $R_F = 0.49$ when developed in petroleum ether and could only be distinguished from *15* by careful chromatography; on alumina plates *16* had $R_F = 0.42$ when developed in petroleum ether-ether (97:3) and was difficult to distinguish from *15* on co-chromatography.

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A Stereoselective Pummerer Rearrangement of an Optically Active Sulfoxide

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By a Pummerer type of rearrangement *o*-benzylsulfinylbenzoic acid (I) and α-phenylsulfinyl-*o*-toluic acid (III), when treated with *N,N'*-dicyclohexylcarbodiimide (DCC), were converted to 2-phenyl-3,1-benzoxathian-4-one (II) and 3-phenylthiophthalide (IV), respectively. The same transformations could be performed by the use of *p*-toluenesulfonic acid. Starting with optically active I the DCC reaction gave active II, while completely racemic II was obtained with *p*-toluenesulfonic acid. The optical purity of II was determined by NMR analysis utilizing the chiral shift reagent tris-(3-heptafluorobuturyl-*d*-camphorato)europium(III), Eu(hfbc)$_3$. The highest degree of stereoselectivity (30 %) and a high yield (91 %) was found with 1,2-dichloroethane as the solvent. In the presence of Eu(hfbc)$_3$, the enantiomeric shift difference (nonequivalence), $\Delta\Delta\delta$, of the enantiotopic 2-methine proton of II has been studied in chloroform and carbon tetrachloride solutions. On comparing II and the corresponding sulfone V in carbon tetrachloride solutions, a larger $\Delta\Delta\delta$ value was obtained for the sulfide.

In an earlier paper we reported the formation of 2-phenyl-3,1-benzoxathian-4-one (II) from *o*-benzylsulfinylbenzoic acid (I) with *N,N'*-dicyclohexylcarbodiimide (DCC) as a condensing agent.[1] Starting with optically active I the reaction was found to proceed stereoselectively with transfer of chirality from sulfur to carbon. This work has now been extended by studies of the solvent dependence of the reaction and the effect obtained by adding orthophosphoric acid as an external proton source. Furthermore, a new synthetic route leading to II and 3-phenylthiophthalide (IV), *viz.* treatment of I and α-phenylsulfinyl-*o*-toluic acid (III) with a catalytic amount of *p*-toluenesulfonic acid (*p*-TsOH), is presented. The reactions can be referred to as Pummerer-type of rearrangements, as the sulfoxide is reduced to a sulfide and concomitantly oxidized at the α-position.[2]

A necessary condition for the stereoselectivity observed in the DCC reaction is the difference in reactivity between the diastereotopic methylene protons at the prochiral benzylic carbon atom in the initially formed DCC-substrate adduct and the retained configuration of the ylide intermediate. Evidence has been presented for a chemical nonequivalence of protons alpha to an asymmetric sulfur atom, especially in sulfoxide chemistry, where the generation and stereochemical preference of α-sulfinylcarbanions in hydrogen exchange reactions[3] and the stereospecific hydroxyalkylation of chloromethyl phenyl sulfoxide[4] have been studied. Diastereotopic reactivity of methylene hydrogens adjacent to a sulfonium center has been studied less, but demonstrated in the case of hydrogen-deuterium exchange.[5] In view of both theoretical and experimental results, it is suggested that the reactions proceed *via* asymmetric carbanion and ylide intermediates and the stereochemical results are mainly interpreted in terms of the stability of the carbanion and ylide conformations.[6]

Sulfonium ylides are capable of exhibiting optical activity, which has been demonstrated by their resolution.[7] Stabilizing substituents at sulfur have less effect upon the stability than an electron-withdrawing group at the α-carbon.[8] Ylide formation will lower the barrier to inversion at sulfur relative to that in the parent sulfonium compound, while the

barrier to pyramidal inversion at carbon is higher in an ylide than that in a carbanion.[6a] In the work of Johnson and Schroeck [9], stable chiral oxosulfonium ylide reagents were used as alkylidene transfer reagents in the preparation of optically active oxiranes and cyclopropanes by asymmetric induction. Intramolecular transfer of chirality from sulfur to carbon *via* a chiral sulfonium ylide as an intermediate has been reported in the [2, 3] sigmatropic rearrangement of an optically active adamantylsulfonium compound with >94 % optical induction.[10] The only reported stereoselective Pummerer rearrangement is the reaction of racemic 2,2-dialkyl-1,3-oxathiolan-5-one *S*-oxides with acetic anhydride.[11] However, the presently described conversion of I to II appears to be the only Pummerer type of reaction in which an intramolecular transfer of chirality has occurred with the formation of an optically active product.

RESULTS AND DISCUSSION

DCC as a condensing agent. When I was treated with DCC at room temperature II was obtained. An excess of DCC which could make purification of II difficult was transferred into dicyclohexylurea (DCU) with acetic acid. DCU was filtered off, the filtrate was evaporated and the residue was purified by column chromatography (silica gel) with benzene as an eluent. The results are summarized in Table 1. The reaction proceeds much faster in acetone and THF than in the more non-polar dichloroethane and benzene. Solvent polarity seems to have a minor influence upon the degree of stereoselectivity. Addition of 1/4 equivalent of orthophosphoric acid increased the rate of the reaction considerably. In THF and acetone solutions it was necessary to perform the reactions at 0 °C to avoid coloured by-products. Addition of the external proton source decreased the stereoselectivity with the exception of acetone as a solvent, where furthermore the sign of rotation surprisingly was changed. In order to investigate the influence of the concentration of orthophosphoric acid upon the reaction, a higher acid concentration (1 equivalent) was used in THF solution. No effect upon the optical purity of the product could be detected but the yield decreased from 89 to 33 %. The reaction is only possible if the sulfoxide contains a carboxylic group in the *ortho* position. Starting with the methyl ester of I, this compound was recovered. In the investigation on the reactions of DCC-DMSO with carboxylic acids, Lerch and Moffatt [12] added orthophosphoric acid as an acidic catalyst and *p*-nitrobenzoic acid was in this manner converted into methylthiomethyl *p*-nitrobenzoate (V) (42 %) and 1-*p*-nitrobenzoyl-1,3-

Table 1. Conditions, optical rotations[a] and yields of II obtained from I,[b]

Pummerer reagent	Solvent	Temp. °C	Reaction time h	Yield %	$[\alpha]_D^{25}$	Optical purity %
DCC	$(CH_2Cl)_2$	25	15	91	−46.3	29.9
DCC/H_3PO_4[c]	$(CH_2Cl)_2$	25	2.5	73	−31.5	20.4
DCC	C_6H_6	25	16	31	−19.4	12.5
DCC/H_3PO_4[c]	C_6H_6	25	2.5	19	−8.7	5.6
DCC	THF	25	2	46	−46.0	29.8
DCC/H_3PO_4[c]	THF	0	0.5	89	−7.9	5.1
DCC/H_3PO_4[d]	THF	0	0.5	33	−7.5	4.9
DCC	$(CH_3)_2CO$	25	2	60	−5.1	3.3
DCC/H_3PO_4[c]	$(CH_3)_2CO$	0	0.5	64	+18.0	11.6
AA	C_6H_6	80	5	91	−30.2	19.5
AA	AA	100	2	95	+17.3	11.2
AA	AA/NaOAc	100	2	98	+8.2	5.3
p-TsOH	$CHCl_3$	62	3	51	0	0
p-TsOH	C_6H_6	80	2	96	0	0

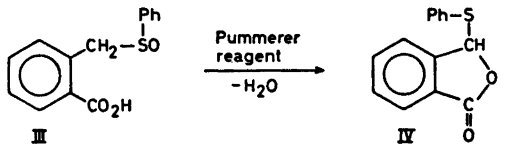
[a] In ethanol, $c=1$. [b] Optically pure, $[\alpha]_D^{25}=+451°$.
[c] 1/4 equivalent of orthophosphoric acid. [d] 1 equivalent of orthophosphoric acid.

Table 2. Formation of IV with different methods.

Method	Solvent	Reaction time h	Temp. °C	Yield %
DCC[a]	$(CH_2Cl)_2$	16	84	32
p-TsOH[a]	C_6H_6	11	80	81
AA	AA	3.5	110	89

[a] 2 equivalents.

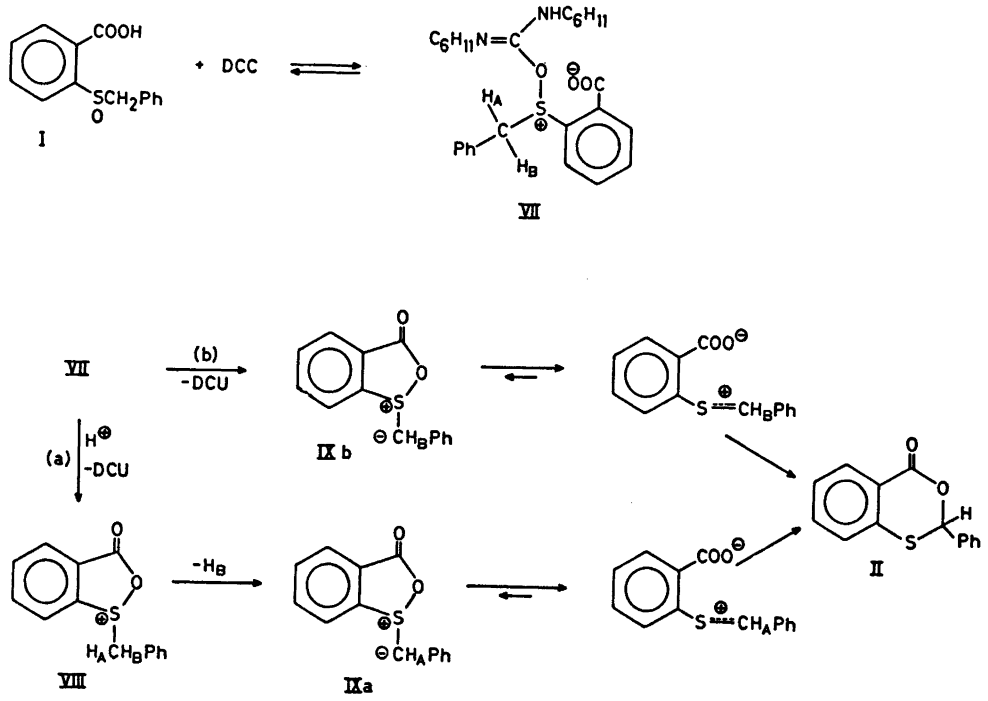
dicyclohexylurea (VI) (40 %). As the results from our reactions show that the carboxylic group is an appropriate proton source, the reaction was reinvestigated in the absence of orthophosphoric acid. Otherwise the conditions were identical. V and VI were isolated in yields of 30 and 21 %, respectively. No attempt was made to optimize the yield but obviously this reaction is also insensitive to the proton source used. In summary, we can say that high yield and optical activity of II are favoured by (1) the use of 1,2-dichloroethane as solvent and (2) the absence of orthophosphoric acid.



III IV

Scheme 1.

The DCC-method was also tested on α-phenylsulfinyl-*o*-toluic acid (III). Analogously to the formation of II, III should be converted to 3-phenylthiophthalide (IV), Scheme 1. In this case it was found that more drastic conditions were needed to accomplish this reaction.

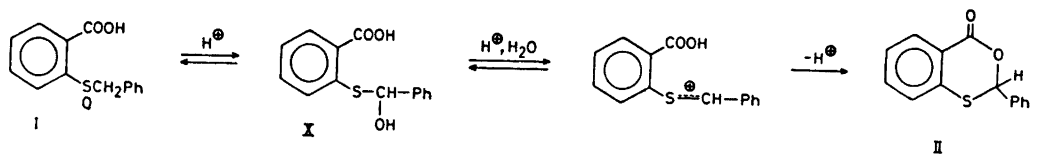


Scheme 2.

When III was subjected to the same treatment as I in 1,2-dichloroethane (with or without orthophosphoric acid), no trace of IV could be detected by TLC analysis. However, boiling under reflux for several hours yielded IV. The results of the preparation of IV with different methods are summarized in Table 2.

The fundamental feature of the mechanism can be formulated based upon the well-known DMSO-DCC reactions developed by Moffatt *et al.*[13] By addition of DMSO to protonated DCC, they propose the initial formation of a DMSO-DCC adduct, a sulfonium isourea, from which the highly insoluble DCU is displaced by an attack from a variety of nucleophilic functional groups with formation of a sulfonium compound, which can readily lose a proton giving a sulfonium ylide. The latter can then directly rearrange or undergo further reactions. The mechanism applied to our system is outlined in Scheme 2 with I as a substrate. We have suggested the formation of the adduct VII as an attack by the sulfoxide oxygen upon protonated DCC. This would lead to an oxysulfonium intermediate well adapted for an intramolecular attack by the carboxylate anion upon the positive sulfur atom. Such a step is supported by the data which illustrate the ease with which alkoxysulfonium salts undergo nucleophilic attack, such as their ready hydrolysis to sulfoxides.[14] Formation of the ylide can occur either directly *via* a concerted cyclic process where the attack by the carboxyl group is accompanied by intramolecular abstraction of a proton from the methylene group by the incipient DCU nitrogen (path b), or in two steps by loss of a proton from the corresponding sulfonium compound, VIII (path a). In this way two enantiomeric ylides, IXa and IXb, may be obtained. Conversion to the product should take place *via* an ion pair with restricted rotation around the partially double C–S-bond. Furthermore, the observed stereoselectivity is consistent with a nonplanar ylide and a carbanion moiety with retained stereochemistry. Preservation of carbanion configuration in an ylide can be assumed, since hydrogen exchange experiments[15] with 1-methylcyclopropylide show no loss of the stereochemical integrity at the carbon atom. The optical rotations of the product II we have obtained, can be reasonably explained if path *b* is the preferred route in the absence of orthophosphoric acid. Addition of the external acid increases the contribution of path *a* in 1,2-dichloroethane, benzene, and THF solutions, but with acetone as a solvent path *a* is now predominant. It has been found that the relative rates of exchange of the diastereotopic methylene hydrogens in benzyl methyl sulfoxide[16] and 1,3-dihydrobenzo[*c*]thiophene-2-oxide[17] are altered by changing base or solvent. Therefore, one can question if our two-way mechanism is needed to interpret the results. However, it is difficult to realize why a catalytic amount of orthophosphoric acid would have an influence on the medium and cause a change of the stereochemistry.

p-*Toluenesulfonic acid as a condensing agent.* The transformation I to II could also be performed using *p*-TsOH as a proton source to catalyze the reaction. The reaction was carried out in refluxing solutions of benzene (water separator connected) or chloroform and the product was purified by column chromatography affording 96 and 51 % yields of II. Starting with optically active I we obtained II with no optical rotation. Under the same conditions the optically active methyl ester of I did neither react nor racemize. The same result was obtained with (+)-I in acetone solution. Therefore, the inactivity of II cannot be explained by racemization of I prior to reaction and a mechanism quite different from that of the DCC-method seems to operate, Scheme 3. The first step involves an acid catalyzed rearrangement of I with the formation of an α-hydroxysulfide (hemithioacetal) X. Support for this assumption is given by the reaction of sulfoxides containing a β-carbonyl function or an other electron-withdrawing group with acidic reagents. Such reactions have been the subject of much mechanistic speculation. Pummerer found that phenylsulfinylacetic acid is cleaved to thiophenol and glyoxylic acid, tentatively *via* the hemithioacetal, in the presence of mineral acids.[18] A mechanism involving a primary cleavage into a mercaptan and a carbonyl fragment followed by hemithioacetal formation has been proposed.[19] However, this cleavage-recombination mechanism is in disagreement with chemical and spectroscopic results.[20] Thus, the mechanism first



Scheme 3.

suggested by Pummerer on intuitive grounds is most consistent with presently available data.

The source of the hydroxyl group in the hemithioacetal is still obscure. Undoubtedly, the first step in the reaction is protonation of the sulfoxide oxygen. An intramolecular migration of the hydroxyl group, attached to the positive sulfur atom, to the adjacent carbon atom has been proposed.[20b] An alternative mechanism to hemithioacetal involving loss of a proton from protonated sulfoxide and a final attack of water upon a reactive ylide intermediate was suggested by Becker.[20a] The formation of α-hydroxysulfides in organic media [19,20b] can be explained according to this consideration as catalytic amounts of water should be sufficient. However, such a mechanism involving a nucleophilic attack upon a carbanionic site of an ylide appears unlikely, as stated by Johnson and Phillips in their investigations of the Pummerer rearrangement of sulfonium salts.[21] Evidence was found for an initial ylide formation and a subsequent α-migration of an alkoxy group by an intermolecular process *via* a sulfur-stabilized carbonium ion. Focusing our attention on the close relationship between this rearrangement and α-hydroxysulfide formation, an attractive thought is to rationalize the latter reaction in terms of the above discussion.[21] As our reaction in benzene solution was performed under anhydrous conditions, the role of water is irrelevant, and the mechanism suggested by Johnson and Phillips is consistent with our results. The last step to II is a lactonization responsible for the racemization. Elimination of water is a reversible process with the equilibrium far shifted to the hydroxy compound, as no formation of II was obtained in the water-soluble solvents acetone, DMSO, THF, acetonitrile, or ethanol.

The carboxyl group of I can also serve as the sole source of protons necessary for catalysis. Heating I under reflux for 4 h in benzene gave II in 16 % yield. No attempts were made to optimize the yield.

Upon treatment with *p*-TsOH in boiling benzene, III was converted to IV. By increasing the amount of *p*-TsOH from 0.15 to 2 equivalents, the yield was raised from 13 to 81 %. The preparation of IV is summarized in Table 2.

Acetic anhydride as a condensing agent. Numata and Oae have reported that I, when treated with a large excess of acetic anhydride, gave II.[22] Utilizing their method with optically active I, we have found this reaction also to be stereoselective.[1] II was obtained with a sign of rotation opposite to that obtained by the DCC-method. However, a small amount of acetic anhydride in benzene gave the same result as with DCC. From the lack of steric effects Numata and Oae formulated the mechanism, different from that of the normal Pummerer reaction, as an initial acetylation of the carboxyl group. The following intramolecular attack of the sulfoxide oxygen upon the anhydride group afforded a cyclic acyloxysulfonium salt, which after removal of an α-proton was rearranged to II. However, we feel that a mechanism analogous to that of the DCC-method (Scheme 2) cannot be ruled out. We found that treatment of optically active I with acetic anhydride at 100 °C and quenching the solution after 3 min, 37 % of the starting sulfoxide was recovered with 38 % of the original activity. In the same way the methyl ester of I was found to racemize much slower than the acid and no formation of II could be detected. The first step in the racemization of sulfoxides by acetic anhydride [23] is the formation of an intermediate acetoxysulfonium compound followed by a rapid acetoxy interchange.[24] As this mechanism accounts for the racemization of the ester of I it could also be valid for I. This implies the formation of an acetoxysulfonium salt of I, which can serve

as the entrance to II according to the mechanism outlined in Scheme 2.

It was found that this method was the best one to prepare IV. By boiling III in acetic anhydride and removing excess acetic anhydride high yield of IV was obtained after recrystallization, Table 2.

Determination of enantiomeric composition. As the rotation of optically pure II is not known, the optical yields of the DCC-reactions cannot immediately be estimated from available data. We have considered the possibility to obtain optically pure II with chemical methods as fruitless. The NMR method based on chemical shift non-equivalence of enantiomers in optically active solvents is limited by the small magnitude of the chemical shift differences induced between corresponding resonances of enantiomers.[25] However, after the discovery [26] and in connection with development [27] of lanthanide shift reagents (LSR), the problem of direct determination of optical purity has been solved in a satisfactory way by the use of chiral LSR.[28] This method was found to be applicable to our system.

Fig. 1 illustrates the influence of tris-(3-heptafluorobuturyl-*d*-camphorato)europium (III), $Eu(hfbc)_3$, on the NMR spectrum of II. Addition of successive small quantities of LSR to a carbon tetrachloride solution of II produces marked modifications in the original spectrum. The lanthanide induced shifts (LIS) of the H_2 and H_5 protons increase with an increase in the LSR/substrate ratio. In esters and lactones the carbonyl oxygen is the preferred coordination site of LSR and therefore II has two potential coordinating groups. However, with one exception,[29] thioethers have been found to complex much less strongly than carbonyl or ether groups with LSR in the vast majority of cases.[30] Therefore, competition between the two functionalities in II

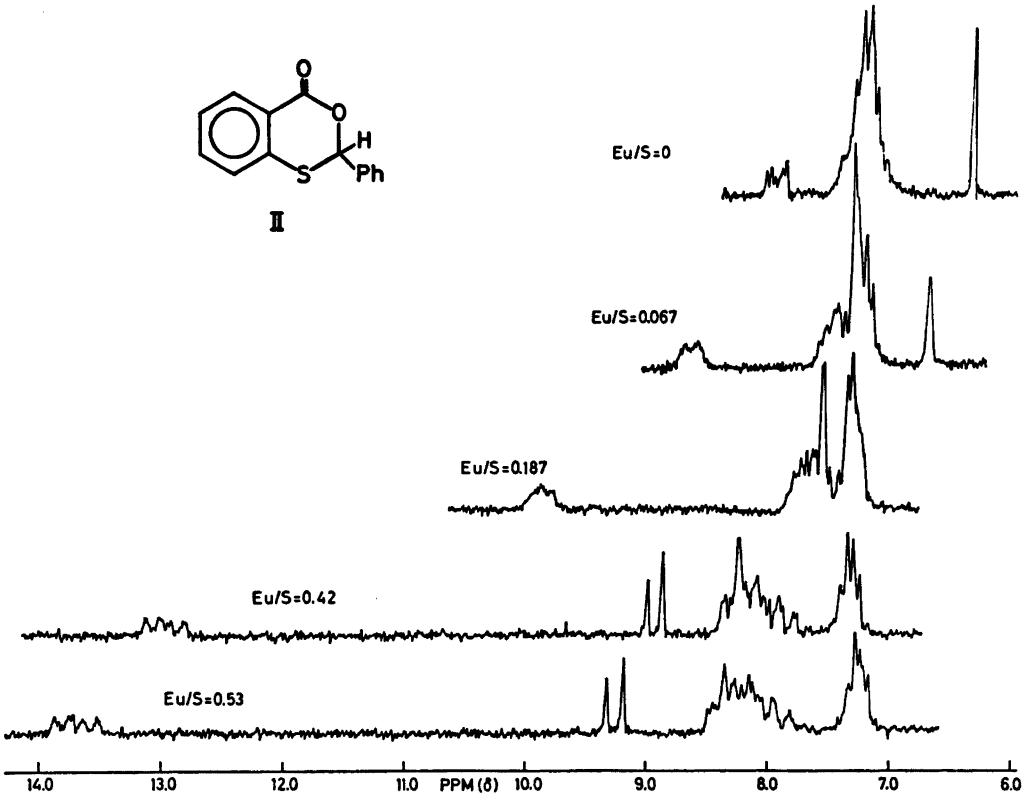


Fig. 1. The NMR spectrum of partially resolved II ($[\alpha]_D^{25} = -28.1°$) as a function of the LSR/substrate molar ratio.

for the shift reagent need not be considered. The latter observations are consistent with our results as the close proximity of H_5 to the coordinating carbonyl group is reflected in the great response of the LIS by addition of LSR.

In the presence of chiral shift reagents enantiomers have non-equivalent NMR spectra, demonstrated in Fig. 1 by the significant splitting of the H_2 proton. This enantiomeric shift difference ($\Delta\Delta\delta$) increases over the range investigated. The resonances are sufficiently separated for direct determination of the enantiomeric composition. This was performed with a partially resolved sample of II ($[\alpha]_D^{25} = -28.1$). A 0.30 M solution of this compound in carbon tetrachloride containing 0.53 equivalents of Eu(hfbc)$_3$ was used. Peak areas of the expanded signals ($\Delta\Delta\delta = 0.15$ ppm) corresponded to 18.2 % optical purity of II. In order to investigate the influence of solvent and substrate concentration on the induced shifts and $\Delta\Delta\delta$, spectra were recorded for solutions in carbon tetrachloride and chloroform and at two different concentrations of II in carbon tetrachloride. By plotting the chemical shifts of the enantiomeric H_2 proton

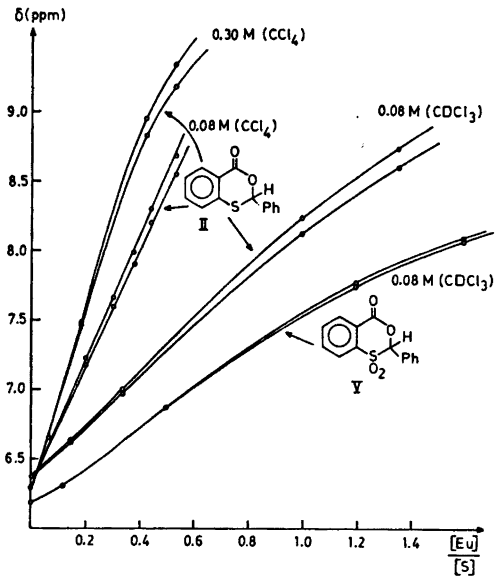


Fig. 2. Illustration of the enantiomeric shift differences obtained for the methine proton with varying solution and concentration conditions.

vs. the Eu(hfbc)$_3$/substrate ratios, straight lines are obtained with different slopes at lower concentrations, but deviation from linearity is apparent at higher ratios in two of the cases, Fig. 2. The slope of a line is a measure of the shifting power of LSR. As can be seen in Fig. 2, this is influenced both by the solvent and by the concentration of the substrate. For comparable concentrations of II (0.08 M), carbon tetrachloride gives significantly larger values for the induced shift as well as $\Delta\Delta\delta$ than does chloroform. The induced shift, but not the magnitude of non-equivalence, is increased by increasing substrate concentration. Fig. 2 also shows the effect of adding Eu(hfbc)$_3$ to a chloroform solution of the sulfone of II. Within the range investigated, the frequency separation between the enantiomeric shifts is less than 0.02 ppm. The sulfone group is a weak donor toward LSR.[31]. Therefore, one can expect the interaction of this molecule with the shift reagent to occur at the carbonyl group. The validity of this assumption is supported by a comparison of the induced shift obtained for a 0.08 M solution of II in chloroform. Inspection of Fig. 2 reveals about the same slope for the two compounds, which indicates the LSR/substrate complexes to be very similar.

EXPERIMENTAL

General. All melting points are uncorrected. The optical activity was measured at 589 nm (D-line) using a Perkin-Elmer model 141 photoelectric polarimeter and 1 ml microcells of 10 cm length with ethanol as a solvent. The IR spectra were recorded on a Perkin-Elmer model 157 spectrophotometer. NMR spectra were obtained with a Varian A-60 D NMR spectrometer. The Eu(hfbc)$_3$ was purchased from Willow Brook Labs., Inc., USA. The shift reagent was stored *in vacuo* over P_2O_5 until just before use, but no extraordinary precautions were taken to exclude water or air during the addition. The chemical shift differences were followed by incremental addition of Eu(hfbc)$_3$, thereby shifting the signals progressively downfield from TMS. Thin layer and column chromatographic procedures were accomplished using silica gel F_{254} plates (Merck) and silica gel 60, 70–230 mesh (Merck), respectively. The column chromatographic separation was followed by the use of a LDC model 1522 ultraviolet detector operating at 280 nm. Elemental analyses were performed by the Analytical Department, Institute of Chemistry,

University of Uppsala. Most of the experimental data are summarized in Tables 1 and 2.

2-Phenyl-3,1-benzoxathian-4-one (II). Method A. (+)-I+DCC. To 0.130 g (0.5 mmol) of finely powdered (+)-I (slightly soluble in benzene and 1,2-dichloroethane) in 5 ml of solvent was added with stirring 0.206 g (1 mmol) of DCC and in some cases 1/4 equivalent of anhydrous orthophosphoric acid. To destroy excess DCC, 0.25 g of acetic acid was added and after stirring for an additional 30 min, the precipitated *N,N'*-dicyclohexylurea (DCU) was removed by filtration and the remaining filtrate evaporated to dryness. The product was then purified by column chromatography on silica gel with benzene as the eluent. TLC-examination showed only a single spot and the optical activity was determined.

Method B. (+)-I + p-*TsOH.* 0.5 mmol of (+)-I and 1/8 mmol of *p*-TsOH was heated under reflux in benzene (water separator connected) or chloroform. The reaction mixture was cooled, washed with a diluted sodium bicarbonate solution and water and dried over anhydrous calcium chloride. The product was purified in the same way as above. The obtained II was found to be racemic. After 2 h a white solid began to precipitate from the chloroform solution. The IR spectrum of this product was identical with that for 2,2'-dithiodibenzoic acid.

If (+)-I or its methyl ester were subjected to the same treatment in acetone for 2 h or benzene for 1 h, no formation of II was detected and the recovered acid and ester had retained optical activities.

Method C. (+)-I + AA. According to Oae and Numata,[22] treatment of I with an excess of acetic anhydride afforded II. Starting with optically pure I, partially active II was obtained.

By this method a quenching study was performed as follows. 0.5 mmol of (+)-I was heated at 100 °C with 1 ml of acetic anhydride. After 3 min, the reaction was quenched by cooling in an ice-bath and 10 ml of 2 M sodium hydroxide solution was added. The solution was extracted repeatedly with chloroform whereupon the water layer was acidified with dilute sulfuric acid. The acid which had precipitated was filtered off and dried. 0.045 g of (+)-I was recovered with $[\alpha]_D^{25} = 171°$.

3-Phenylthiophthalide (IV). Starting with α-phenylsulfinyl-*o*-toluic acid (III),[32] IV was obtained by the methods A, B, and C. The product was recrystallized from benzene-ligroin. M.p. 100–102 °C, lit.[33] 102–103 °C. Experimental data are given in Table 2.

2-Phenyl-3,1-benzoxathian-4-one-1,1-dioxide (V). To 0.97 g (4 mmol) of II dissolved in 40 ml of glacial acetic acid was added a warm solution (50 °C) of potassium permanganate (1.28 g) in 15 ml of water. The reaction mixture was stirred for 15 min. Then a solution of sodium disulfite (1.50 g) in 15 ml of water was added to destroy excess permanganate and manganese dioxide. Ice water was added to the mixture and the precipitated solid collected by filtration. The product was recrystallized from ethanol and the yield of sulfone, melting at 184–185.5 °C, was 1.00 g (91 %). (Found: C 61.34; H 3.71; S 11.66. Calc. for $C_{14}H_{10}O_4S$: C 61.30; H 3.67; S 11.69).

Methyl (−)-o-benzylsulfinylbenzoate. 0.52 g (2 mmol) of (−)-I was suspended by stirring in 10 ml of methanol. After cooling to 0 °C an excess of diazomethane in ether was added. The solvent was then allowed to evaporate at room temperature. Recrystallization from ligroin-acetone yielded 0.42 g (77 %) ester with m.p. 143–144 °C and $[\alpha]_D^{25} = -423.1°$ ($c = 0.8$, ethanol).

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Characterization of Charge Isomers of Yeast Phosphoglycerate Kinase. Evidence for Intracellular Differences

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Three electrophoretic components of phosphoglycerate kinase have been isolated from baker's yeast. The isoionic point of the major component is 7.18 at 10 °C. Corresponding values for the minor ones are 6.91 and 7.48, respectively.

There is a difference of one charge-unit between the isomers 1 and 2, and between the isomers 2 and 3. The release of component 3 from the yeast cells appears in contrast to the isomers 1 and 2 to be promoted by an organic solvent, thus suggesting this component to be bound to the cell-membrane.

The amino-terminal amino acid residue appears to be *N*-acetylated serine in each of the three cases. The carboxyl-terminal ends seem to be identical also with -(Ala, Leu, Val, Lys)-Ala-Lys as the ultimate sequence.

From circular dichroism spectra the contents of α-helix and β-structure were estimated to 15 and 40 – 50 %, respectively. Factors have been determined for transformation and comparison of the specific activities as determined under the various conditions used at different laboratories.

In the last few years intensified studies on phosphoglycerate kinase (ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) from different sources have given evidence for enzyme isomers. Larsson-Raźnikiewicz [1] has found that the yeast enzyme prepared according to the old method of Bücher [2] contains three enzymatically active electrophoretic components. Also the presence of different forms of the enzyme in the crude yeast autolysate is demonstrated by counter-current distribution technique.[3,4] Beutler [5] and Chen *et al.*[6] have found three isomers of phosphoglycerate kinase in homogenates from human erythrocytes. In contrast, the purified erythrocyte enzyme seems to consist of only one component.[7–9] Liver phosphoglycerate kinase [10,11] purified from human and bovine species give three active components each. Genetic studies including starch gel electrophoresis indicate that *Drosophila melanogaster* contains three condominant allels [12] for phosphoglycerate kinase and that *Macropus* species have two forms of this enzyme, of which the major component is autosomally controlled.[13,14]

The preparation method used in the present work is in principal similar but in details different from methods published recently.[15,16] Three electrophoretic components with phosphoglycerate kinase activity were obtained as previously.[1] The present study was undertaken to ascertain the origin of our different components as others [15,16] have not noticed any such heterogeneities in their preparations of yeast phosphoglycerate kinase, when tested by gel electrophoresis. Precautions were taken to exclude possible artifacts caused by proteolytic activity and/or organic solvents. Different chemical and physical methods were used to characterize and compare the three phosphoglycerate kinase components obtained. Two of them have in part been characterized earlier.[1]

MATERIALS AND METHODS

Reagents. The DEAE cellulose used was Whatman DE 23. Amberlite resin MB 3 was obtained from BDH Chemicals. The carboxypeptidases A, B, and C (DFP-treated) were

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products of Worthington Biochemical Corporation. Phenylmethylsulfonyl fluoride came from Sigma Chemical Company. The salt-free DCC-treated crystalline preparation of trypsin was obtained from Miles-Servac. *N*-Acetylserine was a gift from Dr. G. Fölsch (Department of Medical Chemistry, University of Göteborg) and *N*-formylserine was prepared by the method of Sheehan and Yang.[17]

Activity measurements. Phosphoglycerate kinase activity was assayed with Bücher's spectrophotometric method [2] (*cf.* Ref. 1).

Protein determination. Protein concentrations were determined as absorbance at 280 nm. For pure phosphoglycerate kinase $E(1\ \%, 1\ cm) = 5.0$ at 280 nm was used.[2,1]

Isolation of isomers. Autolysis of the yeast cells was carried out in two different ways, IA and IB, respectively, as was the ensuing salt fractionation steps (IIA and IIB). The following steps were identical in both cases. Fig. 1 is representative for a preparation including toluene autolysis (procedure A).

IA. *Toluene autolysis.* Autolysis of the yeast cells was performed according to Westhead and McLain.[18] The autolysate was centrifuged (432 000 *g* min).

IIA. *Precipitation with ammonium sulfate.* The pH of the water phase from IA was adjusted to 7.5 with 5 M KOH. Solid ammonium sulfate (70 g/100 ml) was slowly stirred into the solution. The pH decreased to 7.1. The precipitate

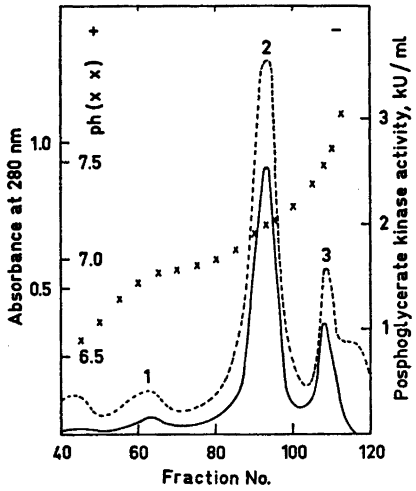

Fig. 1. Isoelectric focusing of phosphoglycerate kinase performed instead of column electrophoresis as the final purification step. The experiment was run for 67 h at 10 °C at 400 V. The (+) and the (−) at the top of the figure indicate the position of the anode and the cathode, respectively. About 60 mg of protein was used. Fraction volumes: 1 ml. − − −, A_{280}; ———, specific activity; ×, pH.

was collected by centrifugation (1 296 000 *g* min) and then dissolved in a minimum volume of 0.1 M Tris-Cl buffer (pH 9.5) whereupon it was dialyzed overnight (15 h) against 5 mM Tris-Cl buffer (pH 9.5) at 12 °C.

IB. *Freeze autolysis.* Two kg of fresh yeast was mixed with an equal quantity of dry ice (w/w) and 50 g of Trizma-base. The mixture was finely dispersed in a Waring Blendor and, after melting, 115 ml of conc. ammonia was slowly added during stirring which was continued overnight. The following morning centrifugation was performed (1 296 000 *g* min).

IIB. *Fractionated ammonium sulfate precipitation.* Ammonium sulfate (30 g/100 ml) was added to the supernatant from IB. After removal of the precipitate by centrifugation, more ammonium sulfate (35 g/100 ml) was added and the pH was adjusted to 7.1. The precipitate was then treated as described above (IIA).

III. *DEAE Cellulose chromatography.* After adjustment of the pH to 9.5 with 1 M Trizma-base, the solution from II was applied to a DEAE cellulose column (5.7 × 60 cm) equilibrated with 50 mM Tris-Cl buffer (pH 9.5) at 12 °C. The column was washed with 2 l of this buffer, containing 8 mM KCl. Phosphoglycerate kinase was then eluted with 60 mM KCl in the same buffer, and pH was adjusted to 8.0 with HCl, whereupon the solution was concentrated to 25 ml in a Diaflo apparatus.

IV. *Gel filtration.* The concentrate from III was applied to a Sephadex G-100 column (5.7 × 105 cm), equilibrated with 50 mM Tris-Cl buffer (pH 8.0) at 12 °C. This buffer was also used for the elution procedure. Fractions containing phosphoglycerate kinase activity were collected and concentrated to a volume of about 3 ml.

V. *Column electrophoresis.* The solution from IV was applied to the top of a water-cooled electrophoresis column with cellulose as supporting medium. The electrophoresis was performed as described earlier.[1]

Amino acid analysis. The amino acid compositions were determined as described in Ref. 1. The tryptophan content was analysed spectrophotometrically by the method of Edelhoch.[19]

Isoelectric focusing. Electrofocusing was performed on an Ampholine column LKB 8101 using a sucrose gradient. The carrier ampholyte had a pH range of 6.5 − 7.5. The experiments were mostly carried out as described elsewhere.[20,21]

Polyacrylamide gel electrophoresis. Sodium dodecylsulfate polyacrylamide gel electrophoresis was run for 180 min (current: 5 mA/gel) at pH 9.5 as described by Maurer.[22] Protein zones were made visible after staining with amido black. Gels were destained by putting them into 7 % acetic acid, which contained Amberlite resin MB 3 (after a suggestion from Dr. L. Henderson).

Fingerprinting. Tryptic digestion of heat-denatured phosphoglycerate kinase was performed for 7 h at 37 °C in a 0.1 M ammonium bicarbonate buffer (pH 8.8). The ratio of phosphoglycerate kinase to trypsin was 50:1 (w/w). After digestion the protein was lyophylized and subjected to high-voltage paper electrophoresis in a Michl apparatus [23] equipped with a water-cooled coil in the cover. The paper used was Whatman 1 or Whatman 3MM (57 × 42 cm). The pH of the pyridine buffer was 6.5 and the electrophoresis was run for 35 min at 52 V/cm. The chromatography was carried out for 15 h in butanol-acetic acid-water, 3:1:1, (v/v). The chromatograms were stained by the cadmium-ninhydrin method.[24] Peptides containing tryptophan residues were identified using Ehrlich's reagent.[25]

Identification of the terminal amino acids of phosphoglycerate kinase. For determination of the *C*-terminal amino acids, phosphoglycerate kinase was heat-denatured for 10 min in 0.2 M bicarbonate buffer (pH 8.0), containing 10 % dimethyl sulfoxide. Digestion of the protein with carboxypeptidase A was performed at 37 °C. The ratio of phosphoglycerate kinase to carboxypeptidase was 40:1 (w/w). Dansylation according to Gray [26] was performed in sodium dodecylsulfate. The procedure of Yoshida [27] was followed for identification of the blocked NH_2-terminal residues.

Circular dichroism measurements. A Cary Model 60 recording spectropolarimeter with circular dichroism attachment was used. The results are expressed in the terms of the mean residue ellipticity, θ, defined as: $\theta = M\theta_{obs}/10cl$, where θ_{obs} is the observed ellipticity in degrees, M is the mean residue weight, l is the optical pathway in cm, and c is the concentration in g/ml. M was calculated to 107 for the enzyme (*cf.* Refs. 1, 15). No corrections for the refractive index of the solvent was made.

RESULTS AND DISCUSSION

Purification of different electrophoretic components of phosphoglycerate kinase. Isolation of the enzyme according to the above methods gives yields of about 60 %. Vigorous stirring in step IA is necessary to get high amounts of the enzyme.

The elution pattern obtained after column electrophoresis of the material from procedure A is almost identical with the one obtained earlier (Fig. 2 in Ref. 1) when quite a different preparation method was employed.[2] Three peaks with phosphoglycerate kinase activity occur, one major (named 2) and two minor (named 1 and 3). The material from component 1 (previously [1] called A) was earlier shown to be heterogeneous in the ultracentrifuge and to possess lower specific activity than the components 2 and 3 (previously [1] called B and C). The present results show that a restrictive collection of the fractions with phosphoglycerate kinase activity is needed after elution of the G-100 column to get a component 1 devoid of contaminating proteins, having molecular weights of 35 000 and 70 000.

The electrophoretic components of phosphoglycerate kinase can be completely separated by isoelectric focusing (Fig. 1). The enzyme content of peak 1 varied from one preparation to another; about 5 % of the protein content of component 2 was obtained as a maximum value. The corresponding value of component 3 was almost constant, 25 – 30 %, if toluene autolysis of the yeast cells was performed. When freeze autolysis was used only traces of form 3 could be detected by isoelectric focusing. Neither did our attempts to prepare isoenzymes according to Scopes [16] give any component 3. The two purification methods giving this component involve treatment of fresh yeast cells with an organic solvent, ethanol [2] or toluene. Possible interconversion of the different enzyme forms, induced by such a solvent, was investigated by incubation of pure component 2 with toluene at pH 7.0 and 25 °C for 6 h. No interconversion of the enzyme could, however, be detected by isoelectric focusing. To find out whether an organic solvent facilitates solubilization of component 3 from the yeast cells, the precipitate from step IB (freeze autolysis) was treated with toluene during continuous stirring for 3 h. The supernatant gained threefold in phosphoglycerate kinase activity by this treatment. The enzyme was isolated as described above (the steps IIA – IV), and was finally applied for isoelectric focusing. The preparation contained about the same amount of the two electrophoretic components 2 and 3, the latter corresponding to about 20 % of form 2 totally prepared from the same yeast cells (*cf.* above). These results show that the components 2 and 3 within the cell have different solubility properties, and may suggest that the component 3 occurs bound to the cell membrane, supporting results by Green *et al.*[28] who demonstrated that glycolytic enzymes are associated to the membranes of yeast and red blood corpuscles.

Isoelectric focusing. The isoionic points [29] of the charge isomers were found to be 6.91, 7.18, and 7.48 for components 1, 2, and 3, respectively, at 10 °C. Treatment with 6 M urea did not change the isoionic points of any of the phosphoglycerate kinase components. Unfolding of the enzyme in urea should normalize possible pK values of amino acid residues being anomalous in the native state.[21] Hence the present results may indicate that the differences in isoionic points are not due to conformationally induced modifications of the ionization constants. However, we cannot exclude the possibility that the unfolding of the protein molecules in urea is incomplete (*cf.* the unavailability of the carboxyl-terminal amino acid in urea, below).

Amino acid composition and molecular weight. No significant difference in amino acid composition was observed between the three forms of the enzyme. These results and sodium dodecylsulfate polyacrylamide gel electrophoresis suggest the molecular weight of isomer 1 to be in the range of 44 600 ± 1600, earlier determined for the isomers 2 and 3 (Ref. 1).

Specific activities and kinetic parameters. The isolation procedure presented in this paper gives three charged isomers of phosphoglycerate kinase with the same specific activity, 990 U/mg at 25 °C and saturation of both the substrates (*cf.* Ref. 1). To facilitate comparisons of the specific activities determined under various conditions at different laboratories, parallel experiments were performed under the conditions used by Krietsch and Bücher,[15] by Scopes,[16] and by ourselves.[1] A factor of 0.78 was found for conversion of reaction rates at 30 to 25 °C. Estimations showed that the reaction velocities measured at the conditions of Krietsch and Bücher and of Scopes should be multiplied by 1.33 and 0.98, respectively, to be comparable with the activities presented in this paper.

The isomers 1 and 3 appear identical to the isomer 2 concerning the K_m values for $MgATP^{2-}$ and 3-*P*-glycerate, and the typical non-linear Lineweaver-Burk plots at 10 mM Mg^{2+} (*cf.* Ref. 30).

Tests for protease activity during the purification procedure. Kunitz' casein method [31] indicated a protease activity in step IA corresponding to a trypsin content not exceeding 1 mg/l of the autolysate. This proteolytic activity could be completely inhibited by addition of phenylmethylsulfonyl fluoride (*cf.* Ref. 32), a final concentration of 0.5 mM, to the autolysate after toluene removal. The electrophoretic pattern was not affected by the inhibitor treatment.

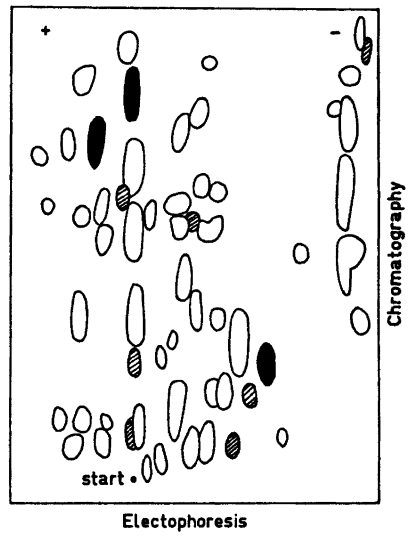

Fig. 2. Tryptic finger print map of phosphoglycerate kinase 2. Details of the experiment are given in the section MATERIALS AND METHODS. Solid patches represent Ehrlich positive peptides, cross-hatched patches represent peptides stained yellow, and unfilled patches represent peptides stained red by the cadmium-ninhydrin method. The (+) and the (−) in the figure indicate the anode and the cathode, respectively.

Fingerprinting. We could not see any differences in the tryptic finger print maps (Fig. 2) of the isomers 2 and 3. The amino acid compositions predict an ideal digest to give about 55 peptides. Around 60 ninhydrin positive spots could be identified for both enzyme components, which is an indication for good tryptic digestions. The same deviations from ideality appear to exist in both cases. Three Ehrlich positive spots were obtained though each protein contains only two tryptophanyl residues.

Carboxyl-terminal and amino-terminal ends. Both in its native state and in 6 M urea the carboxyl-terminal amino acid residue of phosphoglycerate kinase was shown to be unavailable for digestion with the carboxypeptidases A, B, and C. Successful results were obtained

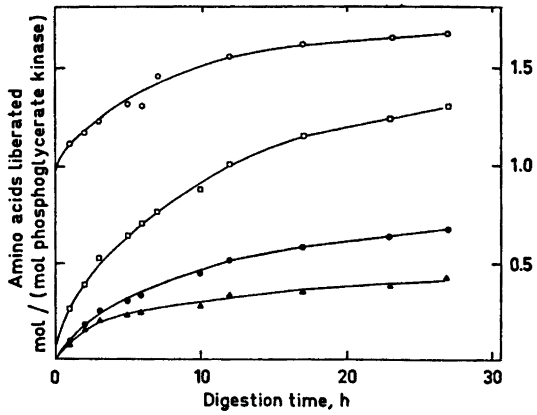

Fig. 3. Liberation of amino acids from phosphoglycerate kinase 2 by carboxypeptidase A. Details of the experimental conditions are given in the section MATERIALS AND METHODS. ○, lysine; □, alanine; ●, valine, ▲, leucine.

after heat-denaturation of the enzyme in 10 % dimethyl sulfoxide. The release of amino acid residues during carboxypeptidase A digestion of phosphoglycerate kinase 2 is seen in Fig. 3, which demonstrates that -(Ala, Leu, Val, Lys)-Ala-Lys is the sequence of the carboxyl-terminal end. Corresponding experiments on the enzyme forms 1 and 3 gave identical results.

Attempts to identify the amino-terminal residues by dansylation in dodecylsulfate,[26] indicated that these residues are blocked in all the three isomers. Yoshida's method [27] for investigation of blocked amino-terminal residues showed that an *N*-acetylated serine occurs at the terminus in all the three cases (the serine recovery in the acid hydrolysis step was 50 % of the total yield).

Circular dichroism spectra. The circular dichroism spectrum of phosphoglycerate kinase 2 is seen in Fig. 4. The spectra of the isomers 1 and 3 appeared to be identical. Calculations according to Greenfield and Fasman [33] give contents of α-helix and β-structure of 15 and 40–50 %, respectively. Bryant *et al.*[34] found in crystallographic studies some 86 residues to be involved in helical segments, which would correspond to 20 % of the protein molecule if a molecular weight of 45 000 is presumed.

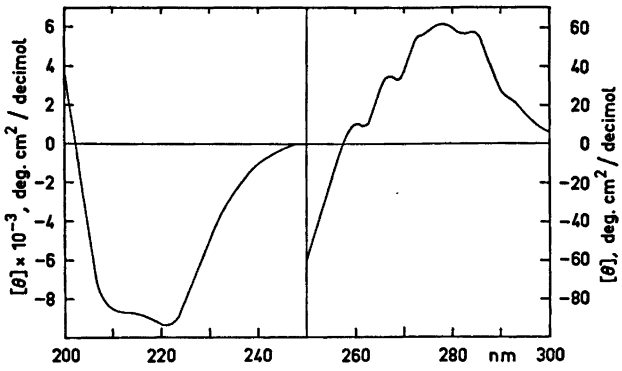

Fig. 4. Circular dichroism spectrum of phosphoglycerate kinase 2. The experiments were performed at room temperature in 1.0 mM Tris-Cl buffer (pH 7.8). The optical pathway was 1 mm in the 200–250 nm region, and 10 mm in the 250–300 nm region. The corresponding enzyme concentrations were 0.7 mg/ml and 2.5 mg/ml, respectively.

Concluding remarks

If toluene autolysis of the yeast cells is utilized, three electrophoretic components with phosphoglycerate kinase activity can be separated from yeast by column electrophoresis or isoelectric focusing. A similar electrophoretic pattern was earlier obtained when quite a different preparation method was used (*cf.* Ref. 1). In preparations using freeze autolysis or autolysis achieved by ammonia, one of the minor components, named 3, otherwise occurring in about 25 % of the major component, is missing.

The present results demonstrate that after freeze autolysis has been performed and the soluble phase has been removed, component 3 can be released from the ruptured cell fragments by toluene treatment. The yield of this enzyme form was the same as if toluene treatment had been performed on the intact cells, indicating that component 3 is more firmly bound to the cell than the components 1 and 2. An extract obtained by treatment of the yeast cells with, *e.g.* ethanol was in the old preparation method the source of the enzyme. Thus it appears that removal of component 3 from the yeast cells is facilitated by an organic solvent.

Calculations according to Edsall and Wyman [35] show that the divergences in isoionic points between the three phosphoglycerate kinase components approximately correspond to one charge-unit per molecule between the forms 1 and 2, and between the forms 2 and 3. For a protein like phosphoglycerate kinase with many of the charged amino acids occurring in such amounts that the error limit for one of these amino acids is ± 1, it is not possible unambigously to exclude a difference in charge corresponding to one amino acid. However, the agreeing finger print maps of the enzyme forms are indications, though no proof, of identical sequences. Phosphoglycerate kinase 1 occurs in variable amount, suggesting this component to be a product of component 2, possibly as a result of a split of one amide bond.

Analyses of the terminal amino acids show that proteolytic digestion as a cause of the charge isomers of yeast phosphoglycerate kinase can be excluded.

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Identification of Volatile Compounds in Seven Edible Fresh Mushrooms

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About 50 volatile compounds were identified in each of the seven edible fresh mushrooms *Cantharellus, cibarius, Gyromitra esculenta, Boletus edulis, Lactarius trivialis, Lactarius torminosus, Lactarius rufus,* and *Agaricus bisporus.*

The relative concentrations of the main aroma compounds were determined and their contribution to the mushroom aroma is discussed.

The studied mushrooms are rich in alcohols and carbonyl compounds containing eight carbon atoms. The main volatile compound in fresh wild mushrooms is 1-octen-3-ol and in cultivated *Agaricus bisporus,* benzyl alcohol.

Among the volatiles of *Gyromitra esculenta,* three previously unknown toxic compounds were identified and their structures revealed.

The special aroma of mushrooms constitutes an essential part of their appeal, but mushrooms as foodstuff are difficult to handle, with the result that their aroma is easily changed during processing and storage.

Many mushroom species possess an odour that can be generally described as "mushroom-like". Other species have, in addition, distinct aromas with which they uniquely flavour foodstuffs.

Only a few studies on the volatile compounds in mushrooms have been made.

Tricholoma matsutake (S. Ito & Imai) Sing (called also *Armillaria matsutake*) contains 1-octen-3-ol,[1] the compound also responsible for the "mushroom-like" off flavour of some dairy products.[2]

The East Asian shiitake mushroom, *Lentinus edodes* (Berk.) Sing, contains the sulfur compound lenthionine, which helps to produce its specific flavour.[3]

About fifteen volatiles, including 1-octen-3-ol, 2-octen-1-ol, phenylacetaldehyde, and benzaldehyde, have been identified in cultivated English champignons, *Agaricus bisporus* (Lange) Sing.[4] Polish champignons have been reported to contain eight volatiles, including *cis*-2-octen-1-ol.[5]

Dried *Boletus edulis* Fr. is known to contain numerous lactones, pyrroles and pyrazines,[6] whereas six volatiles have been reported for the fresh mushroom.[7]

The sulfur compounds in *Phallus impudicus* Pers. give this nonedible mushroom its typical odour.[8]

Cantharelle, *Cantharellus cibarius* Fr., is enthusiastically collected in northern Europe because of its attractive aroma and taste and because it is seldom damaged by insects.

In spite of their toxicity when fresh, false morels, *Gyromitra esculenta* (Pers.) Fr., are likewise widely used. These mushrooms possess a delicious aroma distinctly different from that of cantharelle and other edible mushrooms. Since they contain the toxic compound acetaldehyde *N*-methyl-*N*-formyl hydrazone, known is gyromitrin, however, false morels must be cooked or dried before use.[9]

Boletus edulis is also a delicious wild mushroom and extensively collected.

Among wild edible mushrooms, many *Lactarius* species are abundant.

Lactarius trivialis Fr. is the wild mushroom most often collected in Finland.

Under favourable weather conditions high yields of *Lactarius rufus* Fr. are also obtained, and the mushroom has appeared in surprising amounts, for example, in connection with the drying of marshes.

Table 1. The volatile compounds identified in mushrooms.

Peak No.	Compound, proposed structure	%
	Cantharellus cibarius	
1	ethyl formate [a]	
3	ethyl acetate	
4	methylene chloride	
5	benzene	
6	2-pentanone	
7	chloroform	
8	3-methylbutanal [c]	
9	isobutanal	
10	2-hexanone	
11	butanol	0.1
12	2-methyl-2-penten-4-one	0.4
13	3-methylbutanol	0.1
14	pentanol	
16	3-octanone	0.2
20	1-octen-3-one	0.35
21	4-methylpentanol	
24	hexanol	1.0
28	1-octen-3-yl acetate	
29	3-octanol	0.4
31	*trans*-2-octenal	0.1
34	1-octen-3-ol	66
35	furfural [c]	
37	1-octen-3-yl propionate	
39	benzaldehyde [c]	0.4
42	octanol	0.9
47	*trans*-2-octen-1-ol	24
49	nonanol [c]	
55	2-decanone [c]	
58	1-phenylethanol	
60	benzyl alcohol	
62	2-phenylethanol	
64	β-ionone	0.1
66	benzothiazole	
67	epoxy-β-ionone [b]	
84	diethyl phthalate [b]	
86	dibutyl phthalate [b]	
	Gyromitra esculenta	
1	diethyl ether, pentane, and ethyl formate	
2	methylene chloride and ethyl acetate	
3	1-octen-3-yl formate	
4	3-methylbutanal	
5	pentanal [c]	
6	2-hexanone [c]	
7	hexanal	
8	isobutanol	
9	butanol	
10	3-methylbutanol	2.4
11	limonene	
12	3-octanone	0.4
13	*p*-cumene	
14	1-octen-3-one	0.4
16	2-methyl-3-octanone [c]	
19	hexanol	0.3
20	octanal [c]	
21	1-octen-3-yl acetate	
22	3-octanol	3.9
23	*trans*-2-octenal	0.7
24	1-octen-3-ol	72
25	heptanol [c]	
26	furfural [c]	
28	benzaldehyde	
29	isophorone	
30	acetaldehyde *N*-methyl-*N*-formyl hydrazone	0.9
31	octanol	0.3
36	*trans*-2-octen-1-ol	1.4
37	phenylacetaldehyde	3.0
38	nonanol	
39	lavandulol [c]	
40	benzyl acetate	
41	butyrophenone [c]	
43	3-methylbutanal *N*-methyl-*N*-formyl hydrazone	3.3
44	decanol [c]	
45	pentanal *N*-methyl-*N*-formyl hydrazone	0.1
48	benzyl alcohol	0.2
49	2,6-di-*tert*-butyl-4-methylphenol	
50	β-ionone	
51	hexanal *N*-methyl-*N*-formyl hydrazone	0.6
52	2-phenylethanol	0.6
57	benzothiazole	
58	4-hydroxynonanoic acid lactone [c]	
62	4-hydroxydecanoic acid lactone [c]	
65	diethyl phthalate [b]	
72	dibutyl phthalate [b]	
	Boletus edulis	
1	pentane and diethyl ether	
2	ethyl formate	
4	ethyl acetate	
5	methylene chloride	
10	3-methylbutanal	8
18	hexanal [c]	0.5
20	isobutanol	
22	butanol	
27	3-methylbutanol	17
28	pentanol	
29	3-octanone	0.8
35	1-octen-3-one	8
41	hexanol	
44	3-octanol	0.5
46	*trans*-2-octenal	1
47	1-octen-3-ol	49
51	1-octen-3-yl propionate	
52	benzaldehyde	0.5
53	linalool [c]	
54	octanol	
55	*trans*-2-octen-1-ol	11

Table 1. Continued.

56	phenylacetaldehyde	1
57	nonanol	
65	1-phenylethanol	
66	benzyl alcohol	
69	2,6-di-*tert*-butyl-4-methylphenol	1
71	2-phenylethanol	
72	2-phenyl-2-butenal	1
75	benzothiazole	

Lactarius trivialis

1	pentane, diethyl ether, and 2-pentanone	
3	acetaldehyde[b]	
4	ethyl acetate	
5	methylene chloride	
6	1-octen-3-yl formate[b]	
7	2-hexanone	
10	hexanal	
13	isobutanol	
15	butanol	
16	2-heptanone[c]	
17	3-methylbutanol	
18	pentanol	
19	3-octanone	0.2
20	2-octanone[c]	
22	1-octen-3-one	0.2
26	4-methylcyclohexanone[c]	0.05
27	hexanol	0.2
28	1-octen-3-yl acetate	0.03
29	3-octanol	
30	*trans*-2-octenal	0.2
32	3,3,5-trimethylcyclohexanone	
34	1-octen-3-ol	70
36	furfural	
38	benzaldehyde	0.05
40	isophorone	
42	linalool	
43	linaloyl acetate	
44	octanol	0.4
46	*trans*-2-octen-1-ol	24
47	phenylacetaldehyde[c]	
53	2,4-nonadienal[b]	
55	naphthalene	
58	2,4-decadienal[b]	
59	citronellyl acetate[b]	
60	5-hydroxyheptanoic acid lactone[c]	
61	2-decanone[c]	
62	2,4-undecadienal	
63	β-ionone	
64	4-hydroxynonanoic acid lactone	
67	epoxy-β-ionone[c]	0.2
68	derivative of velleral[b]	0.1

Lactarius torminosus

1	pentane	
2	diethyl ether	
3	ethyl formate	
4	ethyl acetate	
5	methylene chloride	
13	hexanal	
18	isobutanol[c]	
20	butanol	
25	3-methylbutanol[c]	1.5
26	pentanol	
28	3-octanone	0.5
34	1-octen-3-one	
38	3-octanol	0.5
39	*trans*-2-octenal	0.5
42	1-octen-3-ol	90
44	furfural[c]	
45	benzaldehyde[c]	
50	linalool	
51	linaloyl acetate	
52	octanol	
56	methyl decanoate	
58	*trans*-2-octen-1-ol[c]	
68	2,4-nonadienal[b]	
69	1-phenylethanol[c]	
74	2,4-decadienal[b]	
76	benzyl alcohol[c]	
84	4-hydroxynonanoic acid lactone	
93	epoxy-β-ionone[c]	

Agaricus bisporus

1	pentane	
2	diethyl ether	
3	ethyl formate	
4	ethyl acetate	
5	methylene chloride	
7	1-octen-3-yl formate[b]	
8	3-methylbutanal	
12	hexanal	0.5
13	furfuryl acetone[c]	
14	isobutanol	
15	2-heptanone[c]	0.2
16	butanol	
19	3-methylbutanol	11
20	pentanol	
21	3-octanone	4
25	1-octen-3-one	
32	hexanol	
35	1-octen-3-yl acetate	
36	3-octanol	1
37	*trans*-2-octenal	
40	1-octen-3-ol	33
41	heptanol	
42	lavandulol deriv.[c]	
44	1-octen-3-yl propionate	
45	benzaldehyde	3
47	lavandulol deriv.[c]	
48	linalool[c]	
49	linaloyl acetate[c]	
50	octanol	
54	*trans*-2-octen-1-ol	6
56	nonanol	
57	lavandulol[c]	0.5
60	benzyl acetate	
65	1-phenylethanol	

Table 1. Continued.

70	benzyl alcohol	37
71	benzyl butyl ether[c]	
73	2-phenylethanol	
74	benzothiazole	
75	benzyl octyl ether[c]	
76	ester of benzoic acid[c]	
77	dibutyl phthalate[c]	
Lactarius rufus		
1	pentane and diethyl ether	
2	ethyl formate	
3	ethyl acetate	
4	methylene chloride	
11	hexanal	
16	isobutanol	
19	butanol	
22	3-methylbutanol	
27	heptanal[c]	
29	3-octanone	
37	1-octen-3-one	2
45	hexanol	8
52	3-octanol	1.5
53	*trans*-2-octenal[c]	3
57	1-octen-3-ol	72
58	furfural	
62	2-decanone[c]	
64	benzaldehyde	5
67	isophorone	
73	octanol	
80	*trans*-2-octen-1-ol[c]	
89	nonanol[c]	
90	ethyl benzoate[b]	1.5
92	2,4-nonadienal[b]	
99	naphthalene	
103	benzyl alcohol	
106	citronellyl acetate[b]	
114	4-hydroxyoctanoic acid lactone[c]	
120	2,6-di-*tert*-butyl-4-methylphenol[b]	
127	benzothiazole[b]	
132	epoxy-β-ionone[c]	
139	derivative of velleral[c]	

[a] Compounds without index: Identification based on the mass spectrum and comparison with reference compound in GLC. [b] Identification based on MS. [c] Tentative identification.

The attitude towards *Lactarius* species as edible mushrooms differs greatly from country to country. *Lactarius torminosus* (Fr.) Gray, for instance, is considered inedible and even poisonous in Germany. On the other hand, in eastern and northern European countries it is widely collected. Like most other *Lactarius* species, however, *Lactarius torminosus* is bitter tasting when fresh and must be boiled before being eaten.

Champignons, *Agaricus bisporus*, are cultivated on a large scale. Most of the commercial mushroom products are prepared from this mushroom. However, compared with the aroma of wild mushrooms, that of champignons can be considered mild and less attractive.

Wild, fresh or deep frozen *Cantharellus cibarius*, *Gyromitra esculenta*, *Boletus edulis*, *Lactarius trivialis*, *Lactarius rufus*, *Lactarius torminosus* and cultivated *Agaricus bisporus* were studied using a steam distillation, extraction and concentration procedure. The temperature was kept below + 35 °C in order to obtain high yields of the volatile compounds.

RESULTS

The mushrooms studied contained, on average, 5 – 15 ppm volatiles, which amount does not include the acids. The percentages of the major components with respect to the total amount of volatiles are seen in Table 1. The areas (Fig. 1) of the minor peaks can be compared with the areas of the signals reported in Table 1. Table 2 presents the volatile acids and their relative concentrations in the mushroom samples.

The aroma concentrations also contained some unknown components for which proper mass spectra were recorded, but exact interpretation of the spectra failed.

Compound *29* in *Gyromitra esculenta*, MS: *m/e* 111(80), 91(15), 83(25), 70(60), 69(100), 55(85), and the compound *39*, MS: *m/e* 136(2), 124(7), 111(50), 93(14), 81(26), 69(100), 57(36), 55(54), 41(45) showed a similar type of mass spectra fragmentation. The spectrum of compound 39 is nearly identical to that of lavandulol.[10] The mass spectra of γ-isogeraniol and hydrated ionones also contain the fragments 111 and 69.

Based on the mass spectrum, compound *57* in *Agaricus bisporus* was tentatively identified as lavandulol,[10] and the compounds *42*, MS: *m/e* 111(83), 83(14), 81(13), 69(100), 57(38), 55(58), 41(46), and *47*, MS: *m/e* 151(10), 137(16), 111(95), 95(7), 81(12), 69(100), 55(45), 41(40) were tentatively identified as compounds whose structures are closely related to that of lavandulol.

Compound *68*, MS: *m/e* 232(75 %), 217(36), 202(25), 199(50), 189(53), 123(100), 105(21),

Table 2. The volatile acids and their relative concentrations (%) in steam distillates obtained from mushrooms.

Acid	*Cantharellus cibarius*	*Gyromitra esculenta*	*Boletus edulis*	*Lactarius trivialis*	*Lactarius torminosus*	*Lactarius rufus*	*Agaricus bisporus*
Acetic	26	9	4	8	8	35	76
Propionic	5	+	1	0.5	+	3.5	+
Isobutyric	+	6	+	0.5	3	+	+
Butyric	8	+	1	1	+	4.5	+
Isovaleric	+	31	+	1	+	3	+
Valeric	5	+	3	0.5	+	2.5	+
Isocaproic	+	2	+	0.5	−	5.0	+
Caproic	32	23	53	8	2	17	7
Heptanoic	+	7	2	1	1	3.5	+
Octanoic	8	11	2	4	6	8	7
Nonanoic	2	+	2	10	1	2.5	+
Decanoic	1	+	+	57	72	8	4
trans-2-Octenoic	7	7	9	1	+	+	+

+ Small amounts tentatively identified.

95(37), 91(29), 81(51), 43(55) in *Lactarius trivialis*, and compound *139*, MS: *m/e* 232(70 %), 217(35), 199(45), 189(50), 123(100), 95(59), 81(50), 57(60), 43(90), in *Lactarius rufus* were tentatively identified as compounds with structures closely related to those of velleral and isovelleral.[11] These bitter tasting compounds were originally identified from *Lactarius vellereus* (Fr.) Fr.[11,12]

Three new toxic compounds were found among the volatiles of *Gyromitra esculenta*. Structurally (Fig. 2) these compounds proved to be higher homologues of acetaldehyde *N*-methyl-*N*-formyl hydrazone (gyromitrin). In preliminary tests, rabbits fed with 350 mg of pentanal, 3-methylbutanal, or hexanal *N*-methyl-*N*-formyl hydrazone per 1 kg body weight died within 24 h.

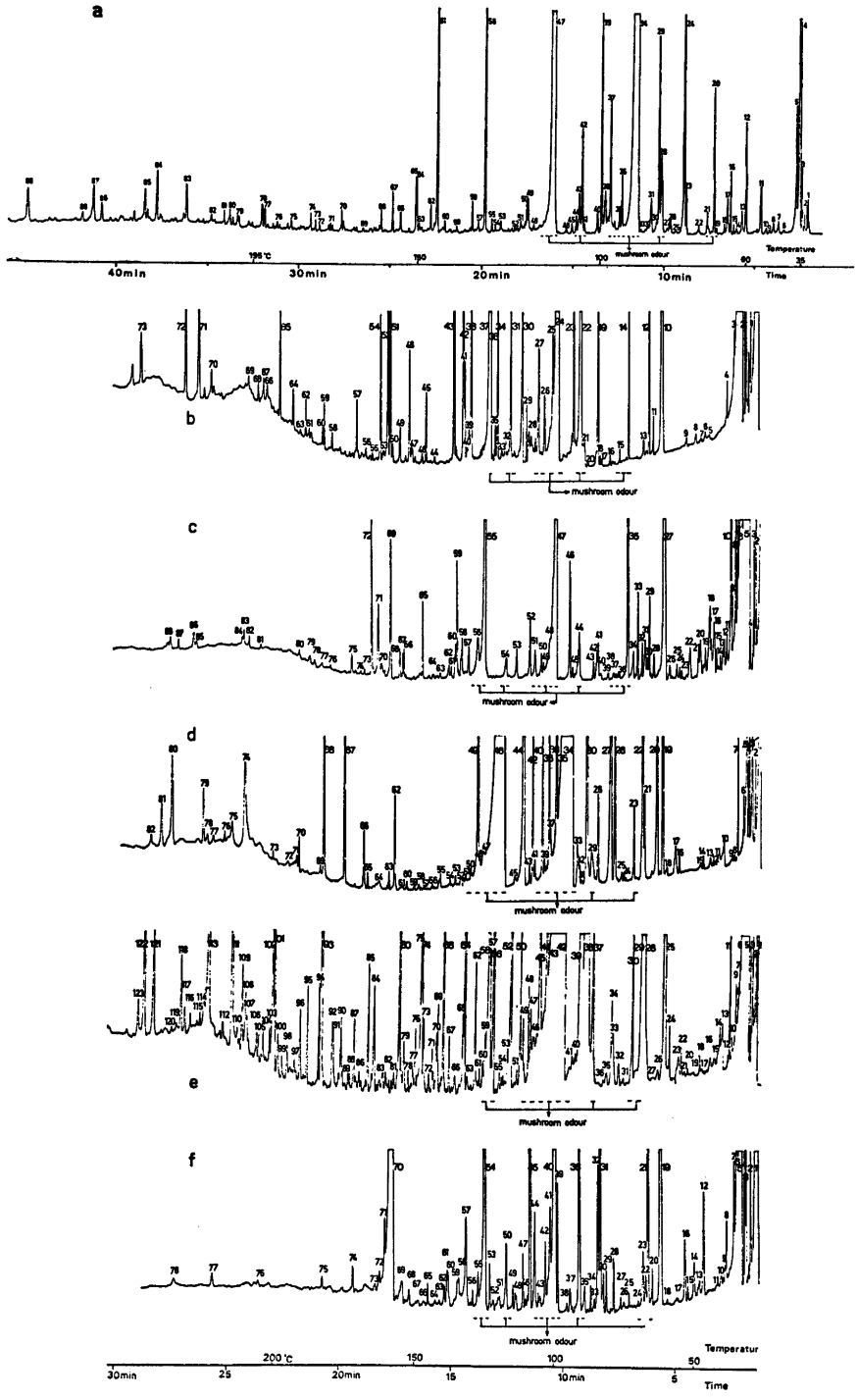
Pentane, diethyl ether and methylene chloride were used as solvents. The phthalates and 2,6-di-*tert*-butyl-4-methylphenol may have originated from the packing materials. Naphthalene, chloroform and benzene probably did not originate from mushrooms.

To establish which compounds contribute to the characteristic odours of the mushrooms, the concentrates were analyzed by preparative GLC with packed FFAP column and four persons familiar with mushrooms were asked to describe the odours eluting from the column. The retention times at which the panelists considered the odours to be "mushroom-like" are seen in Fig. 1.

DISCUSSION

In the mushrooms studied here the main volatiles all have eight carbon atoms. From the descriptions of the odours (Fig. 1) and the tests with synthetic reference compounds,[13] the fresh mushroom-like aroma of the studied mushroom species can be attributed mainly to 1-octen-3-ol, 1-octen-3-one, *trans*-2-octen-1-ol, octanol, nonanol, esters of 1-octen-3-ol, *trans*-2-octenal, and 3-octanol. Many others of the identified compounds, *e.g.*, benzaldehyde, 2,4-dienals, hexanal, and dihydroisophorone, possess strong odours, too. 1-Octen-3-ol gives the general mushroom-like aroma to all mushrooms, while 1-octen-3-one seems to be important in producing the more specific aroma of wild mushrooms.

Dried and cooked *Boletus edulis* contains a great number of pyrazines, pyrroles and lactones,[6] none of which was found in the fresh mushroom. The main volatile in fresh *Boletus edulis*, 1-octen-3-ol, was present in the dried product only in low concentration, while 1-octen-3-one and *trans*-2-octen-1-ol were not found at all in dried *Boletus edulis*.[6] 2-Phenyl-2-butenal was not found in any of the mushrooms studied here except *Boletus edulis*. It has also been isolated from *Phallus impudicus*,[8] a mushroom with an odd odour. 2-Phenyl-2-butenal was synthesized and found to possess


a
b
c
d
e
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mushroom odour
Temperature
Time
40min
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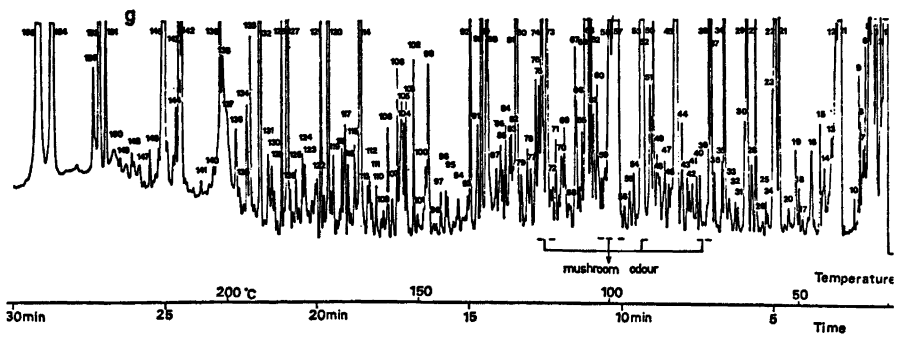
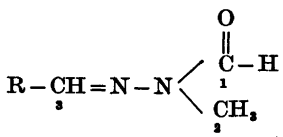


Fig. 1. Gas chromatogram of the volatile extract obtained from (a) fresh *Cantharellus cibarius*, (b) deep frozen, fresh *Gyromitra esculenta*, (c) deep frozen, fresh *Boletus edulis*, (d) deep frozen, fresh *Lactarius trivialis*, (e) fresh *Lactarius torminosus*, (f) fresh *Agaricus bisporus*, and (g) deep frozen, fresh *Lactarius rufus*. Liquid phase FFAP, 40 m × 0.3 mm i.d. glass capillary column, constructed by A. Hesso, University of Helsinki.



(a) $R = CH_3$ (4)

(b) $R = CH_2-CH_2-CH_2-CH_3$ (4, 5, 6, 7)

(c) $R = CH_2-CH(CH_3)-CH_3$ (4, 5, 6; CH_3)

(d) $R = CH_2-CH_2-CH_2-CH_2-CH_3$ (4, 5, 6, 7, 8)

Fig. 2. Structures of the toxic compounds in fresh *Gyromitra esculenta*. (a) Acetaldehyde *N*-methyl-*N*-formyl hydrazone. (b) Pentanal *N*-methyl-*N*-formyl hydrazone. (c) 3-Methylbutanal *N*-methyl-*N*-formyl hydrazone. (d) Hexanal *N*-methyl-*N*-formyl hydrazone.

a strong but not particularly unpleasant flavour.

1-Octen-3-ol is the main volatile component in all three *Lactarius* mushrooms. *trans*-2-Octen-1-ol is present in *Lactarius rufus* and *Lactarius torminosus* in very low concentration, if at all, although it is among the main volatiles in *Lactarius trivialis* and in all the other mushrooms studied. The aroma of *Lactarius rufus* is considered to be milder than that of *Lactarius trivialis* and *Lactarius torminosus*. Hence, the great number of signals in Fig. 1 g does not reflect the higher total concentration of volatiles in *Lactarius rufus* but greater sensitivity of the gas chromatograph used in recording the chromatogram of this mushroom.

The composition of aroma compounds in *Agaricus bisporus* differs from that in wild mushrooms mostly with respect to the high concentration of benzyl alcohol and benzaldehyde. These compounds are the main volatiles only in champignons. The total amount of volatiles in wild mushrooms and in *Agaricus bisporus* was found to be of the same level, 5 – 15 ppm and the mild mushroom-like aroma of champignons can thus be attributed to low concentrations of 1-octen-3-ol, 1-octen-3-one, and *trans*-2-octen-1-ol.

It is evident that processing quickly changes the aroma composition of mushrooms. The steam distillate of English champignons obtained at atmospheric pressure was reported to contain a high concentration of phenylacetaldehyde,[4] which compound was not found in the Finnish champignons. Polish champignons have been reported to contain *cis*-2-octen-1-ol as one of the main volatile components.[5] According to this work, 2-octen-1-ol is in *trans*-form in *Agaricus bisporus* and all the other mushrooms studied. It is possible, however, that the mushrooms also contain low concentrations of *cis*-2-octen-1-ol.

It should be noted that the concentration of 3-methylbutanal *N*-methyl-*N*-formyl hydrazone in *Gyromitra esculenta* is high, even higher than the concentration of acetaldehyde *N*-methyl-*N*-formyl hydrazone. Since hydrazones

easily hydrolyse, the concentrations in the sample made from the steam distillate do not correspond exactly to the concentrations of hydrazones in fresh false morels.

The quantitative determinations of the volatiles (Table 1) are based on peak areas in GLC. Although the GLC showed satisfactory separation power, peak areas determined by integration do not correspond exactly to original concentrations. The volatiles are lost to some extent during preparation of the concentrates and consequently the ratio of the concentrations of the components is changed. Moreover, the splitter in GLC does not split the compounds in exactly the original ratio.

The results of analyses on acids do not exclude the presence of formic acid. Formic acid gives no signal in FID though there is no reason to believe it absent, especially since esters of formic acid were found.

Acids containing an even number of carbon atoms are present in considerably higher concentration than those with odd numbers of carbon atoms. However, isovaleric acid is one of the main acidic components in *Gyromitra esculenta*.

EXPERIMENTAL

The volatile concentrate of *Cantharellus cibarius* was prepared from fresh mushrooms collected in early September in eastern Finland. *Gyromitra esculenta* was collected in May and deep frozen and stored a few months at −30 °C. *Agaricus bisporus* was purchased from a local cultivator. The aroma concentrate was made from fresh champignons, diameter of the pileus less than 3 cm. *Boletus edulis* was collected in August in northern Finland and stored a few days at −30 °C. The aroma concentrate of *Lactarius torminosus* was prepared from fresh mushrooms, collected in August in eastern Finland. *Lactarius trivialis* and *Lactarius rufus* were collected in southern Finland in August and stored a few weeks at −30 °C. Only mushrooms with a diameter of the pileus less than 8 cm were used.

General methods and instruments. Mushrooms (5 kg) were pressed into juice, which was steam distilled in vacuum, not exceeding a temperature of 35 °C. The pH of the distillate was adjusted with sodium bicarbonate to 8 and the volatiles were extracted during 24 h into an ether–pentane mixture 1:1. The extract was concentrated in vacuum to 50 μl. Instruments described by Honkanen *et al.* and Suomalainen *et al.* were used.[14,15] The solvents were commercial chemicals of *pro analysi* quality and were freshly distilled. Cool traps in dry ice or in liquid nitrogen were used to avoid the loss of light volatiles.

The flavours were retained during preparation of the samples, and the final concentrates, as detected by several persons familiar with mushrooms, had the characteristic aromas of the original mushrooms. After extraction of the neutral volatiles, the steam distillate was made acidic (pH = 3) with hydrochloric acid and the acids were extracted into ether–pentane as above. The aroma concentrates were studied with a Perkin-Elmer 270B GLC-MS system equipped with an 8 m stainless steel packed FFAP column and with a Jeol JMS-D100 GLC-MS system equipped with a 90 m glass capillary FFAP column. The gas chromatographs were programmed from 60 to 200 °C, 2 °C per min. The gas chromatograms (Fig. 1) were recorded with a Carlo-Erba Mod. GI-instrument, carrier gas and flow 4 ml H_2 per min, splitting ratio 1:30.

The quantitative estimates of the total amounts of the volatiles were made so that known amounts of the 5–10 largest components were added to the original juices and the peak areas in the GLC made from these samples were compared with those obtained from the samples prepared in a similar way but without added components.

^{1}H NMR spectra were recorded at 60 or 100 MHz in $CDCl_3$ and the scales are in ppm downfield from internal TMS.

Proton noise decoupled ^{13}C NMR spectra were recorded at 25 MHz with a Jeol JNM PFT-100 spectrometer and the chemical shifts from internal TMS in $CDCl_3$ were determined with a 20k EC-20 data system. The assignments in the proton noise decoupled ^{13}C NMR spectra were confirmed through the undecoupled ^{13}C NMR spectra.

The mass spectra (70 eV) were compared with those in standard collections of mass spectra and the retention times in a 90 m FFAP glass capillary column were compared with those of commercially available or synthetic compounds.

The acidic fractions were studied by GLC with a 50 m FFAP glass capillary column. The retention times of the original acids and in some cases also their methyl esters were compared with reference compounds. The mass spectra of the main acidic components were measured as well. The quantitative estimations of the acids (Table 2) are based on their peak areas in GLC.

Preparation of the reference compounds. 1-Octen-3-one was obtained by a potassium dichromate oxidation from commercial 1-octen-3-ol.[16] The mass spectrum of synthetic 1-octen-3-one was identical to that reported by Stark *et al.*[17] *trans*-2-Octenoic acid, b.p. 140–143 °C/15 mmHg, was prepared by Knoevenagel reaction from hexanal and malonic acid.[18]

From the splitting due to the coupling $J_{H_2-H_3}$ = 16Hz in the ^1H NMR spectrum, the compound was identified as the *trans*-isomer.

trans-2-Octen-1-ol, b.p. 95 °C/13 mmHg, was prepared from *trans*-2-octenoic acid by reduction with lithium aluminium hydride in ether. ^1H NMR spectrum: δ 5.35 (m, 2 H, C2-H, C3-H), 3.8 (m, 2 H, Cl-H), 2.6 (s, 1 H, OH), 1.8 (m, 2 H, C4-H), 1.3 (m. 6 H, C5-H, C6-H, C7-H), 0.9 (t, 3 H, C8-H). With the aid of tris(dipivalomethanato)-europium shift reagent, the coupling $J_{H_2-H_3}$ was revealed to be 16 Hz, which indicates the compound to be the *trans*-isomer. The IR spectrum was identical to that reported for *trans*-2-octen-1-ol.[5]

trans-2-Octenal, b.p. 90–95 °C/15 mmHg, was prepared from the corresponding alcohol by oxidation with fresh manganese dioxide,[18] and from the coupling $J_{H_2-H_3}$ = 16 Hz in ^1H NMR spectrum identified as the *trans*-isomer.

Acetaldehyde *N*-methyl-*N*-formyl hydrazone was synthesized according to the method described by List.[9] The structure of this compound was confirmed by MS, IR, ^1H NMR and ^{13}C NMR experiments.[19]

2,4-Undecadienal was obtained from heptanal and malonic acid, from which the compounds 2-nonenic acid, 2-nonen-1-ol, and 2-nonenal were obtained analogously to the method described by Nobuhara *et al.*[18] 2-Nonenal and malonic acid in dry pyridine, piperidine as catalyst, gave 2,4-undecadienoic acid, b.p. 170–175 °C/mmHg.

2,4-Undecadienoic acid was reduced with lithium aluminium hydride in dry ether to 2,4-undecadienol, b.p. 130–135 °C/9 mmHg. 2,4-Undecadienol was oxidized with fresh manganese dioxide in dry ether to 2,4-undecadienal, b.p. 130–135 °C/11 mmHg. ^1H NMR δ 0.95 (t, 3 H, CH_3), 1.35 (m, 8 H, C7-H, C8-H, C9-H, C10-H), 1.35 (m, 2 H, C6-H), 5.5–7.0 (m, 4 H, C2-H, C3-H, C4-H, C5-H) 9.3 (d, 1 H, CHO).

3,3,5-Trimethylcyclohexanone (dihydroisophorone), b.p. 189–189.5 °C, was synthesized from isophorone *via* catalytic reduction with Raney nickel in 1:1 tetrahydrofuran–ethanol mixture. ^1H NMR spectrum: δ 0.9 (d, 3 H, C5-CH_3). 1.05 (s, 6 H, C3-CH_3), 1.1–1.6 (m, 1 H, C3-H), 1.7–2.4 (m, 4 H, C2-H, C4-H). MS: *m/e* 149(9 %), 125(10), 97(4), 83(100), 69(57), 56(31), 55(35), 41(35).

The aliphatic chain γ-lactones were obtained from the corresponding 2-alkenoic acids in 70 % sulfuric acid,[20] and the aliphatic chain δ-lactones from their corresponding δ-hydroxy-acids.[20]

Following List,[8] 2-phenyl-2-butenal, b.p. 120 °C/14 mmHg, was prepared in water–ethanol mixture (1:1) by condensing 24 g phenylacetaldehyde with 8.8 g acetaldehyde in the presence of 10 g sodium acetate during 24 h in an autoclave at 120 °C. The ^1H NMR spectrum was identical to that reported in the literature.[8] Mass spectrum: *m/e* 146(100 %), 118(18), 117(90), 115(64), 91(30), 78(13), 77(8), 65(10), 51(13).

Pentanal, 3-methylbutanal, and hexanal *N*-methyl-*N*-formyl hydrazones were prepared analogously to acetaldehyde *N*-methyl-*N*-formyl hydrazone.[9] Methylhydrazine (0.3 mol) was treated at −20 °C with ethyl formate (0.3 mol) to give 19 g (85 % of theory) *N*-methyl-*N*-formyl hydrazine, which upon addition of the corresponding aldehyde (0.1 mol:0.1 mol) at −20 °C gave *N*-methyl-*N*-formyl hydrazones of pentanal, 3-methylbutanal, and hexanal in 50 % yield, respectively. Pentanal *N*-methyl-*N*-formyl hydrazone: Mass spectrum: *m/e* 142(16), 113(8), 100(48), 85(100), 84(39), 71(25), 69(21), 67(8), 60(19), 59(73), 57(25), 43(50), 41(34). ^1H NMR spectrum: δ 8.5 (s, 1 H, Cl-H), 7.1 (t, 1 H, C3-H), 3.1 (s, 3 H, C2-H), 2.25 (m, 2 H, C4-H), 1.45 (m, 4 H, C5-H, C6-H), 0.95 (t, 3 H, C7-H). Proton noise decoupled ^{13}C NMR spectrum. The scales are in ppm downfield from internal TMS in $CDCl_3$ (δ). Cl: 164.7, C2: 26.5, C3: 144.8, C4, C5 and C6: 32.4, 28.9 and 22.3, C7: 13.8. IR spectrum: 2970, 1660–1700, 1625, 1450, 1400, 1370, 1325, 1100, and 1030 cm^{-1}.

3-Methylbutanal *N*-methyl-*N*-formyl hydrazone: Mass spectrum: *m/e* 142(13 %), 127(9), 100(10), 85(89), 84(52), 71(31), 60(19), 59(100), 57(28), 43(80), 41(56). ^1H NMR spectrum: δ 8.45 (s, 1 H, Cl-H), 7.15 (t, 1 H, C3-H), 3.1 (s, 3 H, C2-H), 2.2 (m, 2 H, C4-H), 1.7–2.3 (m, 1 H, C5-H), 1.0 (d, 6 H, C6-H). ^{13}C NMR spectrum: Cl: 164.7, C2: 26.5, C3: 144.0, C4: 41.4, C5: 26.9, C6: 22.4. IR spectrum: 2950, 1660–1700, 1615, 1450, 1395, 1360, 1315, 1205, 1195, 1020, 910, 830, 695 cm^{-1}.

Hexanal *N*-methyl-*N*-formyl hydrazone: Mass spectrum *m/e* 156(12 %), 139(4), 127(3), 113(10), 100(57), 98(54), 85(100), 71(20), 60(41), 59(78), 55(25), 43(68). ^1H NMR spectrum: δ 8.45 (s, 1 H, Cl-H), 7.1 (t, 1 H, C3-H), 3.1 (s, 3 H, C2-H), 2.25 (m, 2 H, C4-H), 1.45 (m, 6 H, C5-H, C6-H, C7-H), 0.95 (t, 3 H, C8-H). ^{13}C NMR spectrum: Cl: 164.7, C2: 26.5, C3: 144.8, C4, C5, C6 and C7: 32.6, 31.4, 26.5 and 22.4, C8: 13.9. IR spectrum: 2840–2950, 1660–1700, 1615, 1445, 1390, 1355, 1315, 1205, 1135, 1020, 890, 690 cm^{-1}.

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Kinetic and Deuterium-labeling Studies of 2-Aminopyridine Catalyzed 1,3-Proton Transfers

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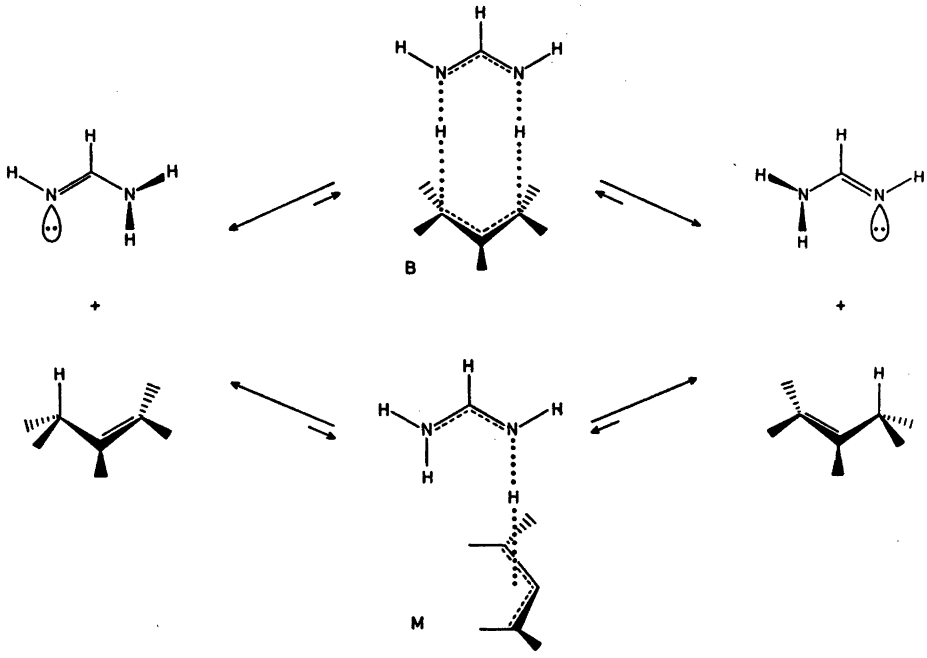
2-Aminopyridine (*1-h*) and *N,N*-dideuterio-2-aminopyridine (*1-d*) have been studied as catalysts for some, 1,3-proton transfer reactions. *β*-9-Fluorenylstyrene (*2*) has been rearranged to 9-*β*-phenylethylidenefluorene (*4*) and 1-methylindene (*3-h*) and 1-deuterio-1-methylindene (*3-d*) to 3-methylindene (*5-h*) and 1-deuterio-3-methylindene (*5-d*), respectively. All rearrangements were carried out in benzene as solvent. Compound *1*, which is a potential bifunctional catalyst, is shown not to act in a concerted bifunctional manner but rather like a monofunctional catalyst. This conclusion is based on the observed absence of H−D exchange in the reactions. The activation parameters, the isotope effect and rate dependence of the base concentration have been measured for the 2-aminopyridine (*1-h*) catalyzed rearrangement of 1-methylindene (*3-h*).

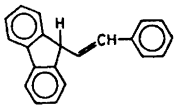
Recently we found in CNDO/2 calculations of the potential energy surfaces for formamidine catalyzed 1,3-proton transfers in propene (Scheme 1) that the bifunctional (B) route is strongly favoured over the monofunctional (M) one.[1,2]

In the present experimental investigation the catalytic mechanism of 2-aminopyridine (*1*),



Scheme 1.

which contains an unsymmetrical structural element resembling formamidine, has been studied in some 1,3-proton transfer reactions. The substrates used in this work contained structural elements related to propene, namely β-9-fluorenylstyrene (*2*), 1-methylindene (*3-h*) and 1-deuterio-1-methylindene (*3-d*). The rearrangement products were 9-β-phenylethylidenefluorene (*4*), 3-methylindene (*5-h*), and 1-deuterio-3-methylindene (*5-d*), respectively. All rearrangements were performed in benzene as solvent.



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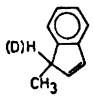
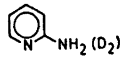
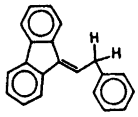


3-h(d)



1-h(d)



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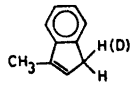


5-h(d)

Earlier we have reported some results of *N,N'*-di-*tert*-butylformamidine catalyzed rearrangements of 1-methyl-3-*tert*-butylindene to 3-methyl-1-*tert*-butylindene.[3] However, the mechanism of this amidine catalyzed rearrangement could not be elucidated due to the interference of a fast H−D exchange process. Presumably this exchange process became competitive with the rearrangement by steric hindrance. Such steric hindrance is avoided by using *1* as catalyst.

2-Aminopyridine (*1*) has been reported to be a bifunctional acid-base (tautomeric) catalyst for the mutarotation of the aldohexose tetramethylglucose (TMG) by Swain and Brown.[4a] However, this result has been questioned by Rony and Neff,[4b] who conclude that *1* is not to be considered a tautomeric catalyst of that reaction.

RESULTS AND DISCUSSION

If 2-aminopyridine (*1*) acts in a bifunctional manner, *i.e.* the catalysis takes place through a B-like transition state (Scheme 1), deuterium labeling in the proper positions of either substrate or catalyst would result in products where the deuterium is found in the base or the substrate, respectively (of structures *1* − *3*). This predicted experimental outcome of bifunctional catalysis is quite different from that expected if *1* is operating as a monofunctional catalyst, *i.e.* with an M-like transition state (Scheme 1). If other processes are not interfering with monofunctional catalysis, a deuterium labeled catalyst will stay labeled when operating on an unlabeled substrate and *vice versa*; *i.e.*, the rearrangement is intramolecular.[5a,b]

Of course, more complex experimental results could be found than the two extreme types suggested above if other reactions are interfering.[5b]

Molecule *1* exists in tautomeric equilibrium with *6* (Scheme 2).

1-h(d) *6-h(d)*

Scheme 2.

This equilibrium is reported to strongly favour *1*. The equilibrium constant (K_{taut}) was estimated to be 5×10^{-6} (at 21 °C, aqueous solution), which corresponds to $\Delta G_{taut} = 31$ kJ mol^{-1} (7.3 kcal mol^{-1}).[6] In each of *1* and *6* either one or both of the nitrogens could act as monofunctional catalytic centers. However, simple resonance theoretical arguments predict the iminonitrogens to be catalytically more active than primary amino-nitrogens. In Table 1 are summarized the experimental conditions as well as some of the results obtained.

In run 2 the reaction of *3-h* with *1-d* was allowed to proceed for $20 \times t_{1/2}$. The reaction products were isolated by a quench-extraction method and analyzed by ^{1}H NMR. The product was exclusively *5-h* and thus no significant deuterium incorporation was observed. Furthermore in run 5, *3-d* was rearranged with *1-h* in benzene and the product analyzed as above. No significant protium incorporation was observed by ^{1}H NMR. These results definitely exclude the concerted bifunctional mechanism of Scheme 1 as being responsible for the catalysis. Instead the results appear to favour the monofunctional mechanism.

Table 1. Rate and product data of 2-aminopyridine (*1*) catalyzed 1,3-proton transfers in benzene. Absolute temp. accuracy ±0.05 °C; relative temp. accuracy ±0.02 °C. Analytical methods: NMR (Run 1–5, 16–18), GLC (Run 6–15).

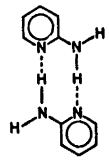
Run No.	Temp./ °C	Substrate	[Substrate]/ M[a]	Catalyst	[Catalyst]/ M[a]	k_{obs} / 10^{-6} s^{-1}	k_{obs}/(Catalyst] / 10^{-6} M^{-1} s^{-1}
1	55.00	*3-h*	0.305	*1-h*	0.968	45.0(36)	46.5(37)
2	55.00	*3-h*	0.329	*1-d*	1.196	—[b]	—[b]
3	55.00	*3-d*	0.327	*1-h*	0.998	10.6(9)	10.6(9)
4	55.00	*3-d*	0.326	*1-h*	0.981	10.3(9)	10.5(9)
5	55.00	*3-d*	0.299	*1-h*	0.911	—[c]	—[c]
6	55.00	*3-h*	0.309	*1-h*	0.976	45.4(10)	46.5(10)
7	55.00	*3-h*	0.313	*1-h*	0.552	22.4(4)	40.5(8)
8	55.00	*3-h*	0.308	*1-h*	0.255	9.00(18)	35.3(7)
9	55.00	*3-h*	0.306	*1-h*	0.123	4.05(8)	32.9(7)
10	55.00	*3-h*	0.305	*1-h*	0.062	1.96(4)	31.7(7)
11	55.00	*3-h*	0.303	*1-h*	0.032	1.00(2)	31.3(6)
12	55.00	*3-h*	0.302	*1-h*	0.016	0.490(8)	30.7(5)
13	75.00	*3-h*	0.105	*1-h*	0.504	80.9(13)	170.2(28)[d]
14	55.00	*3-h*	0.105	*1-h*	0.504	19.8(3)	40.7(6)[d]
15	35.00	*3-h*	0.105	*1-h*	0.504	4.08(8)	8.21(16)[d]
16	35.00	*2*	0.029	*1-h*	0.099	8.38	84.7[e]
17	55.00	*2*	0.044	*1-d*	1.30	—[b]	—[b]
18	35.00	*2*	0.032	*1-d*	0.101	7.62	75.4

[a] Reaction mixtures prepared at 23 °C. Catalyst and substrate conc. refer to this temperature. [b] No deuterium incorporation in product. [c] No protium incorporation in product. [d] The catalyst conc. used in the evaluation of the second order rate constant has been calculated at the running temperature using the benzene volume expansion coefficient $\beta = 1.15 \times 10^{-3}$ K^{-1}. [e] One point kinetics.

Why do these results contrast with those found with the theoretical methods mentioned in the introduction, where bifunctional catalysis was found to be strongly favoured? A possible reason could be that the chosen experimental model system deviates too much from our theoretical one. In the catalyst part of the model system the tautomeric equilibrium (Scheme 2) disfavours a bifunctional mechanism, since *6* is found to be substantially less than *1*. Furthermore, the substrate is more similar to cyclopentadiene than to propene, and it is also much more acidic ($pK_a \sim 20$) than propene ($pK_a = 35.5$).[7] These facts are expected to favour monofunctional rather than bifunctional catalysis.

In order to have a substrate more like propene, β-9-fluorenylstyrene (*2*) was prepared in the pure state. In run 17 compound *2* was rearranged to 9-β-phenylethylidenefluorene (*4*) by *1-d* in benzene. The rearrangement product was found to be exclusively *4*, and the ^{1}H NMR-spectrum revealed no significant incorporation of deuterium. This result seems to indicate that the catalysis of the rearrangement of this propene resembling, but highly acidic compound (*4*) is also of the monofunctional type.

Another reason for observing monofunctional catalysis would be that the catalyst is not a monomer like *1* or *6* but rather mainly a dimer (or polymer) like structure *8*.



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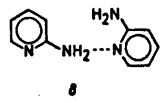

8

Of dimers *7* and *8* only *8* is predicted to be catalytically active through the use of the non-hydrogen bonded imino-nitrogen as catalytic center. The dimer *8* could be classified as a bifunctional catalyst and could be more active than the monomer *1*.

The rearrangement was in all kinetic runs found to be strictly pseudo first-order, *i.e.*

$$\text{rate} = k_{obs}\,[\text{substrate}] \quad (1)$$

If now monomeric as well as dimeric species (assumed to be in equilibrium with each other) are catalytically active, we find that

$$k_{obs} = k_2[M] + k_3K[M]^2 \quad (2)$$

where $K = [D]/[M]^2$, M = monomer and D = dimer, and k_2 is the bimolecular rate constant and k_3 the termolecular one. In order to get further information about the catalysis and the structure of the catalytic system, the rearrangement rate of *3-h* was studied as a function of the 2-aminopyridine concentration (runs 6 – 12). In Fig. 1 is plotted $k_{obs}/[1]$ *vs.* [*1*] using the assumption that the 2-aminopyridine is mainly monomeric. As seen in Fig. 1 the observations fall close to a straight line with non-zero slope. Using eqn. 2 and extrapolation to zero-[*1*] in Fig. 1 yields $k_2 = 30.6 \times 10^{-6}$ M^{-1} s^{-1} and the slope is identified as $k_3K = 15 \times 10^{-6}$ M^{-1} s^{-1}. These results indicate that at 0.5 M of *1* ca 20 % of the catalysis could take place in a termolecular fashion.

On the other hand if it is assumed that the 2-aminopyridine is present mainly in dimeric form, one would expect to observe a curve

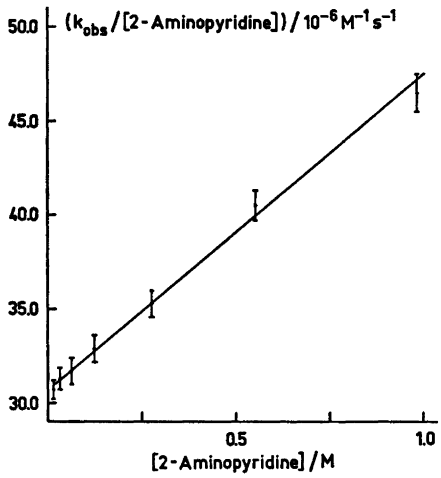

Fig. 1. $k_{obs}/[2\text{-aminopyridine}]$ from runs 6 – 12 in Table 1 plotted *vs.* [2-aminopyridine] (*cf.* eqn. 2).

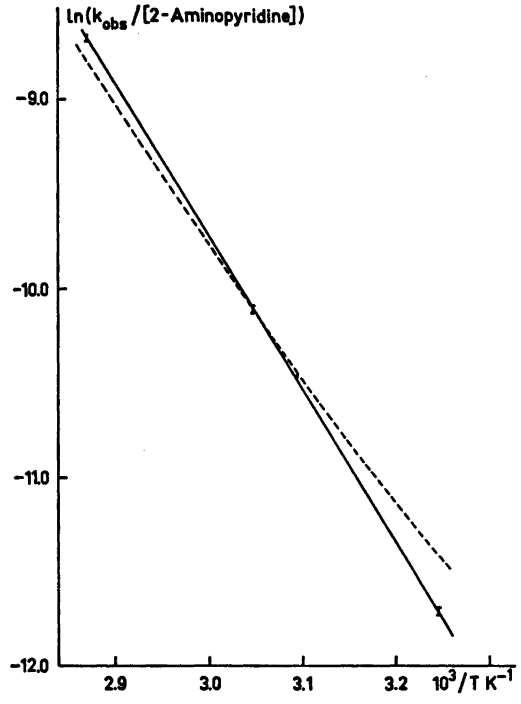

Fig. 2. $\ln(k_{obs}/[2\text{-aminopyridine}])$ *vs.* $1/T$ plots; ———, obtained experimentally (runs 13 – 15); - - -, simulated using assumptions given in the text.

with a [2-aminopyridine]-dependent slope and not a straight line. Since we have been working with quite large concentrations of *1*, we cannot exclude the possibility that a considerable catalyst medium effect could be a cause for the slope in Fig. 1. It has been inferred from freezing point depression measurements that 2-aminopyridine is mainly dimeric in benzene [8] in the concentration range covered by the present work. However, these results may be masked by possible effects of the medium. In order to avoid the influence of medium effects, but still get information about the relative importance of bimolecular and termolecular catalytic pathways, the temperature dependence of the rate of rearrangement was studied.

If it is assumed that ca 20 % of the catalysis is taking place with termolecular reactions and that the dimerization equilibrium ($2M \rightleftharpoons D$) is assumed to have a $\Delta S = -125.5$ J K^{-1}, mol^{-1} (−30 cal K^{-1}, mol^{-1}) and that the dimer and

the monomer have approximately the same activation entropy, the dashed line in Fig. 2 is simulated. This line is found to be significantly curved. However a good straight line is obtained experimentally, *i.e.* no significant curvature was observed (Fig. 2).

Thus it seems most likely that the catalysis either is made by monomeric *1* and that *1* is mainly monomeric or that the catalysis is carried out by a dimer and that *1* is mainly dimerized.

Using the first of these two alternatives we have calculated the activation parameters to be $\Delta G^{\ddagger}$(55 °C) = 108.215 ± 0.042 kJ mol^{-1} (25.864 ± 0.010 kcal mol^{-1}), $\Delta H^{\ddagger}$ = 64.98 ± 0.75 kJ mol^{-1} (15.53 ± 0.18 kcal mol^{-1}), and $\Delta S^{\ddagger}$ = −132.2 ± 2.1 J K^{-1} mol^{-1} (−31.6 ± 0.5 cal K^{-1} mol^{-1}).

The isotope effect k^{H}_{obs}/k^{D}_{obs} for the rearrangement is calculated from runs 3 and 6 in Table 1 to be 4.4 ± 0.5 at 55 °C. This value is similar to 4.2 ± 0.3 (25 °C) obtained with diazabicyclo[2.2.2]octane in the rearrangement of 1,3-dimethylindene in benzene, a rearrangement which is reported[9] to have a $\Delta H^{\ddagger}$ = 64.0 ± 3.8 kJ mol^{-1} (15.3 ± 0.9 kcal mol^{-1}) and $\Delta S^{\ddagger}$ = −109 ± 17 J K^{-1} mol^{-1} (−26 ± 4 cal K^{-1} mol^{-1}).

Other reaction systems which are more similar to the theoretical one mentioned above are under study in our laboratories.

EXPERIMENTAL

All glassware, including calibrated volumetric flasks, used in the kinetics were treated with chromic acid, water, 2 M ammonia and distilled water and finally dried at 120 °C for 24 h and stored in a desiccator.

The ^{1}H NMR spectra were obtained with a Varian A60-D analytical NMR spectrometer.

Preparative GLC was made with a Varian Aerograph 90P and the analytical work with a Perkin-Elmer 990 Gas Chromatograph. The 1-methylindenes were purified with a 0.6 m × 10 mm column containing 15 % Apiezon L on Chromosorb W 60/80 using an N_2 flow of 100 ml/min and an oven temperature of 120 °C.

For analytical purposes a 3 m × 3 mm column packed with 2 % Apiezon on Varaport 30 100/120 was used at 172 kPa N_2 and an oven temperature of 100 °C.

2-Aminopyridine was analyzed on a 2.5 m × 3 mm steel column packed with 20 % UCON-LB 550X, 20 % KOH on Chromosorb P 80/100, 172 kPa N_2 at 100 °C.

Kinetics. All kinetics were performed in HETO 01PT 623 thermostats. Most of the runs were made in ampoules which were broken at proper reaction times and the reaction mixture was quenched by shaking with 1 M HCl twice. The two phases were separated by centrifugation and the benzene layer was analyzed by GLC and occasionally by ^{1}H NMR. Before the ^{1}H NMR analysis the solutions were concentrated in vacuum. The relative amount of product or substrate in the reaction mixture was evaluated by triangulation of GLC peaks. This method as well as the quench technique was calibrated by using substrate/product mixtures of known composition, *i.e.* the mixtures were made by weighing varying amounts of substrate and product.

Calibration of the quench-extraction-GLC method gave maximum errors of ±0.5 % (absolute %) in the mixture composition (expressed as % of one component in the mixture). This error was used in the determination of the error in the rate constant.

When the quench-^{1}H NMR technique was used, error limits are only assigned to those runs where 1-methylindene has been used as a substrate, because only with this substrate was the technique calibrated. Every sample was integrated 5 − 10 times. From these data the mean value was calculated, and the maximum difference between the mean value and the integrals was used in calculation of the error in the rate constant. Calibration of the technique gave maximum errors ±1.5 % (absolute %) in the mixture composition.

All estimated errors are considered to be maximum errors, including both systematic and random errors ($\sim 2\sigma$).

2-Aminopyridine (*1-h*) (Merck) was distilled in vacuum and twice recrystallized from petroleum ether, b.p. 60 − 71 °C. GLC showed purity > 99.9 %.

N,N-*Dideutero-2-aminopyridine* (*1-d*) was prepared from pure 2-aminopyridine. A benzene solution was shaken with D_2O and the phases separated by centrifugation. This procedure was repeated four times. The degree of deuteration was found by NMR to be 94 ± 3 %.

1-Methylindene (*3-h*) was prepared[5a] and purified by preparative GLC. Analytical GLC showed that the purity was > 99.9 %.

1-Deuterio-1-methylindene (*3-d*) was prepared,[5a,b] purified and analyzed as above and was found to have a purity > 99.9 % and by NMR it was found to be > 99.5 % deuterated. In this last analysis a ^{13}C satelite was employed.

β-9-Fluorenylstyrene (*2*) was prepared[10] and analyzed by NMR and TLC. No trace of impurities was noticed.

3-Methylindene (*5-h*) was prepared[5c] and purified by preparative GLC. Purity was found to be > 99.9 % by analytical GLC.

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Synthetic Studies in the Alkaloid Field. Part III.* Selective Alkaline Decarboalkoxylative Cyclization of Some *N*-Alkyldihydro- and *N*-Alkyltetrahydropyridines

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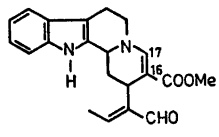
Selective alkaline decarboalkoxylative cyclization of three partially hydrogenated 1-[2-(3-indolyl)ethyl]-3,5-dimethoxycarbonylpyridine derivatives is described. The convenience of the method for the preparation of vallesiachotamine models is discussed.

In continuation of our studies concerning the preparation of indole alkaloid models of vallesiachotamine *1*[1] type we have examined the alkaline decarboalkoxylative cyclization[2] of three recently described,[3] partially hydrogenated 1-[2-(3-indolyl)ethyl]-3,5-dimethoxycarbonylpyridine derivatives *2*, *3*, and *4*. Since heretofore no dimethoxycarbonylpyridine derivative containing both methoxycarbonyl groups directly attached to the heterocyclic ring had been employed in the alkaline decarboalkoxylative cyclization, the preparation of indoloquinolizine derivatives from compounds of this type was of special interest. We hoped to accomplish selective decarboalkoxylation of the methoxycarbonyl group at position 3 of the starting materials while preserving the methoxycarbonyl group at position 5. Moreover, owing to the better conjugation possibility, it was hoped that in compound *4* the double bond would isomerize to the 3,4-position (corresponding to the 16,17-position in the biogenetic nomenclature of vallesiachotamine *1*) of the formed tetracyclic product.



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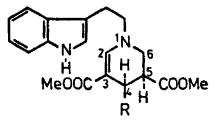

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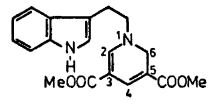

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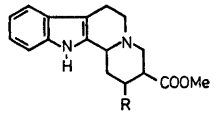
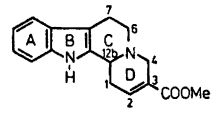


5 R = H

6 R = CH_3



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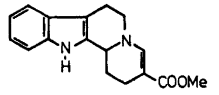

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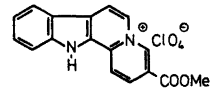

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Heating of the hydropyridine derivatives *2*, *3*, and *4* with aqueous alkali, a reaction known[2] to cause decarboalkoxylative cyclization (hydrolysis, decarboxylation, and cyclization), fol-

* Part II. Lounasmaa, M. and Johansson, C.-J. *Acta Chem. Scand. B 29* (1975) 655.

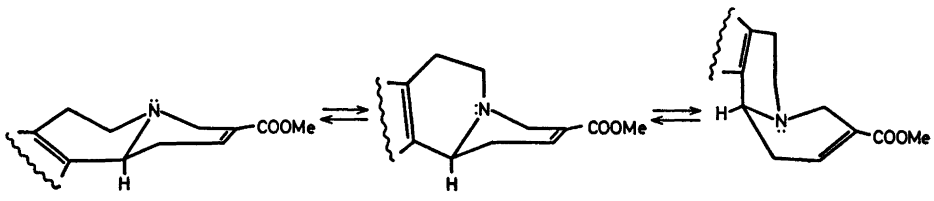
lowed by reesterification with methanolic acid, led to basic compounds which were purified by column chromatography. Compound *2* yielded two diastereoisomers of *5*, designated as *5a* and *5b*, whereas in the case of compound *3* only one of the possible diastereoisomers of 6, designated as *6a* was isolated. Compound *4* afforded *7*.

The correctness of the gross structures proposed for *5a*, *5b*, and *6a* was determined by direct comparison with samples of these substances from unambiguous syntheses.[4] A discussion concerning the stereochemistry of compounds *5a*, *5b*, and *6a* will be published later.

Dehydrogenation of compound *8*, prepared by sodium dithionite reduction of the recently described 1-[2-(3-indolyl)ethyl]-3-methoxycarbonyl pyridinium bromide [3] followed by acid-induced cyclization,[4] with palladium in aqueous maleic acid solution [5] yielded a hexadehydro compound, isolated as the perchlorate *9*. Reduction of the latter with sodium borohydride afforded a sample of *7* (*cf.* Ref. 5), which proved to be identical with *7* obtained from the alkaline decarboalkoxylative cyclization and reesterification of *4*. On the other hand, catalytic hydrogenation of the perchlorate *9*, which this time was prepared by palladium-maleic acid dehydrogenation of compound *7*, afforded compound *8*. Thus a reciprocal conversion between compounds *7* and *8* was accomplished.

Compound *7* can exist in three conformations, which are in equilibrium by nitrogen inversion and half-chair ring interconversion (Scheme 1, only one enantiomer is illustrated). The conformation with a *trans* diaxial C/D ring juncture is not possible. In all conformations examined, rings C and D are assumed by analogy with cyclohexene to be in the half-chair conformations.[6] Strictly speaking, however, this is a simplification, because rings C and D cannot both be at the same time exactly in the half-chair conformation. This becomes evident if one considers, for instance, conformer *a* (Scheme 1) where, with no deformation of the valence angles and overlooking the small difference between the C–N and C–C bond distances, the relationship between the C(12b) hydrogen and the lone electron pair of the basic nitrogen would be simultaneously *trans* diaxial (considering ring D) and *trans* pseudoaxial-axial (considering ring C). Thus, a slight deformation of the ideal half-chairs is to be expected.

Compounds of the above indoloquinolizine-type, possessing in their preferred conformation the C(12b) hydrogen and at least one more adjacent C hydrogen *trans* diaxial to the lone electron pair of the basic nitrogen, are known [7–11] to exhibit so-called Bohlmann bands in their IR spectra. The preference of the ring C of indoloquinolizines for the half-chair conformation (*cf.* above) tends to force the C(12b) substituents to pseudoaxial and pseudoequatorial positions. As a consequence, for most indoloquinolizines in the conformation where the relationship between the C(12b) hydrogen and the lone electron pair of the basic nitrogen is said to be *trans* diaxial, the relationship is not exactly *trans* diaxial but slightly deformed. This deformation, which is dependent *inter alia* on the ring D and its substituents, may have a slight influence on the intensity of the Bohlmann bands. However, this deformation has no practical meaning when the Bohlmann bands are being used for the determination of preferred conformations. The presence of Bohlmann bands in the IR spectrum of compound *7* indicates that conformer *a*, which possesses *trans*-



a b c

Scheme 1.

quinolizine juncture of the C/D rings, dominates the conformational equilibrium between *a*, *b*, and *c* (Scheme 1).

A study of the nonbonded interactions of each conformer, made with the aid of Dreiding models, reveals that in conformer *b* there is an interaction between the C(1) pseudoaxial hydrogen and the C(6) axial hydrogen, and in conformer *c* between the C(4) pseudoaxial hydrogen and the C(7) pseudoaxial hydrogen, whereas in conformer *a* no appreciable nonbonded interactions are present.

The preponderance of conformer *a* is also supported by the ^{1}H NMR. The absence of any signal downfield from δ 3.8 that could be assigned to the C(12b) hydrogen is characteristic of *trans*-quinolizine juncture (conformer *a*).[8, 10–13]

The selective decarboalkoxylation of the methoxycarbonyl groups at C(3) of the starting materials *2*, *3*, and *4*, with the preservation of the methoxycarbonyl groups at C(5), has thus been accomplished in all three cases. On the other hand, the desired direct isomerization of the double bond of compound *4* to the 3,4-position (enamine position) of the formed tetracyclic product did not take place. This isomerization could not be achieved by various modifications of the reaction conditions, nor could it be induced by treatment of the tetracyclic product *7* with *t*-BuOK in DMSO under carefully controlled conditions.[14] However, the conversion of compound *7* to compound *8* by dehydrogenation with palladium in aqueous maleic acid followed by catalytic hydrogenation indicates the applicability of the selective alkaline decarboalkoxylative cyclization method described above to the preparation of vallesiachotamine *1* models containing a $\Delta^{3(4)}$ double bond.

EXPERIMENTAL

The UV spectra were measured on a Perkin-Elmer 137 UV apparatus and the IR spectra on a Perkin-Elmer 237 apparatus. The NMR spectra were taken with either a Varian A-60 instrument or a Jeol JNM-PMX 60 instrument using TMS as internal standard. The mass spectra were recorded on a Perkin-Elmer 270 mass spectrometer at 70 eV using direct sample insertion into the ion source whose temperature was 80–90 °C. The melting points were determined in a capillary melting point apparatus (Büchi) and are uncorrected.

Decarboalkoxylative cyclizations

General procedure: A mixture of the tetrahydro- or dihydropyridine derivative and potassium hydroxide in methanol and water was refluxed with stirring under nitrogen for 45 h. The solvents were evaporated under vacuum and the residue dried in a vacuum desiccator for 18 h. The residue was then dissolved in abs. methanol and the solution saturated with dry hydrogen chloride gas during 2 h. The mixture was allowed to stand for 70 h and then slowly poured into a suspension of excess of sodium hydrogencarbonate in 200 ml of dichloromethane. The mixture was filtered and the filtrate evaporated under vacuum. The residue was extracted with dichloromethane and purified by column chromatography (Al_2O_3; act. IV).

Tetrahydropyridines. Reaction between 388 mg of diester *2* and 2.0 g of potassium hydroxide in 10 ml of methanol and 10 ml of water gave a mixture of two isomers which were separated by column chromatography.

5a. Yield 165 mg. M.p. 192–194 °C (methanol). IR, UV, NMR, MS, and TLC were identical with those of an authentic sample.[4]

5b. Yield 44 mg. M.p. 223–225 °C (methanol). IR, UV, NMR, MS, and TLC were identical with those of an authentic sample.[4]

Reaction between 334 mg of diester *3* and 2.0 g of potassium hydroxide in 10 ml of methanol and 10 ml of water gave a mixture which was fractionated by column chromatography and preparative layer chromatography.

6a. Yield 87 mg. M.p. 173–175 °C (methanol). IR, UV, NMR, MS, and TLC were identical with those of an authentic sample.[4]

A small fraction, which proved to be identical (IR, NMR, MS, and TLC) with the 60/40 mixture of the two diastereoisomers recently described (Ref. 3., gross structure XXIII), was also isolated.

Dihydropyridine. Reaction between 580 mg of diester *4* and 2.0 g of potassium hydroxide in 10 ml of methanol and 10 ml of water gave a tarry mixture which was fractionated by column chromatography and preparative layer chromatography.

7. Yield 28 mg. M.p. 180–182 °C (methanol). IR (KBr) NH 3370 (s), Bohlmann bands 2820 and 2765, C=O 1695 (s), C=C 1650 (m) cm^{-1}. IR ($CHCl_3$) Bohlmann bands 2820 and 2770, C=O 1710 (s), C=C 1660 (m) cm^{-1}. UV (EtOH 94 %) λ_{max} 205 (infl.) (ε 29 200), 224 (ε 40 700), 284 (ε 9140), and 292 (ε 8230) nm. λ_{min} 206 (infl.), 250, and 289 nm. NMR ($CDCl_3$) δ 3.75 (3H, s, $-COOCH_3$) and δ 7.78 (1 H, br s, ind. N–H). MS M^+ at *m/e* 282

corresponding to $C_{17}H_{18}N_2O_2$. Other important peaks at *m/e* 281, 170, and 169.

Small amounts of *5a*, *5b*, and 3,5-dimethyl dinicotinate (Ref. 3., structure VII) were also found, due to disproportionation.

Conversion between compounds *7* and *8*.

Salt 9. A mixture of 150 mg of *8*,[4] 130 mg of maleic acid and 150 mg of palladium-charcoal (10 %) in 15 ml of water was refluxed for 18 h under a stream of nitrogen. The solution was filtered and the filtrate evaporated under vacuum. The residue was dissolved in a saturated solution of sodium perchlorate to yield 90 mg of the salt *9*. M.p. 274–276 °C (dec.) (methanol). IR (KBr) NH 3280 (m), C=O 1730 (s), C=C 1635 (s) and 1555 (s) cm^{-1}.

A similar treatment of a mixture of 54 mg of *7*, 48 mg of maleic acid and 54 mg of palladium-charcoal (10 %) in 5 ml of water yielded 48 mg of the salt *9*.

1,4,6,7,12,12b-Hexahydro-3-methoxycarbonylindolo[2,3-a]-quinolizine 7. 130 mg of the salt *9* was dissolved in 30 ml of methanol and 300 mg of sodium borohydride was added portionwise. The mixture was stirred for *ca.* 2.5 h and the solution was evaporated under vacuum. The residue was dried in a vacuum desiccator overnight and then dissolved in 50 ml of abs. methanol. Dry hydrogen chloride gas was passed into the solution for 2 h and the mixture was allowed to stand at room temperature for 60 h. The solution was neutralized in a mixture of dichloromethane and sodium hydrogencarbonate. The inorganic materials were filtered off and the filtrate evaporated under vacuum. The residue was chromatographed on alumina (act. IV) to yield 12 mg of ester *7*. M.p. 180–182 °C (methanol). IR, UV, NMR, MS, and TLC were identical with those of the sample above.

1,2,6,7,12,12b-Hexahydro-3-methoxycarbonylindolo[2,3-a]-quinolizine 8. A mixture of 110 mg of the salt *9*, 200 mg of palladium-charcoal (10 %) and 0.2 ml of triethylamine in 200 ml of abs. methanol was hydrogenated for 24 h at atmospheric pressure. The catalyst was filtered off, the filtrate evaporated under vacuum and the residue extracted with dichloromethane. The extract was washed with water, dried over anhydrous sodium sulfate and evaporated under vacuum. The residue was chromatographed on alumina (act. IV) to yield 20 mg of ester *8*. M.p. 170–172 °C (methanol). IR, UV, NMR, MS, and TLC were identical with those of an authentic sample.[4]

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Lanthanide-induced Chemical Shifts in Proton NMR Spectra of 5,5-Dimethyl-2-oxo-1,3,2-dioxaphosphorinanes

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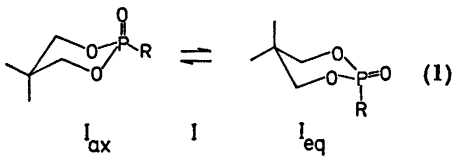
The conformational preference of the R substituent in twelve 5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinanes (I) is discussed on the basis of lanthanide shift NMR data. The results show that although lanthanide induced shifts might not be conclusive when considered isolated, they are of great value in connection with other NMR parameters, *i.e.* coupling constants and chemical shifts. In fact, the chemical shifts induced in the substrate upon complexation in some cases indicate the presence of conformers which cannot easily be detected by interpretation of coupling constants and ordinary chemical shifts. As far as only chair conformers are considered, it is concluded that the P=O bond is mainly axial for R = CH_3, CH_2Ph, Ph, $C(CH_3)_3$, CPh_3, $N(CH_3)_2$ and mainly equatorial for R = OCH_3, $OC(CH_3)_3$, OPh, SCH_3, Cl, F. There is evidence, however, that non-chair conformers might contribute to the true average conformation for I, R = $C(CH_3)_3$, CPh_3.

Information about the conformation in 2-oxo-1,3,2-dioxaphosphorinanes has been obtained by a number of different techniques [1] such as X-ray crystallography, dipole moment studies, IR spectroscopy, NMR chemical shifts and coupling constants. Most of the available information supports a chair conformer with the substituent on phosphorus axial.[2] There is evidence, however, that a conformational equilibrium exists in solution.[3]

For 5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinanes (I), the conformational equilibrium is given by (1).



I_{ax} I I_{eq} (1)

The use of lanthanide shift reagents (LSR) in structural and conformational analysis has been successful in a number of cases.[3] The strong electron donor ability of the P=O oxygen in I opens the possibility that equilibrium (1) can be studied by observing the chemical shifts induced in I upon complexation with an LSR. In fact, it was shown previously that lanthanide induced chemical shifts (LIS) allowed qualitative conclusions regarding the stereochemistry around phosphorus in I.[4] The purpose of this work is to obtain information about the orientation of the substituent at the phosphorus atom.

RESULTS AND DISCUSSION

Existing data on the conformation of I in solution agree with the equilibrium shown in (1).[2] In the discussion to follow it is presumed, unless otherwise stated, that the chair forms I_{ax} and I_{eq} are the only conformers contributing to the true average conformation of the molecule. On the basis of the available X-ray structural data this is a reasonable assumption.[5–7] Non-chair geometries have, however, been shown to exist in a few special cases.[8,9]

The application of LIS are based on the pseudocontact shift equation [10]

$$\Delta\nu_i = K(3\cos^2\theta_i - 1)R_i^{-3} \qquad (2)$$

where K is a constant, $\Delta\nu_i$ is the incremental chemical shift induced in proton H_i on complex-

ation, R_i the internuclear distance between H_i and the lanthanide atom, and θ_i the angle between the vector R_i and the symmetry axis of the LSR. Although eqn. 2 has been applied quantitatively,[11] its approximate nature should be kept in mind.

The chemical shifts induced in the hydrogens in I will depend on the relative contribution from I_{ax} and I_{eq}.[4] The shifts induced in the methylene protons should be particularly informative with respect to the position of equilibrium (1). A qualitative application of eqn. 2 shows that the difference in the chemical shifts induced in the two types of methylene protons should be larger for conformer I_{ax} than for I_{eq}. Apart from these shifts, additional information can be obtained from the LIS of other protons in the molecule. Useful information regarding the position of the conformational equilibrium is also provided by the *POCH* coupling constants.[8,12]

In this study the LSR europium(III)-tris-1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedione, $Eu(fod)_3$,[13] was applied to carbon tetrachloride or deuteriochloroform solutions of I. In all cases the induced chemical shifts were observed as a function of the molar ratio $\varrho = [LSR]/[substrate]$ at a constant substrate concentration, normally at 0.100 M. Representative LIS plots are shown in Fig. 1. Common for all LIS plots obtained is an approximately linear ν *vs.* ϱ dependence for low values of ϱ. In this region the ν/ϱ slope can be taken as a measure of $\Delta\nu$ caused by complexation. Assuming that the stoichiometry of the complex is one molecule of LSR to two molecules of I, it can be shown that the initial ν/ϱ slope (S) is approximately equal to twice the limiting shift of the 1:2 complex.[14] Whether the stoichiometry of the complex is 1:2 or 1:1 is not essential as the arguments given only depend on the existence of the same type of complex in the series of compounds studied, a reasonable assumption. In addition to geometry, S will depend on the strength of the interaction. While the LIS within a molecule reflects the difference in orientation with respect to the lanthanide in the complex, this need not be the case when comparing S-values obtained for different compounds. However, the qualitative picture of the LIS plots should be similar for compounds of type I if their conformation is the same, even if the strength of the interaction with the LSR differs between the members of the series.

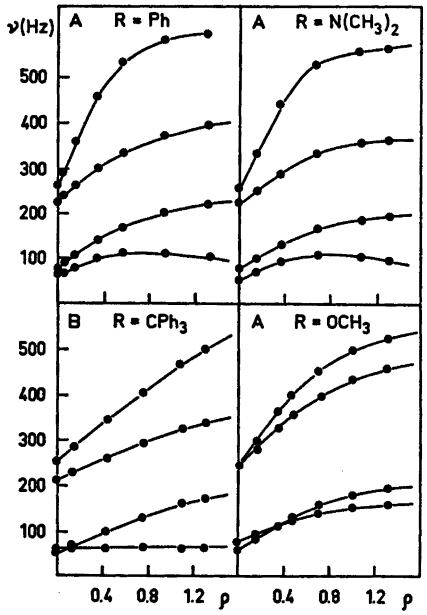


Fig. 1. Examples of ν/ϱ-curves for the methyl (high field) and methylene (low field) protons in compounds of type I. A and B denote carbon tetrachloride and deuteriochloroform, respectively.

The obtained S-values, chemical shifts and coupling constants are listed in Tables 1 and 2. The indices 1, 2, 3, and 4 denote, respectively, the highfield methyl signal, the low field methyl signal, the high field methylene signal, and the low field methylene signal. For the purpose of internal comparison of chemical shifts, the ratios S_3/S_1 and S_4/S_2 are also included in the table.

I [$R = CH_3$, CH_2Ph, *Ph*, $C(CH_3)_3$, $N(CH_3)_2$]. The qualitative LIS pictures of these compounds are very similar and therefore indicate a common conformation for these molecules in solution.

The LIS behaviour of 5,5-dimethylmethylenesulfite (II) is very similar to that observed for compounds *1–3*.[4] Since there is compelling evidence for the axial orientation of the S=O bond in this compound,[15–17] it can tentatively be concluded that I_{ax} is the main contributing conformer for compounds *1–5*.

Table 1. Initial ν/ϱ slopes (S-values)[a] for 0.1 M solutions of I at 25 °C.

Compound	R	Solvent[b]	S_1	S_2	S_3	S_4	S_2/S_1	S_4/S_3
1	CH_3	A	85	155	205	435	1.82	2.12
		B	55	115	145	295	2.09	2.03
2	CH_2Ph	A	70	110	150	310	1.57	2.07
		B	50	100	130	250	2.00	1.92
3	Ph	A	110	190	220	600	1.73	2.73
		B	35	135	145	315	3.36	2.17
4	$C(CH_3)_3$	A	110	150	200	490	1.36	2.45
		B	30	120	140	280	4.00	2.00
5	$N(CH_3)_2$	A	110	150	180	490	1.36	2.72
		B	100	110	140	290	1.10	2.07
6	OCH_3	A	170	110	250	350	0.65	1.40
		B	130	90	~240		0.69	
7	$OC(CH_3)_3$	A	120	80	170	290	0.67	1.70
8	OPh[c]	A	120	80	200	270	0.67	1.35
		B	90	60	~180		0.67	
9	SCH_3	A	120	90	170	240	0.75	1.41
10	Cl	A	110	160	220	260	1.45	1.18
		B	95	135	160	210	1.42	1.31
11	F	A	130	170	~240		1.31	
12	CPh_3	B	0	105	120	195		1.63
II		A	160	230	340	770	1.44	2.26

[a] In Hz/mol ratio shift reagent. [b] A and B denote carbon tetrachloride and deuteriochloroform, respectively. [c] 0.05 M in carbon tetrachloride solution.

Table 2. Chemical shifts[c] (ν) and *POCH* coupling constants[a] for compounds *1–12*.

Compound	Solvent	ν_1	ν_2	ν_3	ν_4	ν_{2-1}[b]	ν_{4-3}[c]	$\varrho=0$ $J(POCH)_3$	$\varrho=0$ $J(POCH)_4$	$\varrho=1$ $J(POCH)_3$	$\varrho=1$ $J(POCH)_4$
1	A	57.0	71.9	219	254	14.9	35	17	6	20	3
	B	60.7	69.7	227	254	9.0	27	15	8	19	3
2	A	50.2	53.3	214	251	3.1	37	17	5	20	2
	B	49.1	56.4	220	252	7.3	32	15	7	19	3
3	A	62.0	76.8	225	260	14.8	35	17	6	20	3
	B	67.9	71.2	233	259	3.3	26	13	10	19	5
4	A	52.2	74.0	216	261	21.8	41	18	3	19	2
	B	52.5	74.7	222	262	22.2	40	18	3	19	2
5	A	52.6	74.7	219	256	22.1	37	21	2	22	—
	B	53.9	74.2	227	258	20.3	31	19	4	21	—
6	A	55.5	75.3			19.8				~11	~11
	B	54.4	76.1			21.7					
7	A	54.7	73.1			18.4				14	~11
8	A	52.0	80.1			28.1				~11	~13
	B	55.0	80.4			25.4					
9	A	57.2	78.4			21.2				~11	15
	B	53.6	76.7			23.1					
10	A	58.0	82.0			24.0		27	3	27	3
	B	56.6	81.0			24.4		27	3	27	2
11	A	54.2	79.2			25.0					
	B	54.3	78.8			24.5					
12	B	48.7	60.4	209	255	11.7	46	14	6	19	2
II	A	53.2	79.3	199	275	26.1	75				

[a] In Hz at 60 Mz. [b] $\nu_{2-1}=\nu_2-\nu_1$. [c] $\nu_{4-3}=\nu_4-\nu_3$.

This conclusion is primarily based on the differentiation between the lanthanide induced chemical shifts of the methylene protons, as measured by the S_4/S_3 ratios. As seen from Table 1, this ratio is of similar magnitude for compounds *1 – 5* and clearly different from the values obtained for other members of the series, as for instance I (R = Cl). This is the case whether the solvent is carbon tetrachloride or deuteriochloroform.

Although the methyl protons are not expected to be particularly sensitive to the stereochemistry at the phosphorus atom, their LIS behaviour are in no contradiction to an axial orientation of the P = O bond.

There is generally a change to smaller S_4/S_3 ratios when changing the solvent from carbon tetrachloride to deuteriochloroform. This is in accordance with a change in the conformational equilibrium towards I_{eq} and that conformers like I_{eq} have a larger dipole moment than conformers like I_{ax}.[18] There is also a solvent effect on the S_3/S_1 ratios, which with the exception of compound *5* is towards higher values on going from carbon tetrachloride to chloroform. No attempt will be made to interpret this variation apart from noting that the largest effect is observed for the compound with the bulkiest substituent, $C(CH_3)_3$. The existance of non-chair conformers is therefore indicated.

The stereospecificity of the *POCH* coupling constants in the 2-oxo-1,3,2-dioxaphosphorinane series is now well documented.[3] Typical values for the considered compounds are 2 and 20 Hz, respectively, for axially and equatorially situated methylene protons. The large difference between the *POCH* coupling constants for compounds *1 – 5*, Table 2, is thus in agreement with the predominance of one conformer. The fact that these values converge when changing the solvent from CCl_4 to $CDCl_3$ corresponds to the observed displacement of the conformational equilibrium towards I_{eq} as was detected by the solvent induced variation of the S_4/S_3 ratios. There is also consistency between the LIS behavior and the *POCH* couplings in the sense that the smallest coupling constant is found for the low field methylene signal and *vice versa*.

The very small solvent induced variation of the *POCH* coupling constant for *4* is interpretable in terms of a complete dominance of I_{ax}.

With reference to the anisotropy effect caused by an axially oriented S = O bond in methylenesulfites,[17] it is reasonable to expect an analogous effect of the P = O bond in conformer I_{ax}. The relatively large chemical shift difference for compounds *1 – 5* can therefore be taken as additional evidence for the domination of the conformer with an axially oriented P = O bond.

A solvent induced variation of the ν_{3-1} and ν_{4-3} values clearly exists for compounds *1*, *2*, *3*, and *5*, Table 2. This is in accordance with a displacement of the conformational equilibrium, *i.e.* in correspondence with the effect of solvent on the LIS parameters and *POCH* coupling constants. In the case of compound *4*, however, no significant solvent effect is observed. This finding parallels the virtual constancy of the *POCH* coupling constants on variation of the solvent. If only conformers I_{ax} and I_{eq} are considered for compound *4*, it seems that the conformational equilibrium is totally displaced to the left. However, because of the steric requirements of the $C(CH_3)_3$ group, non-chair conformers cannot be excluded.

The dominant contribution of I_{ax} to the conformation of compounds *1 – 5* in solution agrees with infrared studies by Majoral *et al.* on derivatives *3* and *5*.[19,20]

Where meaningful comparison is possible, the axial preference of the P = O bond in the solution state is, with the possible exception of *5*,[3] in contrast to results obtained from X-Ray crystallography. Compound *3*, for instance, exists in the solid state in the form of a chair with an equatorially oriented P = O bond.[5] While there thus appears to be a lack in correspondence between the conformations adopted by *3* in the solid and the solution state, this is not the case for the phosphorus-sulfur analogue of *1*. ^1H, ^{13}C and ^{31}P NMR showed this compound to have the same conformation in solution[21] as in the solid state,[22] namely a chair with an axial P = S bond.

I [*R* = *OCH*$_3$, *OC(CH*$_3$*)*$_3$, *OPh*, *SCH*$_3$]. As can be seen from Fig. 1 and Table 1, the LIS behaviour of compounds *6 – 9* is very similar, indicating a common solution conformation for these compounds. The crossing of ν/ϱ-curves for the methyl signals is characteristic.

The small LIS discrimination of the methylene protons, as measured by the S_4/S_3 ratio, is another feature which is different from that observed for compounds *1–5*. On this basis it can be concluded, if only chair conformers are considered, that the contribution from I_{ax} cannot be dominant. Due to the small chemical shift separation between methylene protons, the effect of solvent on the S_4/S_3 ratio could not be measured. However, the S_2/S_1-values are not markedly affected by changing the solvent from CCl_4 to $CDCl_3$. Contrary to what was observed for compounds *1–5*, this observation indicates that the conformational equilibrium is not drastically changed upon solvent variation.

Due to the small chemical shift difference of the methylene hydrogens in compounds *6–9*, the *POCH* coupling constants could not be determined by first order analysis of the NMR spectrum. The values obtained in the presence of $Eu(fod)_3$ in CCl_4 solution are, however, of the same magnitude and therefore indicate competing contributions from I_{ax} and I_{eq}. Because of the possible effect of $Eu(fod)_3$ on the conformational equilibrium (as discussed below), the conformer ratio I_{ax}/I_{eq} might very well be smaller in the absence of the shift reagent. That this is at least the case for derivatives *7* and *8*, is shown by the available *POCH* coupling constants for these compounds, obtained by detailed analysis of their NMR spectra. The pairs of coupling constants were found to be (21.0 Hz, 3.3 Hz) [8] and (22.5 Hz, 1.7 Hz),[23] respectively, for *7* and *8* in deuteriochloroform solution, *i.e.* in agreement with an equatorially oriented P=O bond.

In analogy with the S_2/S_1 ratios, the ν_{2-1} values are very similar in the solvents CCl_4 and $CDCl_3$. This is in contrast to the solvent variation observed for compounds *1*, *2*, *3*, and *5* and indicates that the conformational equilibrium is not significantly changed upon solvent variation.

The fact that the chemical shifts for the methylene protons cannot be resolved is in agreement with the absence of an anisotropy effect of the P=O bond and consequently with a nonpredominating contribution of I_{ax}.

The P=O stretching frequencies observed in the infrared spectrum of *6* and *8* have by Majoral *et al.* been interpreted in terms of relative contributions from conformers I_{ax} and I_{eq}.[19] It was concluded that the compounds in benzene and pyridine solution were present largely in the form of I_{eq}, i.e. in agreement with the results above.

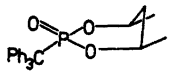
Available structural data on compounds related to I(R=OR′) together with the results above, indicate that the conformational situation is not drastically changed on going from solution to the solid state. In particular, 2-oxo-2-phenoxy-1,3,2-dioxaphosphorinane, was in the solid state shown to exist as a chair with an equatorial P=O bond.[7]

I (R=Cl, F). The S_4/S_3 value for compound *10* indicated a very small, if any, contribution from I_{ax}, and must therefore, if only chair conformers are considered, be present in solution as I_{eq}. The domination of one conformer is strongly supported by the very different *POCH* coupling constants, Table 2. Further evidence for this conclusion is provided by the near constancy of ν_{3-1}, the invariance of coupling constants on changing the solvent from CCl_4 to $CDCl_3$ and the fact that its infrared spectrum shows only one band which can be assigned to the P=O stretching frequency.[19]

Addition of LSR to a carbon tetrachloride solution of *11* did not result in chemical shift separation between methylene protons large enough to allow determination of S_3 and S_4. This small chemical shift discrimination induced by the LSR is similar to the situation for compound *10* in which case S_4/S_3 was found to be 1.18. The nearly identical values of ν_{3-1} for *10* and *11* are also consistent with a common conformation for these two compounds. By analogy therefore, it is concluded that *11* exists in solution as I_{eq}.

I (R=CPh$_3$). For solubility reasons compound *12* could not be studied in carbon tetrachloride solution. The S_4/S_3 value is in correspondance with a predominant contribution from I_{ax}. This is also consistent with the observed *POCH* coupling constants and the chemical shift difference between the methylene protons in the absence of shift reagent. However, the LIS behavior of the methyl signals, which are different from that observed for the other compounds studied whether the P=O bond is axially or equatorially oriented, indicates the presence of non-chair conformers. It is interesting to note that the type of LIS

behaviour observed for the methyl groups in *12* is also observed for *4* in $CDCl_3$ solution, although to a smaller degree. The fact that a bulky substituent is present in both these compounds supports the assumption of a contribution from non-chair conformers to the average conformation of the molecule. The relevance of steric effects for this type of substituents is demonstrated by the solid state conformation of compound III. X-Ray structural studies showed this compound to exhibit a "chaise longue" conformation.[24]



III

The effect of shift reagent on the conformational equilibrium. From the observation that a change of solvent from CCl_4 to $CDCl_3$ might result in displacement of the conformational equilibrium (1), the possible influence of the shift reagent on this equilibrium should be considered. Such an influence has previously been noted for *1–3* [4] and some related compounds.[25–27] As seen from Table 2, the difference in the observed *POCH* coupling constants for compounds *1–5* and *12* is increased upon addition of the shift reagent. Similarly, a convergence of coupling constants was noted for derivatives *7* and *8*. In both cases this means that equilibrium (1) is displaced towards I_{ax}. The effect can be explained in terms of a non-negligible difference in the basicity of the P=O oxygens in conformer I_{ax} and I_{eq}. Support for this explanation is provided by the fact that the P=O stretching frequency for compounds of the considered type have been shown to be lower for I_{ax} than for I_{eq}.[19] This is in correspondance with a greater polarity of the P=O bond in I_{ax}, a result which is in agreement with molecular orbital CNDO/2 calculations.[28] The effect of $Eu(fod)_3$ on equilibrium (1) can therefore be interpreted in terms of a larger equilibrium constant for the interaction of the LSR with I_{ax} as compared to I_{eq}, thus leading to a displacement of (1) to the left.

This finding is related to the effect of BF_3 on the relative contribution from conformers I_{ax} and I_{eq}.[29] Thus for compounds *3*, *5* and *8* it was shown that addition of BF_3 induced changes in the ^1H and ^{31}P NMR parameters which were consistent with displacement of equilibrium (1) towards I_{ax}.

Although quantitative results cannot be obtained in the cases where the applied shift reagent distorts the equilibrium under study, the qualitative conclusions drawn above should still be valid. It can therefore be concluded that lanthanide induced chemical shifts can be a valuable tool for gaining information about the solution conformation in compounds of type I.

EXPERIMENTAL

Synthesis and reagents. Compounds *1–12* were prepared according to well-known procedures [30] and characterized by their melting points and ^1H NMR spectra.

Spectroscopic grade carbon tetrachloride and deuteriochloroform were stored over molecular sieves. The shift reagent $Eu(fod)_3$ was kept over P_2O_5 in vacuum before use.

Instrumentation. ^1H NMR spectra were recorded on a JEOL JNM-C-60H spectrometer operating at 60 Mz.

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Fluorescence Characteristics of Lignin Model Compounds. I. Styrene Derivatives

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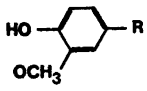
The UV fluorescence and excitation spectra of some lignin models with styrene skeleton (*2* – *9*) have been investigated. Auxochromic groups in the β-position cause minor changes in the spectroscopic properties of the styrene chromophore, whereas *para*-substitution significantly affects both the position of the 0 – 0 band and the Franck-Condon transition. A methoxyl group in the *meta*-position to the propene side chain alters the vibrational pattern of the spectrum, but the energy shifts remain unaltered. The intensity of emission is great upon excitation in the α-band, but shows a major decrease on excitation in the *A*-band for all the compounds. It is concluded that an increase in the rate constants for some similar nonradiative processes is the reason for the fall in emissive intensity on scanning the exciting wavelength from the α-band into the *A*-band. A corrigendum to previously reported pK_a^*-values for isoeugenol *7* and coniferyl alcohol *9* is included. They are estimated to be 4.0 and 3.1 ± 0.5, respectively.

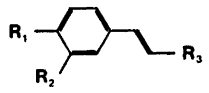
Most aromatic elements in softwood lignins are of the guaiacyl type (1-hydroxy-2-methoxyphenyl type) carrying different substituents in the 4-position (*1*).



1

Substances with related structures are frequently used as lignin models.[1] This is a report on the UV-fluorescence characteristics of lignin models with a styrene skeleton (*2* – *9*).

A preliminary communication on the room temperature fluorescence spectra of these compounds is given in Ref. 2.



2-9

	R_1	R_2	R_3
2	H	H	H
3	H	H	CH_3
4	H	H	CH_2OH
5	OCH_3	H	CH_2OH
6	OCH_3	H	CH_3
7	OH	OCH_3	CH_3
8	OCH_3	OCH_3	CH_3
9	OH	OCH_3	CH_2OH

EXPERIMENTAL

Emission and excitation spectra were measured on a Hitachi Perkin-Elmer spectrofluorimeter model MPF-2A. The spectra were corrected by a procedure described previously.[3] The excitation spectra were measured as the total emissive intensity perpendicular to incident light on solutions (5 – 20 ppm) in ethanol and cyclohexane at 298 K in 1 cm cells, and ni ethanol at 77 K in a cylindrical cell. The solvents were checked for spectral purity before use. The spectra were measured on freshly prepared deaerated solutions. The ethanol was Alko AaS spectrograde. The cyclohexane was purified by procedures outlined by Perrin *et al.*[4] Styrene *2*, manufactured by Shell Ltd. (Holland), was chromatographically pure. *trans*-1-Phenyl-1-propene *3* was Fluka *purissimum* grade. Cinnamic alcohol *4* and anethole *6* were Fluka *purum* grade, purified by recrystallization and distillation *in vacuo*, respectively. Isoeugenol methyl ether (1,2-dimethoxy-4-

propenylbenzene) *8* was Merck *purissimum* grade, purified by distillation *in vacuo*. 4-Methoxycinnamic alcohol *5* was synthesized by standard procedures and purified by recrystallization from ether-petroleum ether, m.p. 80 °C. Isoeugenol *7* and coniferyl alcohol (3-(4′-hydroxy-3′-methoxy)-2-propene-1-ol) *9* were purified as described previously.[5]

RESULTS AND DISCUSSION

In the UV-absorption spectra of the compounds under investigation two bands can be recognized at wavelengths greater than 220 nm.[5] The strong bands at about 250 – 270 nm have been assigned to interaction between the conjugated side chain and the benzene nucleus, or $p-\pi^*$ bands resulting from the transfer of p-electrons from oxygen substituents (in OH and OCH_3) to the benzene ring, or combinations of these bands, whereas the weak bands at 270 – 310 nm are due to transitions of the local excitation type occurring in the benzene nucleus ($\pi-\pi^*$ benzenoid bands).[7,8] The former band is referred to as the A-band, ^1L_a (K-band), the latter as the α-band, ^1L_b (B-band).[9] The α-band shows a well-defined vibrational structure in absorption spectra of styrene *2*, 1-phenylpropene *3* and cinnamic alcohol *4* at room temperature. The corresponding vibrational pattern of the α-band is recognizable in excitation spectra of the ring substituted compounds measured at 77 K in ethanol solution.

Inspection of the fluorescence, absorption and excitation spectra with respect to the mirror symmetry wave number [10] reveals the following facts. In spectra of a particular compound measured in ethanol at 77 K or in cyclohexane at 298 K the 0 – 0 bands coincide within the limits of error. The Franck-Condon transitions in the emission spectra are sensitive to the variations in solute-solvent interaction.[11] In Table 1 the Stokes' losses in spectra measured in cyclohexane solution at room temperature are given. They are defined as the energy difference between the observed 0 – 0 bands and the Franck-Condon transitions ($\bar{\nu}_{max}$ of fluorescence). The Stokes' loss in ethanol is greater than in cyclohexane. For compounds *4*, *5*, *6*, and *8*, which contain no phenolic hydroxyl group, this increase is small, 0 – 100 cm^{-1}, but it amounts to 250 cm^{-1} for isoeugenol *7* and 600 cm^{-1} for coniferyl alcohol *9* (Table 1).

The characteristics of the fluorescence spectra measured in ethanol solution at 77 K are also listed in Table 1. Vibrational fine structure is found in all the spectra, a pattern of five bands can be recognized, either as shoulders or as maxima. It is also noteworthy that the 0 – 0 band is recognizable in the spectra. In spectra of the polar solutes run at higher temperatures in ethanol or cyclohexane solution the vibrational structure is obscured. Typical fluorescence spectra at 77 K in ethanol are seen in Fig. 1 – 3. No phosphorescence could be detected for the compounds investigated.

For most molecules in condensed media, the fluorescence quantum yield is independent of the wavelength of the exciting light, and an excitation spectrum gives the same information as an absorption spectrum.[12] Fluorescence quantum yield dependence of the wavelength of the exciting light has been found for retinals.[13,14]

Table 1. Fluorescence characteristics of lignin model compounds with a styrene skeleton.

	Maxima (italics) or shoulders in fluorescence emission spectra in ethanol at 77 K (cm^{-1})					Stokes loss in cyclohexane at 298 K (cm^{-1})	Increase of Stokes loss in ethanol solution at 298 K with respect to Stokes loss in cyclohexane (cm^{-1})
2	*34 246*	*33 222*	*32 786*	32 258	31 746		
3	*34 013*	*33 003*	*32 467*	31 847	31 446		
4	*34 130*	*33 003*	*32 467*	31 948	*31 545*	1557	
5	32 787?	*32 258*	*30 960*		29 761	2206	94
6	32 280?	*32 051*	30 864	*30 581*	29 585	1885	92
7	32 467	*31 446*	30 910	*30 120*	28 818	2437	541
8	32 786	*31 847*	*31 152*	*30 487*	29 940	2840	90
9	32 362	*31 348*	*30 864*	*30 030*	28 901	2332	660
	±200	±200	±200	±200	±200		

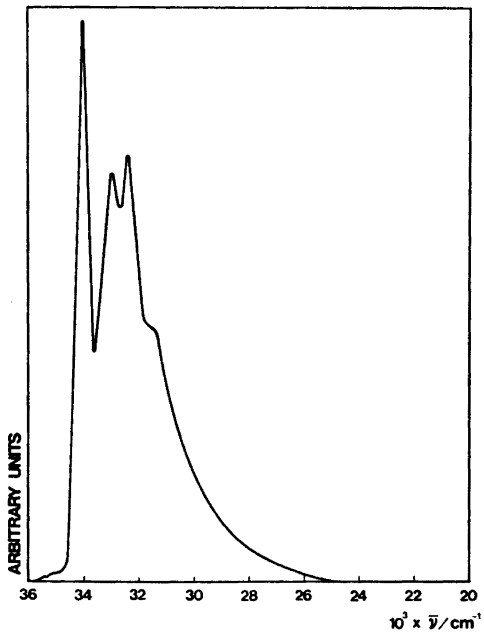


Fig. 1. Fluorescence emission spectrum of *trans*-1-phenyl-1-propene *3* at 77 K in ethanol solution.

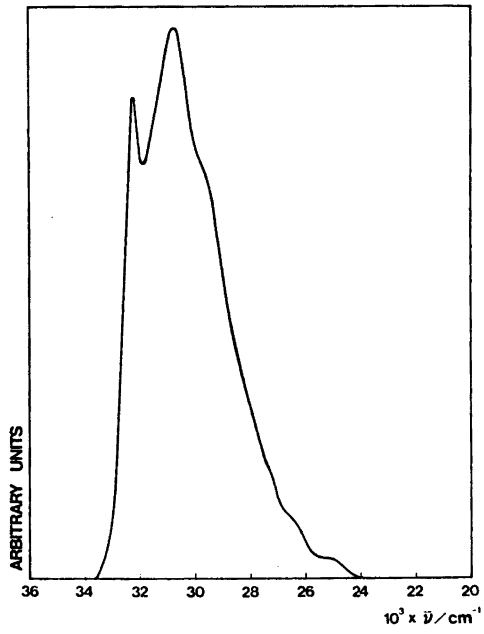


Fig. 2. Fluorescence emission spectrum of anethole *6* at 77 K in ethanol solution.

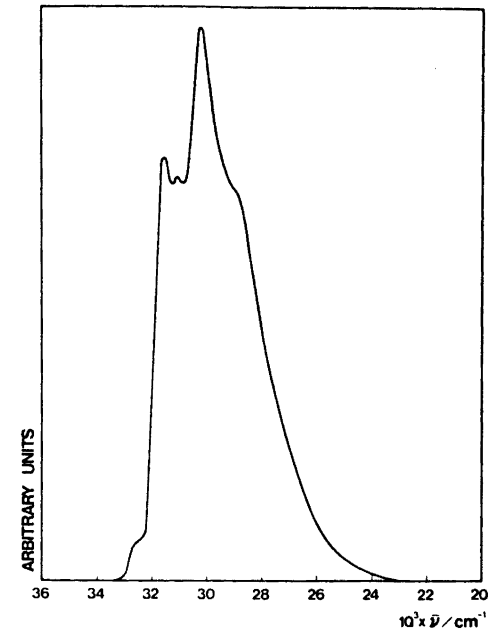


Fig. 3. Fluorescence emission spectrum of coniferyl alcohol *9* at 77 K in ethanol solution.

In gas phase investigations on the *cis-trans* isomerization of 1-phenyl-propene Rockley and Salisbury [8] found that the quantum yield of fluorescence is strongly dependent on the excitation wavelength, remaining constant throughout the α-band, and falling to approximately zero in the *A*-band. They assume that a rapid increase in the rate constants for some non-radiative process(es) is the reason for the rapid fall in the fluorescence quantum yield as the excitation wavelength moves from the α-band into the *A*-band.

In the present investigation the excitation spectra of compounds *2* – *9* were measured in cyclohexane at room temperature and in ethanol at 77 K. In all the spectra it was found that the intensity of emission is strong on excitation in the α-band, and that the emission shows a minimum about the λ_{max} absorption wavelength in the *A*-band. The possibility that this behaviour of model compounds *2* – *9* is due to impurities is excluded on the same grounds as discussed by Christensen and Kohler.[13]

It has been shown that there is little, if any, increased interaction between the double bond and the benzene ring on excitation in the α-band of styrene.[15] It was found that the excita-

Table 2. Excitation characteristics of lignin model compounds with a styrene skeleton.

	Maxima in the α-band of excitation spectra of compounds *2* – *9* at 77 K in ethanol (cm^{-1})		
2	36 496	35 460	34 365
3	36 101	35 088	34 013
4	36 300	35 211	34 130
5		34 013	32 787
6	34 364	33 670	32 467
7	33 898	33 222	32 573
8	33 300	32 788	32 088
9	33 113	32 573	31 847

tion spectra of *2*, *3* and *4* are similar. Compounds *5* – *9* with substituents on the aromatic ring show the same type of difference in absorption and excitation spectra as compounds *2* – *4*. Evidently the slightly electron donating groups in *para*-position to the side chain and the slightly withdrawing groups in the *meta*-position do not affect the interaction between the propene side chain and the aromatic ring. It is concluded that in the solution spectra of styrene *2* and its derivatives *3* – *9* an increase in the rate constants for some nonradiative process(es) is the reason for the rapid fall in emissive intensity on scanning the excitation wavelength from the α-band into the *A*-band, and that the nature of the non-radiative process(es) probably is similar in all the cases.

The characteristics of the excitation spectra measured in ethanol solution at 77 K are listed in Table 2.

The excitation spectra show some vibrational fine structure which is not seen in the absorption spectra due to overlap between the α- and *A*-bands. It is found that the vibrational spacing of the bands in the excitation spectra corresponds to the spacing of bands found in the fluorescence emission spectra.

In previous papers [5,16] the excited state pK_a-values (pK_a*-values) for a number of lignin phenols have been reported. The calculation was performed using the Förster cycle [17] on values from the structureless absorption and emission spectra in aqueous alcoholic solution. In accordance with the above discussion it seems more justified to estimate the pK_a*-values for isoeugenol *7* and coniferyl alcohol *9* with the wavenumber of the center of gravity of the α-band in the excitation spectra as the value of $\bar{\nu}_A$ and $\bar{\nu}_A'$, respectively. The corrected pK_a*-values of isoeugenol *7* and coniferyl alcohol *9* will be 4.0 and 3.1 ± 0.5, respectively.

The model compounds contain auxochromic groups of three different types: (i) substituent groups attached to the β-carbon atom of the side chain, (ii) substituent groups in the *para*-position to the side chain in the aromatic ring, and (iii) substituents in the *meta*-position of the aromatic ring.

The incorporation of an auxochromic substituent into the styrene chromophore causes significant changes in the fluorescence spectra as well as in the absorption spectra. The substituents of the first type (i) cause only small increases in Stokes loss, and the position of the 0 – 0 band remains almost unaltered as compared to styrene. Substituents of cathegory (ii) shift the position of the 0 – 0 band more than 1300 cm^{-1} (10 nm) towards smaller wavenumbers. The shift is largest for the phenolic compounds *7* and *9*, 2000 cm^{-1} (17 – 18 nm), a fact correlating with the greatly enhanced acidity of these compounds in the excited state. The similarity between a hydroxyl group and a methoxyl group as regards auxochromic properties is great enough to make compounds 5 – 9 analogous with respect to fluorescence emission.

The introduction of a group (iii) substituent, the *meta*-methoxyl group typical for softwood lignins, causes only a minor shift of the 0 – 0 band and a small change in the Stokes loss in the spectra. The vibrational pattern is, however, altered by introduction of the *meta*-methoxyl group. This corresponds to the findings of Aulin-Erdtman and Sandén.[7] The effect is related to the effect of the *meta*-methoxyl groups on the excited state acidity of lignin phenols reported previously.[16]

The fluorescence intensity of the investigated compounds is linearly dependent on concentration within the range 0.01 – ~50 ppm. Even smaller concentrations can be detected by fluorescence measurements. No sign of excimer formation could be detected within this concentration range.

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Cathodic *t*-Butylation of Pyrene

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Cathodic reduction of pyrene in *N*,*N*-dimethylformamide in the presence of *t*-butyl chloride yields 1-*t*-butylpyrene in 52 % yield. Besides this compound minor amounts of 4-*t*-butyl-4,5-dihydropyrene, 1,6-di-*t*-butylpyrene and 1,8-di-*t*-butylpyrene are formed. Autoxidation of the intermediately formed dihydropyrenes is observed. Variations in the product composition have been noted depending on supporting electrolyte and water content.

The reaction between aromatic anion radicals, formed by reduction of the aromatic compound with alkali metal [1–7] or electrolytically,[8] and alkyl halides has been studied extensively. The reaction mechanism for the chemical and electrochemical reductive alkylation has been shown generally to involve a coupling between the alkyl radical and the aromatic anion radical.

The coupling reaction and the reduction of the alkyl radical by the anion radical are competing reactions,[7,8] and it has been shown that during the electrolytic reductive coupling the catalytic reduction of the alkyl halide is more pronounced for primary alkyl halides than for tertiary.[8]

Tertiary halides are thus well suited for reductive alkylation reactions, and anthracene, stilbene, and naphthalene have previously been *t*-butylated by this means.[8]

1-*t*-Butylpyrene (*1*) has been found difficult to prepare by classical methods; thus Friedel-Crafts alkylation of pyrene with *t*-butyl chloride leads exclusively to 2,7-di-*t*-butylpyrene,[9] whereas a Friedel-Crafts reaction using pivaloyl chloride gives a mixture of 2-*t*-butylpyrene and 1-pivaloyl pyrene.[10] Attempts to prepare *1* from 1-fluoropyrene and *t*-butyllithium following a procedure analogous to the one used by Huisgen[11] to prepare 1-*t*-butylnaphthalene gave only a very small amount of a difficultly separable mixture of 1- and 2-*t*-butylpyrene.[10]

The purpose of the present study was to investigate whether compounds which could easily be transformed into 1-*t*-butylpyrene could be prepared by electrolytic reductive alkylation.

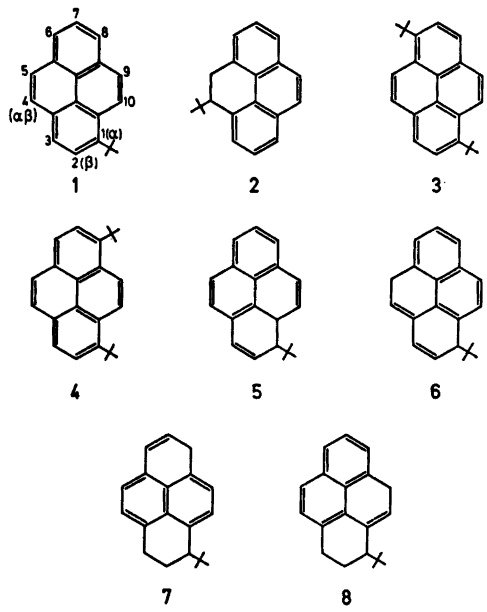
RESULTS AND DISCUSSION

Pyrene gives in cyclic voltammetry in *N*,*N*-dimethylformamide (DMF) two cathodic peaks [$E_p^1 = -2.13$ V; $E_p^2 = -2.74$ V (SCE)]; to the first one corresponds an anodic peak, whereas it has not yet been possible to obtain an anodic peak corresponding to the reoxidation of the pyrene dianion. The first redox reaction, the formation and reoxidation of the anion radical, is reversible, and the anion radical is stable for a long time as most other anion radicals of aromatic hydrocarbons.[12]

Addition of *t*-butyl chloride to pyrene in DMF containing tetrabutylammonium iodide (TBAI) gave only a small increase in the peak height of the first wave, indicating that the reaction between the pyrene anion radical and *t*-butyl chloride is a rather slow one. A new peak at -2.6 V may be due to a coupled product or may be a prepeak to the second peak.

Controlled potential reduction in anhydrous DMF/0.1 M TBAI of pyrene in the presence of *t*-butyl chloride at -2.0 V (SCE) gave a mixture of 1-*t*-butylpyrene (*1*), 4-*t*-butyl-4,5-dihydropyrene (*2*), 1,6-di-*t*-butylpyrene (*3*), and 1,8-di-*t*-butylpyrene (*4*), which were subjected to column chromatography.

1–*4* were identified by ^{1}H NMR, IR,[13] and



MS spectroscopy, and *2* was, furthermore, oxidized to 4-*t*-butylpyrene by 2,3-dicyano-5,6-dichlorobenzoquinone (DDQ).

On reductive alkylation generally hydro derivatives are obtained; the isolation of *1*, *3*, and *4* points to an oxidative aromatization during work up. Such an oxidation might also be responsible for the observed extensive tar formation that seems to occur on exposure of the reaction mixture to air. Aromatization by chloranil in the reduction cell immediately after reduction leads to good yields of the compounds (*1* – *4*), and hardly any tar formation is observed.

Work-up under oxygen-free conditions revealed the presence of dihydropyrenes rather than the fully aromatic pyrenes. The dihydropyrenes have tentatively been assigned the structures 1-*t*-butyl-1,10a-dihydropyrene (*5*) and 1-*t*-butyl-1,5-dihydropyrene (*6*) on the basis of ^{1}H NMR spectra. They are stable under oxygen-free conditions, but are oxidized in contact with air within a few hours. Besides the stable 4,5-dihydropyrene only the unstable 1,9-dihydropyrene, prepared by reduction of pyrene with lithium in liquid ammonia, is known.[14]

The formation of *1* (through *5* and/or *6*) and *2* from pyrene is easily explained from the general reaction scheme given previously.[8]

This scheme is supported by the fact that in the presence of a 4-fold excess of naphthalene no butylated naphthalene derivatives were formed. This observation makes reactions of the *t*-butyl radical with the nonreduced aromatic species less probable.

Addition of small amounts of water (0.2 – 1.0 %) did not change the product composition much although more highly reduced compounds occurred and a decrease in the amount of *2* with increasing amounts of water is observed. When D_2O was added (1 %) instead of water no incorporation of deuterium in the products *1* – *4* occurred. These findings support the assumption that *t*-BuCl is the proton donor.

The mechanism of the formation of the dibutylated pyrenes is less clear. The anion, *t*-BuPy$^-$, Py = pyrene, could react with *t*-BuCl in a S_N-manner with subsequent aromatization, but it seems more likely that it is oxidized to *1*, which could then be reductively alkylated following the general scheme. The latter possibility was strongly supported by the fact that *3* and *4* besides a series of *t*-butylated dihydropyrenes were produced by reductive alkylation of *1*. However, no evidence of the mechanism of an aromatization in the catholyte is available.

When LiCl was used as supporting electrolyte the product distribution changed because of the different ion pairing and solvation properties of Li^+ as compared with Bu_4N^+. Besides *2* the only major compounds were the *t*-butyl derivative of each of the two tetrahydropyrenes: 1,2,3,8 and 1,2,3,9, respectively, as shown by the ^{1}H NMR spectra. They are formed in the ratio 2:1. The *t*-butyl group is not in an aromatic or vinylic position. Most probably it occupies position 1 in both compounds as shown in *7* and *8*. On exposure to air, a solution of these compounds turns green to greenish brown. In the oxidized mixture considerable amounts of *1* are formed.

Compounds with similar properties are formed on reduction of pyrene by sodium in liquid ammonia:[15] 1,2,3,8-tetrahydropyrene and 1,2,3,9-tetrahydropyrene are formed in the ratio 2:1. Exposure to air of these compounds leads, however, to a stable 6,7,8-trihydropyrenyl radical.[14]

Reductive alkylation of pyrene in DMF/0.1 M LiCl with 0.5 % D_2O added gave *1* – *4* with 20 – 40 % incorporation of deuterium (MS,

^{1}H NMR), reflecting the competition between *t*-BuCl and hydrated Li^+ as proton donors. The occurrence of deuterium in position 9 in *1* points towards 1-*t*-butyl-1,9-dihydropyrene as a likely intermediate in this reaction. In *2* incorporation took place in position 5.

About the same ratio of α- to α,β-substituted compounds was found whether TBAI (4:1) or LiCl (3:1) served as supporting electrolyte. Thus, coupling takes place preferentially at the position of highest electron density [16] in the pyrene radical anion.

Steric factors have been observed to be important in the reductive alkylation of naphthalene and anthracene.[8] Steric effects, therefore, would be expected to make coupling at the β-positions in pyrene more favourable, but no β-substituted products are observed.

The results reported indicate that electrolytic reductive alkylation is a feasible method for preparation of 1-*t*-butylpyrene. By variation of the procedure with respect to work-up, supporting electrolyte, water content, or potential, further reduced products can be obtained.

EXPERIMENTAL

Electrolytic reductive alkylation. The electrolytic cell described by Iversen [17] was used. *N,N*-Dimethylformamide (DMF) was dried over Molecular Sieves (4A).

The reduction potential was kept near the polarographic half-wave potential of pyrene rather than at the plateau of the wave in order to avoid further reduction of the dihydropyrenes which are reduced at potentials 0.2 – 0.3 V more negative than that of pyrene.

1. *Pyrene. TBAI as electrolyte.* Pyrene (2.02 g, 0.01 mol) was reduced at −1.5 V (*vs.* Ag/AgI, 0.1 M TBAI) in 170 ml of DMF containing TBAI (0.1 M) and *t*-butyl chloride (20 ml, 0.18 mol); $n = 2 - 2.5$ F/mol. The reduction completed, chloranil (4.92 g, 0.02 mol) was added to the catholyte. The stirred solution was allowed to stand for 2 h and then poured into water and acidified. The mixture was extracted five times with 200 ml portions of toluene. After washing and drying the toluene was evaporated. The dark redbrown oily product was cleared of tarry materials and chloranil hydroquinone on a short column (alumina) and subsequently separated by column chromatography on silica mixed with 10 % caffeine.[18] As eluent served petrol ether (b.p. below 50 °C). The following products were isolated in the order *2*, *3*, *4*, *1* (yields in mol % relative to pyrene):

1-t-*Butylpyrene* (*1*) (52 %), m.p. 100.0 – 100.5 °C. ^{1}H NMR (CS_2): δ 1.70 (s, 9 H), 7.4 – 8.1 (m, 5 H), 7.81 (s, 2 H), 7.89 (d, $J = 9.8$, 1 H), 8.57 (d, $J = 9.8$, 1 H). IR (KBr) cm^{-1} (intensity): 835 (s), 820 (m), 812 (m), 750 (m), 720 (m), 672 (m). MS: 258 (M^+), 243 (−15).

4-t-Butyl-4,5-dihydropyrene (*2*, mixed with *3*) (14 %). ^{1}H NMR (CS_2): δ 0.65 (s, 9 H), 2.87 (1 H), 3.35 (1 H), 3.41 (1 H), 7.10 – 7.75 (m, 6 H), 7.51 (s, 2 H). MS: no molecular ion was observed.

1,6-Di-t-butylpyrene (*3*, mixed with *2*) (2.5 %). ^{1}H NMR (CS_2): δ 1.71 (s, 18 H), 7.78 (d, $J = 9.8$, 2 H), 7.92 (4 H), 8.57 (d, $J = 9.8$, 2 H). MS: 314 (M^+), 299 (−15).

1,8-Di-t-butylpyrene (*4*) (0.8 %) m.p. 186 – 187 °C. ^{1}H NMR (CS_2): δ 1.77 (s, 18 H), 7.81 (s, 2 H), 7.95 (4 H), 8.58 (s, 2 H). IR (KBr) cm^{-1} (intensity): 845 (s), 825 (m), 807 (w), 760 (w), 731 (m). MS: 314 (M^+), 299 (−15). A reduction was performed with zinc dust in the anode compartment to avoid a possible diffusion of oxidants from the anode compartment to the catholyte, but the yield of dibutylated products did not change.

In order to observe the dihydropyrenes *5* and *6* the catholyte, in a separate experiment, was poured directly into a saturated solution of sodium dithionite. The extraction, washing and evaporation was performed in a nitrogen atmosphere. No separation was attempted.

2. *Pyrene. LiCl as electrolyte.* Pyrene (2.02 g, 0.01 mol) was reduced at −1.55 V (*vs.* Ag/Ag^+, 0.1 M TBAI) in 170 ml DMF containing LiCl (0.1 M) and *t*-butyl chloride (20 ml, 0.18 mol); $n = 2.5 - 3$ F/mol. The catholyte was worked up as described above except that no chloranil was added. The reaction mixture was not separated, but investigated as such. Ratios between *2*, *7*, and *8* have been estimated from the ^{1}H NMR integrals of the *t*-butyl groups. No satisfactory integration of the other parts of the spectrum was obtained.

Mixture of *1*-t-*butyl-1,2,3,8-tetrahydropyrene* (*7*) and *1*-t-*butyl-1,2,3,9-tetrahydropyrene* (*8*) (75 %). ^{1}H NMR (CS_2): δ 0.80 (s), 0.82 (s), 1.5 – 2.7 (m), 3.85 (broad s), 5.53 (t, $J = 4.0$), 5.73 (dt, $J_1 = 9.8$, $J_2 = 4.0$), 6.40 (dt, $J_1 = 9.8$, $J_2 = 2.0$), 6.70 – 7.20 (m). Compound *2* (25 %).

3. *1-t-Butylpyrene. TBAI as electrolyte. 1* (0.525 g, 0.002 mol) was reduced and worked up as for pyrene except for the addition of chloranil after reduction. Separation yielded the following hydrocarbons (yields, in mol %, include also amounts in mixed fractions as estimated from the NMR-spectra): *3* (8 %), *4* (19 %), *1,3,6-tri-t-butylpyrene* (6 %), *1,4-di-t-butyl-4,5-dihydropyrene* (a), *1,5-di-t-butyl-4,5-dihydropyrene* (b), and *1,9-di-t-butyl-9,10-dihydropyrene* (c), a + b + c = 23 %). Traces of the last three compounds were also seen in the products from pyrene.

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Protonation and Deprotonation of Enamines. A Convenient Way of Separating Isomeric Enamines Obtained from Methyl Isopropyl Ketone and Morpholine

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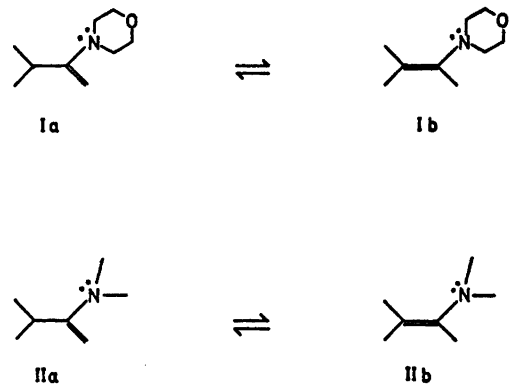
Enamines from methyl isopropyl ketone were converted to the corresponding immonium salts using hydrogen chloride or trifluoroacetic acid. The salts were characterized by their ^{1}H NMR, ^{13}C NMR and mass spectra. The morpholine immonium salt is readily deprotonated to the least substituted enamine isomer by treatment with sterically hindered base. By careful dosage of acid the most substituted morpholine enamine isomer can be left unreacted in solution, and can easily be separated from the other isomer. A convenient procedure for this separation is described.

Preparation of enamines from unsymmetric ketones usually affords mixtures of tautomeric enamines. In some cases it is possible to obtain one isomer in a pure state, using titanium tetrachloride and a hydrocarbon solvent.[1] The yield using this method is moderate due to the conditions necessary for avoiding rearrangement of the kinetically favoured product. Immonium salts may be intermediates in the formation of an enamine from a ketone and a secondary amine. The aim of the present investigation is to study if proton abstraction from the immonium salt primarily yields one isomer, *viz* the same isomer as using titanium tetrachloride and *excess* of secondary amine. Model substances were the morpholino and dimethylamino enamines from methyl isopropyl ketone (Scheme 1).

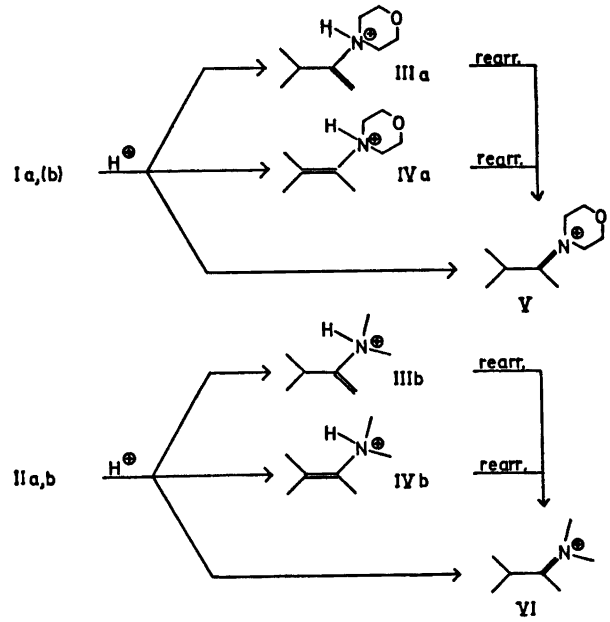


Scheme 1.

RESULTS AND DISCUSSION

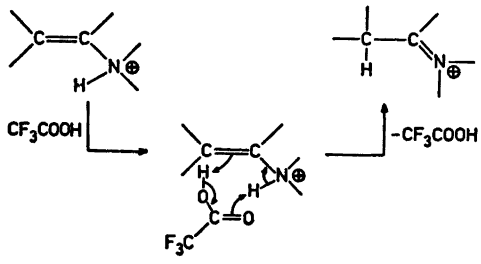
The enamines in dry pentane were treated with hydrogen chloride at −78 and 0 °C, or with trifluoroacetic acid at 0 °C. White crystals precipitated when dry hydrogen chloride was bubbled through the enamine solution. As reported before,[2] they were found to be a mixture of *β-C* and *N*-protonated species (IIIa, IIIb, IVa, IVb, V, VI). The crystals were isolated and kept in a desiccator at room temperature. After 7 − 10 days a complete rearrangement was found to have taken place (NMR) (Scheme 2). Using trifluoroacetic acid only *β-C*-protonated species (V, VI) were obtained. This difference in behaviour may lie in bifunctional catalysis by trifluoroacetic acid on the nitrogen to carbon transfer (Scheme 3).

The immonium salts were characterized by their spectra. Some features of the ^{1}H NMR spectra ought to be mentioned. The morpholine appeared as a broad multiplet at δ 4.4 − 3.9 in the immonium salts and not as two distinct



Scheme 2.

multiplets, as in the free enamine. A general downfield shift of 0.2 – 1.5 ppm was observed in the immonium salts compared with the corresponding enamines. In the ^{13}C NMR spectra a resonance signal at δ 195.0 – 194.5 was observed, and is assigned to $C=N^+$ in the immonium structure. The resonance signal from the parent ketone C = O carbon appeared at lower field. Samples of the immonium salts, partially hydrolyzed to the ketone, showed both signals. It was not possible to obtain mass spectra of the immonium salts due to early decomposition in the instrument. The largest fragments were the corresponding enaminic molecule ions.



Scheme 3.

The immonium chlorides (V, VI) were quite stable, and could be stored in a desiccator for months at room temperature without decomposition. This is in striking contrast to the immonium salts from aldehyde enamines and enamines from cyclic ketones.[3–5] Immonium trifluoroacetates (V, VI) were less stable and decomposed on standing.

Surprisingly, the most substituted isomer Ib could be readily separated in a pure state from a mixture of Ia and Ib. This was done simply by introducing hydrogen chloride or trifluoroacetic acid in an amount equivalent to the less substituted isomer Ia. Mass spectra analysis was performed on immonium salts and supernatant liquid consisting of Ib in dry pentane, from experiments using deuterium chloride or trifluoroacetic acid-d_1. A considerable deuterium incorporation was found in the immonium salts, but could not be detected in Ib. This indicates that no conversion of Ia to Ib *via* the immonium salt occurs during the reaction. The method for separation described here was not successful for separation of IIa from IIb. A more laborious method for separating Ia from a mixture has been re-

ported previously, using 1,3-dipolar cycloaddition.[6]

The isolated immonium salts were dissolved in chloroform or suspended in pentane and treated with an equivalent amount of diisopropylamine. The morpholine immonium salt V was completely deprotonated to Ia, and no trace of Ib could be detected in the ^{1}H NMR spectrum. The dimethylamine immonium salt VI yielded a mixture of IIa and IIb under the same reaction conditions. Sterically less hindered bases, *e.g.* trimethylamine and triethylamine, showed less selectivity, yielding isomeric mixtures of both morpholine and dimethylamino enamines.

EXPERIMENTAL

^{1}H NMR spectra were recorded on a JEOL-C 60 HL spectrometer, and the ^{13}C NMR spectra were obtained using a JEOL PFT-60 HL spectrometer. Deuterated chloroform was used as solvent (*ca.* 1 M solutions) using TMS as an internal standard. Mass spectra were obtained on a LKB-9000 mass spectrometer. The enamines were prepared according to White and Weingarten.[1]

Immonium chlorides were prepared by bubbling a slow stream of dry gaseous hydrogen chloride through a solution of the enamines in dry pentane at −78 °C. The precipitated salt was collected by filtration under nitrogen.

Deuterium chloride was prepared by adding D_2SO_4 to a calculated amount of dry potassium chloride.

Immonium trifluoroacetates were prepared by adding an equivalent amount of trifluoroacetic acid dissolved in dry pentane to a solution of the enamine in dry pentane at 0 °C. The precipitate was recovered as above.

Separation of Ib. A typical procedure was: 3.1 g (0.02 mol) of a mixture of Ia and Ib (1:1) was treated with 225 ml (0.01 mol) of gaseous hydrogen chloride as above. After filtration, the filtrate was evaporated *in vacuo*, yielding 1.3 g (84 %) of Ib in a pure state.

Separation of Ia from a mixture using trifluoroacetic acid. To 7.2 g of a mixture of Ia and Ib (6:1) in dry pentane was added 4.6 g (0.04 mol) of trifluoroacetic acid in 50 ml of dry pentane with stirring at 0 °C. The immonium salt separated as an oil, which crystallized on scratching. The supernatant liquid was decanted and the residue washed with a small portion of cooled pentane. The combined pentane solutions were evaporated *in vacuo* yielding 0.9 g (88 %) of Ib. The immonium salt was suspended in 150 ml of cooled pentane, and 4.1 g (0.04 mol) of diisopropylamine in 10 ml of cooled pentane was added with stirring at 0 °C. The reaction was complete within 1 h. The precipitated diisopropylammonium trifluoroacetate was removed by filtration and the filtrate was evaporated *in vacuo* yielding 4.5 g (73 %) of Ia. No trace of Ib could be detected by NMR.

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Short Communications

A New One Step Synthesis of Diazooctane from Octylamine

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Precursors for higher homologs of diazomethane are not readily available. Their preparation is therefore time-consuming, and they are not much used in organic synthesis. Diazooctane was reported as an intermediate in the deamination of octylamine with nitrosyl chloride at −70 °C.[1,2] However, an excess of primary amine was necessary if reduction of the yield due to side-reactions between nitrosyl chloride and the diazoalkane was to be avoided. This constitutes a practical disadvantage in the case of less readily available amines. The excess primary amine presumably acts as a base in the diazoalkane formation and the use of a readily available alternative base might allow reduction of the excess of primary amine.

Tertiary amines seemed a possible choice as "foreign base". Reactions between nitrosyl chloride and tertiary amines have been reported,[3] but at low temperatures complex formation predominates.[4,5] Trialkylamines[4] and pyridine[5] were possible candidates, while *N*-alkylated anilines might be subject to substitution by nitrosyl chloride.[3] We now wish to report the influence of added tertiary amines on the reaction between a primary amine and nitrosyl chloride. Octylamine was chosen as a model substance for the deamination.

The reactions were run at −70 °C by adding gaseous nitrosyl chloride to the mixture of octylamine and tertiary amine in the chosen solvent. Formation of diazooctane was established qualitatively by IR spectroscopy. A quantitative estimate was provided by determination of the octyl acetate formed on addition of acetic acid to the reaction mixture.

Three tertiary amines were tried: triethylamine, pyridine and triethylenediamine (1,4-diazabicyclo[2,2,2]octane). Deaminations with triethylamine were most thoroughly studied. The yield of diazooctane was optimized by variations in the excess of triethylamine and nitrosyl chloride and in the reactant concentrations. The optimum condition, an 80 mM solution of octylamine deaminated with nitrosyl chloride (160 mM) in the presence of triethylamine (400 mM) resulted in a 43 % yield of diazooctane (determined as octyl acetate).

Diazooctane proved to be less stable in the preparations with triethylamine than in those with excess octylamine, and to obtain a maximum yield acetic acid should be added within 10 min of the addition of nitrosyl chloride.

The two other tertiary amines tried as bases gave lower yields of octyl acetate than triethylamine: triethylenediamine 25 % and pyridine 15 %. The low yield with triethylenediamine can be explained by its low solubility in ether at −70 °C, and that with pyridine by its lower base strength.

Four different solvents were tried: ethyl ether, tetrahydrofuran (THF), methylene chloride, and triethylamine itself. The etheral solvents gave the best yields[2] (ethyl ether 43 %, THF 35 %, methylene chloride 8 %, and triethylamine 3 %).

In one experiment triethylamine in ethyl ether was allowed to react with nitrosyl chloride before addition of octylamine, but this resulted in a lower yield of diazooctane (30 %) than obtained by the normal procedure.

In addition to octyl acetate, several other compounds were detected from the deamination. The identified substances are given in Table 1.

The presence in the product mixture of octyl chloride and octene has been discussed earlier.[1] Ethyl octyl ether has also been reported,[1] and was now unambiguously identified by MS. Octanol may have been formed in a reaction between the intermediate diazonium ion and hydroxide ions or water. The latter possibility was confirmed by addition of excess water to the deamination product at −70 °C, which increased the yield of octanol from 3 to 13 %. While the identification of pentylcyclopropane was not conclusive due to the low yield, its presence was not unexpected in view of the work of Friedman *et al.*[6]

The ways of formation of octanal and octanal oxime are obscure at present. Octanenitrile was not present as such in the reaction mixture, but formed from precursors in the gas chromatograph. Although octanal oxime gives octane nitrile on gas chromatography, the oxime was not present in sufficient concentration to explain the yield of octane nitrile. It should be noted that octanal, octanal oxime and octane nitrile were only formed when the deaminations were run in the presence of triethylamine.

Table 1. Substances identified from deaminations of octylamine in ethyl ether with triethylamine present (octylamine – triethylamine – nitrosyl chloride 1:5:2).

Substance	Yields (in % of octylamine) After addition of acetic acid	Without acetic acid
Octyl chloride	8[a]	6[a]
Octene	5[b]	1,5[b]
Ethyl octyl ether	< 2[b]	~ 2[b]
Octanol	5 – 6[b]	3[b]
Pentylcyclopropane	< 0.3[d]	trace
Octanal	< 2[b]	~ 2[a](17 – 19[c])
Octanal oxime		< 1[a]
Octane nitrile	~ 2[c]	12 – 13[c]
Octyl nitrate		4[a]
Nitrooctane	2 – 3[a]	2 – 3[a]

[a] Isolated; IR, NMR, GC/MS. [b] Identified and determined by GC/MS. [c] Identified by GC/MS only. Formed in GC. [d] Identified by GC only.

When a fourfold excess of octylamine in ethyl ether was used,[1] only traces of octanal or octane nitrile were detected by gas chromatographic analysis. This suggests that the deaminations in the presence of triethylamine proceed partly by other pathways than those it its absence. Octyl nitrate and nitrooctane may have been formed from traces of nitrogen tetroxide contaminating the nitrosyl chloride. Exclusion of air was without effect on the yield of these two substances.

Two other substances, with molecular formulae $C_9H_{15}NO_2$ and $C_{16}H_{33}NO$, were also formed. While their structures have not yet been elucidated, the former was neither nitrooctene nor nitrosoxy octene, and the latter was different from α-heptyl-*N*-octylnitrone, 2-octyl-3-heptyl-oxaziridine[11] and octanal oxime *O*-octyl ether. Nitrones have been reported to form from diazoalkanes and nitrosoalkanes;[7] both types of substances have been found in aprotic deaminations.[1,2] α-Heptyl-*N*-octylnitrone was synthesised from octanal oxime and octyl bromide.[8] Nitrones have been reported both as dimers[9] and monomers.[7,9,10] Our product was found to be monomeric from spectroscopic evidence.

The deamination of octylamine in the presence of triethylamine thus produces several by-products whose way of formation and significance in the reaction are obscure at present.

Nevertheless, by running the deamination under these conditions, it is possible to transform some aliphatic amines in one step and in acceptable yields to the corresponding diazoalkanes.

Experimental. The general instrumentation used has been described earlier.[12] GC separations were performed on a 3 m × 3 mm, 28 % Pennwalt 223, 4 % KOH, column. Quantitative GC determinations were carried out using 2-heptanone as internal standard. Most of the deaminations were run in a flask equipped with an Ascarite tube and a rubber septum. Nitrosyl chloride was added as gas by a syringe filled directly from a commercial gas flask (Matheson). The described products were not formed from reactions of nitrosyl chloride with 1-octene or triethylamine.

Acknowledgements. Professor N. A. Sørensen is thanked for providing laboratory facilities and Dr. G. Francis for linguistic corrections.

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Structural Studies of the Capsular Antigen from *Hæmophilus influenzæ* Type b

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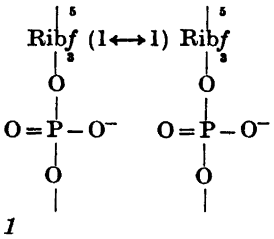
The structure (*1*) proposed by Zamenhof *et al.*[1] for the capsular antigen from *Hæmophilus influenzæ*, type b, is at variance with established principles for the biosynthesis of teichoic acids and related polymers.[2] The structure must therefore be incorrect or the polymer is synthesized by a route, differing from those previously outlined. In order to resolve this matter, a reinvestigation of the structure was undertaken. During the course of this investigation, Crisel *et al.*[3] showed that the structure was, indeed incorrect and demonstrated that the polymer is composed of repeating units having the structure *2*. We now report some results from our own studies, which support this structure and give further structural details.



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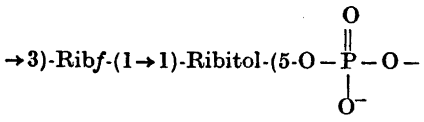
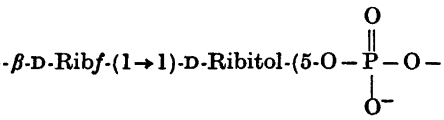


2

In agreement with Crisel *et al.* we find that the polymer is composed of equimolecular parts of D-ribose, ribitol and phosphate. On dephosphorylation, by treatment first with alkali and then with phosphatase, a ribosylribitol is produced. Methylation analysis [4] of this disaccharide gives a mixture of 2,3,5-tri-*O*-methyl-D-ribose and 1,2,3,4-tetra-*O*-methylribitol, demonstrating that the disaccharide is a 1-*O*-D-ribofuranosylribitol. The MS of the fully methylated disaccharide [5] is in agreement with this proposal. The optical rotation of the disaccharide, $[\alpha]_{589}^{25}$ −28°, indicates that it is a β-D-ribofuranoside.

We recently prepared 1-*O*-β-D-galactopyranosyl-D-ribitol and the corresponding L-ribitol derivative.[6] The optical rotation of the former showed a large negative shift in molybdate at pH 5.5[7] and a corresponding positive shift was observed for the L-isomer. Since it has been established that molybdate complexes with the ribitol moiety and not the glycosyl moiety,[8] we may conclude that the shifts are typical for 1-*O*-substituted D- and L-ribitol derivatives, respectively. The 1-*O*-β-D-ribofuranosylribitol from *Hæmophilus influenzæ* type b shows $[\alpha]_{589}^{25}$ −80°, $[\alpha]_{578}^{25}$ −83°, $[\alpha]_{546}^{25}$ −102°, $[\alpha]_{436}^{25}$ −204° and $[\alpha]_{365}^{25}$ −417° in sodium molybdate buffered to pH 5.5, demonstrating that the ribitol moiety has the D-configuration. The phosphate, linked to the other primary position in the ribitol moiety,[3] is consequently linked to O−5 in D-ribitol. This is in agreement with the postulated biosynthetic route [2] in which ribitol phosphate most probably derives from cytidine diphosphate ribitol, in which D-ribitol is phosphorylated at O−5. The previous results on the structure of the capsular antigen from *Hæmophilus influenzæ* type b are therefore supplemented and a complete structure of the repeating unit *3* is proposed.



3

Experimental. Solutions were concentrated under reduced pressure at bath temperatures not exceeding 40 °C. For GLC, a Perkin-Elmer 990 instrument fitted with flame ionisation detectors was used. Separations were performed on columns of 3 % OV-1 on Gas-Chrom Q and 3 % ECNSS-M on Gas-Chrom Q. For GLC-MS, a Varian Mat-311-SS 100, MS computer system was used. Preparative PC was performed on Whatman No. 1 paper, employing butanol−pyridine−water, 6:4:3, as the solvent system. Alkaline silver nitrate was used for detection.

Capsular antigen (60 mg) from *Hæmophilus influenzæ* type b, strain RAB, prepared as previously described,[9] was treated with 0.5 M aqueous sodium hydroxide (10 ml) at 100 °C for 4 h and worked up as described by Armstrong *et al.*[10] The product in sodium hydrogen carbonate buffer of pH 10.4 (10 ml) was treated with alkaline phosphatase (10 mg, Sigma Chemical Company) for 3 days at 37 °C. The hydrolysate was fractionated on a Sephadex G-15 column (2.6 × 100 cm) irrigated with water, the separation being followed by dif-

ferential refractometry. The two components in the disaccharide region were separated by PC ($R_{ribitol}$ 0.13 and 0.86). NMR of the fast component (6 mg) showed, *inter alia*, a signal at δ 5.03, $J_{1,2}$ 1.5 Hz, assigned to the anomeric proton. The component showed $[\alpha]_{589}^{25}$ −28° (*c* 0.6, water) and $[\alpha]_{589}^{25}$ −80°, $[\alpha]_{578}^{25}$ −83°, $[\alpha]_{546}^{25}$ −102°, $[\alpha]_{436}^{25}$ −204°, and $[\alpha]_{365}^{25}$ −417° (*c* 0.2, 0.037 M sodium molybdate at pH 5.5). The oligosaccharide (2 mg) in dimethyl sulfoxide (1 ml) was treated with 2 M sodium methylsulfinyl anion in dimethyl sulfoxide (1 ml) for 4 h at room temperature and methyl iodide (1 ml) was added under external cooling with ice. The solution was diluted with chloroform (5 ml) and extracted with water (6 × 2 ml). The chloroform phase on GLC-MS gave a single peak with the MS expected [5] for fully methylated 1-*O*-β-D-ribofuranosylribitol, and showing, *inter alia*, the following ions: 45(63), 55(6), 59(23), 71(36), 74(5), 75(11), 83(5), 87(5), 88(6), 89(17), 99(7), 101(100), 102(7), 103(5), 111(6), 115(7), 133(2), 143(8), 145(3), 175(4), 177(1), 191(4) and 207(1).

The fully methylated disaccharide was hydrolysed with 0.25 M sulfuric acid (0.5 ml) at 100 °C for 16 h, reduced ($NaBH_4$), acetylated and analysed by GLC-MS. Two components, with MS corresponding to 1-*O*-acetyl-2,3,4,5-tetra-*O*-methylribitol and 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylribitol, were obtained.

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A Photolytic Study of Ethoxy(diphenylmethylene)sulfonium Ion and Hydroxy(diphenylmethylene)sulfonium Ion. Alkylation of Benzophenone, Thiobenzophenone and Thiobenzophenone-*S*-oxide

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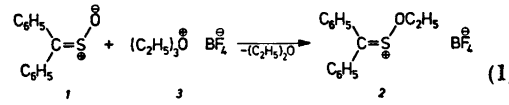
In connection with our investigations on the photolytic transformation of sulfines (thiocarbonyl-*S*-oxides) into the thermally unstable oxathiiranes,[1] we have studied the photolysis of thiobenzophenone-*S*-oxide (*1*) in concentrated sulfuric acid. In addition, with the aim of eventually obtaining more stable derivatives, we have examined the photochemistry of the hitherto unknown ethoxy(diphenylmethylene)sulfonium tetrafluoroborate (*2*).

Compound *2* is prepared by heating a mixture of *1* and triethyloxonium tetrafluoroborate in the solid state; it is analyzed as described in the experimental section. The yellow, crystalline, highly hygroscopic compound exhibits a UV absorption maximum at 369 nm ($\varepsilon = 1.77 \times 10^4$ M^{-1} cm^{-1}).



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On hydrolysis of *2*, elemental sulfur is formed along with benzophenone in greater than 90 % yield. In analogy with the acid catalyzed hydrolysis of *1* reported by Strating *et al.*[2] the hydrolysis may be formulated as shown in eqn. 2.

(2)

Compound *2* may be dealkylated to the starting sulfine in 25 % yield with triphenylphosphine in methylene chloride.

When *1* was dissolved in concentrated sulfuric acid an orange solution was obtained. The absorption bands of *1* [3] were replaced by a band with maximum at 364 nm ($\varepsilon = 1.59 \times 10^4$ M^{-1} cm^{-1}), close to that of *2* indicating protonation of *1* at oxygen. Cautious addition of water (cooling) afforded *1* in quantitative yield.

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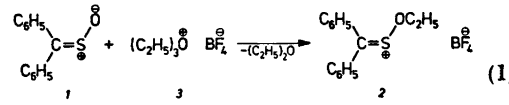

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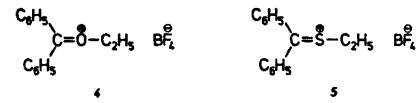
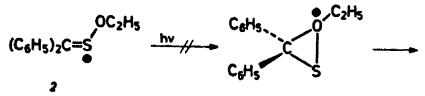
The ethyl salts of benzophenone (*4*) [4] and thiobenzophenone (*5*) were prepared and analyzed in the same way as *2*. In both cases yellow highly hygroscopic products were obtained exhibiting UV absorption maxima at 337 and 384 nm for *4* and *5*, respectively.



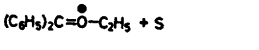
4 *5*

Photolytic reactions of hydroxy(diphenylmethylene)sulfonium ion and ethoxy(diphenylmethylene)sulfonium ion. A 10^{-4} M solution of *1* in concentrated sulfuric acid was irradiated ($\lambda = 360$ nm) at room temperature for 3 h. No significant change in concentration was observed during this time. This is in striking contrast to the rapid transformation of *1* in ethanol upon photolysis under similar conditions.[1] The reasons for this apparent inactivity are not known but one explanation may be a photolytic deprotonation (in analogy with the below mentioned dealkylation of *2*) followed by reprotonation.[5]

When a 10^{-4} M solution of *2* in methylene chloride was irradiated ($\lambda = 370$ nm) and the conversion followed by UV spectroscopy, an apparently complicated transformation took place. Benzophenone was observed as the end product in 75 % yield. Formation of compound *4* was not observed which rules out a mechanism involving an *O*-alkylated oxathiirane (eqn. 3) analogous to the photolytic rearrangement of sulfines [1] (eqn. 4). Compound *4* is stable under the prevailing conditions.



2 (3)



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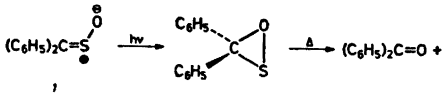
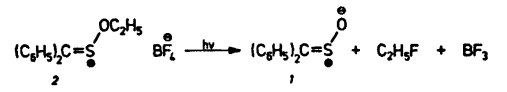


1 (4)

A sample of *2* in methylene chloride showed no decomposition when stored in the dark for several days.

On irradiation of *2* monitored with IR spectroscopy, formation of benzophenone was also observed. Weak absorptions at 1105 and 1005 cm^{-1} characteristic of *1* [3] might correspond to a steady state concentration of this compound. By monitoring the reaction by NMR, new signals were observed at δ 1.39 (t) and 4.15(q). These are well within the region reported for ethyl fluoride.[6] The formation of this compound was further demonstrated by use of GLC-MS. From the NMR data ethyl fluoride was estimated to be formed in almost quantitative yield. It is inferred from these experiments that the photochemical reaction of *2* as the primary step most probably is dealkylation with formation of ethyl fluoride and *1* (eqn. 5). The latter, which is photochemically active was observed in small concentrations only as it undergoes a facile transformation into benzophenone and sulfur [1] (eqn. 4).



2 *1* (5)

Experimental. IR spectra in methylene chloride were recorded on a Perkin-Elmer 337 Grating Infrared Spectrophotometer, UV spectra on a Pye Unicam SP-800, and ^{1}H NMR spectra on a Varian A-60A spectrometer. GLC-MS analysis was carried out on a Varian Aerograph model 2700 in combination with a Finnigan 1015 S/L mass spectrometer on a 2 m, 3 mm column with 3 % SE-30 (J. J.'s Chromatography Ltd.) on chromosorb W 80/100 mesh (Johns-Manville Products Corp.). Irradiations were performed with a Bausch and Lomb SP-200 mercury point source equipped with monochromator (typical bandwidth 20 nm).

Alkylation procedure. Approximately 2 mmol of the compound to be ethylated (*ca.* 5 % excess) was mixed with triethyloxonium tetrafluoroborate and heated in the solid state to melting of the mixture. After cooling, the tetrafluoroborates were obtained on digestion with dry ether. Yields around 50 % were obtained. Because of their highly hygroscopic nature elemental analysis could not be performed. However, equivalent weights were determined by titration with sodium hydroxide after hydrolysis according to eqn. 2. *O-Ethyl thiobenzophenone-S-oxide.* M.p. 80 – 85 °C (closed tube), equiv. weight 329 ± 3, calc. 330. NMR (CH_2Cl_2): δ 4.76 (CH_2, q), 1.59 (CH_3, t). The shift of the methylene protons are well within the range where $-OCH_2-$ signals are normally found.[7] *O-Ethyl benzophenone* M.p. (closed tube) 113.5 – 115 °C, equiv. weight 292 ± 3, calc. 298. *S-Ethyl thiobenzophenone.* Oil, equiv. weight 316 ± 3, calc. 314.

Acknowledgement. We are grateful to the Danish Natural Science Research Council for financial support.

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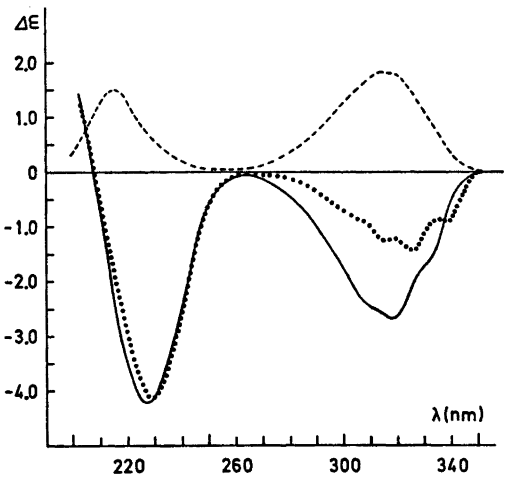

Fig. 1. CD curves of methyl dihydroketo-picrotoxinate (*3*, ——), methyl keto-β-picrotoxinate (*4*, · · ·) and methyl dihydroketo-β-picrotoxinate (*5*, - - -) in methanol.

Studies on Orchidaceae Alkaloids. 41.* The Configuration at C-4 in δ-Nobilonine and Dihydroketopicrotoxinic Acid

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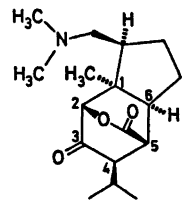
To gain further evidence concerning the configuration at C-4 in *1* and *2*, the picrotoxinin derivatives *3*, *4*, and *5* have now been synthesised and their CD curves recorded. In *4* and *5* an epimerisation at C-4 is impossible due to the ether linkage between C-6 and



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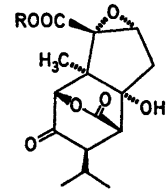
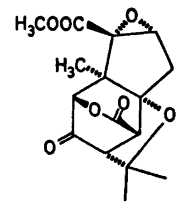


2:R=H
3:R=CH_3



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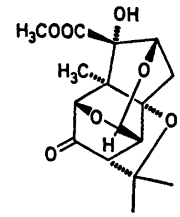

5

C-12. Compounds *1*, *2* and *3*, which have the same chromophore as *4*, exhibit negative Cotton effects at ~315 nm which are almost twice that of *4*. This large difference in amplitude indicates that the isopropyl groups in *1*, *2* and *3* make a significant negative contribution to the Cotton effect associated with the $n \rightarrow \pi^*$ transitions of the C-3 carbonyl group. Hence *1* and *2* have the 4*R* configuration.

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* For Paper 40 in this series, see Ref. 1.

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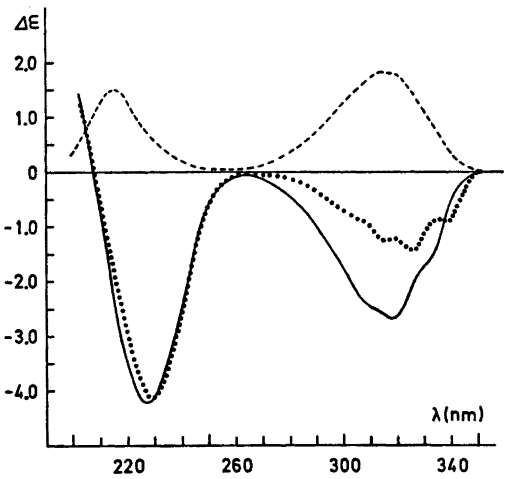

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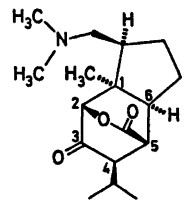

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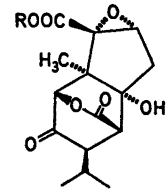

2:R=H
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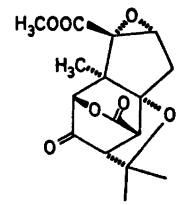

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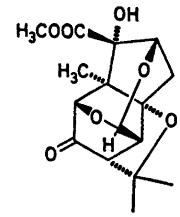

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but of opposite sign. It has been shown that oxygen-containing substituents, such as hydroxyl or acetoxyl, in the α-position to a carbonyl group give a contribution to the Cotton effect opposite to that predicted by the octant rule.[3] Little is known, however, about the amplitude contributions of these substituents and great care should be exercised in applying the octant rule to compounds containing such systems, *e.g.* *1* – *5*.

The proposed configuration (*R*) at C-4 was further supported by NMR studies on *3* using tris(dipivalomethanato)europium [Eu(DPM)$_3$] as shift reagent. It has been shown that complex formation preferentially occurs at hydroxyl groups, rather than at ester, lactone, ketone or epoxide groups.[4] In sterically hindered alcohols, however, complex formation at other sites may compete.

The magnitudes of the induced chemical shifts were investigated and found to vary linearly with the molar ratio of *3* to Eu(DPM)$_3$, when this ratio was varied between 0.20 – 0.45. The largest shifts were observed for those hydrogen atoms which are located closest to the hydroxyl group, with the exception of H-8. It thus seems probable that the shift reagent forms a complex mainly with the hydroxyl group. The large induced chemical shift for H-8 is probably due to competing complex formation with the ester group, which also explains why the changes in shifts for the two hydrogen atoms at C-7 are the same.

The small induced chemical shifts for the hydrogen atoms of the isopropyl group indicate that these atoms are located at a longer distance from the europium complex than H-4, H-5 and the methyl group at C-1. This is only possible if *3* has the 4*R* configuration.

Experimental. Melting points are corrected. Optical rotations were measured on a Perkin-Elmer 141 polarimeter, IR spectra on a Perkin-Elmer 257 instrument, NMR spectra on a Varian XL-100 spectrometer and CD spectra on a Jasco J-40 spectropolarimeter. Concentrations were performed under reduced pressure, at bath temperatures not exceeding 40 °C. Elemental analyses were carried out at Lantbrukshögskolan, Uppsala, Sweden.

Methyl dihydroketopicrotoxinate (3). Dihydroketopicrotoxinic acid[2] (*2*, 35 mg) was dissolved in methanol and esterified with diazomethane. Evaporation of the solvent and crystallisation of the residue from ethyl acetate – hexane (1:2) gave *3* (19 mg), m.p. 85 – 86 °C. $[\alpha]_{578}^{22}$ – 93° (*c* 0.48, methanol). Anal. $C_{16}H_{20}O_7$: C,H. IR (KBr): 3420(s), 1755(s), 1740(s), 1725(s) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.98 (d, 3 H, *J* 7 Hz), 1.05 (d, 3 H, *J* 7 Hz), 1.45 (s, 3 H) 2.22 (m, H-12, J_1 6.5 Hz, J_2 7 Hz), 2.57 (d, H-7′, *J* 16 Hz) and 2.74 (dd, H-7, J_1 16 Hz, J_2 2 Hz) AB part of an ABX system, 3.05 (dd, H-4, J_1 2 Hz, J_2 6.5 Hz), 3.36 (d, H-5, *J* 2 Hz), 3.60 (s, 3 H), 4.08 (d, H-8, *J* 2 Hz), 5.55 (s, H-2). The following induced chemical shifts, in ppm, were observed at the molar ratio 0.45 of *3* to Eu(DPM)$_3$: H-2 +1.4, H-4 +2.9, H-5 +2.7, H-7α +1.5, H-7β +1.5, H-8 +2.0, H-12 +0.4, CH_3-1 +1.8, CH_3-12 +0.2 and −0.2.

Methyl keto-β-picrotoxinate (4). Methyl-β-picrotoxinate[5] (256 mg) was dissolved in acetone (25 ml) and an excess of Jones reagent[6] was added. After stirring the solution at room temperature for 45 min, sodium hydrogen sulfite was added to destroy the excess of chromic acid. The reaction mixture was filtered and evaporated to dryness. The residue was crystallised from ethyl acetate – hexane (1:2) giving *4* (182 mg), m.p. 170 – 171 °C. $[\alpha]_{578}^{22}$ – 93° (*c* 0.96, methanol). Anal. $C_{16}H_{18}O_7$: C, H. IR (KBr): 1777(s), 1750(s), 1729(s) cm^{-1}. ^{1}H NMR (pyridine-d_5): δ 1.23 (s, 3 H), 1.33 (s, 6 H), 2.37 (d, H-7, *J* 15 Hz) and 2.59 (dd, H-7′, J_1 15 Hz, J_2 3 Hz) AB part of an ABX system, 3.12 (d, H-4, *J* 3.5 Hz), 3.60 (s, 3 H), 4.02 (d, H-5, *J* 3.5 Hz), 4.11 (d, H-8, *J* 3 Hz), 5.17 (s, H-2).

Methyl dihydroketo-β-picrotoxinate (5). Methyl dihydro-β-picrotoxinate[5] (375 mg) was oxidised in the same way as described above for methyl-β-picrotoxinate. Crystallisation from ethyl acetate-hexane (1:1) gave *5* (301 mg), m.p. 243 – 245 °C. $[\alpha]_{578}^{22}$ – 3.0° (*c* 0.60, methanol). Anal. $C_{16}H_{20}O_7$: C,H. IR (KBr): 3400(s), 1735(s) cm^{-1}. ^{1}H NMR (pyridine-d_5): δ 1.32 (s, 3 H), 1.33 (s, 3 H), 1.36 (s, 3 H), 1.94 (dd, H-7, J_1 11 Hz, J_2 1.4 Hz), 2.63 (d, H-4, *J* 3.8 Hz), 2.94 – 3.18 (m, 2 H), 3.62 (s, 3 H), 4.34 (s, H-2), 4.72 (dd, H-8, J_1 2.4 Hz, J_2 1.4 Hz), 5.72 (d, H-15, *J* 3.5 Hz), 8.44 (broad, −OH).

Acknowledgements. We thank Professor Torbjörn Norin for valuable discussions and advice, Professor Salo Gronowitz for the spectropolarimetry facilities placed at our disposal, and Mr. Jan Glans and Dr. Rolf Håkansson for the measurement of circular dichroism. Support from the Swedish Natural Science Research Council is gratefully acknowledged.

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Structural Analogues of GABA. A New Convenient Synthesis of Muscimol

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Muscimol (5-aminomethyl-3-isoxazolol) (*8*), a centrally active constituent of *Amanita muscaria*, is a semirigid analogue of γ-aminobutyric acid (GABA) [1] of considerable pharmacological and neurochemical interest. Various widely different routes for the synthesis of muscimol (*8*) have been published.[2–5] This paper presents a new and more convenient synthesis of *8*.

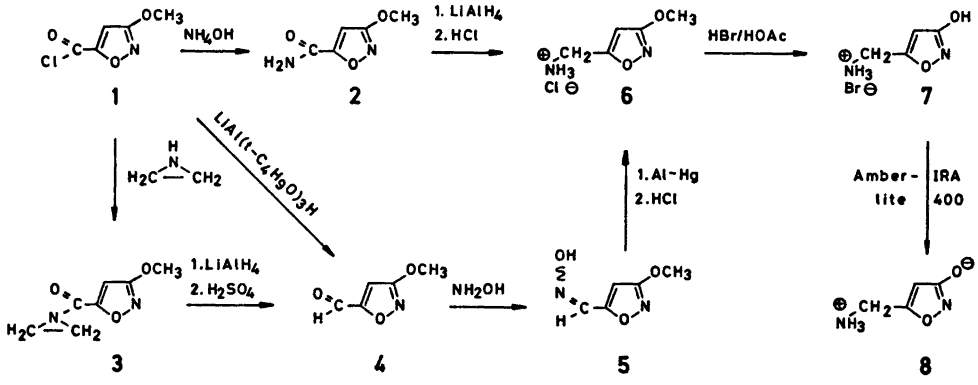
Attempts to convert *2*, prepared from *1*, into 3-methoxy-5-aminomethylisoxazole hydrochloride (*6*) *via* reduction with lithium aluminium hydride gave *6* in very low yields. Attempts to convert *1* into *4* by treatment with lithium tri-*t*-butoxyaluminium hydride according to a general procedure [6] were unsuccessful. Finally the acid chloride *1* was transformed into muscimol hydrobromide (*7*) (Scheme 1) without purification of intermediate products. A crude product of the unstable 1-acylaziridine *3* was treated with lithium aluminium hydride, followed by hydrolysis, to give 3-methoxy-5-isoxazolecarboxaldehyde (*4*) in a good yield. However, contrary to previous findings from analogous reactions,[5,7,8] satisfactory yields of *4* could only be obtained by using equimolar amounts of *3* and the reagent. Kishida *et al.* have reported a synthesis of *4* based on 3,3-diethoxypropyn through several steps.[9] The mixture of isomeric oximes *5* was reduced by aluminium amalgam, and 3-methoxy-5-aminomethylisoxazole was isolated as the hydrochloride *6*, which was transformed into *7*. The total yield of *7* from *1* was 35 %.

The structure determinations of *3*, *5*, and *7*, which are new compounds, are based on spectroscopic methods. The IR, UV, and ^{1}H NMR data of *2* and *6*, which have not previously been reported, are described. The product *5* consists of a mixture of the *Z*- and *E*-forms of the aldoxime as established by TLC and ^{1}H NMR spectroscopy in agreement with the findings for a series of similarly prepared ketoximes.[10,11] The structure of *7* was determined by conversion of the compound into muscimol (*8*), the identity of which was established.

Experimental. 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.[12] Mass spectra were measured on a Finnigan 3500D mass spectrometer at 70 eV. TLC was carried out using silica gel GF_{254} plates (Merck).

3-Methoxy-5-isoxazolecarboxamide (2). 3-Methoxy-5-isoxazolecarbonylchloride (*1*) [10] (970 mg; 6 mmol) was added drop by drop to aqueous ammonia (2 ml; ϱ 0.88) at 0 °C. Upon standing at 0 °C for 15 min the mixture was evaporated *in vacuo*. Recrystallization (water) afforded 700 mg (82 %) of *2*, m.p. 175.0 – 176.0 °C (Ref. 3, m.p. 174 – 175 °C). λ_{max} 221 nm (CH_3OH) ($\varepsilon = 1.25 \times 10^4$). IR data (KBr) cm^{-1}: 3360 (m), 3195 (m), 3150 (m), 1680 (s), 1625 (m), 1610 (m), 1530 – 1515 (s). ^{1}H NMR data (DMSO – d_6): δ 8.4 – 7.7 (two broad s, 2 H); 6.75 (s, 1 H); 3.91 (s, 3 H).

N,N-Ethylene-3-methoxy-5-isoxazolecarboxamide (3). To a solution of aziridine (900 mg; 21 mmol) and triethylamine (2.4 g; 24 mmol) in ether (120 ml) was slowly added at 0 °C a solution of *1* (3.4 g; 21 mmol) in ether (80 ml). After stirring for 10 min at 0 °C the mixture was filtered to give an ether solution of *3*, an analytical sample of which was evaporated *in vacuo*. Recrystallization (benzene-petroleum ether) gave *3*, m.p. 56.0 – 57.0 °C (decomp.). λ_{max} 231 nm (CH_3OH) ($\varepsilon = 1.14 \times 10^4$). IR data (KBr) cm^{-1}: 3130 (m), 3010 (w), 2950 (w), 1675 (s), 1610 (m), 1530 (m), 1515 (s). ^{1}H NMR



Scheme 1.

data ($CDCl_3$): δ 6.47 (s, 1 H); 3.97 (s, 3 H); 2.50 (s, 4 H).

3-Methoxy-5-isoxazolecarboxaldehyde (4). To the above ether solution of crude *3* was added at 0 °C lithium aluminium hydride (722 mg; 21 mmol). After stirring the mixture at 0 °C for 2 h an aqueous solution of sulfuric acid (40 ml; 20 %) was added. The organic phase was isolated and the aqueous phase extracted with three 40 ml portions of ether. The combined ether phases were dried and concentrated *in vacuo* to give 2.1 g of crude *4*. An analytical sample was distilled *in vacuo* in a "kugelrohr" to give *4*, the IR and ^1H NMR data of which were consistent with those published for *4*.[9]

3-Methoxy-5-hydroxyiminomethylisoxazole (5). A solution of crude *4* (2.1 g; *ca.* 17 mmol), sodium acetate trihydrate (2.5 g; 18 mmol), and hydroxylammonium chloride (1.3 g; 18 mmol) in aqueous ethanol (50 ml; 50 %) was refluxed for 30 min. The solution was evaporated *in vacuo* and upon addition of water (10 ml) the mixture was extracted with three 20 ml portions of ether. The combined ether phases were dried and evaporated *in vacuo* to give 1.8 g of crude *5*, which was shown by TLC to consist of two compounds with $R_F = 0.05$ and $R_F = 0.03$ (eluent: methylene chloride). ^1H NMR data of crude *5* (DMSO-d_6), which are consistent with a mixture (*ca.* 2:3) of the isomeric oximes: δ 12.5 and 12.0 (two broad signals, 0.4 H and 0.6 H, respectively); 8.08 and 7.60 (two s, 0.6 H and 0.4 H, respectively); 6.72 and 6.38 (two s, 0.4 H and 0.6 H, respectively); 3.93 and 3.91 (two s, a total of 3 H).

3-Methoxy-5-aminomethylisoxazole hydrochloride (6). To a solution of crude *5* (1.8 g; *ca.* 13 mmol) in aqueous methanol (80 ml; 50 %) was added aluminium amalgam, prepared by treatment of aluminium strips (3.9 g; 144 mmol) with an aqueous solution of mercuric chloride (200 ml; 5 %) for 30 s followed by washing with ethanol. After stirring for 4 h at 25 °C the mixture was filtered. Upon addition of hydrochloric acid (4 ml; 4 N) the filtrate was evaporated *in vacuo* to give 2.5 g of crude *6*. Recrystallization (2-propanol) of an analytical sample afforded *6*, m.p. 176–177 °C (decomp.) (Ref. 3, m.p. 175–177 °C). $\lambda_{max} < 210$ nm (CH_3OH). IR data (KBr) cm^{-1}: 3500–3350 (m), 3130 (m), 3050–2400 (s), 1625 (s), 1580 (w), 1510 (s). ^1H NMR data (DMSO-d_6): δ 9.6–8.4 (broad s, 3 H); 6.45 (s, 1 H); 4.17 (broad s, 2 H); 3.94 (s, 3 H).

5-Aminomethyl-3-isoxazolol hydrobromide (7). A solution of crude *6* (2.5 g; *ca.* 15 mmol) in glacial acetic acid (30 ml) containing 43 % of hydrogen bromide was refluxed for 10 min. The solution was evaporated *in vacuo*. Recrystallization (methanol-ether) afforded 1.49 g of *7*, m.p. 179–181 °C (decomp.). Anal. $C_4H_7BrN_2O_2$: C, H, N, Br. $\lambda_{max} < 210$ nm (CH_3OH). IR data (KBr) cm^{-1}: 3530–3300 (m), 3200–2300 (s), 1635 (s), 1595 (m), 1580 (m), 1545 (s), 1505 (s). ^1H NMR data (DMSO-d_6): δ 10.3–9.0 (broad s, 4 H); 6.25 (s, 1 H); 4.15 (s. 2 H).

5-Aminomethyl-3-isoxazolol (Muscimol) (8). An analytical sample of *7* was passed through a column containing ion exchange resin [Amberlite IRA 400, (OH), 5 ml] using acetic acid (1 M) as an eluent. The fractions containing *8* were concentrated *in vacuo*, and recrystallization (water-ethanol) of the residue gave muscimol (*8*), m.p. 171–172 °C (decomp.) [Ref. 3, m.p. 172–174 °C (decomp.)]. The IR and mass spectra of *8* were identical with those of an authentic sample.

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Regulation and Metabolic Background of Polyketide Formation. I. Effects of (—)-Hydroxycitrate and Metabolic Roles of Citrate and Malate in Fatty Acid and Polyketide Formations

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The effects of (−)-hydroxycitrate on fatty acid and alternariol syntheses from glucose and acetate in *Alternaria alternata* were investigated.

Fatty acid synthesis from glucose and acetate was inhibited by (−)-hydroxycitrate. The inhibition could partly be removed by the addition of malate or isocitrate. Alternariol synthesis from glucose was also inhibited by (−)-hydroxycitrate. This inhibition was not influenced to the same extent by malate addition as was that of fatty acid synthesis.

It is shown that ATP-citrate lyase is essential not only for the production of cytoplasmic acetyl-CoA but also for the production of NADPH by supplying malate *via* oxaloacetate.

Fatty acid synthesis in animal tissue depends on mitochondrially formed acetyl-CoA which is transported to the cytoplasm as citrate. The cytoplasmic citrate is cleaved by ATP-citrate lyase (EC 4.1.3.8, ATP:citrate oxaloacetate-lyase) into oxaloacetate and acetyl-CoA which is used for fatty acid synthesis.[1]

ATP-citrate lyases from the fungi *Penicillium* [2] and *Mortierella* [3] were recently reported. The fungus *Alternaria alternata* forms in addition to fatty acids also the polyketide alternariol from acetyl-CoA.[4] The biosyntheses of fatty acids and polyketides differ only in the reduction steps which are present in fatty acid synthesis but not in polyketide synthesis.[4] It was therefore decided to test the ATP-citrate lyase system and its influence on the formation of these two groups of compounds of acetyl-CoA origin in *A. alternata*.

(−)-Hydroxycitrate, a powerful inhibitor of ATP-citrate lyase,[5] inhibits fatty acid synthesis in the rat.[6–8] It is assumed that the inhibition depends on a shortage of cytoplasmic acetyl-CoA and Barth *et al.*[8] show that acetate can overcome this inhibition, probably by forming cytoplasmic acetyl-CoA independently of ATP-citrate lyase.

In the present investigation the effects of (−)-hydroxycitrate on fatty acid and polyketide biosyntheses have been studied by incorporation experiments with labelled glucose and acetate.

MATERIALS AND METHODS

Cultural conditions. Alternaria alternata (tenuis) (CMI 89 343) was obtained from the Commonwealth Mycological Institute (Kew, Surrey). Stock cultures of this organism were maintained at 4 °C on agar as described by Jerebzoff.[9] Submerged cultures were incubated from the agar slants in conical flasks of 500 ml capacity containing 150 ml modified Czapek-Dox medium of the following composition: $NaNO_3$, 1.0 g; NH_4Cl, 0.25 g; KH_2PO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.25 g; NaCl, 0.25 g; $ZnSO_4.7H_2O$, 0.01 g; $FeSO_4.7H_2O$, 0.01 g; yeast extract, 1.0 g; glucose, 40 g and distilled water, 1000 ml. The mycelium was harvested by filtration after 5 to 10 days and washed with modified Czapek-Dox medium containing 5 g glucose per 1000 ml but deficient of nitrogen substrates.

Incubations. Portions of wet mycelium (0.50 g) were incubated in 25 ml conical flasks containing 5 ml modified nitrogen-free Czapek-Dox medium with 5 g glucose per 1000 ml and 2 mM sodium acetate. The flasks were incuba-

ted for 6 h on a table rotary shaker at room temperature. Further additions to the medium were done to the following concentrations and as sodium salts, 4 mM (−)-hydroxycitrate; 2 mM malate; 2 mM isocitrate. The following labelled compounds were used: either 1 μCi [U-^{14}C] glucose and 5 μCi 2-^{14}C-acetate in separate incubations or a combination of 1 μCi [U-^{14}C] glucose and 2.5 μCi 2-^{3}H-acetate in a single incubation. The latter combination was preferred in the later stage of the experiments, saving time and increasing precision.

Incorporation of label. Lipids and alternariol were extracted from the mycelium using Floch's method [10] with chloroform-methanol (2:1, by vol.) with 10 ml solvent for 0.5 g wet mycelium. The filtrate obtained from the extraction was shaken with 2 ml water, and the lower phase washed with 2 ml methanol-water (1:1, by vol.). The organic phase containing lipids and alternariol was evaporated to dryness. The residue was chromatographed on thin layer silica gel in benzene-dioxane-acetic acid (95:25:4, by vol). Lipids (R_F 0.65 − 0.85) and alternariol (R_F 0.36) were localized with a radiochromatogram scanner and observation of fluorescense. The activity was eluted from the gel and counted in a Packard Tri-Carb Scintillation Spectrometer.

Scintillation fluid used was toluene containing 4 g PPO and 50 mg dimethyl-POPOP per 1000 ml. Alternariol was dissolved in 500 μl methanol before adding the scintillation fluid.

Material. 2-^{3}H-Acetate was obtained from the Radiochemical Centre, Amersham, England and [U-^{14}C] glucose and 2-^{14}C-acetate from New England Nuclear, Dreieichenhain, W. Germany. (−)-Hydroxycitrate was prepared from *trans*-aconitic acid by a modification of the method of Martius and Maué.[11] Silica gel plastic sheets were obtained from Schleicher and Schüll, W. Germany.

RESULTS AND DISCUSSION

All results are calculated as percentage of a control incubation without inhibitor or other additions. This procedure allows comparison between different cultures. Means and errors are calculated on the basis of the calculated percentages.

Inhibition with (−)-hydroxycitrate. On addition of (−)-hydroxycitrate to the incubation medium the syntheses of fatty acids and alternariol from glucose are inhibited (Table 1). As (−)-hydroxycitrate inhibits ATP-citrate lyase the inhibitions of the biosyntheses of these compounds demonstrate that this enzyme is essential in *A. alternata* for the production of cytoplasmic acetyl-CoA originating from glucose. With exogenous acetate as precursor fatty acid synthesis is inhibited but not alternariol synthesis (Table 1). The unchanged alternariol synthesis shows that (−)-hydroxycitrate has no influence on the formation of acetyl-CoA and malonyl-CoA from acetate. The inhibition of fatty acid synthesis from acetate must therefore be due to (−)-hydroxycitrate affecting the availability of cytoplasmic NADPH. The main difference between fatty acid and alternariol syntheses, as pointed out earlier, is the demand for NADPH in fatty acid synthesis.

ATP-citrate lyase provides in addition to acetyl-CoA also oxaloacetate. This oxaloacetate is reduced by cytoplasmic NADH to malate with malate dehydrogenase (EC 1.1.1.37, L-malate: NAD^+ oxidoreductase). The cytoplasmic malate is partly utilized in a shuttle system for transport of citrate out of the mitochondria. Inside

Table 1. Effects of (−)-hydroxycitrate, malate, and isocitrate on fatty acid and alternariol syntheses *in vivo* in *Alternaria alternata*. The results are the mean ± S.E.M. of the number of experiments indicated by the figures in parentheses.

Addition	Fatty acid synthesis from glucose %	Fatty acid synthesis from acetate %	Alternariol synthesis from glucose %	Alternariol synthesis from acetate %
Control	100	100	100	100
Hydroxycitrate	40 ± 2 (10)	60 ± 6 (6)	54 ± 4 (9)	107 ± 6 (4)
Hydroxycitrate + malate	68 ± 4 (5)	98 ± 19 (2)	67 ± 2 (4)	118 ± 5 (2)
Hydroxycitrate + isocitrate	66 ± 6 (2)	92 ± 16 (2)	–	112 ± 34 (2)
Malate	93 ± 13 (5)	92 (1)	119 ± 2 (5)	109 (1)

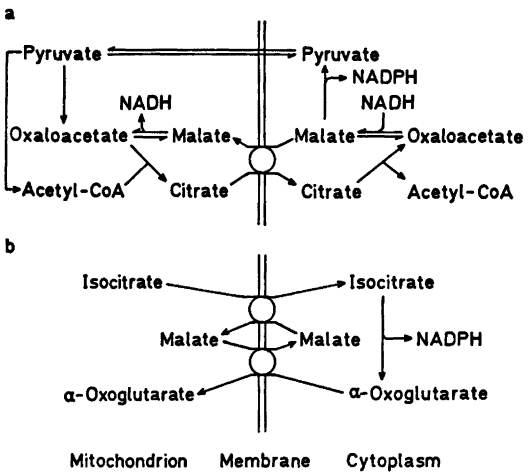

Fig. 1. The roles of malate in cytoplasmic NADPH regeneration processes and mitochondrial shuttle system. a: Formation of cytoplasmic acetyl-CoA from mitochondrial acetyl-CoA. Malic enzyme deprives citrate transport of malate. b: Formation of NADPH by cytoplasmic isocitrate dehydrogenase.

the mitochondria the malate is reoxidized to oxaloacetate and subsequently reutilized for citrate formation (Fig. 1a).

The inhibition caused by (−)-hydroxycitrate can be influenced by the addition of exogenous malate (Table 1). On addition of malate to (−)-hydroxycitrate inhibited cells glucose is incorporated more efficiently into both fatty acids and alternariol. The inhibition of fatty acid synthesis from acetate is entirely removed on addition of malate to (−)-hydroxycitrate inhibited cells. These results are interpreted in the following manner. Since malate alone does not influence fatty acid and alternariol syntheses in the same way as when malate is added to inhibited cells, (−)-hydroxycitrate is likely to induce a shortage of malate. It seems that the addition of malate to inhibited cells mainly influences the production of cytoplasmic NADPH since fatty acid synthesis is the one that is most promoted by malate addition. Besides being involved in the mitochondrial transport of citrate and reducing equivalents, malate thus seems to play a role in the production of NADPH.

Cytoplasmic malic enzyme [EC 1.1.1.40, L-malate:NADP+ oxidoreductase (oxaloacetate-decarboxylating)] will split malate to pyruvate and carbon dioxide with the concomitant formation of NADPH. Malate can also participate in NADPH formation in a more indirect way by being used for transport of isocitrate [12] out of the mitochondria (Fig. 1b). Cytoplasmic isocitrate dehydrogenase [EC 1.1.1.42, *threo*-D-isocitrate:NADP+ oxidoreductase (decarboxylating)] will oxidize isocitrate to α-oxoglutarate and provide NADPH. α-Oxoglutarate is then transported into the mitochondria in exchange for malate.[12] A reduced malate concentration would thus reduce the transport rate of isocitrate and possibly also the production of cytoplasmic NADPH. The participation of isocitrate dehydrogenase *in vivo* is demonstrated by the reduction of the inhibition of the fatty acid synthesis on the addition of isocitrate to (−)-hydroxycitrate inhibited cells (Table 1).

Exogenous isocitrate is able to dilute the radioactivity of citrate formed from labelled glucose in the reaction catalysed by aconitase [EC 4.2.1.3, citrate (isocitrate) hydro-lyase]. This enzyme, however, is inhibited by (−)-hydroxycitrate [13] and such a dilution effect should thus be of no significance.

It is important to take into consideration the role of malate for the transport of citrate, isocitrate and α-ketoglutarate in fatty acid and polyketide syntheses. Every citrate molecule leaving the mitochondria must be exchanged with one malate molecule. If malic enzyme splits malate in the cytoplasm this leads to a loss of malate for transport of citrate and isocitrate. This loss must be met by a cytoplasmic synthesis or by transport of malate from the mitochondria. It is therefore possible that isocitrate dehydrogenase is the principal NADPH producer, depending on but not consuming malate, whereas malic enzyme would mainly have a regulating function by affecting the malate concentration. The inhibition of malate formation induced by (−)-hydroxycitrate thus causes the observed restriction of NADPH formation.

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Ring-opening Reactions of Heterocyclic Organometallics. VI.* The Regio- and Stereospecific Synthesis of Alkylthiovinyl Acetylenes

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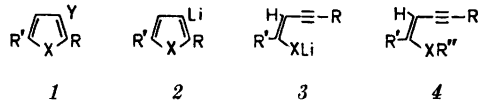
It has been found that 2,5-dialkyl-3-thienyllithium derivatives, prepared through halogen-metal exchange between the corresponding bromo or iodo heterocycles and alkyllithium reagents, ring-open to thioenynes (*3*), which with the alkyl halide formed in the halogen-metal exchange and added in excess give alkylthiovinyl acetylenes (*4*). If the halogen-metal exchange is carried out with phenyllithium, alkylation of *3* can be carried out with other alkylating agents (dimethyl sulfate, ethyl bromoacetate, benzyl chloride).

From ring-opening experiments with 3-bromo-2-ethyl-5-methyl- and 3-bromo-5-ethyl-2-methylthiophene, as well as with 3-bromo-2-*t*-butyl-5-methyl- and 3-iodo-5-*t*-butyl-2-methylthiophene, it was demonstrated that these reactions formally correspond to a strictly regio- and stereospecific addition of thiolates to unsymmetrically substituted diacetylenes. It is also shown that the addition of ethylthiolate to 2,5-heptadiyne is not regiospecific. From 2,4,5-trimethyl-3-thienyllithium a fully substituted vinylacetylenic thioether, which cannot be prepared by thiolate addition to diacetylenes, was obtained.

By-products formed in the ring-opening reactions have been identified.

The synthesis of the thiophenes used as starting materials is described.

Some years ago, we discovered that *2a*, prepared through halogen-metal exchange between 2,5-dimethyl-3-iodoselenophene (*1a*) and ethyllithium, ring-opened even at −70 °C to give *3a*, which was alkylated to yield *4a*, by the ethyl iodide formed in the halogen-metal exchange.[1] We also found that when *2b*, prepared from *1b* and methyllithium, was refluxed in ether for 2 h and then hydrolyzed, a 42 % yield of *4b* was obtained *via* the thiolate *3b*.[2] It followed from experiments with 3-selenienyllithium and 3-thienyllithium in the presence of ethyl bromide, which gave (*Z*)-1-ethylseleno-1-buten-3-yne and (*Z*)-1-ethylthio-1-buten-3-yne, that the ring-opening was stereospecific.[3]



1 *2* *3* *4*

Scheme 1.

a, R=R′=CH_3, R″=C_2H_5, X=Se, Y=I;
b, R=R′=CH_3, R″=CH_3, X=S, Y=I;
c, R=R″=C_2H_5, R′=CH_3, X=S, Y=Br;
d, R=CH_3, R′=R″=C_2H_5, X=S, Y=Br;
e, R=$C(CH_3)_3$, R′=CH_3, R″=C_2H_5, X=S, Y=Br;
f, R=CH_3, R′=$C(CH_3)_3$, R″=C_2H_5, X=S, Y=I.

The formation of *4a* and *4b* formally corresponds to the addition of ethaneselenol and methanethiol respectively to a symmetrically disubstituted diacetylene (*i.e.* 2,4-hexadiyne). The addition of ethanethiol to such diacetylenes under alkaline conditions has been studied by Russian workers.[5–7] In this way *4b* (R″=C_2H_5) was prepared but the stereochemistry of the addition was not discussed. It is known from the work of Truce *et al.*[8,9] that this addition is predominantly *trans* with monoacetylenic compounds. However, it has been shown that in substituted diacetylenes containing one polar group such as carboxyl [10] or hydroxymethyl,[11] the *trans*-stereospecificity of the addition was

* Part V. see Ref. 36.
** To whom correspondence should be addressed.
*** Taken in part from the Ph.D. thesis of T. Frejd, Lund 1975.

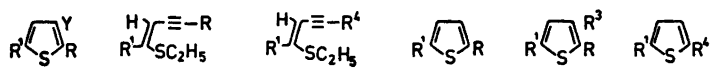
lost, leading to mixtures of *Z*- and *E*-thioenynes. However, the regiospecificity was good and determined by the electronic properties of the substituent, the nucleophile attacking the most electron-deficient terminal carbon of the triple bond array.[10,11] Petrov *et al.*[7] also claimed that ethanethiolate added exclusively to the acetylenic carbon bearing the *t*-butyl and isopropyl group of 6,6-dimethyl-2,4-heptadiyne and 6-methyl-2,4-heptadiyne. (However, *cf.* below.)

Ring-opening reactions. We were therefore interested in investigating the general applicability of the ring-opening reaction for the synthesis of alkylthiovinyl acetylenes, corresponding to a formal regio- and stereospecific addition of thiolates to unsymmetrically disubstituted diacetylenes.

By adding ethyllithium rapidly to *1c* at room temperature, and then adding an excess of ethyl iodide to the reaction mixture, a 60 % yield of *4c* could be isolated. In an analogous way, *1d*, *1e*, and *1f* gave *4d*, *4e*, and *4f*, respectively. The yields obtained are given in Table 1.

We have also found that the addition of ethylthiolate to an unsymmetrically disubstituted diacetylene, in which the two substituents are similar, does not occur regiospecifically. Thus, addition of ethylthiolate to 2,4-heptadiyne, following the procedure of Petrov *et al.*,[7] gave a mixture of 60 % of *4c* and 40 % of *4d* according to GLC analysis. The fact that ethylthiolate shows a weak preference for the methyl bearing carbon of 2,4-heptadiyne, made us therefore doubt the previously mentioned results obtained by Petrov *et al.*[7] These authors claimed that only *4f* was obtained in the addition of ethylthiolate to 6,6-dimethyl-2,4-heptadiyne through an attack on the *t*-butyl carrying carbon. They suggested this structure as they considered the observed methyl long-range coupling to be too small to be that expected for *4e*. They explained this result by assuming that the propagation of the stronger +I-effect of the branched alkyl groups through the π-bond system made the methyl-bearing carbon more electron-rich than the *t*-butyl-bearing carbon. Steric effects on the orientation were not considered. However, we found that the IR spectrum of the product published by these authors was identical with that of *4e* and not of *4f* prepared by

Table 1. Reaction product analysis of the ring-opening experiments on compounds *1c*–*1f* with equivalent amounts of ethyllithium at 21 °C. Reaction time: 4 h. Conditions: A, a fivefold excess of ethyl iodide; B, no ethyl iodide added. Uncalibrated GLC values; isolated yields in parentheses.



1 *4* *5, 8* *6, 10* *11, 12* *7, 9*

Starting material	Cond.	Products and yield (%)			
R=C_2H_5, R^1=CH_3, R^4=$CH(CH_3)C_2H_5$, Y=Br					
1c	A	*4c* 75 (60)	*5* 20	*6* 3	*7* 2
	B	*4c* 31	*5* 3	*6* 57	*7* 9
R=CH_3, R^1=C_2H_5, R^4=C_3H_7, Y=Br					
1d	A	*4d* 79 (64)	*8* 11	*6* 8	*9* 2
	B	*4d* 24	*8* 6	*6* 51	*9* 19
R=$C(CH_3)_3$, R^1=CH_3, R^3=C_2H_5, Y=Br					
1e	A	*4e* 90		*10* 10	*11*
	B	*4e* 40		*10* 55	*11* 2
R=CH_3, R^1=$C(CH_3)_3$, R^3=C_2H_5, X=I					
1f	A	*4f* 50 (35)		*10* 4	*12* 46 (33)
	B	*4f* 23		*10* 60	*12* 17

us. Their experiment was repeated and it was found by careful GLC analysis that *4e* was formed exclusively and in 65 % yield. We found that the coupling constant of the ethylenic methyl protons and the ethylenic proton of *4e* was 1.4 Hz (allylic coupling) and that of the acetylenic methyl protons and the ethylenic proton of *4f* was 2.4 Hz.

It is obvious that the ring-opening reactions are of value for the synthesis of the sterically most unfavourable of two possible isomers of alkylthiovinyl acetylenes.

6,6-Dimethyl-2,4-heptadiyne was prepared in 58 % yield by treating *21* with two equivalents of ethereal ethyllithium and ethyl iodide. When the same reagents were applied to 3,4-diiodo-2,5-dimethylthiophene,[13] a 54 % yield of 2,4-hexadiyne was obtained. This type of ring-opening was first observed by Wittig and Rings[14] with 3,4-diiodo-2,5-diphenylthiophene.

Since several by-products were formed in some of the ring-openings, a more detailed product analysis was undertaken in the experiments with *1c–1f*. Two sets of experimental conditions were used: one with an excess (fivefold) of ethyl iodide (A) and the other without added ethyl iodide (B). All experiments were run with one equivalent of ethyllithium per equivalent of haloheterocycle at room temperature for 4 h whereupon water was added. The results are given in Table 1. An excess of ethyl iodide must apparently be used to obtain acceptable yields of *4e–4f*. The dehalogenated heterocycles *6* and *10*, which become the main products if excess ethyl iodide is not used, most probably arise from protonation of the enynethiolates *3c–3f*. Intramolecular addition of the resulting thiols to the triple bond then leads to *6* and *10*. It has been demonstrated by NMR studies that the ring-opening of the 3-thienyllithium derivatives (*2b*, *2e–f*) is complete[15] and it is therefore unlikely that *6* and *10* are formed by protonation of *2c–f*. It is evident that the S_N2 *S*-alkylation of *3c–f* is relatively slow so that, in the absence of excess ethyl iodide, alkylation is far from complete after 4 h.

In the reaction of *1f* large amounts of *12* were formed, and isolated when excess ethyl iodide was used. The reason for this became evident when a kinetic study of the ring-opening reaction was carried out.[15] The ring-opening of *2f* was rather slow, so that Wurtz-Fittig coupling of *2f* with ethyl iodide could successfully compete. This side-reaction could also occur in other cases, where the ring-opening is slow. It should, however, be possible to avoid it by carrying out the halogen-metal exchange with phenyllithium (*cf.* below). It has been previously demonstrated that 2-thienyllithium undergoes Wurtz-Fittig coupling with various alkyl halides.[16] A few percent of the coupling product were also formed in the reaction of *1e*. However, the presence of *11* was only indicated by combined GLC-MS spectrometry. The mass spectrum was rather weak, which made the interpretation of the fragmentation pattern unreliable, and the structure is accordingly uncertain.

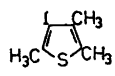
With the ethyl derivatives *1c* and *1d* other types of by-products were observed. Thus in the reaction of *1c* with ethyllithium and excess ethyl iodide, 20 % of *5* and 2 % of *7* were obtained. When equivalent amounts of ethyl iodide were used, the relative amounts were reversed and 3 % of *5* and 9 % of *7* were formed. Similarly, *8* and *9* were formed in the reaction of *1d* and showed the same variation with the amount of ethyl iodide as mentioned above (*cf.* Table 1).

The formation of *5* and *7* is certainly due to deprotonation of a propargylic CH_3 group by ethyllithium or the thienyllithium present, followed by *C*- and *S*-alkylation by ethyl iodide. It is well known that propargylic CH_3 groups are quite acidic.[17] The fact that *7* and *9* are formed in larger amounts when only equivalent amounts of ethyl iodide are used indicates that *C*-alkylation of the propargylic anion is more rapid than *S*-alkylation, the homologous enynethiolates ring-closing to the thiophenes *7* and *9*, upon hydrolysis.

The evidence for the formation of *5* was only its mass spectrum and its slightly longer GLC retention time compared to *4c*. The compound *8*, on the other hand, was isolated and gave a satisfactory ^1H NMR spectrum showing the propyl grouping. Its mass and IR spectra also confirmed the structure. The formation of *9* was proven by comparing its mass spectrum with literature data as well as by comparing its retention time (GLC) with that of an authentic sample. In the case of *7*,

only the mass spectrum was taken as evidence for its formation. The mass spectrum of the isomeric *16* was, however, quite different from that of *7*.

In order to demonstrate that 3-thienyllithium derivatives with substituents in the 4-position could also be utilized for the synthesis of thioenynes, *13* was treated with ethyllithium and ethyl iodide at room temperature. As expected, *14* was formed (50 %). During the progress of this work, Jakobsen [4] reported the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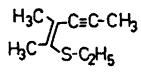
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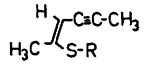
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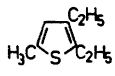
a R = $CH_2C_6H_5$
b R = CH_2CO_2H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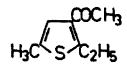
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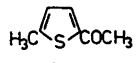
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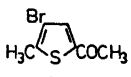
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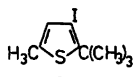
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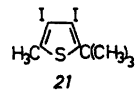
21

ring-opening of 4-methyl-3-thienyllithium in the presence of butyl bromide, which afforded (*Z*)-1-butylthio-2-methyl-1-buten-3-yne. These results show the synthetic possibility of preparing thioenynes with substituents in all positions. It should be pointed out that compounds like the above-mentioned thioenynes cannot be synthesized by adding thiolates to diacetylenes, but more elaborate routes must be used.

It should be stressed that our approach is not limited to the use of alkylating agents corresponding to the alkyl halide formed in the halogen-metal exchange. Thus the reaction of *1b* with ethereal phenyllithium at room temperature yielded *2b*, which ring-opened smoothly to *3b*, which of course does not react with iodobenzene. Addition of dimethyl sulfate gives a 63 % yield of *4b*. This yield is higher than that obtained if *1b* is reacted only with methyllithium (42 %).[2] In this connection it should be mentioned that halogen-metal exchange between 2,5-dimethyl-3-bromothiophene and methyllithium is slow. After 21 h at room temperature only 10 % halogen-metal exchange took place and 90 % of the starting bromo derivative was recovered. Obviously methyllithium is not suitable for exchange with bromoarenes. This has previously been observed in the benzene series. [18] When benzyl chloride was added to the ethereal solution of *3b* a 52 % yield of *15a* [19] was obtained. With ethyl bromoacetate *3b* gave a 58 % yield of *15b* after hydrolysis of the initially formed ethyl ester.

Syntheses of starting materials. Compound *1c* [12] was used for the synthesis of *16*, which was needed as a reference compound. Halogen-metal exchange between *1c* and butyllithium at −70 °C followed by *N*,*N*-dimethylacetamide gave *17* in 42 % yield. The relatively low yield is probably due to steric hindrance from the ethyl group, leading to deprotonation of the *N*,*N*-dimethylacetamide by the thienyllithium derivative.[21] Wolff-Kishner reduction [22] of the acetyl derivative *17* then gave the desired diethyl derivative. Starting from 2-*t*-butylthiophene, *1e* was prepared in the same way as *1c* according to the procedure of Lantz and Hörnfeldt.[12] Also *1d* was prepared according to Ref. 12 by bromination of *18* with bromine and aluminium trichloride to give *19*, followed by Wolff-Kishner reduction.

By again following the description of Lantz and Hörnfeldt,[12] *1f* was synthesized from *10* by the iodine-iodic acid method.[23] It was found that a mixture containing 90 % of *1f* and 10 % of its isomer *20* was formed (GLC). A convenient way of eliminating *20* was to treat the mixture with 0.2 equivalent of butyllithium followed by hydrolysis. In this way *1f* was obtained free from the isomer although some of it (10 %) was lost. It is possible that less than 0.2 equivalent of butyllithium is necessary, as it can be expected that the iodine of *20* being *ortho* to the *t*-butyl group, is the most reactive one, due to release of steric strain, when the iodine is replaced by lithium. Such effects have been observed in halogen-metal exchange of some alkylsubstituted bromothiophenes.[24] It was also possible to achieve diiodination of *10* with the iodine-iodic acid method. Prolonged reaction at 80 °C with two equivalents of iodine-iodic acid gave a 43 % yield of *21*.

2,3,5-Trimethylthiophene was prepared from *1b*[13] by halogen-metal exchange with butyllithium at −70 °C followed by reaction with dimethyl sulfate. Iodination with iodine-iodic acid then gave *13* in 59 % yield.

A number of workers have prepared 2,4-heptadiyne by several methods.[25–27] However, the yields were low and this compound was first fully characterized in 1965.[27] The synthesis of diacetylene from 1,4-dichloro-2-butyne has been described by Armitage *et al.*,[28] who used sodium amide in liquid ammonia for the elimination of hydrogen chloride. The alkylation of the diacetylene anion gave various mono- and disubstituted diacetylenes, depending on the conditions. A detailed description of the preparation of 1,3-hexadiyne starting from 1,4-dichloro-2-butyne has been given by Brandsma.[29] By subsequent anion formation of 1,3-hexadiyne with butyllithium in hexane followed by methyl iodide in hexamethylphosphoric triamide we obtained 2,4-heptadiyne in 59 % yield.

Mass spectra were extensively used for molecular weight determination and for distinguishing between alkylthiophenes and the isomeric vinylacetylenic thioethers. The molecular ion fragment was the base peak for most of the enyne thioethers. Exception to this rule was found for *8* (base peak at M − 57). However, the molecular ion was still 89 rel. %, which is not in agreement with the fragmentation of propylthiophenes or ethylthiophenes. These compounds should give base peaks at M − 29 and M − 15, respectively.[30] It is well known that most simple alkylthiophenes give a base peak due to β-cleavage,[31] with a molecular ion of relatively low abundance (20 − 40 %). Accordingly, it was no problem to differentiate between alkylthiophenes and enyne thioethers when all compounds formed in the reactions were not isolated. Quite abundant fragments of the ethylthioenynes were those originating from the CH_3CS ion and from the loss of SC_2H_5. The above mentioned fragmentations were almost negligible for the alkylthiophenes. The fragment at *m/e* 91 ($C_7H_7^+$, tropylium) was prominent for enyne thioethers with seven-carbon chains, while this was not so in the cases of the isomeric thiophenes.

EXPERIMENTAL

General remarks. All experiments with organometallic compounds were performed under a dry oxygen-free nitrogen atmosphere and in solvents (ether, hexane) which were dried and distilled from sodium wire. The nitrogen gas was bubbled through a wash bottle with a basic pyrogallol solution to remove oxygen and then a wash bottle with conc. sulfuric acid to remove moisture. Hexamethylphosphoric triamide (HMPA) was purified according to Ref. 32. Dimethyl sulfate (DMS) and dimethylformamide (DMF) were used freshly distilled, and the iodobenzene (used for the phenyllithium preparations) was distilled twice with an efficient column.

The ethereal organometallic reagents (C_2H_5Li, C_6H_5Li and C_4H_9Li) were all titrated on the total base content with 0.1 M HCl and the real titre of the ethyllithium and butyllithium solutions was obtained by the double titration procedure.[33]

GLC analyses were performed with a Perkin-Elmer 900 gas chromatograph or a Varian 1400 gas chromatograph, both equipped with flame ionization detectors. The areas of the peaks of the gas chromatograms were evaluated with a Varian 480 digital integrator connected to the PE 900 or simply by the triangle approximation. It appeared that the discrepancy between the two methods was less than ±5 rel. %, which was considered small, since calibration was not performed in any case. However, the chromatograms could be reproduced within ±2 rel. %, and therefore fairly accurate comparisons of changes in the product distributions could be made. The columns were made of stainless steel (2.0 mm i.d., 1.9 m), and nitrogen was used as carrier gas (flow rate 35 ml/min). The carriers for the stationary phases were Chrom. W (80/100, for NPGS and BDS), Gas Chrom. Q (80/100, for OV 17 and OV 1) and Diatomite C1Q (100/120, for SE 30). For preparative GLC a Perkin-Elmer F 21 preparative gas chromatograph was used, equipped with a 20 % BDS column (8.0 mm i.d., 2.7 m).

Mass spectra were recorded on an LKB 9000 mass spectrometer with an ionization energy of 70 eV. ^1H NMR spectra were in most cases recorded on a Varian A-60 NMR spectrometer, and in a few cases on a Varian XL 100-15 NMR spectrometer. IR spectra were recorded on a Perkin-Elmer Grating Infrared spectrometer. The melting points were determined with a Reichert melting point microscope and are uncorrected. Elemental analyses were performed by the Department of Analytical Chemistry at the University of Lund, Miss Ilse Beetz, Mikroanalytisches Laboratorium, Kronach, West Germany and by Dornis und Kolbe, Mikroanalytisches Laboratorium, Mülheim a.d. Ruhr, West Germany.

The isolations of the pure acetylenic derivatives were in most cases performed by TLC. Glass plates with an activated 1 mm silica gel layer (Kieselgel GF 254 nach Stahl, 10–40 μ, Merck) were used, and the crude products were applied on the plates as 50 % acetone solutions by the aid of a Camag Chromatocharger. The zones were made visible with a UV lamp (360 nm) and eluted with ether. The purity of the substances was checked with VPC, NMR and elemental analyses.

3-Acetyl-2-ethyl-5-methylthiophene (17). A solution of 10.2 g (49.7 mmol) of 3-bromo-2-ethyl-5-methylthiophene (*1e*) in 100 ml of ether was cooled to −70 °C, and 42 ml (55 mmol) of 1.30 M butyllithium in hexane was added. The mixture was stirred for 1 h at this temperature, whereupon 4.8 g (55 mmol) of *N,N*-dimethylacetamide in 50 ml of ether was added. The reaction mixture was allowed to reach room temperature, stirred overnight and poured into 5 N HCl/ice. After stirring for 1 h, the aqueous layer was extracted with ether and the ethereal portions were washed with water and dried. Evaporation of the solvent and distillation gave 3.5 g (42 %) of the title compound, b.p.$_{0.6}$ 60–65 °C. NMR (CCl_4): δ 6.90 (q, 1 H, 4-H), 2.33 (s, $COCH_3$), 2,40 (bs, 5-CH_3), 3.08 (q, 2 H, 2-C_2H_5), 1.25 (t, 3 H, 2-C_2H_5). $J_{4H,5CH_3}$ = 1.0 Hz; $J_{CH_2-CH_3}$ = 7.0 Hz. [Found: C 63.5; H 7.34; S 19.0. Calc. for $C_{10}H_{12}OS$ (168.26): C 64.25; H 7.19; S 19.06].

2,3-Diethyl-5-methylthiophene (16). A mixture of 3.5 g (21 mmol) of *17* and 5 ml of 99.5 % hydrazine hydrate in 20 ml of ethylene glycol was gradually heated to 140 °C. Water and hydrazine were distilled off. After cooling, 5 g of KOH pellets was added and the mixture was heated to 90–110 °C until the nitrogen evolution ceased (2 h), whereupon the mixture was cooled and poured into 200 ml of 2 N HCl and extracted with ether. The ethereal portions were washed with water and dried. Evaporation and distillation gave 0.6 g (19 %) of the title compound, b.p.$_{12}$ 94–96 °C. Mass spectrum: *m/e* = 154, 35 % (M^+); *m/e* = 139, 100 % ($[M-15]^+$). NMR (CCl_4): δ 6.32 (q, 1 H, 4-H), 2.33 (d, 5-CH_3), 2.2–2.8 and 0.9–1.4 (C_2H_5). $J_{4H,5CH_3}$ = 1.4 Hz. [Found: C 70.13; H 9.05; S 20.65. Calc. for $C_9H_{14}S$ (154.28): C 70.07; H 9.15; S 20.78].

5-t-Butyl-3-iodo-2-methylthiophene (1f). From 45.8 g (0.297 mol) of 2-*t*-butyl-5-methylthiophene (*10*), 27.9 g (0.110 mol) of iodine, 13.9 g (0.0790 mol) of iodic acid, 150 ml of acetic acid, 60 ml of water, 60 ml of CCl_4 and 2 ml of conc. H_2SO_4, 84.0 g of a crude product was obtained, following the procedure described in Ref. 12. The product contained 90 % of the title compound and 10 % of its isomer 2-*t*-butyl-3-iodo-5-methylthiophene (*20*), according to combined GLC-MS analysis (column OV 1, 3 %, 100–210 °C, 10 °C/min). Distillation gave 64.0 g of material still containing *20*, b.p.$_{12}$ 129–130 °C. To a solution of 60.0 g (0.214 mol) of the distillate in 250 ml of ether, 31 ml (0.043 mol) of 1.40 M butyllithium was added at −70 °C. After 30 min the reaction mixture was hydrolyzed with methanol. The usual work-up and distillation gave 35.6 g (43 %) of isomer-free *1f*, b.p.$_{12}$ 128–129 °C, n_D^{20} 1.5704 (lit.[12] b.p.$_{10}$ 120–122 °C, n_D^{20} 1.5715).

5-t-Butyl-3,4-diiodo-2-methylthiophene (21). A mixture of 15.4 g (0.0998 mol) of *10*, 20.6 g (0.0811 mol) of iodine, 7.90 g (0.0449 mol) of iodic acid, 40 ml of acetic acid, 15 ml of water, 20 ml of CCl_4 and 1.0 ml of conc. H_2SO_4 was stirred vigorously at 80 °C for 8 h and worked up as described for *13* below. After distillation, 17.3 g (43 %) of the title compound was obtained, b.p.$_{0.01}$ 113–123 °C, n_D^{20} = 1.6430. NMR (CCl_4): δ 2.48 (s, 3 H, CH_3), 1.48 (s, 9 H, $C(CH_3)_3$). [Found: C 26.70; H 3.02. Calc. for $C_9H_{12}I_2S$ (406.07): C 26.62; H 2.98].

A forerun, 7.3 g, b.p.$_{0.01}$ 65–75 °C, was mainly 5-*t*-butyl-3-iodo-2-methylthiophene (*1f*) (NMR).

2,3,5-Trimethylthiophene. A solution of 50.0 g (0.210 mol) of 2,5-dimethyl-3-iodothiophene [13] (*1b*) in 100 ml of ether was cooled to −70 °C, and 146 ml (0.22 mol) of 1.5 M butyllithium in hexane was added, followed by 27.7 g (0.220 mol) of dimethyl sulfate in 100 ml of ether (very slowly). The mixture was stirred at −70 °C for 1.5 h, whereupon the cooling bath was removed and the reaction mixture was allowed to reach room temperature. Conc. ammonium hydroxide was added and the ethereal layer was separated, washed with 2 N HCl, water and dried. Evaporation and distillation gave 14.9 g (56 %) of the title compound, b.p. 161–163 °C (lit.[34] b.p. 163 °C).

4-Iodo-2,3,5-trimethylthiophene (13). A mixture of 12.6 g (0.100 mol) of 2,3,5-trimethylthiophene, 35 ml of acetic acid, 40 ml of water, 35 ml of CCl_4, 4.40 g (0.025 mol) of iodic acid and 4 drops of conc. H_2SO_4 was warmed to 40 °C, whereupon 12.7 g (0.0500 mol) of iodine was added in portions (vigorous stirring). When the addition was complete, the temperature was raised to 60 °C. After 2 h the reaction mixture was poured into aqueous sodium thiosulfate and extracted with CCl_4. The organic portions were washed with water, dried, evaporated and distilled, giving 15.0 g (59 %) of the title compound, b.p.$_{0.7}$ 67–68 °C. NMR (CCl_4): δ 2.07 (s, 3 H, CH_3) and 2.30 (bs, 6 H, CH_3). [Found: C 33.41; H 3.63; S 12.75. Calc. for C_7H_9IS (252.12): C 33.35; H 3.60; S 12.72].

General method for the ring-opening of 3-lithioheterocycles (G)

Nitrogen gas was supplied for 30 min to the predried, hot (110 °C) apparatus, consisting

of a three-necked round-bottomed flask fitted with a condenser (drying tube, $CaCl_2$), stirrer, dropping funnel and a neck for the gas inlet. The dropping funnel was also supplied with nitrogen gas. The 3-halo heterocyclic compound was dissolved in ether and ethereal ethyllithium was added rapidly. After 10 min, an excess of ethyl bromide or iodide was added all at once, and the reaction mixture was kept at +21 °C (room temperature) for 4 h. The reaction mixture was hydrolyzed with water and the aqueous phases were extracted three times with ether. The collected ethereal portions were washed with water to neutral reaction and dried with $MgSO_4$. After the evaporation of the solvent the crude product was purified by distillation or by TLC.

(Z)-2-Methylthio-2-hexen-4-yne (4b). To 100 ml (0.10 mol) of 1.0 M ethereal phenyllithium, 23.8 g (0.100 mol) of *1b* in 100 ml of ether was added at room temperature. After 1/2 h, 13.9 g (0.110 mol) of dimethyl sulfate in 50 ml of ether was slowly added. Ammonia was added after 1 h and the ethereal layer was washed with water, 2 N HCl and water to neutral reaction. After drying and evaporation of the solvent, the residue was distilled, to yield 7.9 g (63 %) of the title compound with the same properties as described previously (NMR, IR, m.p.).[2]

The reaction between 2,5-dimethyl-3-bromothiophene and methyllithium. A solution of 15.0 g (0.0785 mol) of 2,5-dimethyl-3-bromothiophene in 100 ml of ether was cooled to −70 °C, whereupon 100 ml (0.0810 mol) of 0.81 M methyllithium was added dropwise, followed by 5.68 g (0.0400 mol) of methyl iodide. The mixture was then allowed to reach room temperature. A sample was poured onto solid carbon dioxide in ether but no carboxylic acid could be isolated. After 21 h, water was added to the reaction mixture and the ethereal layer was washed with water and dried. GLC analysis (column BDS, 10 %, 105 °C) showed, upon comparison of the retention times with authentic samples, that the starting material amounted to ~90 % and compound *4b* to ~10 %. IR of the evaporated crude product: C≡C 2220 cm⁻¹.

(Z)-2-Hexen-4-yne-2-ylthioacetic acid (15b). To 100 ml (0.100 mol) of 1.0 M phenyllithium, 23.8 g (0.100 mol) of *1b* in 100 ml of ether was added at room temperature. After 1/2 h the reaction mixture was forced over (with nitrogen gas) to a dropping funnel and added to a solution of 16.7 g (0.100 mol) of ethyl bromoacetate in 100 ml of ether (water cooling). The reaction mixture was stirred for 1 h at room temperature, whereupon it was poured into 500 ml of 2 N NaOH and stirred for 2 h. The aqueous layer was separated, extracted with ether and acidified with 5 N HCl. An oil precipitated, which subsequently crystallized. Thus, 10.7 g of the crude title compound was collected by filtration. The pure acid, 9.8 g (58 %), was obtained after recrystallization from ethanol:water, m.p. 84.5 −86.5 °C. IR: C=O 1700 cm⁻¹, C≡C 2210 cm⁻¹. NMR ($CDCl_3$): δ 5.50 (m, 1 H, 4-H), 2.08 (m, $C=CCH_3$), 2.01 (bd, $C\equiv CCH_3$), 3.65 (s, 2 H, $-CH_2-$), 11.87 (s, 1 H, COOH). [Found: C 56.51; H 5.95; S 18.83. Calc. for $C_8H_{10}O_2S$ (170.23): C 56.45; H 5.92; S 18.84].

(Z)-2-Benzylthio-2-hexen-4-yne (15a). With the same amounts of reagents as above, the title compound was prepared by replacing ethyl bromoacetate with 13.9 g (0.110 mol) of benzyl chloride in 50 ml of ether. The reaction mixture was stirred overnight and was subsequently hydrolyzed. The ethereal layer was washed with water and dried. After evaporation of the solvent, the residue was distilled to give 10.5 g (52 %) of *15a*, b.p. $_{2.0}$ 141 − 142 °C, m.p. 53 − 55 °C (lit.[19] m.p. 55 °C). IR: C≡C 2220 cm⁻¹. NMR (CCl_4): δ 7.0 − 7.5 (m, 5 H, C_6H_5), 3.97 (s, 2 H, $-CH_2-$), 5.32 (m, 1 H, 3-H), 1.93 (bs, 6 H, 1-H and 6-H).

(Z)-2-Ethylthio-2-hepten-4-yne (4c). The general method G was followed. From 10.3 g (0.0500 mol) of 3-bromo-2-ethyl-5-methylthiophene (*1c*) in 100 ml of ether, 100 ml (0.060 mol) of 0.60 M ethereal ethyllithium and 39.0 g (0.250 mol) of ethyl iodide, 4.6 g (60 %) of the title compound was obtained by distillation, b.p.$_{13}$ 106 − 107 °C. Combined GLC-MS analysis (column BDS, 10 %, 130 °C) of the washed and dried reaction mixture showed the following compounds: *4c* (*m/e* = 154; calc. for $C_9H_{14}S$ = 154), *(Z)*-2-ethylthio-6-methyl-2-octen-4-yne (*5*) (*m/e* = 182; calc. for $C_{11}H_{18}S$ = 182), 2-ethyl-5-methylthiophene (*6*) (*m/e* = 126; calc. for $C_7H_{10}S$ = 126) and 2-*sec*-butyl-5-methylthiophene (*7*) (*m/e* = 154; calc. for $C_9H_{14}S$ = 154). See Table 1, condition A. IR: C≡C 2220 cm⁻¹. NMR (CCl_4): δ 1.99 (m, 3 H, 1-H), 5.32 (m, 1 H, 3-H), 2.32 (bq, 6-H), 1.37 − 1.05 (7-H), 2.81 and 1.37 − 1.05 (q, *S*-C_2H_5). $J_{SCH_2-CH_3} = J_{6H,7H} = 7.0$ Hz. [Found: C 70.10; H 9.13; S 20.71. Calc. for $C_9H_{14}S$ (154.28): C 70.07; H 9.15; S 20.78]. The reaction was repeated, but ethyl iodide was not added to the reaction mixture, which changed the component distribution of the reaction product as shown in Table 1, condition B.

(Z)-3-Ethylthio-3-hepten-5-yne (4d). The general method G was followed, and the same amounts of reagents as in the preceding experiment were used. Thus, 4.9 g (64 %) of *4d*, b.p.$_{2.0}$ 74 − 75 °C was obtained from 3-bromo-5-ethyl-2-methylthiophene (*1d*). Combined GLC-MS analysis (column BDS, 10 %, 130 °C) of the washed and dried reaction mixture showed the following compounds: *4d* (*m/e* = 154; calc. for $C_9H_{14}S$ = 154), *(Z)*-3-ethylthio-3-nonen-5-yne (*8*) (*m/e* = 182; calc. for $C_{11}H_{18}S$ = 182), 2-ethyl-5-methylthiophene (*6*) (*m/e* = 126; calc. for $C_7H_{10}S$ = 126) and 2-ethyl-5-propylthiophene (*9*) (*m/e* = 154; calc. for $C_9H_{14}S$ = 154). See Table 1, condition A. IR: C≡C 2220 cm⁻¹.

NMR (CCl_4): δ 1.38–0.97 (1-H), 2.25 (q, 2-H), 5.39 (m, 1 H, 4-H), 1.98 (d, 7-H), 2.80 (q, 2 H) and 1.38–0.97 (*S*-C_2H_5). $J_{SCH_2-CH_3} = J_{1H,3H} = 7.0$ Hz. [Found: C 70.21; H 9.17; S 20.65. Calc. for $C_9H_{14}S$ (154.28): C 70.07; H 9.15; S 20.78].

During the distillation, a few mg of almost pure (*Z*)-3-ethylthio-3-none-5-yne (*8*) was collected at 88–89 °C, 2.0 mmHg. NMR (benzene, XL-100 spectrum): δ 1.18–0.87(1-H), 2.06 (q, 2-H), 5.54 (m, 4-H), 2.22 (t, d, 7-H), 1.63 –1.34 (pent, 8-H), 1.15–0.87 (9-H), 2.61 (q) and 1.15–0.87 (*S*-C_2H_5). The small splitting of the signal originating from the terminal $-CH_2-$ group of the propyl group (7 H) was 2.3 Hz. The experiment was repeated, but no ethyl iodide was added to the reaction mixture, which changed the component distribution of the reaction product as shown in Table 1, condition B.

(Z)-2-Ethylthio-6,6-dimethyl-2-hepten-4-yne (4e). The general method G was followed. From 4.66 g (0.0200 mol) of 3-bromo-2-*t*-butyl-5-methylthiophene (*1e*) in 50 ml of ether, 38 ml (0.023 mol) of 0.60 M ethereal ethyllithium and 15.6 g (0.100 mol) of ethyl iodide, 3.13 g (86 %) of crude *4e* was obtained. Combined GLC-MS analysis (column SE 30, 3 % 100 –200 °C, 10 °C/min) showed the following compounds: 2-*t*-butyl-5-methylthiophene (*10*) ($m/e = 154$; calc. for $C_9H_{14}S = 154$) and *4e* ($m/e = 182$; calc. for $C_{11}H_{18}S = 182$). See Table 1, condition A. Isolation of *4e* through distillation was unsuccessful due to vigorous foaming and therefore a pure sample was obtained through preparative TLC (1 mm silica gel, hexane, R_F 0.35–0.16); n_D^{21} 1.5110. IR: C≡C 2220 and 2190 cm^{-1}, C=C 1585 cm^{-1}. NMR (CCl_4): δ 1.97 (d, 3 H, 1-H), 5.34 (q, 1 H, 3-H), 1.25 (s, $C(CH_3)_3$), 2.83 (q, 2 H) and 1.1–1.3 (*S*-C_2H_5). $J_{1H,3H} = 1.4$ Hz. [Found: C 72.50; H 10.00; S 17.52. Calc. for $C_{11}H_{18}S$ (182.33): C 72.46; H 9.95; S 17.59].

The experiment was repeated, but no ethyl iodide was added to the reaction mixture, which changed the component distribution of the reaction product as shown in Table 1, condition B.

(Z)-3-Ethylthio-2,2-dimethyl-3-hepten-5-yne (4f) *and 5-t-butyl-3-ethyl-2-methylthiophene* (12). The general method G was followed. From 28.0 g (0.100 mol) of 5-*t*-butyl-3-iodo-2-methylthiophene (*1f*) in 200 ml of ether, 200 ml (0.11 mol) of 0.55 M ethereal ethyllithium and 78.0 g (0.500 mol) of ethyl iodide, 16.6 g (91 %) of a crude mixture of *4f* and *12* was obtained. Combined GLC-MS analysis (column SE 30, 3 %, 100–200 °C, 10 °C/min) of the washed and dried reaction mixture showed the presence of the following compounds: 2-*t*-butyl-5-methylthiophene (*10*) ($m/e = 154$; calc. for $C_9H_{14}S = 154$), *12* ($m/e = 182$; calc. for $C_{11}H_{18}S = 182$) and *4f* ($m/e = 182$; calc. for $C_{11}H_{18}S = 182$). See Table 1, condition A.

Also in this experiment, distillation was unsuitable due to foaming. Compound *12* was obtained pure by preparative GLC (column BDS, 20 %, 100–200 °C, 10 °C/min). Thus, 5.00 g of the crude product gave 1.8 g (33 %) of *12*. NMR (CCl_4): δ 6.35 (s, 4-H), 2.23 (s, 2-CH_3), 1.30 (s, $C(CH_3)_3$), 2.40 (q) and 1.13 (t, 3-C_2H_5). $J_{CH_2-CH_3} = 7$ Hz. [Found: C 72.2; H 9.88; S 17.6. Calc. for $C_{11}H_{18}S$ (182.33): C 72.46; H 9.95; S 17.59].

Compound *4f* was obtained pure by preparative TLC (1 mm silica gel, hexane, R_F 0.52 –0.64). Compound *12* had R_F 0.18–0.36, but was not isolated in this way. Thus, 1.00 g of the crude product gave 0.38 g (35 %) of *4f*, $n_D^{21} = 1.5149$. IR: C≡C 2220 and 2040 cm^{-1}, C=C 1580 cm^{-1}. NMR (CCl_4): δ 1.14 (s, $C(CH_3)_3$), 5.75 (q, 1 H, 4-H), 1.99 (d, 3 H, 7-H), 2.98 (q, 2 H) and 1.09–1.33 (*S*-C_2H_5). $J_{SCH_2-CH_3} = 7.5$ Hz; $J_{4H,7H} = 2.4$ Hz. [Found: C 72.2; H 9.87; S 17.3. Calc. for $C_{11}H_{18}S$ (182.33): C 72.46; H 9.95; S 17.59].

The experiment was repeated, but no ethyl iodide was added to the reaction mixture, which changed the component distribution as shown in Table 1, condition B.

(Z)-2-Ethylthio-3-methyl-2-hexen-4-yne (14). The general method G was followed. From 10.0 g (0.0397 mol) of 4-iodo-2,3,5-trimethylthiophene (*13*) in 100 ml of ether, 67 ml (0.040 mol) of 0.60 M ethereal ethyllithium and 21.8 g (0.200 mol) of ethyl bromide, 5.50 g of crude product was obtained. Distillation gave 3.12 g (51 %) of *14*, b.p.$_{0.6}$ 52–54 °C. IR: C≡C 2220 and 2040 cm^{-1}. NMR (CCl_4): δ 1.82 (s, 3 H, CH_3), 1.97 (bs, 6 H, CH_3), 2.72 (q, 2 H) and 1.21 (t, 3 H, *S*-C_2H_5). $J_{SCH_2-CH_3} = 7$ Hz. [Found: C 70.00; H 9.10; S 20.85. Calc. for $C_9H_{14}S$ (154.28): C 70.07; H 9.15; S 20.78].

2,4-Heptadiyne. To a solution of 14.0 g (0.179 mol) of 1,3-hexadiyne [29] in 100 ml of ether, 130 ml (0.185 mol) of 1.42 M butyllithium in hexane was added at –50 °C followed by 28.5 g (0.200 mol) of methyl iodide after 10 min. The reaction was followed by GLC (column OV 17, 3 %, 70–150 °C, 10 °C/min) of hydrolyzed samples. Since no conversion had occurred after 1/2 h, 50 ml of dry HMPA was added. An exothermic reaction took place and the starting material was completely consumed 5 min after the addition of HMPA. The reaction mixture was poured into ice-water, extracted with ether, and dried. The ethereal solution was filtered and 50 ml of decalin was added. The ether was distilled off at ordinary pressure, whereupon the pressure was lowered to 100 mmHg. A fraction was collected between 60 and 70 °C, and it was redistilled to give 9.2 g (56 %) of the title compound, b.p.$_{70-90}$ 75–85 °C (lit.[26] b.p.$_{32}$ 59–60 °C). NMR (CCl_4): δ 1.90 (t, 3 H, 1-H), 2.25 (q with fine structure, 2 H, 6-H), 1.17 (t, 3 H, 7-H). $J_{1H,6H} = 1.0$ Hz; $J_{6H,7H} = 7.0$ Hz.

The addition of ethanethiolate to 6,6-dimethyl-2,4-heptadiyne. A mixture of 1.10 g (9.17 mmol)

of 6,6-dimethyl-2,4-heptadiyne, 1.13 g (18.2 mmol) of ethanethiol and 0.11 g of KOH in 4 ml of methanol was heated to 120 °C for 3.5 h in a 10 ml Pyrex ampoule. The reaction mixture was poured into water, which was extracted several times with ether. GLC analysis (column OV 1, 3 %, 110–260 °C, 10 °C/min) of the dried ethereal solution showed mainly one component (~70 %) with the same retention time as *4e* together with 30 % of starting material. Evaporation of the solvent and the remaining starting material yielded 1.1 g (65 %) of crude *4e*. NMR (CCl_4) of the crude product was identical with that of *4e* but different from that of *4f*. The same was true for an IR spectrum.

The addition of ethanethiolate to 2,4-heptadiyne. With the same method as in the preceding experiment, a mixture of *4c* and *4d* in the proportions 60:40 (GLC, column OV 17, 3 %, 80–200 °C, 10 °C/min) was obtained from 3.69 g (40.1 mmol) of 2,4-heptadiyne, 5.0 g (81 mmol) of ethanethiol and 0.3 g of KOH in 6 ml of methanol. The identification of the components was performed by comparison of the retention times (GLC) with those of authentic samples of *4c* and *4d* ("mixing" analysis). Evaporation of the solvent and the remaining starting material gave 3.7 g (52 %) of crude product.

6,6-Dimethyl-2,4-heptadiyne. To 12.2 g (0.0300 mol) of 2-*t*-butyl-3,4-diiodo-5-methylthiophene (*21*) in 100 ml of ether, 100 ml (0.065 mol) of 0.65 M ethereal ethyllithium was added at −70 °C followed by 4.7 g (0.030 mol) of ethyl iodide. After 1 h, the reaction mixture was allowed to reach room temperature and kept there for 4 h, whereupon it was poured onto solid carbon dioxide in ether. No carboxylic acid could be isolated from the basic extracts upon acidification. The neutral ethereal phase was dried and the solvent was removed by distillation at ordinary pressure. The title compound was obtained at reduced pressure, b.p.$_{14}$ 57–59 °C, 2.1 g (58 %). n_D^{22} 1.4780 (lit.[7] b.p.$_{10}$ 51–51.5 °C, n_D^{20} 1.4802). IR: C≡C 2195, 2155 and 2040 cm^{-1} (lit.[7] 2192, 2151 and 2030 cm^{-1}).

2,4-Hexadiyne. With the same procedure as in the preceding experiment, the title compound was obtained from 36.4 g (0.100 mol) of 3,4-diiodo-2,5-dimethylthiophene[18] in 1 l of ether, 250 ml (0.20 mol) of 0.80 M ethereal ethyllithium and 15.6 g (0.100 mol) of ethyl iodide. The dried neutral ethereal extract was concentrated to half its volume and the residue was cooled to −70 °C, whereupon the crystals were collected by suction. Repeated concentration of the filtrates and cooling gave a total of 5.0 g (54 %) of the title compound, m.p. 67–68 °C (lit.[35] 66–67 °C).

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Studies on Orchidaceae Glycosides 4.* The Structures and Absolute Configurations of *cis*- and *trans*-Crassinodine, Two Glucosides from *Dendrobium crassinode* B. & Rf.

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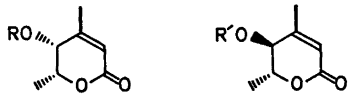
Two glucosides, *cis*- and *trans*-crassinodine (*1* and *2*), have been found in *Dendrobium crassinode* B. & Rf. Compound *1* was isolated as its tetraacetate. The glucosides *1* and *2* are shown to be the 4*R*, 5*R* and 4*S*, 5*R* isomers, respectively, of 4-β-D-glucopyranosyloxy-3-methylhex-2-en-5-olide.

Two glucosides, *cis*- and *trans*-crassinodine (*1* and *2*) have been found in *Dendrobium crassinode* B. & Rf. *trans*-Crassinodine (*2*) was isolated from the glucosidic mixture by fractional crystallisation. The *cis* isomer (*1*), which could not be obtained in a pure state, was isolated and characterised as its tetraacetate (*3*).



1: R = β-D-glucopyranosyl
2: R' = β-D-glucopyranosyl
3: R = 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl
4: R' = 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl
5: R = H
6: R' = H
7: R' = propionyl

Spectrochemical and elemental analyses indicate that *cis*- and *trans*-crassinodine (*1* and *2*) have the composition $C_{13}H_{20}O_8$. Sugar [2,3] and methylation [4] analyses show *1* and *2* to be glucopyranosides. Since β-glucosidase (emulsin) hydrolyses the glucosides, the sugar moieties in *1* and *2* must be D-glucose, β-linked to the aglycones. The β-configuration of the glucosidic bonds in *1* and *2* is also evident from the chemical shifts (δ 4.66 and 4.64) and the large coupling constants (8 Hz) of the anomeric protons in the corresponding tetraacetyl derivatives (*3* and *4*).[5]

Enzymatic hydrolysis of the mixture of glucosides with emulsin gave the aglycones *5* and *6*, which were separated by preparative TLC. When *2* was treated with emulsin, only aglycone *6* was obtained. The spectral properties (UV, IR and NMR) of *5* and *6* are in accordance with those reported for racemic *cis*- and *trans*-3-methyl-4-hydroxyhex-2-en-5-olide, respectively.[6]

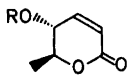
The propionyl derivative of *6* (*7*) has a specific rotation of +98° (in methanol). Since the 4*R*, 5*S* isomer of 3-methyl-4-propionyloxyhex-2-en-5-olide has been reported [7] to have the specific rotation −94.1° (in ethanol), it follows that the aglycone *6* has the 4*S*,5*R* configuration, and hence that the glucoside *2* is (4*S*,5*R*)-4-β-D-glucopyranosyloxy-3-methylhex-2-en-5-olide.

The influence of an allylic oxygen atom on the sign of the Cotton effect associated with the $\pi \rightarrow \pi^*$ transition in UV, located in the 205−230 nm region for enelactones, has been described.[8] A right-handed chirality in the allylic oxygen-olefinic double bond system results in a positive contribution to the CD curve, while a left-handed chirality results in a negative contribution. This sign/chirality re-

* For paper 3 in this series, see Ref. 1.

lationship is illustrated by the CD curves of *2*, *4* and *6* which show a positive effect at 226.5, 224 and 231.5 nm, respectively, consistent with the 4*S* configuration. In the similar compound (4*R*,5*S*)-4-hydroxyhex-2-en-5-olide, osmundalactone [9] (*8*), the corresponding effect at 238 nm is negative.

The CD curves of *3* and *5* show negative Cotton effects at 220 and 218 nm, respectively, which indicate a 4*R* configuration. This assignment is further supported by the application of Mills' rule for cyclic allylic alcohols [10] to *5* and *6*.[11] This rule states that an allylic alcohol with a left-handed chirality of the allylic hydroxyl-double bond helix is more laevorotatory at the sodium D line than its epimer. The aglycones *5* and *6* have specific rotations of −259 and 0°, respectively. Hence the aglycone *5* has the 4*R*,5*R* configuration and the glucoside *1* is (4*R*,5*R*)-4-β-D-glucopyranosyloxy-3-methylhex-2-en-5-olide.



8 : R = H
9 : R = ß-D-glucopyranosyl

Lactones similar to *5* and *6* have been found in some fungi [12–14] and higher plants.[9,15] One of these, osmundalactone (*8*), occurs as the aglycone in the glucoside osmundalin (*9*), isolated from two ferns, *Osmunda japonica* and *O. regalis*.[9] Those of fungal origin show some antibiotic activity [12,14] whereas osmundalin (*9*) may be carcinogenic.[9]

EXPERIMENTAL

General conditions were the same as in an earlier communication.[16] The 60 MHz NMR spectra were measured on a Varian A-60A spectrometer and the CD spectra on a Jasco J-40 spectropolarimeter.

Isolation of the glucosidic mixture (1 *and* 2). Fresh plants of *Dendrobium crassinode* B. & Rf. (2.5 kg) were extracted with methanol (9.5 l). The extract was concentrated to 0.5 l, acidified (pH 4) with hydrochloric acid and washed with carbon tetrachloride (7 × 60 ml). The aqueous phase was made alkaline (pH 8) with sodium hydrogen carbonate, washed with ether (3 × 50 ml), neutralized with hydrochloric acid and evaporated to dryness. The residue was extracted with methanol (2 × 300 ml) and the combined extracts were concentrated to 200 ml. Ethanol (400 ml) was added and the precipitate filtered off. The ethanolic solution was evaporated and the residue was extracted with ethanol-methanol (3:1, v/v, 300 ml). The extract was evaporated and the residue filtered through a column of silica gel (5 × 15 cm, methanol). The eluate was evaporated giving an amorphous residue (18 g).

A portion (5 g) of this residue was chromatographed on silica gel (5 × 15 cm, chloroform-ethanol 2:1, v/v) yielding a mixture of *1* and *2* (800 mg).

Characterisation of 2. Part of this mixture (400 mg) was recrystallised four times from ethyl acetate-methanol giving *2* as needles (40 mg) which started to melt at 94.5 °C, solidified, and melted again at 177.5–182 °C; $[\alpha]_{578}^{25}$ +39° (*c* 0.7, methanol). UV, λ_{max} (methanol) (log ε): 215.5 (4.0) nm. CD, $\lambda_{extrema}$ (methanol) (Δε): 226.5 (+8.45), 256 (−1.92) nm. IR, ν_{max} (KBr): 3480 (s), 3280 (s), 1730 (s), 1640 (w) cm^{-1}. ^{1}H NMR, 60 MHz, δ (pyridine-d_5): 1.46 (d, 3 H), 2.08 (m, 3 H), 3.58–5.00 (m, 9 H), 5.70–7.25 (4 H, exchangeable in D_2O), 5.92 (m, 1 H). (Found: C 51.0; H 6.9; O 42.2. Calc. for $C_{13}H_{20}O_8$: C 51.3; H 6.6; O 42.1).

Acetylation of the glucosidic mixture. A solution of the glucosidic mixture (28 mg), pyridine (1 ml) and acetic anhydride (0.5 ml) was left at room temperature for 20 h. The reaction mixture was poured into ice-water and extracted with chloroform (2 × 3 ml). The chloroform extracts were combined, dried and evaporated. The acetylated glucosides were separated by preparative TLC on silica gel. The plate was developed twice with benzene-acetone (4:1, v/v), giving two bands, R_F 0.45 (fraction I) and R_F 0.50 (fraction II).

Tetra-O-*acetyl*-cis-*crassinodine* (3). Fraction I (10 mg) was crystallised from ethyl acetate giving *3* (6 mg), m.p. 162–163 °C; $[\alpha]_{578}^{21}$ −101° (*c* 0.2, methanol). UV, λ_{max} (methanol) (log ε): 214.5 (4.04) nm. CD, $\lambda_{extrema}$ (methanol) (Δε): 220 (−15.7), 257.5 (−1.48) nm. IR, ν_{max} ($CHCl_3$): 1759 (s), 1650 (w) cm^{-1}. ^{1}H NMR, 100 MHz, δ ($CDCl_3$): 1.41 (d, 3 H, J=7 Hz), 1.96–2.24 (15 H), 3.56–3.82 (m, 1 H), 4.13–4.27 (m, 1 H), 4.19 (d, 2 H, J=4 Hz), 4.46 (m, 1 H), 4.66 (d, 1 H, J=8 Hz), 4.91–5.36 (m, 3 H), 5.91 (m, 1 H). (Found: C 53.3; H 5.9; O 40.6. Calc. for $C_{21}H_{28}O_{12}$: C 53.4; H 6.0; O 40.6).

Tetra-O-*acetyl*-trans-*crassinodine* (4). Fraction II (23 mg) was crystallised from ethyl acetate-light petroleum giving *4* (20 mg), m.p. 171–173 °C: $[\alpha]_{578}^{25}$ +28° (*c* 0.3, methanol). UV, λ_{max} (methanol) (log ε): 213.5 (4.06) nm. CD, $\lambda_{extrema}$ (methanol) (Δε): 201 (−7.45), 224 (+10.1), 254.5 (−1.32) nm. IR, ν_{max} (KBr): 1757 (s), 1725 (m), 1660 (w) cm^{-1}. ^{1}H NMR, 100 MHz, δ ($CDCl_3$): 1.43 (d, 3 H, J=7 Hz), 1.95–2.12 (15 H), 3.60–3.84 (m, 1 H), 4.08

(d, 1 H, $J=7.5$ Hz), 4.22 (d, 2 H, $J=4$ Hz), 4.44 (m, 1 H), 4.64 (d, 1 H, $J=8$ Hz), 4.90–5.40 (m, 3 H), 5.84 (m, 1 H). (Found: C 53.5; H 6.1; O 40.6. Calc. for $C_{21}H_{28}O_{12}$: C 53.4; H 6.0; O 40.6).

Enzymatic hydrolysis of the glucosidic mixture. A mixture of the glucosides (*1* and *2*, 52 mg) was dissolved in a potassium hydrogen phthalate-sodium hydroxide buffer solution (pH 5.2, 4 ml) and emulsin (5 mg) was added. The mixture was kept at 37 °C for 12 h, and then extracted with ether (6 × 1 ml). The ether solutions were combined, dried and evaporated, leaving a mixture (16 mg) of the aglycones *5* and *6*. These were separated by preparative TLC on silica gel. The plate was developed twice with chloroform-ethyl acetate (1:1, v/v), giving two bands, R_F 0.45 (*5*) and R_F 0.50 (*6*).

(4R,5R)-4-Hydroxy-3-methylhex-2-en-5-olide (5). Needles, m.p. 101–102 °C (toluene); $[\alpha]_D^{25}$ −259°, $[\alpha]_{578}^{25}$ −278° (*c* 0.11, methanol). CD, $\lambda_{extrema}$ (methanol) ($\Delta\varepsilon$): 218 (−11.0), 256.5 (−1.83) nm. The UV, IR and NMR spectra were in accordance with those of the racemate.[6] (Found: C 58.9; H 7.1; O 33.4. Calc. for $C_7H_{10}O_3$: C 59.1; H 7.1; O 33.8).

(4S,5R)-4-Hydroxy-3-methylhex-2-en-5-olide (6). Plates, m.p. 121–122 °C (toluene); $[\alpha]_{578}^{26}$ −1.0° (*c* 0.5, methanol); $[\alpha]_{578}^{26}$ −38° (*c* 0.5, acetone). CD, $\lambda_{extrema}$ (methanol) ($\Delta\varepsilon$): 207 (−11.2), 231.5 (+5.28), 256 (−3.49) nm. The UV, IR and NMR spectra are in accordance with those of the racemate.[6] (Found: C 59.0; H 7.0; O 33.5. Calc. for $C_7H_{10}O_3$: C 59.1; H 7.1; O 33.8).

(4S,5R)-3-Methyl-4-propionyloxyhex-2-en-5-olide (7). A solution of *6* (19 mg), pyridine (0.5 ml) and propionyl chloride (0.5 ml) was left at 0 °C for 1 h. Ice-water was added and the reaction mixture was extracted with ether (3 × 1 ml). The ether extracts were combined, washed with hydrochloric acid (0.5 M, 1 ml), dried and evaporated. The residue was purified by preparative TLC on silica gel (chloroform). The plate was developed twice and *7* was isolated as an oil (14 mg); $[\alpha]_D^{21}$ +98° (*c* 1.4, methanol) ($[\alpha]_D^{26}$ −94.1° (ethanol) for the enantiomer[7]). CD, $\lambda_{extrema}$ (methanol) ($\Delta\varepsilon$): 223 (+8.52), 252.5 (−1.06) nm. The IR and NMR spectra were in accordance with those of the enantiomer.[7]

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Synthesis of Methyl 3-*O*-(3,6-Dideoxy-α-D-*xylo*-hexopyranosyl)-α-D-mannopyranoside

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The synthesis of methyl 3-*O*-(3,6-dideoxy-α-D-*xylo*-hexopyranosyl)-α-D-mannopyranoside, required for immunological studies of *Salmonella*, is described. The glycosylation was performed by means of the Helferich procedure and the influence of a *p*-nitrobenzoyl group or a non-participating benzyl group in the 2-position in the 3,6-dideoxyhexosyl bromide used, was investigated.

The disaccharide unit 3-*O*-α-abequopyranosyl-α-D-mannopyranosyl (3-*O*-(3,6-dideoxy-α-D-*xylo*-hexopyranosyl)-α-D-mannopyranosyl) corresponds to the *Salmonella* O-factor 4,[1] which when covalently linked to a protein would provide an antigen of immunological interest.[2] Its methyl glycoside (title compound) is of interest as an O-4 inhibitor and was required for immunological studies. In these studies we have explored two different pathways to the title substance; the more efficient of these has subsequently been used in the synthesis of *p*-isothiocyanatophenyl 3-*O*-abequopyranosyl-α-D-mannopyranoside, which can be covalently linked to suitable proteins. For these exploratory studies we chose the methyl rather than the *p*-isothiocyanatophenyl mannoside, since the former is the more readily accessible. In addition, the products of glycosylation using the methyl mannoside aglycone described in this work are more readily purified and identified than those from the corresponding *p*-isothiocyanato mannoside aglycone described in Ref. 11.

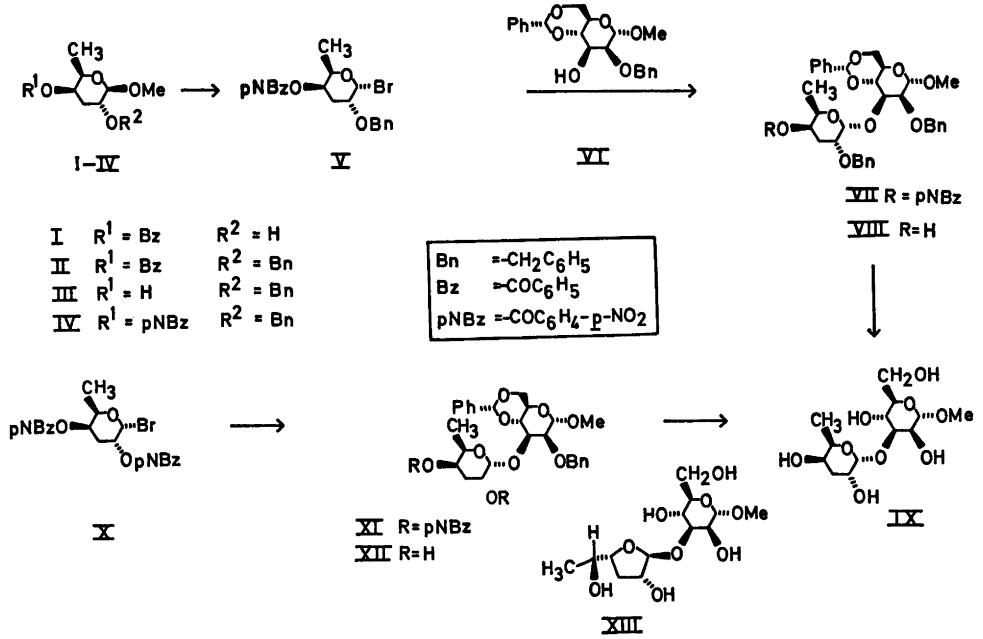
Fucopyranosyl bromides containing a non-participating benzyl group in the 2-position are useful for the synthesis of α-D- (α-L-) fucopyranosides[3] and we thus anticipated that abequopyranosyl bromides benzylated in the 2-position would give similar results. We have previously obtained methyl 4-*O*-benzoyl-β-abequopyranoside from methyl β-D-fucopyranoside *via* treatment of the 3,4-*O*-benzylidene acetal with *N*-bromosuccinimide[4] and hydrogenation of the resulting 3-bromo compound.[5] Benzylation of the remaining free 2-position of methyl 4-*O*-benzoyl-β-abequopyranoside is most efficiently carried out using benzyl trifluoromethanesulfonate.[6]

The glycosylation of a 2,4-di-*O*-acylabequosyl halide offers a more direct route, providing the yield of the desired α-abequopyranoside is high enough. A *p*-nitrobenzoyl group in the axial position in fucopyranosyl halides containing a non-participating benzyl group in the 2-position has previously been shown to favour the formation of α-glycosides.[3,7] In 2,4-di-*O*-*p*-nitrobenzoylabequosyl bromide, a potential precursor for α-abequopyranosides, competing participation from the 2-position might, however, interfere and give a β-glycopyranoside. Indeed, 2,4-di-*O*-*p*-nitrobenzoyl-3,6-dideoxy-D-*ribo*-hexopyranosyl bromide, with an equatorial group in the 4-position yields the β-glycoside exclusively.[8] In the present work, however, the controlling influence of the axial 4-*O*-*p*-nitrobenzoyl group in the 2,4-di-*O*-*p*-nitrobenzoylabequosyl bromide is indicated by the fact that the α-pyranoside was the major product.

Methyl 4-*O*-benzoyl-β-abequoside (I)[5] was benzylated with benzyl trifluoromethanesulfonate;[6] the 2-*O*-benzyl ether II was obtained in 51 % yield. Removal of the benzoyl group in II afforded the hydroxy compound III in 85 % yield and acylation of III with *p*-nitrobenzoyl

chloride afforded the 4-*O*-*p*-nitrobenzoate IV in 87 % yield. Treatment of the abequoside IV with hydrogen bromide in dichloromethane afforded the crude bromide V, used directly in the next step. Condensation of the bromide V with methyl 2-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside [9] (VI) in nitromethane-benzene using mercury(II) cyanide as promotor [10] afforded, after chromatographic purification, the α-linked disaccharide derivative VII in 37 % yield. Debenzoylation of VII afforded the disaccharide derivative VIII in 87 % yield, the latter was converted into the title disaccharide IX in 90 % yield by catalytic hydrogenation.

In the alternative synthesis, 2,4-di-*O*-*p*-nitrobenzoyl-α-abequosyl bromide (X), prepared as described in Ref. 11, was condensed with the above mannoside VI in nitromethane-benzene using mercury(II) cyanide as promotor.[10] Chromatographic purification afforded the disaccharide derivative XI in 34 % yield. Debenzoylation of XI afforded XII in 87 % yield. The latter was hydrogenated to IX in 93 % yield. The final product IX was identical to that produced in the first-mentioned synthesis. A minor component isolated from the reaction mixture was shown to be methyl 2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-(3,6-dideoxy-2,5-di-*O*-*p*-nitrobenzoyl-β-D-*xylo*-hexofuranosyl)-α-D-mannopyranoside and was converted into methyl 3-*O*-(3,6-dideoxy-β-D-*xylo*-hexofuranosyl)-α-D-mannopyranoside (XIII) by debenzoylation followed by hydrogenation. Hydrolysis of the disaccharide and conversion into the per-acetylated alditols produced abequitol and mannitol acetates. From the methylation analysis [12,13] (GLC-MS), 1,4-di-*O*-acetyl-2,5-di-*O*-methylabequitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol were identified. These findings show the presence of an abequosyl-mannosyl disaccharide containing a furanosidic abequose moiety. NMR and optical rotation established a β-linkage. The β-furanoside is presumably formed from the 3,6-dideoxy-2,5-di-*O*-*p*-nitrobenzoyl-D-*xylo*-hexofuranosyl bromide present as a by-product of the treatment of methyl 3,6-dideoxy-*p*-nitrobenzoyl-α-D-*xylo*-hexopyranoside with hydrogen bromide in dichloromethane. Similar rearrangements have been observed by Bock and Pedersen in the treatment of acetylated methyl glycopyranosides with hydrogen bromide in acetic acid.[14]



The NMR assignments given in the experimental part were, where necessary, corroborated by spin decoupling experiments. Thus, the signal given by the anomeric proton of the abequosyl unit in the disaccharide derivative VII was assigned as follows: Irradiation at the H-3, H-3′ frequency (abequose residue) caused a simplification of the signals at δ 3.7 and those at δ 5.00–5.16. From chemical shift considerations the former of these is due to H-2, attached to benzyloxy carbon and the latter to H-4, attached to *p*-nitrobenzoyloxy carbon. This was confirmed by irradiating the H-6 doublet at δ 1.05, locating the H-5 signal of the abequose unit and then irradiating at the H-5 frequency. Since H-2 (abequose unit) had the same resonance frequency as H-5, this irradiation caused both simplification in the H-4 signal and collapse of the H-1 doublet into a singlet. Irradiation at the H-1 resonance frequency conversely caused a simplification of the H-2 signal. The coupling constant of the H-1 (abequose) doublet of 4 Hz indicates an α-linkage to the mannose unit.

EXPERIMENTAL

General methods were the same as those reported in a recent paper [15] and in Ref. 11, respectively.

Methyl 4-O-benzoyl-2-O-benzyl-3,6-dideoxy-β-D-xylo-hexopyranoside (II). Trifluoromethanesulfonic anhydride (0.55 ml) was dissolved in anhydrous dichloromethane (2 ml) and cooled to −60 °C. A solution of benzyl alcohol (0.44 ml) and 2,6-lutidine (0.44 ml) in anhydrous dichloromethane (3 ml) was added at this temperature. After 30 min, a solution of methyl 4-*O*-benzoyl-3,6-dideoxy-β-D-*xylo*-hexopyranoside (I) (300 mg) and 2,6-lutidine (0.27 ml) in anhydrous dichloromethane (2 ml) was added during 30 min at −60 °C. The reaction mixture was allowed to stand for 60 h at this temperature. TLC (toluene–ethyl acetate, 4:1) showed the presence of starting material and a faster-moving component (II) The reaction mixture was diluted with dichloromethane and washed with water. The organic layer was dried over sodium sulfate, filtered and concentrated. The two components were separated by column chromatography on silica gel to yield chromatographically pure II (205 mg) $[\alpha]_D$ +4° (chloroform). 60 MHz NMR ($CDCl_3$): δ 1.25 (3 H, d, $J_{5,6}$ 7 Hz, H-6), 1.5–2.7 (2 H, m, H-3, H-3′), 3.62 (3 H, s, methoxyl protons), 4.38 (1 H, d, $J_{1,2}$ 8 Hz, H-1), 4.63, 4.83 (2 H, AB spectrum, $J_{H,H}$ 12 Hz, benzylic protons), 5.10–5.24 (1 H, m, H-4), 7.1–7.4 (8 H, m) and 8.0–8.2 (2 H, m, aromatic protons).

Methyl 2-O-benzyl-3,6-dideoxy-β-D-xylo-hexopyranoside (III). The above product II (200 mg) in methanol (50 ml) containing barium oxide (150 mg) was refluxed for 30 min. The mixture was neutralized with solid carbon dioxide and concentrated. The residue was taken up in ethyl acetate (50 ml) and filtered. The filtrate was concentrated to a syrup which was purified by silica gel column chromatography (toluene–ethyl acetate, 4:1) to yield chromatographically pure III (120 mg) $[\alpha]_D$ −23° (chloroform). The material crystallized from diethyl ether, m.p. 84–85 °C. (Found: C 66.5; H 7.87. $C_{14}H_{20}O_4$ requires: C 66.6; H 7.99.) 60 MHz NMR: δ 1.26 (3 H, d, $J_{5,6}$ 7 Hz, H-6), 1.5–2.5 (3 H, m, H-3, H-3′, OH), 3.57 (3 H, s, methoxyl protons), 4.30 (1 H, d, $J_{1,2}$ 8 Hz, H-1), 4.63, 4.80 (2 H, AB spectrum, $J_{H,H}$ 12 Hz, benzylic protons), 7.33 (5 H, aromatic protons).

Methyl 2-O-benzyl-3,6-dideoxy-4-O-p-nitrobenzoyl-β-D-xylo-hexopyranoside (IV). The benzyl ether III (110 mg) was dissolved in pyridine (15 ml) and cooled to 0 °C. *p*-Nitrobenzoyl chloride (110 mg) in pyridine (5 ml) was added with stirring. The reaction mixture was kept at room temperature for 4 h. Ice-water was added and the mixture extracted with chloroform. The organic layer was washed with water, dried over sodium sulfate and concentrated to a syrup which was purified by silica gel column chromatography (toluene–ethyl acetate, 3:2) to yield chromatographically pure IV (155 mg $[\alpha]_D$ +50° (chloroform). 60 MHz NMR ($CDCl_3$) δ 1.26 (3 H, d, $J_{5,6}$ 7 Hz, H-6), 1.4–2.7 (2 H, m, H-3, H-3′), 3.62 (3 H, s, methoxyl protons), 4.42 (1 H, d, $J_{1,2}$ 8 Hz, H-1), 4.64, 4.86 (2 H, AB spectrum, $J_{H,H}$ 12 Hz, benzylic protons), 5.13–5.30 (1 H, m, H-4), 7.30 (5 H) and 8.25 (4 H), aromatic protons.

2-O-Benzyl-3,6-dideoxy-4-O-p-nitrobenzoyl-α-D-xylo-hexopyranosyl bromide (V). A solution of the glycoside IV (160 mg) in dichloromethane (10 ml) was cooled to −20 °C. A saturated solution of hydrogen bromide in dichloromethane (10 ml) was added at −20 °C. The reaction was monitored by TLC (toluene–ethyl acetate, 4:1). After 30 min at −20 °C, the solution was concentrated and used immediately in the next reaction step, $[\alpha]_D$ +137° (chloroform). 60 MHz NMR ($CDCl_3$): δ 6.74 (1 H, $J_{1,2}$ 4 Hz, H-1.

Methyl-2-O-benzyl-3-O-(2-O-benzyl-3,6-dideoxy-4-O-p-nitrobenzoyl-α-D-xylo-hexopyranosyl)-4,6-benzylidene-α-D-mannopyranoside (VII). A solution of methyl 2-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside (VI) [9] (160 mg) in nitromethane–benzene (1:1, 8 ml) was boiled until 2.5 ml solvent had distilled off. The solution was cooled to room temperature. Mercury(II) cyanide (140 mg) and the hexosyl bromide V (prepared from 160 mg glycoside IV) dissolved in dry dichloromethane, were

added during 5 min. The mixture was stirred at room temperature under nitrogen for 16 h and then diluted with toluene. The solution was washed with water, saturated aqueous sodium hydrogen carbonate and finally water, dried over sodium sulfate, filtered and concentrated to a syrup. Silica gel column chromatography (toluene – ethyl acetate, 4:1) yielded chromatographically homogeneous VII (120 mg), R_F 0.63, TLC in the same solvent, $[\alpha]_D$ +129° (chloroform). (Found: C 66.4; H 5.98; N 1.82. $C_{41}H_{43}O_{12}$ requires: C 66.4; H 5.84; N 1.89) 100 MHz NMR ($CDCl_3$): δ 1.05 (3 H, d, $J_{5,6}$ 7 Hz, H-6, abequose residue), 2.0 – 2.3 (2 H, m, H-3, H-3′, abequose residue), 3.43 (3 H, s, methoxyl protons), 4.80 (3 H, s, benzylic protons and H-1, mannose residue), 5.00 – 5.16 (1 H, m, H-4), 5.42 (1 H, d, $J_{1,2}$ 4 Hz, H-1, abequose residue), 5.46 (1 H, s, benzylidene proton), 6.9 – 7.5 (15 H, aromatic protons), 8.05 and 8.19 (2 H each, each d, each $J_{H,H}$ 8 Hz, *p*-nitrobenzoyl protons).

*Methyl-2-*O*-benzyl-3-*O*-(2-*O*-benzyl-3,6-dideoxy-*α*-*D*-xylo-hexopyranosyl)-4,6-*O*-benzylidene-*α*-*D*-mannopyranoside (VIII).* The above disaccharide derivative VII (50 mg) in methanol (25 ml) containing barium oxide (50 mg) was refluxed for 30 min. The mixture was neutralized with solid carbon dioxide and concentrated. The residue was taken up in ethyl acetate and filtered. The filtrate was concentrated to a syrup which was purified by silica gel column chromatography (toluene – ethyl acetate, 1:1) to yield chromatographically pure VIII (35 mg) $[\alpha]_D$ +94° (chloroform). 60 MHz NMR ($CDCl_3$): δ 1.11 (3 H, d, $J_{5,6}$ 7 Hz, H-6, abequose residue), 1.4 – 1.6 (3 H, m, H-3, H-3′ and OH), 3.42 (3 H, s, methoxyl protons), 4.83 (3 H, s, benzyl protons and H-1, mannose residue), 5.40 (1 H, d, $J_{1,2}$ 4 Hz, H-1, abequose residue), 5.47 (1 H, s, benzylidene proton), 7.0 – 7.5 (15 H, aromatic protons).

*Methyl-3-*O*-(3,6-dideoxy-*α*-*D*-xylo-hexopyranosyl)-*α*-*D*-mannopyranoside (IX).* (a) From VIII: The above disaccharide derivative XIII (30 mg) was hydrogenated at room temperature and atmospheric pressure in ethanol using a catalytic amount of 10 % palladium on carbon. After 5 h, the catalyst was removed by filtration and the filtrate concentrated to a chromatographically pure syrup, IX (14 mg), $[\alpha]_D$ +103° (water). 100 MHz NMR (D_2O): δ 1.14 (3 H, d, $J_{5,6}$ 7 Hz, H-6), 1.86 – 2.12 (2 H, m, H-3, H-3′), 3.42 (3 H, s, methoxyl protons) 4.75 (1 H, d, $J_{1,2}$ 1.5 Hz, H-1, mannose residue), 5.06 (1 H, d, $J_{1,2}$ 4 Hz, H-1, abequose residue). An aliquot of IX was converted into the corresponding pentaacetate with acetic anhydride and pyridine. (Found for IX pentaacetate: C 51.4; H 6.24. $C_{23}H_{34}O_{14}$ requires: C 51.7; H 6.41). An aliquot of IX was hydrolyzed with 0.25 M aqueous sulfuric acid for 12 h at 100 °C. The hydrolyzate was reduced with sodium borohydride and the mixture of alditols was fully acetylated.[16] GLC-MS showed the presence of two components, indistinguishable from abequitol tetraacetate and mannitol hexaacetate, respectively. Another aliquot of IX was methylated with dimethylsulfinyl anion and iodomethane in dimethyl sulfoxide.[12] The product was hydrolyzed, reduced with sodium borohydride and the alditols converted into their per-acetates.[16] The alditol acetates thus obtained were indistinguishable by GLC-MS [13] from 1,5-di-*O*-acetyl-2,4-di-*O*-methylabequitol and 1,3,5-tri-O-acetyl-2,4,6-tri-*O*-methyl-mannitol, respectively.

(b) From XII (see below): Methyl 2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-(3,6-dideoxy-*α*-*D*-*xylo*-hexopyranosyl)-*α*-*D*-mannopyranoside (XI) (70 mg) was hydrogenated and worked up as described above under (a). Chromatographically pure IX (42 mg) was obtained, $[\alpha]_D$ +95° (water), with NMR data identical to those found for IX. Hydrolysis and conversion to the corresponding alditol acetates [15] of an aliquot of the material (see above) afforded abequitol and mannitol acetates. Likewise, methylation analysis [12,13] yielded the same methylated alditol acetates as described above under (a). Conversion of IX obtained from both routes into the fully trimethylsilylated products gave materials with identical data on both GLC and MS.

Methyl 2-O-*benzyl-4,6-*O*-benzylidene-3-*O*-(3-6-dideoxy-2,4-di-*O*-p-nitrobenzoyl-*α*-*D*-xylo-hexopyranosyl-*α*-*D*-mannopyranoside (XI).* A solution of the mannoside VI (312 mg) in nitromethane – benzene (1:1, 12.4 ml) was boiled until 3.7 ml solvent had distilled off. The solution was cooled to room temperature. Mercury(II) cyanide (360 mg) and 3,6-dideoxy-2,4-di-*O*-*p*-nitrobenzoyl-*α*-*D*-*xylo*-hexopyranosyl bromide (X) (prepared from 400 mg methyl 3,6-dideoxy-2,4-di-*O*-*p*-nitrobenzoyl-*α*-*D*-*xylo*-hexopyranoside [17] and used immediately) in dry dichloromethane (2 ml) were added. After stirring at room temperature for 24 h the mixture was diluted with toluene and the solution washed with water, saturated aqueous sodium hydrogen carbonate and finally water. The solution was dried over sodium sulfate, filtered and concentrated to a syrup. Silica gel column chromatography (toluene – ethyl acetate, 8:1) yielded chromatographically pure XI (230 mg), R_F 0.34, TLC in the same solvent. The material crystallized from diethyl ether – light petroleum, m.p. 105 – 108 °C, $[\alpha]_D$ +90° (chloroform). (Found: C 61.3; H 5.19; N 3.40. $C_{41}H_{40}O_{15}N_2$ requires: C 61.5; H 5.04; N 3.50.) 100 MHz NMR ($CDCl_3$): δ 1.16 (3 H, d, $J_{5,6}$ 7 Hz, H-6), abequose residue), 2.1 – 2.7 (2 H, m, H-3, H-3′, abequose residue), 3.44 (3 H, s, methoxyl protons), 7.0 – 8.4 (18 H, m, aromatic protons).

*Methyl-2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-(3,6-dideoxy-*α*-*D*-xylo-hexopyranosyl)-*α*-*D*-mannopyranoside (XII).* The above disaccharide derivative XI (220 mg) in methanol (50 ml) containing barium oxide (100 mg) was debenzo-

ylated and worked up as described above for VIII. Purification by silica gel column chromatography (toluene–ethyl acetate, 3:2) afforded chromatographically pure XII (138 mg) $[\alpha]_D$ +29° (chloroform). 100 MHz NMR: δ 1.09 (3 H, d, $J_{5,6}$ 7 Hz, H-6, abequose residue), 1.6–2.5 (4 H, m, H-3, H-3′, OH, abequose residue), 3.34 (3 H, s, methoxyl), 4.70 (3 H, s, H-1, mannose residue and benzyl protons), 5.02 (1 H, d, $J_{1,2}$ 4 Hz, H-1, abequose residue), 5.58 (1 H, benzylidene proton), 7.2–7.5 (10 H, m, aromatic protons).

*Methyl 3-O-(3,6-dideoxy-β-*D*-xylo-hexofuranosyl)-α-*D*-mannopyranoside (XIII).* A minor component with R_F 0.30 (TLC, toluene–ethyl acetate, 8:1) was obtained in the preparation of XI (above). This material, presumably, methyl 2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-(3,6-dideoxy-2,5-di-*O*-*p*-nitrobenzoyl-β-D-*xylo*-hexofuranosyl)-α-D-mannopyranoside was debenzoylated and the product hydrogenated as described above for VIII and IX, respectively. The product (XIII) had $[\alpha]_D$ +3° (water). 100 MHz NMR (D_2O): δ 1.23 (3 H, d, $J_{5,6}$ 7 Hz, H-6, abequose residue), 1.64 (1 H, J 3.0, 6.9 and 15.5 Hz, H-3, abequose residue), 2.53 (1 H, J 6.9, 8.6 and 15.5 Hz, H-3′, abequose residue), 3.48 (3 H, s, methoxyl protons), 4.85 (1 H, d, $J_{1,2}$ 1.5 Hz, H-1, mannose residue), 5.21 (1 H, s, $J_{1,2}=0$ Hz, H-1, abequose residue). An aliquot of XIII was hydrolyzed and converted into the corresponding alditol acetates. These were shown to be indistinguishable from abequitol and mannitol acetates by GLC-MS. Another aliquot was subjected to methylation analysis as described above and yielded 1,4-di-*O*-acetyl-2,5-di-*O*-methylabequitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, respectively. On GLC 1,4-di-*O*-acetyl-2,5-di-*O*-methylabequitol had a relative retention time of 0.31 (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, $r=1$) as compared to that of 0.32 for 1,5-di-*O*-acetyl-2,4-di-*O*-methylabequitol (column: 3 % ECNSS-M on Gas-Chrom Q). Pertinent mass spectral peaks: *m/e* (rel. intensity): 43(65), 59(100), 69(7), 97(20), 101(8), 117(8), 129(20), 189(2).

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Synthesis of *p*-Isothiocyanatophenyl 3-*O*-(3,6-Dideoxy-α-D-*xylo*-hexopyranosyl)-α-D-mannopyranoside

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A synthesis of the disaccharide derivative *p*-isothiocyanatophenyl 3-*O*-(3,6-dideoxy-α-D-*xylo*-hexopyranosyl)-α-D-mannopyranoside, corresponding to the Salmonella O-factor 4, is described. The *p*-isothiocyanatophenyl group is useful for attaching the abequosylmannosyl disaccharide unit to free amino groups in a protein. The glycosylation step in the synthetic sequence was performed using the Helferich procedure and 2,4-di-*O*-*p*-nitrobenzoyl-α-abequosyl bromide as the glycosylating reagent.

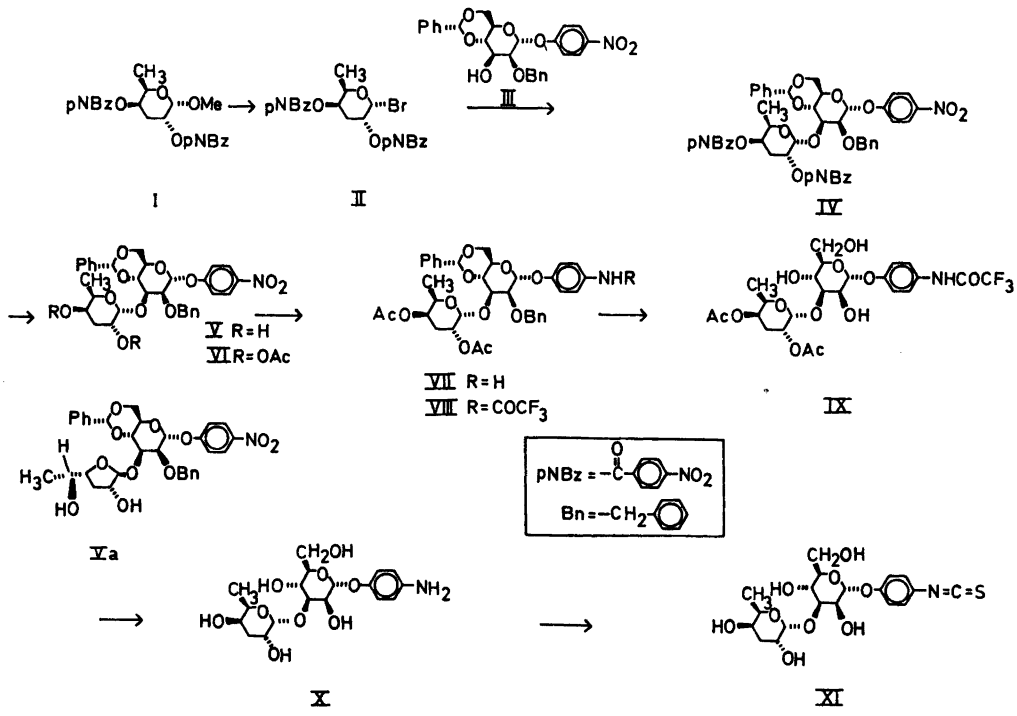
Access to α-D-3,6-dideoxyhexosyl-(1→3)-α-D-mannosides in which the dideoxyhexose residue is abequose, paratose or tyvelose and with the mannose residue linked to an aglycone containing a functional group suitable for attachment to proteins is of considerable immunological interest.[1] These three antigens include the majority of the more severe types of Salmonellosis from a diagnostic point of view and are of potential value for prophylaxis.

We have previously synthesized *p*-isothiocyanatophenyl 3-*O*-α-tyvelosyl-α-D-mannopyranoside [2] and have demonstrated its immunological potential.[1] We now describe the synthesis of the analogous abequosyl disaccharide XI, corresponding to Salmonella O-factor 4.[3]

In Ref. 4 we describe the synthesis of an α-abequosyl disaccharide, using two different abequosyl bromides, one with a non-participating benzyl group in the 2-position and a *p*-nitrobenzoyl group in the 4-position, and the other with *p*-nitrobenzoyl groups in both positions. These exploratory studies showed that a non-participating group at C-2 was unneccessary for obtaining a good yield of the desired α-abequosyl disaccharide and the more readily accessible 2,4-di-*O*-*p*-nitrobenzoyl-α-abequosyl bromide (II) was therefore used in the present work. A complication, however, arose in the use of II in abequoside synthesis. Treatment of methyl 2,4-di-*O*-*p*-nitrobenzoyl-α-abequoside (I) with hydrogen bromide in dichloromethane to obtain II and the subsequent use of the crude product in glycosylation also produced a β-furanoside as a minor component.[4] Bock and Pedersen have shown that treatment of acetylated methyl glycopyranosides with hydrogen bromide in acetic acid gives glycofuranosyl bromides in addition to the expected, glycopyranosyl bromides.[5] In the present work a minor component of glycosylation yields NMR data, suggesting, by analogy [4,5] the presence of an abequosyl unit in the β-furanose ring form.

The major product of treatment of methyl 2,4-di-*O*-*p*-nitrobenzoyl-α-abequoside [6] (I) with hydrogen bromide in dichloromethane is the α-bromo sugar II. The α-configuration is indicated by the optical rotation ($[\alpha]_D + 181°$) and by NMR ($J_{1,2}$ 4 Hz).

Mercury cyanide in benzene-nitromethane was used to promote the condensation of bromo sugar II with the mannoside III.[7] All hydroxyl groups on the latter were protected except for the one at C-3.[2] After chromatography the disaccharide derivative IV was obtained in a 42 % yield. The assignment of the anomeric configuration is based on optical rotation and NMR. The anomeric signals of the abequosyl and mannosyl residues were identified and differentiated by comparison with NMR results,



including spin decoupling experiments, obtained for the analogous derivative of methyl 3-*O*-α-abequosyl-α-D-mannopyranoside.[4]

The following steps, from IV to XI, were analogous to those previously described for the corresponding tyvelosyl disaccharide:[2] The *p*-nitrobenzoyl groups in the abequose residue in IV were removed (V) and replaced by acetyl groups (VI). The nitro group in VI was reduced by hydrogenation with Adams catalyst (VII) and the resulting amino group was protected with a trifluoroacetyl group (VIII). Benzyl and benzylidene groups were removed by catalytic hydrogenation (IX). Treatment of IX with methanolic ammonia afforded the product X, which was converted into the isothiocyanatophenyl [8] compound XI. The conversion of V to XI proceeded in yields exceeding 85 % in the individual steps.

EXPERIMENTAL

General methods were the same as those recently reported.[2] NMR spectra were recorded using a Varian XL-100 instrument, in deuteriochloroform unless otherwise stated. Chemical shifts were recorded in ppm downfield from tetramethylsilane as internal standard. NMR spectra were recorded for all new compounds and were in agreement with postulated structures. Only selected NMR data are therefore presented below.

3,6-Dideoxy-2,4-di-O-(p-*nitrobenzoyl*)-α-D-xylo-*hexopyranosyl bromide (II).* A solution of methyl 2,4-di-*O*-*p*-nitrobenzoyl-α-abequoside (I) [6] (0.5 g) in dichloromethane (25 ml) was cooled to −20 °C and then saturated at this temperature with hydrogen bromide. The reaction was monitored by TLC (toluene – ethyl acetate 4:1). After 2 h at −20 °C when no starting material remained, the solution was concentrated to a syrup which was used directly in the next step. The optical rotation of the syrup was $[\alpha]_D$ +181°. NMR: δ 1.30 (3 H, d, $J_{5,6}$ 7 Hz, H-6), 2.2–2.8 (2 H, m, H-3, H-3′), 4.44 (1 H, q, $J_{5,6}$ 7 Hz, H-5), 5.16-5.48 (2 H, m, H-2, H-4), 6.84 (1 H, d, $J_{1,2}$ 4 Hz, H-1).

p-*Nitrophenyl* 2-O-*benzyl-4,6*-O-*benzylidene-3*-O-*(3,6-dideoxy-2,4-di*-O-p-*nitrophenyl*-α-D-xylo-*hexopyranosyl)*-α-D-*mannopyranoside (IV).* A solution of *p*-nitrophenyl 2-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside (III) [2] (505 mg) in nitromethane-benzene 1:1 (20 ml) was boiled until 6 ml solvent mixture had distilled off and then it was cooled to room temperature. Mercury(II) cyanide (353 mg) and the glycosyl halide II (prepared from 500 mg of the glycoside

I and used immediately) dissolved in dry dichlormethane (2 ml) were added and the solution was stirred at room temperature under nitrogen for 24 h. The mixture was diluted with toluene and washed with water, saturated aqueous sodium hydrogen carbonate and finally water. The solution was dried over anhydrous sodium sulfate, filtered and concentrated to a syrup (970 mg). Column chromatography on silica gel (toluene – ethyl acetate 16:1) yielded chromatographically homogeneous IV, R_F 0.45 (TLC, same solvent system) (406 mg). The material crystallized from ethanol, m.p. 127 – 129 °C, $[\alpha]_D$ +123° (chloroform). (Found: C 60.9; H 4.66; N 4.84. $C_{46}H_{41}N_3O_{17}$ requires: C 60.9; H 4.53; N 4.63). NMR: δ 1.18 (3 H, d, $J_{5,6}$ 7 Hz, H-6), 1.6 – 2.7 (2 H, m, H-3, H-3'), 4.83, 4.93 (2 H, AB spectrum, $J_{H,H'}$ 12 Hz, benzylic protons), 5.20 (1 H, $J_{1,2}$ 4 Hz, H-1, abequose residue), 5.52 (1 H, s, benzylidene proton), 5.75 (1 H, d, $J_{1,2}$ 1.5 Hz, H-1, mannose residue), 7.0 – 8.4 (22 H, aromatic protons).

p-Nitrophenyl 2-O-benzyl-4,6-O-benzylidene-3-O-(3,6-dideoxy-α-D-xylo-hexopyranosyl)-α-D-mannopyranoside (V). The above disaccharide derivative IV (406 mg) in methanol (100 ml) containing barium oxide (200 mg) was refluxed for 1 h. The mixture was neutralized with solid carbon dioxide and concentrated. The residue was taken up in ethyl acetate (100 ml) and filtered. The filtrate was concentrated to a syrup which was purified on a silica gel column (toluene-ethyl acetate 2:3) to yield chromatographically pure V (195 mg), $[\alpha]_D$ +114° (chloroform). NMR: δ 1.17 (3 H, d, $J_{5,6}$ 7 Hz, H-6 abequose residue), 1.84 (2 H, broad s, OH), 1.9 – 2.5 (2 H, m, H-3 and H-3' abequose residue), 4.79 (2 H, s, benzylic protons), 5.13 (1 H, d, $J_{1,2}$ 4 Hz, H-1, abequose residue), 5.61 (2 H, nearly s, benzylidene and H-1, mannose residue), 7.08 and 8.19 (2 H each, both d, both $J_{H,H}$ 9.5 Hz, p-$NO_2C_6H_4O$-protons), 7.2 – 7.5 (10 H, other aromatic protons).

In a separate experiment the above major component IV was isolated together with a minor one. Debenzoylation of this mixture as described above for IV afforded a product containing V and the presumed furanoside Va in a ratio of 92:8. Chromatographically homogeneous Va (TLC, toluene – ethyl acetate 1:1) had $[\alpha]_D$ +40° (chloroform). NMR for Va: δ 1.17 (3 H, d, $J_{5,6}$ 7 Hz, H-6, abequose residue), 1.6 – 2.6 (2 H, m, H-3 and H-3', abequose residue), 4.70, 4.84 (2 H, AB spectrum, $J_{H,H}$ 12 Hz, benzylic protons), 5.00 [1 H, $J_{1,2}$ 0 Hz, H-1, abequose (furanosidic residue)], 5.55 (1 H, s, benzylidene proton), 5.58 (1 H, d, $J_{1,2}$ 1.5 Hz, H-1, mannose residue), 5.07 and 8.18 (2 H each, d, each $J_{1,2}$ 9 Hz, p-$NO_2C_6H_4O$-protons).

p-*Nitrophenyl-2-O-benzyl-4,6-O-benzylidene-3-O-(2,4-di-O-acetyl-3,6-dideoxy-α-D-xylo-hexopyranosyl)-α-D-mannopyranoside* (VI). V (771 mg) was acetylated with acetic anhydride (5 ml) in pyridine (7 ml) at room temperature overnight. The solution was concentrated yielding the diacetate VI (815 mg) which crystallized from diethyl ether-light petroleum, m.p. 95 – 98 °C, $[\alpha]_D$ +107° (chloroform). (Found: C 62.0; H 5.67; N 1.97. $C_{36}H_{39}O_{13}$ requires: C 62.3; H 5.67; N 2.02). NMR: δ 1.07 (3 H, d, $J_{5,6}$ 7 Hz, H-6 abequose residue), 1.62 (3 H, s, 2-OAc), 2.12 (3 H, s, 4-OAc), 1.8 – 2.4 (2 H, m, H-3 and H-3' abequose residue), 4.75, 4.85 (2 H, AB spectrum $J_{H,H}$ 13 Hz, benzylic protons), 5.32 (1 H, d, $J_{1,2}$ 4 Hz, H-1, abequose residue), 5.58 (1 H, s, benzylidene proton), 5.70 (1 H, d, $J_{1,2}$ 1.5 Hz, H-1, mannose residue, 7.13 and 8.21 (1 H each, both d, both $J_{H,H'}$ 9 Hz), p-$NO_2C_6H_4O$-protons), 7.3 – 7.5 (10 H, other aromatic protons).

p-*Trifluoroacetamidophenyl 3-O-(2,4-di-O-acetyl-3,6-dideoxy-α-D-xylo-hexopyranosyl)-2-O-benzyl-4,6-O-benzylidene-α-D-mannopyranoside (VIII).* The foregoing compound VI (850 mg) was hydrogenated at room temperature and atmospheric pressure in ethyl acetate (50 ml) using Adams catalyst (80 mg). When sufficient hydrogen to account for the conversion of a nitro to an amino group had been consumed, trifluoroacetic anhydride (0.8 ml) and pyridine (1.9 ml) were added and the mixture was kept at 60 °C for 30 min. The catalyst was removed by filtration and the filtrate was concentrated. The residue was dissolved in toluene and shaken with water. The organic layer was dried over sodium sulfate, filtered and concentrated to a syrup (848 mg) which crystallized from methanol, m.p. 105 – 107 °C, $[\alpha]_D$ +74° (chloroform). A satisfactory elemental analysis could not be obtained for the compound. NMR: δ 1.07 (3 H, d, $J_{5,6}$ 7 Hz, H-6 abequose residue), 1.8 – 2.3 (2 H, m, H-3 and H-3', abequose residue), 1.62 (3 H, s, 2-OAc), 2.12 (3 H, s, 4-OAc), 4.70, 4.84 (2 H, AB spectrum, $J_{H,H'}$ 13 Hz, benzylic protons), 5.32 (1 H, d, $J_{1,2}$ 4 Hz, H-1, abequose residue), 5.57 (1 H, s, benzylidene proton), 5.60 (1 H, d, $J_{1,2}$ 1.5 Hz, H-1, mannose residue), 7.05 and 7.50 (2 H each, both d, both $J_{H,H'}$, 9 Hz, p-$CF_3CONHC_6H_4O$-), 7.2 – 7.6 (10 H, other aromatic protons).

p-*Trifluoroacetamidophenyl 3-O-(2,4-di-O-acetyl-3,6-dideoxy-α-D-xylo-hexopyranosyl)-α-D-mannopyranoside (IX).* The syrupy product VIII (780 mg) from the above reaction was hydrogenated at atmospheric pressure in ethanol (30 ml) using 10 % palladium on charcoal (300 mg) as catalyst. When hydrogen consumption had ceased, the catalyst was removed by filtration and the filtrate was concentrated to yield IX as a syrup (611 mg). This material was chromatographically pure on TLC (chloroform-methanol 3:1). A small amount was purified by column chromatography on silica gel and crystalline IX was obtained from ethyl acetate-light petroleum, m.p. 191 – 194 °C, $[\alpha]_D$ +145° (methanol). (Found: C 50.2; H 5.39; N 2.37; F 9.70. $C_{24}H_{30}NO_{12}F_3$ requires: C 49.6; H 5.20; N 2.41; F 9.80. NMR: δ 1.12 (3 H, d, $J_{5,6}$ 7 Hz, H-6 abequose residue), 1.8 – 2.6 (2 H, m, H-3, H-3' abequose residue), 2.08

(3 H, s, 2-OAc), 2.14 (3 H, s, 4-OAc), 5.29 (1 H, d, $J_{1,2}$ 4 Hz, H-1 abequose residue), 5.49 (1 H, d, $J_{1,2}$ 1.5 Hz, H-1, mannose residue), 7.15 and 7.56 (2 H each, both d, both $J_{H,H'}$ 9 Hz, *p*-$CF_3CONHC_6H_4O-$).

p-*Isothiocyanatophenyl 3-O-(3,6-dideoxy-α-D-xylo-hexopyranosyl)-α-D-mannopyranoside (XI).* The above pure disaccharide derivative IX (98 mg) was dissolved in saturated methanolic ammonia (10 ml) in a capped serum bottle and kept at room temperature for 48 h. Concentration afforded a syrup which on TLC (chloroform – methanol 3:1) showed the presence of one component only (X). The syrup was dissolved in 80 % aqueous ethanol (20 ml) and the pH was adjusted to about 8 by adding barium carbonate and maintained at this pH level throughout the reaction by adding more barium carbonate as required. Thiophosgene (0.1 ml) was added and the reaction was stirred for 30 min. Filtration and concentration gave a syrup which was purified by TLC (chloroform – methanol 3:1) to give pure XI (67 mg), $[\alpha]_D$ +158°. IR (KBr) showed a broad absorption band centred at 2120 cm^{-1}. The material crystallized to a low-melting hygroscopic material with m.p. too diffuse for useful characterization. NMR (CD_3OD): δ 1.16 (3 H, d, $J_{5,6}$ 7 Hz, H-6 abequose residue), 1.80 – 2.30 (2 H, m, H-3, H-3' abequose residue, 5.06 (1 H, d, $J_{1,2}$ 4 Hz, H-1, abequose residue), 5.49 (1 H, d, $J_{1,2}$ 1.5 Hz, H-1, mannose residue), 7.00 – 7.30 (4 H, aromatic protons).

An aliquot of XI was hydrolyzed with 0.25 M aqueous sulfuric acid for 12 h at 100 °C. The product was reduced with sodium borohydride and acetylated.[9] The abequitol triacetate and mannitol pentaacetate thus obtained were indistinguishable from authentic materials on GLC [9] and MS.[10]

Another aliquot of IX was methylated,[11] hydrolyzed, reduced with sodium borohydride and acetylated.[9] The two methylalditol acetates thus obtained were indistinguishable from authentic 1,5-di-*O*-acetyl-3,6-dideoxy-2,4-di-*O*-methyl-D-*xylo*-hexitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol, respectively, on GLC and MS.[12]

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Studies on Orchidaceae Glycosides. 5.* The Absolute Configuration of (+)-*erythro*-Isobutyltartaric Acid, a Component of the Glucoside Loroglossine

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(+)-*erythro*-Isobutyltartaric acid, a component of the glucoside loroglossine, is shown to have the 2*R*,3*S* configuration. Syntheses of the 2*S*,3*R* and 2*R*,3*R* isomers of isobutylerythritol and dimethyl (2*S*,3*S*)-isobutyltartrate from 2,3-*O*-isopropylidene-D-glyceraldehyde are described. The circular dichroism curves of the molybdate complexes of the 2*R*,3*S* and the 2*S*,3*S* isomers of isobutyltartaric acid are discussed.

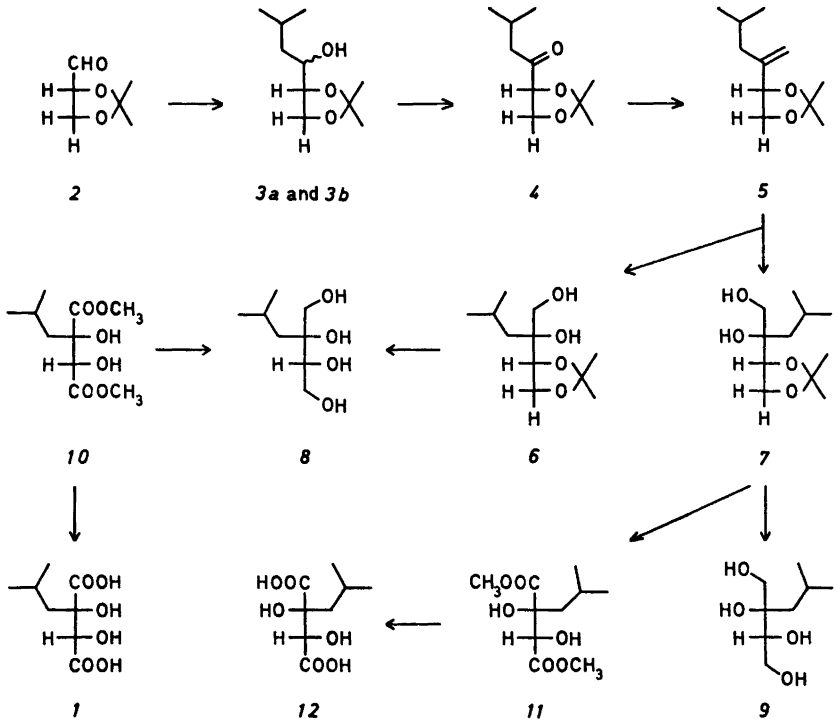
In a recent paper [2] the structures of the Orchidaceae glucosides loroglossine and militarine were reported. Loroglossine was shown to be a diester of isobutyltartaric acid and 4-*O*-β-D-glucopyranosylbenzyl alcohol. The relative configuration of the acid was demonstrated by synthesis to be *erythro*, but its absolute configuration was not determined.

In this communication we report a determination of the absolute configuration of the diacid part of loroglossine, (+)-*erythro*-isobutyltartaric acid (*1*), by correlation with D-glyceraldehyde.

2,3-*O*-Isopropylidene-D-glyceraldehyde(*2*),[3] obtained from D-mannitol, gave on reaction with isobutylmagnesium bromide two diastereomeric alcohols *3a* and *3b*. The crude mixture of the alcohols was transformed to the ketone *4* by oxidation with dimethyl sulfoxide in the presence of dicyclohexylcarbodiimide, pyridine and trifluoroacetic acid.[4] In a Wittig reaction with methylenetriphenylphosphorane,[5] *4* gave the olefin *5*, which on hydroxylation with osmium tetroxide [6] yielded the diols *6* and *7* in the ratio 2:3. These were separated by preparative TLC on silica gel. Acid hydrolysis of *6* and *7* gave the isobutylerythritols *8* and *9*, respectively. The former was found to be indistinguishable from the isobutylerythritol obtained by reduction of dimethyl (+)-*erythro*-isobutyltartrate (*10*).[2] Since *8* is derived from D-glyceraldehyde, it follows that (+)-*erythro*-isobutyltartaric acid (*1*) has the 2*R*,3*S* configuration.

In order to compare the CD curves of the molybdate complexes of (2*R*,3*S*)- and (2*S*,3*S*)-isobutyltartaric acid (*1* and *12*), the latter acid was synthesized in a low yield from *7* by oxidation with nitric acid.[7] Attempts to prepare (2*R*,3*S*)-isobutyltartaric acid (*1*) from *6* in the same way or by oxidation of *8* with oxygen in the presence of platinum [8] were unsuccessful. Instead, this acid (*1*) was obtained by hydrolysis of its dimethyl ester (*10*). From Fig. 1 it is apparent that the configuration at C-2 in *1* and *12* has a dominant influence on the CD curves of their molybdate complexes. Thus a 2*R* configuration gives rise to a strong positive Cotton effect at about 210 nm and a weaker negative effect at 260 – 270 nm (for the conditions used, see Experimental). The CD curves also show two negative Cotton effects at about 230 nm and 300 – 330 nm which, however, are too weak to be useful for the assignment of the configuration at C-3 in alkyltartaric acids (*cf.* Ref. 13). It is interesting to note that the CD curve of the molybdate

* For Paper 4 in this series, see Ref. 1.



complex of *1* is similar to that of (*R*)-2-isobutylmalic acid, which shows positive Cotton effects at 215 and 250 nm, and negative ones at 238 and 270 nm.[9]

Other alkyltartaric acids than *1* have been found in Nature. 4-Hydroxybenzyltartaric acid (piscidic acid) and 2-(3,4-dihydroxybenzyl)-tartaric acid (fukiic acid) have been isolated from *Piscidia erythrina* and *Petasites japonicus*, respectively. Both acids were shown to have the 2*R*,3*S* configuration by application of Horeau's method and degradation into hibiscus acid.[10,11] Recently a methyl derivative of fukiic acid, 2-(4-hydroxy-3-methoxybenzyl)tartaric acid, has been isolated from *Piscidia erythrina*.[12] Isopentyltartaric acid, which is a component of the alkaloid isoharringtonine, has also been shown to possess the 2*R*,3*S* configuration. This assignment was based on a comparison of the CD spectra of the molybdate complexes of isopentyltartaric acid, piscidic acid and its hexahydro derivative.[13]

EXPERIMENTAL

General conditions were the same as in an earlier communication.[2] The CD spectra were measured on a Jasco J-40 spectropolarimeter. Preparative GLC. Column: 0.35 × 180 cm, 10 % Carbowax 20 M on Chromosorb W (60 – 80 mesh), Varian 1400 chromatograph. Carrier gas: N_2, 40 ml/min. Plates precoated with silica gel F_{254} (Merck) were used for TLC, and silica gel 60 was used (230 – 400 mesh, Merck) for column chromatography.

(1R,2R)- and (1S,2R)-1-Isobutyl-2,3-O-isopropylideneglycerol (3a *and* 3b *respectively*). A solution of 2,3-*O*-isopropylidene-D-glyceraldehyde *2*, (0.07 mol) in ether (20 ml) was added dropwise with stirring to an ethereal solution of isobutylmagnesium bromide (0.18 mol, 250 ml). The reaction mixture was stirred at room temperature for 3 h, poured into ice-water and extracted with ether. The evaporation residue of the dried etheral layer (8.2 g) contained two epimeric alcohols in the ratio 1:3. Small amounts of the two epimers were isolated by preparative GLC (115 °C, retention times: major product 12 min, minor product 19 min).

Major product. $[\alpha]_{578}^{22}$ −15.4° (*c* 2.20, methanol). NMR ($CDCl_3$): δ 0.92 (d, 3 H, *J* 6.5 Hz), 0.96 (d, 3 H, *J* 6.6 Hz), 1.39 (s, 3 H), 1.45 (s, 3 H), 1.10 – 1.52 (8 H), 1.62 – 2.10 (m, 1 H), 2.10 (broad, 1 H, exchangeable in D_2O), 3.68 – 4.14 (4. H).

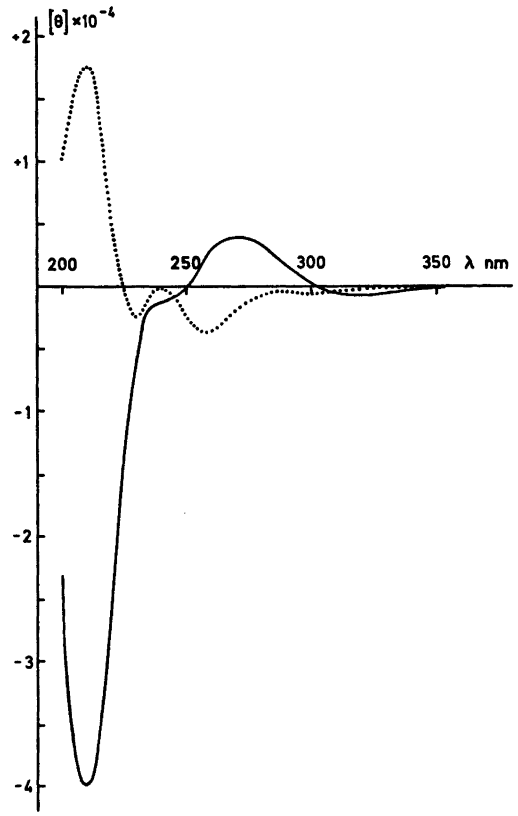


Fig. 1. CD curves of molybdate complexes of (2*R*,3*S*)-2-isobutyltartaric acid (*1*,···) and (2*S*,3*S*)-2-isobutyltartaric acid (*12*, ——) in water.

Minor product. $[\alpha]_{578}^{22}$ +32.8° (*c* 1.79, methanol). NMR ($CDCl_3$): δ 0.89 (d, 3 H, *J* 6.5 Hz), 0.96 (d, 3 H, *J* 6.7 Hz), 1.37 (s, 3 H), 1.44 (s, 3 H), 1.20–2.04 (8 H), 2.11 (d, 1 H, exchangeable in D_2O, *J* 5 Hz), 3.40–4.30 (4 H).

(R)-Isovaleryl-2,2-dimethyldioxolane (4). Dicyclohexylcarbodiimide (0.47 mol), pyridine (0.16 mol) and trifluoroacetic acid (0.09 mol) were carefully added to a cooled mixture of benzene (195 ml) and dimethyl sulfoxide (195 ml). A solution of the crude mixture of the epimers *3a* and *3b* (0.03 mol) in benzene (130 ml) was added to this reagent. The reaction mixture was heated to 50 °C for 17 h. A small amount of a crystalline precipitate was filtered off and ether (650 ml) was added to the filtrate. The solution was washed with a saturated solution of oxalic acid in water (2 × 100 ml). During this procedure a crystalline precipitate formed, which was filtered off and washed with ether (2 × 50 ml). The combined ethereal layers were washed with a saturated aqueous solution of sodium hydrogen carbonate (2 × 100 ml) and by water (100 ml), dried and concentrated. The residue was combined with the two precipitates above. Chromatography on silica gel (6.5 × 75 cm, dichloromethane) gave *4* (4.15 g) as a pale yellow oil.

A pure sample was obtained by preparative GLC (125 °C, retention time 5 min). $[\alpha]_{578}^{22}$ +43.3° (*c* 0.46, methanol). IR, ν_{max} ($CHCl_3$): 1715 cm^{-1}. NMR ($CDCl_3$): δ 0.91 (d, 3 H, *J* 6.4 Hz), 0.98 (d, 3 H, *J* 6.7 Hz), 1.41 (*q*, 3 H, *J* 0.6 Hz), 1.50 (*q*, 3 H, *J* 0.6 Hz), 1.90–2.70 (3 H), 3.80–4.50 (3 H).

(S)-4-Methyl-2-(2,2-dimethyldioxolanyl)-1-pentene (5). Triphenylmethylphosphonium bromide (0.012 mol) was added in portions with stirring to a solution of butyllithium (0.013 mol, 6 ml of a 20 % solution in hexane) in ether (30 ml).[5] The mixture was stirred at room temperature for 3.5 h. (*R*)-Isovaleryl-2,2-dimethyldioxolane (*4*, 0.010 mol) in ether (50 ml) was added dropwise after which the solution was refluxed for 15 h, cooled and filtered. The filtrate was washed with water, dried and concentrated. Chromatography of the residue on silica gel (4.6 × 24 cm, chloroform) gave *5* (0.7 g) as an oil.

A pure sample was obtained by preparative GLC (80 °C, retention time 10 min). $[\alpha]_{578}^{22}$ +32.6° (*c* 0.97, methanol). NMR ($CDCl_3$): δ 0.92 (d, 6 H, *J* 6 Hz), 1.41 (s, 3 H), 1.46 (s, 3 H), 1.5–2.2 (m, 3 H), 3.58 (dd, 1 H, $J_1 = J_2$ 8.0 Hz), 4.10 (dd, 1 H, J_1 8.0 Hz, J_2 6.5 Hz), 4.49 (dd, 1 H, J_1 6.5, J_2 8.0 Hz), 4.87 (s, broad, 1 H), 5.19 (s, broad, 1 H).

(2R,3S)1,2-O-Isopropylidene-3-isobutyleryth-ritol (6) and (2R,3R)-1,2-O-isopropylidene-3-isobutylerythritol (7). A mixture of osmium tetroxide (3.23 mmol) and *4* (3.21 mmol) in pyridine (13 ml) was stirred at room temperature for 2 h.[6] A solution of sodium hydrogen sulfite (1.48 g), water (25 ml) and pyridine (16 ml) was added and the mixture was stirred for 30 min. The reaction mixture was extracted with chloroform (5 × 10 ml), and the organic phase was dried and concentrated to a syrup (0.56 g). A part of this syrup (140 mg) was chromatographed on a preparative silica gel plate, (chloroform–methanol, 19:1). Three fractions were obtained: A (R_F 0.38, 25 mg) contained the pure 3*S* epimer (*6*), B (60 mg) a mixture of the 3*S* and 3*R* epimers and C (R_F 0.45, 40 mg) the pure 3*R* epimer (*7*).

3S Epimer (6): needles from hexane at +4 °C, m.p. 36–38 °C $[\alpha]_{578}^{20}$ +7.1° (*c* 0.34, methanol). (Found: C 60.1; H 10.2; O 29.4. Calc. for $C_{11}H_{24}O_4$: C 60.5; H 10.2; O 29.3). NMR (pyridine-d_5): δ 1.07 (d, 3 H, *J* 6.7 Hz), 1.14 (d, 3 H, *J* 6.4 Hz), 1.44 (s, 3 H), 1.53 (s, 3 H), 1.91 (d, 2 H, *J* 6 Hz), 2.00–2.40 (m, 1 H), 3.96 and 4.02 (AB part of an ABX system, J_{AB} 12 Hz, $J_{AX} = J_{AB}$ 5.2 Hz, collapses to an AB system on addition of D_2O), 4.12–4.77 (3 H), 5.20 (s, 1 H, exchangeable in D_2O), 6.30 (t, 1 H, exchangeable in D_2O, *J* 5.2 Hz).

3R Epimer (*7*): $[\alpha]_{578}^{22}$ +9.8° (*c* 0.43, methanol). NMR ($CDCl_3$): δ 0.94 (d, 3 H, *J* 6.4 Hz), 1.00 (d, 3 H, *J* 6.2 Hz), 1.26 (d, 2 H, 6.1 Hz), 1.38 (s, 3 H), 1.44 (s, 3 H), 1.52–2.08 (m, 1 H), 2.70 (s, 1 H, exchangeable in D_2O), 3.56 and 3.72 (AB system, J_{AB} 11 Hz), 3.88–4.24 (3 H).

(2S,3R)-2-Isobutylerythritol (8). A. From (2*R*,3*S*)-1,2-*O*-isopropylidene-3-isobutylerythritol (*6*): A solution of *6* (15 mg) in acetic acid (70 %) was heated at 70 °C for 15 min. The reaction mixture was evaporated and the residue was purified by chromatography on silica gel (1.5×7 cm, chloroform–methanol 9:1). *8* (11.5 mg) was obtained as a syrup, $[\alpha]_{578}^{23}$ +6.1° (*c* 1.15, methanol). NMR (pyridine-d_5): δ 1.09 (d, 3 H, *J* 7 Hz), 1.15 (d, 3 H, *J* 5 Hz), 1.68–2.50 (3 H), 4.00–4.52 (5 H), 4.9–6.6 (broad, exchangeable in D_2O).

B. From dimethyl (+)-*erythro*-2-isobutyltartrate [2] (*10*): A solution of *10* (38.5 mg) in ether (10 ml) was treated with lithium aluminium hydride (60 mg) for 100 min at room temperature. The reaction mixture was diluted with water and filtered. The filtrate was passed through a mixture of equal amounts of Dowex 50 W-X8 (H^+) and Amberlite IRA 400 (OH^-) (1×11 cm). Evaporation of the eluate and chromatography of the residue on silica gel (1×5 cm, chloroform-methanol 9:1) gave *8* (13 mg), $[\alpha]_{578}^{23}$ +6.9° (*c* 1.30, methanol). The NMR spectrum of this sample was indistinguishable from that described under A.

(2R,3R)-2-Isobutylerythritol (9). A solution of *7* (30 mg) in acetic acid (70 %, 10 ml) was heated at 75 °C for 35 min. The solvent was evaporated and the residue was purified on silica gel (1.5×10 cm, chloroform–methanol 9:1). *(2R,3R)*-2-Isobutylerythritol (*9*) was obtained as a syrup (11.8 mg), $[\alpha]_{578}^{21}$ +13.9° (*c* 1.18, methanol). NMR (pyridine-d_5): δ 1.12 (d, 3 H, *J* 7 Hz), 1.16 (d, 3 H, *J* 7 Hz), 1.64–2.55 (3 H), 4.18 (s, 2 H), 4.24–4.50 (3 H), 4.95, 5.16 and 6.3 (broad, 4 H, exchangeable in D_2O).

(2R,3S)-2-Isobutyltartaric acid (1). Dimethyl (+)-*erythro*-2-isobutyltartrate [2] (*10*, 27.8 mg) was refluxed with aqueous hydrochloric acid (2 M, 10 ml) for 70 h. Evaporation of the reaction mixture gave *1* as an amorphous solid, $[\alpha]_{578}^{22}$ +18.8° (*c* 0.69, methanol). NMR (CD_3OD): δ 0.90 (d, 3 H, *J* 6 Hz), 0.96 (d, 3 H, *J* 6 Hz), 1.45–2.10 (3 H), 4.33 (s, 1 H), 4.90 (s, OH). The acid was used without further purification in the preparation of the solution for CD measurement (water; 3.0 mM solution with respect to *1* and 2.7 mM with respect to sodium molybdate. Acidification with hydrochloric acid to pH 3.0). The CD curve is shown in Fig. 1.

Dimethyl(2S,3S)-2-Isobutyltartrate (11). A solution of *7* (103 mg) in nitric acid [7] (32 %, 0.6 ml) was heated at 55 °C for 24 h and then concentrated to a syrup. Water (0.5 ml) was added and the solution was again concentrated. This procedure was repeated twice and the residue was then dried under reduced pressure, dissolved in methanol (1.5 ml) and treated with an excess of diazomethane in ether. The methylation product was purified by preparative GLC (160 °C, retention time 13 min) giving *11* (9.8 mg), m.p. 38–44 °C, $[\alpha]_{578}^{22}$ +12.5° (*c* 0.98, methanol). The NMR spectrum of *11* was indistinguishable from that of the racemic compound.[2]

(2S,3S)-2-Isobutyltartaric acid (12). A solution of *11* (9 mg) in aqueous hydrochloric acid (2 M, 10 ml) was refluxed for 68 h. Evaporation of the reaction mixture gave *12* as an amorphous solid (7.8 mg), $[\alpha]_{578}^{22}$ +9.9° (*c* 0.39, methanol). Before measuring its NMR spectrum, the hydroxylic protons were replaced by deuterium. NMR (CD_3OD): δ 0.90 (d, 3 H, *J* 6 Hz), 0.96 (d, 3 H, *J* 6 Hz), 1.45–2.10 (3 H), 4.25 (s, 1 H). The solution for the CD measurement was prepared as above. The CD curve is shown in Fig. 1.

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Ring-opening Reactions of Heterocyclic Organometallics. VII.* The Regio- and Stereospecific Synthesis of Alkylselenovinyl Acetylenes

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Ring-opening of 3-selenienyllithium derivatives (*2*) with nonidentical substituents in the α-positions leads to enyneselenolates (*3*), which are alkylated by ethyl bromide to give ethylselenovinyl acetylenes (*4*) with *Z*-configuration and with the ethylseleno function attached to either of the terminal carbons of the vinylacetylenic moiety.

The 3-selenienyllithium derivatives (*2*) were prepared by halogen-metal exchange between the corresponding 3-bromoselenophenes (*1*) and ethyllithium. The synthesis of the starting materials is described.

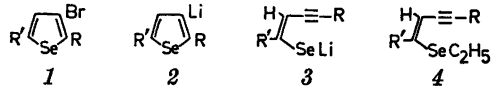
RING-OPENING REACTIONS

We have previously described the synthetic possibilities of the ring-opening reaction of some 3-thienyllithium derivatives with nonidentical substituents in the α-positions in order to prepare alkylthiovinyl acetylenes with controlled stereochemistry (*Z*-configuration), and with the alkylthio function attached to either of the terminal carbon atoms of the vinylacetylenic moiety.[1]

Since 3-selenienyllithium derivatives show a tendency to ring-open much faster than the corresponding 3-thienyllithium derivatives,[2-4] it could be expected that 3-selenienyllithium derivatives with non-identical substituents in the 2- and 5-positions would give alkylselenovinyl acetylenes in analogy with the 3-thienyllithium derivatives.

It should be mentioned that ethanethiolate adds to 2,4-heptadiyne in a non-specific manner to give a mixture of 2-ethylthio-2-hepten-4-yne and 3-ethylthio-3-hepten-5-yne,[1] both probably with *Z*-configuration (*cf.* Ref. 5). The use of ethaneselenolate instead of ethanethiolate would probably not give a significantly more specific reaction. Thus, the ring-openings of the lithium derivatives *2b* and *2a* could be expected to provide convenient routes to *4b* and *4a*, respectively.

Therefore *1b* and *1a*, the synthesis of which will be described below, were treated with ethyllithium followed by ethyl bromide in ether for 4 h at room temperature. After hydrolysis of the reaction mixture, *4b* and *4a* were isolated in 54 and 60 % yield, respectively. The crude products contained only minor amounts of by-products, which makes the reaction of synthetic value. However, upon the isolation of the compounds (distillation) much decomposition took place, explaining the relatively moderate yields.



1 *2* *3* *4*

Scheme 1. a, R′= C_2H_5, R=CH_3; b, R′= CH_3, R=C_2H_5.

The reaction path by which these compounds are formed is most likely initiated by a halogen-metal exchange between *1a* and *1b* with ethyllithium to give *2a* and *2b*, respectively. Then, *2a* and *2b* ring-open as described previously [1]

* Part VI, see Ref. 1.
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*** Taken in part from the Ph.D. thesis of T. Frejd, Lund 1975.

to form the enyne selenolates *3a* and *3b*, which eventually react with ethyl bromide at selenium (S_N2 reaction) to give *4a* and *4b*.

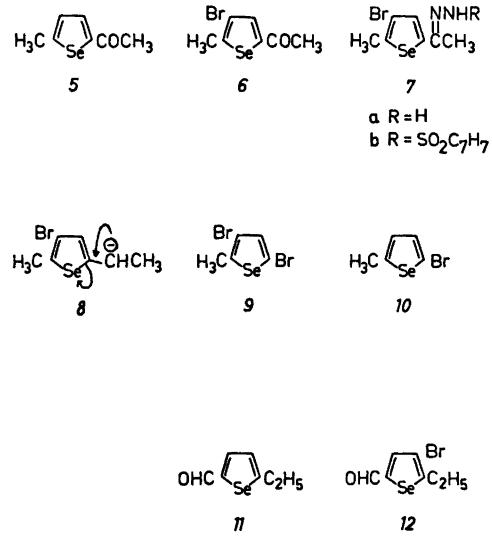
The structures of these compounds were deduced from IR, NMR and mass spectra as well as from elemental analyses (see experimental part). In the mass spectra of *4a* and *4b* the fragment at $m/e = 93$ was quite abundant (32 and 66 %, respectively).

Since the coupling constant between vinylic protons and the protons of the vinylic methyl or methylene groups (the allylic coupling constant) is of the same magnitude for both *cis* and *trans* arrangements,[6] it could not be determined whether *4a* and *4b* had the *Z*- or *E*-configuration with the aid of this tool. However, it has been shown by us that the products originating from the ring-opening of 3-thienyl- and 3-selenienyllithium had the *Z*-configuration.[4] Independently, Jakobsen showed that the ring-opening of 2-methyl-3-thienyllithium also gave a thioenyne with *Z*-configuration.[7] Thus it seems quite likely that the ring-opening takes place in a *trans* manner leading to thio- and selenoenynes with the heteroatom and the triple bond in a *cis* arrangement.

SYNTHESIS OF THE STARTING MATERIALS

The synthesis of *1b* was performed as follows. First, 2-ethylselenophene [18] was treated with butyllithium followed by dimethylformamide at −70 °C to give *11*. Bromination of *11* with the "swamping catalyst" method [9] (bromine in aluminium trichloride) gave *12*, which was subsequently reduced with the Wolff-Kishner method to give *1b*. This synthetic route is essentially analogous to that used in the thiophene series for the preparation of 3-bromo-2-ethyl-5-methylthiophene,[10] except that the formyl group was introduced by Vilsmeier formylation in the latter case.

Then it seemed natural to apply an analogous route to obtain *1a* as well. By the "swamping catalyst" method, *5* [11] was converted to *6* without difficulty. However, when the modified Wolff-Kishner reduction [8] was attempted on *6*, no reduction took place. A yellow precipitate was formed when hydrazine was heated together with *6* in ethylene glycol. The subsequent base treatment did not lead to nitrogen evolution, even at 160 − 180 °C. At this temperature red elemental selenium started to form, and therefore the experiment was stopped and a small amount of the yellow crystals was isolated. The most heavy fragment was around $m/e =$ 280, which indicated the presence of the hydrazone, *7a*. Despite several recrystallisations, the substance was not obtained pure as judged from its broad melting interval. Lithium aluminium hydride reduction of the tosyl hydrazone *7b* was also unsuccessful. Red selenium precipitated when the excess of lithium aluminium hydride was destroyed with moist ether. Since base treatment of hydrazones and tosyl hydrazones may form carbanions, it is suggested that an anion such as *8* is involved. We have shown in previous work that ring-opening from the side-chain is possible in the thiophene series [12] and *8* may also undergo ring-opening as indicated in the figure. It should be pointed out, however, that 2-acetylselenophene and *12* were successfully converted to 2-ethylselenephene and *1b*, respectively, with the Wolff-Kishner procedure as mentioned. Further work is necessary in order to reveal the causes of these results.



Consequently, another approach had to be used for the synthesis of *1a*. Starting from 2,5-dibromoselenophene, *10* was prepared in 67 % yield by halogen-metal exchange with butyllithium followed by dimethyl sulfate. Bromina-

tion of *10* with bromine in acetic acid gave a 53 % yield of *9*. This route was used as it was found that the reaction of 2-methylselenophene with two equivalents of bromine in acetic acid led to extensive tar formation. The decomposition was almost instantaneous when only a small amount of bromine had been added. The corresponding reaction with 2-methylthiophene gives 3,5-dibromo-2-methylthiophene in good yield.[13] It was also found that 2-methylselenophene could be brominated at room temperature in 55 % yield, when two equivalents of *N*-bromosuccinimide in acetic acid were used.[14] These experiments indicate that it was 2-methylselenophene or some intermediate that decomposed in the presence of strong acids (*e.g.* HBr) and thus illustrate the greater acid sensitivity of selenophenes compared to thiophenes.

When 2-methylselenophene was treated with bromine in carbon disulfide at −20 °C, a brown oil was obtained, which darkened rapidly. An exothermic reaction took place when potassium hydroxide pellets were added and a 41 % yield of *9* could be isolated after distillation (decomposition of 9 took place to a great extent during all attempts at distillation). Thus it seems probable that an addition product was initially formed between 2-methylselenophene and bromine and that the elimination of hydrogen bromide started the decomposition in the absence of base.

Halogen-metal exchange between *9* and butyllithium followed by reaction with diethyl sulfate gave the desired compound *1a*. Unfortunately it could not be obtained pure by distillation or by TLC. The diethyl sulfate, left from the reaction, showed an amazing tendency to adhere to *1a*. However, the preparation was considered pure enough (90 %, NMR) to be used in the ring-opening experiments.

EXPERIMENTAL

General remarks. See Ref. 1.

2-Ethyl-5-selenophenealdehyde (11). To 20.0 g (0.126 mol) of 2-ethylselenophene [18] in 100 ml of ether, 80 ml (0.13 mol) of 1.60 M butyllithium in hexane was added at such a rate that gentle reflux was maintained. After the addition, the reaction mixture was cooled to 0 °C and 9.5 g (0.13 mol) of DMF in 25 ml of ether was added. The mixture was allowed to reach room temperature and poured onto ice/conc. HCl. The aqueous layer was extracted with ether and the collected ethereal phases were washed with water, dried and evaporated, leaving 19.9 g of crude product. Distillation gave 15.0 g (64 %) of the title compound, b.p.$_{11}$ 121–123 °C. NMR (CCl_4): δ 7.75 (d, 1 H, 3-H), 7.05 (d, t, 1 H, 4-H), 2.93 (bq, 2 H, -CH_2-) and 1.33 (t, 3 H, CH_3), 9.58 (s, 1 H, CHO), $J_{3H,4H}$ 4.0 Hz, $J_{4H,5\text{-}CH_2-}$ = 1.1 Hz. [Found: C 44.80; H 4.52; Se 42.04. Calc. for C_7H_8OSe (187.10): C 44.94; H 4.31; Se 42.20].

3-Bromo-2-ethyl-5-selenophenealdehyde (12) was prepared in a way analogous to that used for *6* from 14.0 g (0.0748 mol) of *11*, 25.0 g (0.187 mol) of dry $AlCl_3$ and 13 g (0.081 mol) of bromine. After distillation, 12.6 g (63 %) of the title compound was obtained, b.p.$_{1.8}$ 113–115 °C. NMR (CCl_4): δ 7.77 (s, 1 H, 4-H), 2.84 (q, 2 H, -CH_2-) and 1.32 (t, 3 H, CH_3), 9.60 (s, 1 H, CHO), $J_{CH_2-CH_3}$ 7.0 Hz. [Found: C 31.61; H 2.65; Se 29.69. Calc. for C_7H_7BrOSe (266.00): C 31.61; H 2.65; Se 29.68].

3-Bromo-2-ethyl-5-methylselenophene (1b). A mixture of 10.0 g (0.0376 mol) of *12*, 6 ml of 99.5 % hydrazine hydrate and 30 ml of ethylene glycol was heated gradually to 140 °C, during which time water and hydrazine were distilled off. After cooling to room temperature, 6.0 g of KOH pellets was added, and the mixture was heated to 90–116 °C. The solution became red and was kept at 116 °C for 2.5 h, until nitrogen formation had subsided. After steam distillation, extraction of the distillate with ether, drying and evaporation of the solvent 6.0 g of crude *1b* remained. Distillation gave 5.0 g (53 %) of the pure title compound, b.p.$_{12}$ 99–102 °C. NMR (CCl_4): δ 6.65 (q, 1 H, 4-H), 2.46 (m, 3 H, 5-CH_3), 2.73 (q, 2 H, -CH_2-) and 1.22 (t, 3 H, CH_3), $J_{4H,5CH_3}$ 1.3 Hz, $J_{CH_2-CH_3}$ 7.0 Hz. [Found: C 33.34; H 3.69; Se 31.38. Calc. for C_7H_9BrSe (252.01): C 33.36; H 3.60; Se 31.33].

2-Acetyl-5-methylselenophene (5). A mixture of 25.0 g (0.172 mol) of 2-methylselenophene,[15] 40 g (0.39 mol) of acetic anhydride and 8 drops of perchloric acid (70 %) was refluxed for 1 h, cooled and poured into ice-water. The aqueous phase was extracted with ether and the ethereal portions were dried. Evaporation and distillation yielded 16.5 g (51 %) of the title compound, b.p.$_{13}$ 122–124 °C (lit.[11] b.p.$_{12}$ 114–116 °C). NMR (CCl_4): δ 7.61 (d, d, 1 H, 3-H), 6.90 (d, q, 1 H, 4-H), 2.55 (d, d, 3 H, 5-CH_3), 2.39 (s, 3 H, $COCH_3$), $J_{3H,4H}$ 3.6 Hz, $J_{4H,5CH_3}$ 1.0 Hz, $J_{3H,5CH_3}$ 0.4 Hz.

2-Acetyl-3-bromo-5-methylselenophene (6). To 33 g(0.25 mol) of aluminium trichloride, 18.7 g (0.100 mol) of *5* was added with stirring, cooling the reaction flask in an ice bath. The cooling bath was removed and after 30 min 19.2 g (0.120 mol) of bromine was added rapidly. The reaction mixture was stirred for 1 h, whereupon it was decomposed by treatment

with ice/conc. HCl. The aqueous phase was extracted several times with ether and the ethereal portions were washed with water to neutral reaction and dried. Evaporation and distillation gave the title compound, 18.4 g (69 %), b.p.$_{14}$ 108–111 °C. NMR (CCl_4): δ 7.60 (bs, 1 H, 4-H), 2.45 (bs, 2-CH_3; $COCH_3$). The integral of the methyl band represented 6 H. [Found: C 31.57; H 2.70; Se 29.67. Calc. for C_7H_7BrOSe (266.00): C 31.61; H 2.65; Se 29.69].

Attempted reduction of 5-acetyl-3-bromo-2-methylselenophene (6). (a) When the procedure used for the preparation of *1b* (see above) was applied to *6*, yellow crystals were formed but no trace of the title compound was found. When the reaction mixture was heated with potassium hydroxide gradually to 170–180 °C, red selenium was formed, but no nitrogen evolution was noticed. The reaction was stopped and the crystals were collected by suction and recrystallised from ethanol twice, but the melting interval was unchanged; 100–105 °C. IR: N-H 3320, 3180 cm^{-1}; $m/e = 280$; calc. for the hydrazone (*7a*) ($C_7H_9{}^{79}BrN_2{}^{80}Se$) = 280. NMR ($CCl_4$): δ 6.88 (s, 1 H, 4-H), 2.36 (s, 3 H, CH_3), 2.00 (s, 3 H, CH_3), 5.11 (2 H, NH_2).

(b) The tosylhydrazone *7b* was prepared according to a procedure described in Ref. 16, from 2.66 g (0.0100 mol) of *6* and 2.80 g (0.0150 mol) of tosylhydrazide in 50 ml of methanol; yield 3.4 g (78 %), m.p. 191–193 °C from methanol in the cold (–25 °C). [Found: C 38.4; H 3.54; N 6.78. Calc. for $C_{14}H_{15}BrN_2O_2SSe$ (434.23): C 38.72; H 3.48; N 6.45].

The tosylhydrazone, 3.0 g (0.0069 mol), was treated with 2.7 g (0.071 mol) of $LiAlH_4$ in 50 ml of refluxing THF for 18 h (*cf.* Ref. 16). Upon hydrolysis of the reaction mixture with moist ether, red selenium precipitated and 0.3 g of a brown, uncharacterised oil remained after work-up and evaporation.

2,5-Dibromoselenophene. To a solution of 50.0 g (0.382 mol) of selenophene in 50 ml of acetic acid, 126 g (0.788 mol) of bromine in 80 ml of acetic acid was added (ice cooling). After stirring overnight the reaction mixture was poured into water. The aqueous phase was neutralised with sodium hydrogen carbonate and extracted with ether several times. The ethereal portions were washed with water to neutral reaction and dried. Evaporation and distillation yielded 77.7 g (70 %) of the title compound, b.p.$_{13}$ 101–104 °C, n_D^{20} 1.6665 (lit.[17] b.p.$_{0.03}$ 42 °C, n_D^{20} 1.667).

2-Bromo-5-methylselenophene (10). To a solution of 28.9 g (0.100 mol) of 2,5-dibromoselenophene in 200 ml of ether, 65 ml (0.10 mol) of 1.60 M butyllithium in hexane was added at –50 °C, followed by 13.0 g (0.103 mol) of dimethyl sulfate in 50 ml of ether at –30 °C. The reaction mixture was allowed to reach room temperature and was kept there for 1 h, whereupon conc. ammonium hydroxide was added. Extraction with ether, drying, evaporation and distillation gave 15.0 g (67 %) of the title compound, b.p.$_{11}$ 79–80 °C. NMR (CCl_4): δ 6.90 (d, 1 H, 3-H), 6.52 (d, q, 1 H, 4-H), 2.46 (d, 3 H, 5-CH_3), $J_{3H,4H}$ 3.8 Hz, $J_{4H,5CH_3}$ 1.2 Hz [Found: C 26.75; H 2.28; Br 35.66; Se 35.20. Calc. for C_5H_5BrSe (223.96): C 26.82; H 2.25; Br 35.68; Se 35.26].

3,5-Dibromo-2-methylselenophene (9). (a) To a solution of 7.0 g (0.048 mol) of 2-methylselenophene[15] in 50 ml of carbon disulfide, 16 g (0.10 mol) of bromine in 20 ml of acetic acid was added at –20 °C. When the addition was complete, the mixture was stirred at room temperature overnight and then poured into water and extracted with ether. Drying and evaporation of the solvent gave 16.8 g of a brown fuming oil, which reacted with solid KOH (4 g) under heat evolution. After the base treatment, the residue was distilled, giving 6.0 g (41 %) of the title compound having the same physical data as described earlier.[14]

(b) To a solution of 11.2 g (0.0500 mol) of *10* in 50 ml of acetic acid, 8.0 g (0.050 mol) of bromine in 25 ml of acetic acid was added at room temperature. The mixture was stirred for 1.5 h and then poured into water. The aqueous phase was neutralised with dil. aq. NaOH and extracted with ether. The ethereal portions were dried and the solvent evaporated, giving 12.9 g of a crude product, which was distilled to yield 8.0 g (53 %) of the title compound.

3-Bromo-5-ethyl-2-methylselenophene (1a). A solution of 9.10 g (0.300 mol) of *9* in 150 ml of ether was cooled to –70 °C and 20 ml (0.30 mol) of 1.50 M butyllithium in hexane was added drop-wise, followed by 9.25 g (0.0600 mol) of diethyl sulfate in 25 ml of ether after 10 min. The reaction mixture was slowly heated and an exothermic reaction took place between +17 and +25 °C, after which it was left overnight. Conc. ammonium hydroxide was added and the mixture was stirred for 1 h. The ethereal layer was separated, washed with 2 N HCl, water and dried. Evaporation and distillation gave 2.8 g (37 %) of almost pure title compound, b.p.$_{12}$ 101–104 °C. GLC (column BDS 10 %, 150 °C) and NMR analysis showed that some diethyl sulfate (≈10 %) was present. Repeated distillation and preparative TLC (1 mm silica gel; hexane) did not remove this impurity nor did prolonged treatment with ammonium hydroxide. Combined GLC-MS showed the molecular fragment at m/e 252 (100 %) with the isotopic distribution typical for 1 Br and 1 Se. Calc. for $C_7H_9{}^{79}Br^{80}Se$ 252. NMR (CCl_4): δ 6.66 (bt, 1 H, 4-H), 2.40 (bs, 3 H, 2-CH_3), 2.79 (q, 2 H, -CH_2-) and 1.25 (t, 3 H, CH_3); $J_{CH_3-CH_2}$ 7.5 Hz. [Found: C 34.89; H 3.72; Se 30.66. Calc. for C_7H_9BrSe (252.00): C 33.66; H 3.60; Se 31.33].

(Z)-2-Ethylseleno-2-hepten-4-yne (4b). To 2.52 g (0.0100 mol) of *1b* in 50 ml of ether, 17 ml (0.010 mol) of 0.60 M ethereal ethyllithium was added followed by 5.45 g (0.0500 mol) of ethyl

bromide. GLC analysis (column BDS 10 %, 170 °C) of the washed and dried ethereal solution showed 98 % of *4b*. The crude product was distilled from paraffin oil (to prevent decomposition of the enyne to some extent), b.p.$_{12}$ 108–109 °C, 1.1 g (54 %). IR: C≡C 2220 cm^{-1}. Mass spectrum, *m/e* (%); selenium-containing fragments: 202 (100), 187 (8), 173 (12), 159 (27), 145 (8), 133 (7), 119 (2), 117 (4), 107 (33). Fragments not containing selenium: 93 (66), 92 (40), 91 (73), 79 (10), 78 (23), 77 (43), 67 (3), 66 (3), 65 (23), 64 (3), 63 (10), 62 (3), 55 (3), 53 (10), 52 (3), 51 (17), 50 (3), 44 (3), 41 (17), 40 (3), 39 (23). NMR (CCl_4): δ 2.13 (m, 3 H, 1-H), 5.58 (m, 1 H, 3-H), 2.35 (bq, 6-H), 1.67–0.92 (7-H), 2.78 (q, 2 H) and 1.67–0.92 (SeC_2H_5), $J_{6H,7H}$ 7.0 Hz. [Found: C 53.70; H 7.10; Se 39.25. Calc. for $C_9H_{14}Se$ (201.16): C 53.73; H 7.01; Se 39.25].

(Z)-3-Ethylseleno-3-hepten-5-yne (4a). As above, the title compound was prepared from 2.45 g (9.60 mmol) of *1a* in 50 ml of ether, 16 ml (9.6 mmol) of 0.60 M ethereal ethyllithium and 5.45 g (50.0 mmol) of ethyl bromide. GLC analysis (conditions as above) of the washed and dried ethereal solution showed about 90 % of *4a*, b.p.$_{12}$ 107–108 °C, 1.2 g (60 %). (The distillation was performed from paraffin oil.) IR: C≡C 2220 cm^{-1}. Mass spectrum *m/e*, (%); selenium-containing fragments: 202 (100), 187 (5), 173 (9), 159 (16), 145 (5), 133 (4), 131 (5), 119 (4), 117 (4), 107 (9). Fragments not containing selenium: 93 (32), 92 (40), 91 (100), 79 (12), 78 (24), 77 (80), 67 (4), 66 (4), 65 (28), 64 (4), 63 (12), 62 (4), 55 (4), 53 (16), 52 (4), 51 (16), 50 (4), 44 (4), 41 (20), 40 (4), 39 (28). NMR (CCl_4): δ 1.67–0.83 (1-H), 2.33 (q, 2-H), 5.67 (m, 4-H), 1.95 (m, 7-H), 2.77 (q) and 1.67–0.83 (SeC_2H_5), $J_{SeCH_2-CH_3} = J_{1H,2H} = 7.0$ Hz. [Found: C 53.67; H 6.89; Se 39.22. Calc. for $C_9H_{14}Se$ (201.16): C 53.73; H 7.01; Se 39.25].

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Strained Heterocyclic Compounds. 10. Introduction of an Amide Side Chain in Place of Halogen in 7-Halo-8-oxo-1-azabicyclo[4.2.0]octane

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The nucleophilic displacement reaction between thallium(I) phthalimide and the bromo-β-lactams *2a*—*b* has been studied. The corresponding phthalimido-β-lactams *3a*—*b* were obtained in good yields with 90 % stereoselectivity (inversion at the C-7 carbon atom). The chloro-β-lactam *2c* reacted only in very low yield under the same conditions. Hydrazinolysis of the phthalimido-β-lactam *3a* and subsequent acylation gave a β-lactam *5a* with an amido side chain, appropriate for a penicillin analogue.

In the effort of synthesising penicillin analogs with modified nuclei [1] we have developed a general method for the synthesis of α-halo-β-lactams (*2*) by thermolysis of phenyl mercury compounds (*1*).[2] Other routes to halo-β-lactams are the addition of haloketenes to imines [3] and the base-induced bromination of cepham derivatives.[4]

These methods all give predominantly the thermodynamically more stable *trans*-isomer of the halo-β-lactams [2—4] (*i.e trans*-configuration of the hydrogens at the carbon atoms C-6 and C-7, *cf.* Fig. 1). However, in all the biologically active penicillins and cephalosporins the configuration at the corresponding sites is *cis*.[5] We have therefore searched for a general displacement reaction, exchanging the halogen for an amino function, that proceeds with inversion at the C-7 carbon atom.

After trying various reagents we found that *N*-phthalimidothallium fulfilled the requirements.[6] This reagent effected substitution of the halo-β-lactams *2a*—*b* in fairly good yields and with 90 % stereospecificity. Hydrazinolysis of the phthalimido-β-lactams *3a*—*b* gave the corresponding amino-β-lactams *4a*—*b*, which were acylated to the amido-β-lactams *5a*—*b*.

Similar substitutions have been attempted by several workers.[4,7—9] In one successful case 7-bromocephams were reacted with sodium azide to yield azido-β-lactams, which were reduced to yield the corresponding amino-β-lactams.[4] This method was not successful with the compounds *2a*—*b*.[6]

RESULTS AND DISCUSSION

Reaction between the halo-β-lactams 2a—c *and N-phthalimidothallium.* The formation of the phthalimido-β-lactams *3a*—*b* from *N*-phthalimidothallium and the *trans*-bromo-β-lactam *2a* in DMSO was studied in some detail. The reaction temperature showed a strong influence on the yield (*cf.* Table 1). At the optimum temperature of 150 °C the highest yield of *3* was obtained and after a 10 h reaction time 80 % of the starting material could be accounted for. The product β-lactams *3* were thermally quite stable to isomerisation and decomposition at 150 °C while the starting bromo-β-lactam *2a* decomposed rapidly when heated in DMSO at this temperature. *N*-Phthalimidothallium, however, stabilized the bromo-β-lactam due to its ability to trap released hydrogen bromide.

The stereoselectivity of the displacement reaction was also dependent on the temperature, the ratio of phthalimido-β-lactams *3a* to *3b* in the product decreasing from >10 at

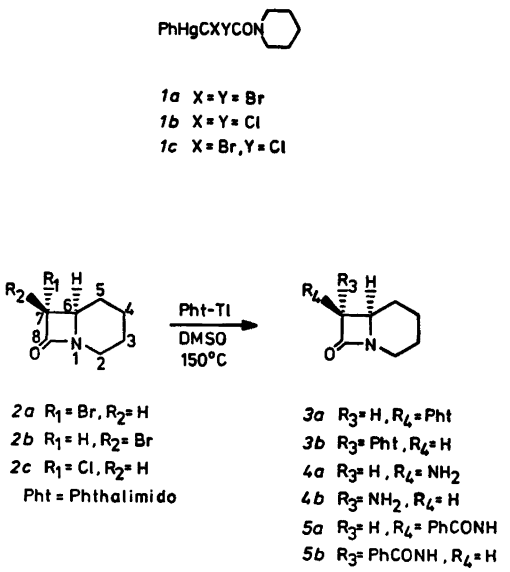

Fig. 1.

100 °C to 2 at 189 °C (*cf.* Table 1). The epimerisation observed may be due to isomerisation prior to substitution of the starting bromo-β-lactam *2a*. Further, as *cis*-phthalimidopenicillanates are known to epimerise irreversibly to their corresponding *trans*-isomers in the presence of bases [10] it is also possible that *N*-phthalimidothallium was sufficiently basic to cause the isomerisation of the β-lactam *3a* to yield *3b*. This would also explain the lack of epimerisation product in the formation of the *trans*-phthalimido-β-lactam *3b* from the *cis*-bromo-β-lactam *2b* (*cf.* below).

The configurations of the respective phthalimido-β-lactams *3a–b* were determined by ^{1}H NMR methods. Thus for the isomer *3a* the coupling constant for the C-6 and C-7 protons was larger than the corresponding coupling constant for the isomer *3b*. Accordingly the isomer *3a* was assigned the *cis*-configuration and the isomer *3b* the *trans*-configuration.[11]

The reaction time was also of importance both for the yield and the stereoselectivity (*cf.* Table 2), as longer reaction times are expected to favour both isomerisation and decomposition as well as substitution.

The reaction between the *cis*-bromo-β-lactam *2b* and *N*-phthalimidothallium in DMSO at 150 °C proceeded in considerably lower yield. The product *trans*-phthalimido-β-lactam *3b* was free from the *cis*-isomer *3a* according to ^{1}H NMR. Thus a reaction time of 2 h yielded 7 % of the product *3b* while 35 % of the *cis*-bromo-β-lactam *2b* was recovered. Extension of the reaction time to 11 h increased the yield of *3b* to 24 % while no bromo-β-lactam was recovered.

Substitution of the more stable *trans*-chloro-β-lactam *2c* was less successful (*cf.* Ref. 8). Thus the β-lactam *2c*, when reacted with *N*-phthalimidothallium in DMSO at 150 °C for 11 h gave only a 4 % yield of the product *3a*. Other polar, aprotic solvents were tried, *e.g* sulfolane and HMPA, but the yields of *3a* were still low (~5 %) when all the starting chloro-β-lactam was consumed.

As potassium phthalimide was more reactive than *N*-phthalimidothallium towards *N*-(haloacetyl)piperidines [12] this reagent was tried in the chloro-β-lactam system. However, after 2 h at 150 °C in DMSO only a trace of the product *3a* was obtained, while more than 50 % of the starting chloro-β-lactam was consumed.

Table 1. Temperature as an influencing factor for the isolated yield of phthalimido-β-lactams *3a–b* and the ratio of isomers *3a* to *3b* in the product from the reaction of *N*-phthalimidothallium with the *trans*-bromo-β-lactam *2a* in DMSO.

Temp. (°C)	Time (h)	Yield (%)	Ratio *3a/3b*
50	18	0	
100	4	5	> 10/1
150	10	55	10/1
189	1	38	2/1

Table 2. Reaction time as an influencing factor for the isolated yield of the phthalimido-β-lactams *3a–b* and the ratio of isomers *3a* to *3b* in the product from the reaction of the *trans*-bromo-β-lactam *2a* with *N*-phthalimidothallium in DMSO at 150 °C.

Time (h)	Yield (%)	Ratio *3a/3b*
2	18	> 10/1
7	34	> 10/1
10	55	10/1
16	36	3/1

Transformation of the phthalimido-β-lactams 3a–b *to the benzamido-β-lactams* 5a–b. Reaction of the β-lactam *3a* with anhydrous hydrazine in dichloromethane at room temperature [13] afforded the phthalic hydrazide salt of the amino-β-lactam *4a*. The free amino-β-lactam was obtained by the decomposition of this salt by treatment with dilute hydrochloric acid,[14] followed by removal of the phthalic hydrazide and subsequent basification. The yield of the crude amino-β-lactam *4a* by this method was 64 % and is not optimized. No purification was attempted of the amino-β-lactam, which was characterized by its spectroscopic data. Other methods for the hydrazinolysis were tried; thus treatment with hydrazine hydrate in refluxing dioxane [15] caused partial destruction of the β-lactam. The use of tetrahydrofuran or dioxan as the solvent at room temperature afforded lower yields of the amino-β-lactam.

Acylation of the crude amino-β-lactam *4a* to the benzamido-β-lactam *5a* could be effected with benzoyl chloride and triethylamine in tetrahydrofuran [16] or with benzoic acid and *N,N'*-dicyclohexylcarbodiimide in dichloromethane,[17] the last method giving a slightly better yield (47 % and 53 %, respectively). Thus the overall yield for the transformation of the phthalimido-β-lactam *3a* to an amido-substituted β-lactam *5a* was 26 %.

No isomerisation of the β-lactams *3–5* was detected throughout the reaction sequence, which is important for retaining the desired *cis*-configuration in the penicillin analogs. Thus the amino-β-lactam *4a* showed the typical coupling constant of 4 Hz for the H-7 and H-6 proton coupling of *cis*-β-lactams.[11] For the amido-β-lactam *5a* the coupling pattern for the H-7 proton was more complex due to the additional coupling of this proton with the NH-proton with a coupling constant of 7 Hz, characteristic of the NH and H-7 coupling constant in this type of compound.[18] By effecting the same transformations on the *trans*-phthalimido-β-lactam *3b* the amino-β-lactam *4b* and the benzamido-β-lactam *5b* were obtained. In these compounds the chemical shifts of the H-7 protons were at higher field than in the corresponding *cis*-compounds *4a* and *5a*, respectively. They also showed the characteristically smaller coupling constant (1.5-2 Hz) for the C-6 and C-7 protons of *trans*-β-lactams,[11] while in *5b* the coupling constant for the NH and C-7 protons were of the same magnitude (7 Hz) as in *5a*.

EXPERIMENTAL

All melting points were determined on a micro hot stage apparatus and are uncorrected. Elemental analyses were carried out by Mikroanalyslaboratoriet, Uppsala, Sweden. IR-spectra were recorded using a Perkin-Elmer Model 421 spectrophotometer. ^{1}H NMR spectra were recorded using a Varian A60 and a Varian EM360 instrument. The chemical shifts are given as δ-values relative to TMS as internal standard. Mass spectra were recorded using an LKB 9000 mass spectrometer. Column chromatography was performed using silica gel (Merck 0.05–0.2 mm). Preparative thin layer chromatography was performed using silica gel (Merck Fertigplatten 2 mm). All the DMSO used was distilled prior to use.

N-*Phthalimidothallium*. To a solution of 2.95 g (0.02 mol) phthalimide in 50 ml of dry THF was added 5.0 g (0.02 mol) of thallium(I) ethoxide. The reaction mixture was stirred for 3 h at room temperature. The precipitate formed was collected by filtration and washed with dry benzene. Yield 6.8 g (97 %), m.p. 310–312 °C (dec.). IR, λ_{max} (KBr): 3030, 1680, 1610–1530 (broad) cm^{-1}. ^{1}H NMR (DMSO): δ 7.43 (s, aromatic protons). MS, *m/e*: 349, 351 (M^+), 203, 205 (Tl^+).

cis-*7-Phthalimido-8-oxo-1-azabicyclo[4.2.0]-octane* (3a). *trans*-7-Bromo-8-oxo-1-azabicyclo-[4.2.0]octane (*2a*) [2a] (0.48 g, 2.3 mmol) and *N*-phthalimidothallium (0.84 g, 2.3 mmol) were heated in DMSO (30 ml) at 150 °C for 10 h. The solvent was evaporated *in vacuo* and the residue was dissolved in chloroform. The solution was washed with water and a saturated $NaHCO_3$ solution, dried over $CaCl_2$ and the solvent was evaporated. Separation of the reaction products in the residue was accomplished by chromatography on a column of silica gel, cooled to −20 °C by circulating ethanol. Elution with dry diethyl ether afforded phthalimide (0.055 g, 16 %), *trans*-7-bromo-8-oxo-1-azabicyclo[4.2.0]octane (*2a*) (0.114 g, 24 %), *trans*-7-phthalimido-8-oxo-1-azabicyclo[4.2.0]octane (*3b*) (0.035 g, 5 %), and the required product *cis*-7-phthalimido-8-oxo-1-azabicyclo[4.2.0]octane (*3a*) in 50 % yield (0.311 g). M.p. 174–176 °C. (Found: C 66.3; H 5.2; N 10.0. Calc. for $C_{15}H_{14}N_2O_3$: C 66.6; H 5.2; N 10.3). IR, λ_{max} (KBr): 1760, 1725 (>C=O) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.4–2.0 (m, H-3, H-4, H-5), 2.5–4.2 (m, H-2, H-6), 5.39 (two doublets $J=1.5$ and $J=4.5$ Hz, H-7) and 7.96 (m, aromatic protons). MS, *m/e*: 270 (M^+), 123 ($M-C_6H_5NO_2$).

trans-7-Phthalimido-8-oxo-1-azabicyclo[4.2.0]-octane (3b). *cis*-7-Bromo-8-oxo-1-azabicyclo-[4.2.0]octane (*2b*)[2a] (0.20 g, 1 mmol), freshly purified by preparative TLC and *N*-phthalimidothallium (0.35 g, 1 mmol) were heated in DMSO (10 ml) at 150 °C for 11 h. Work-up as above followed by separation of the products by preparative TLC with diethyl ether as eluent gave *trans*-7-phthalimido-8-oxo-1-azabicyclo[4.2.0]octane (*3b*) (0.067 g, 24 %). M.p. 183–184 °C. (Found: C 66.5; H 5.2; N 9.9. Calc. for $C_{15}H_{14}N_2O_3$: C 66.6; H 5.2; N 10.3). IR, λ_{max} (KBr): 1760, 1725 (>C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.2–2.2 (m, H-3, H-4, H-5), 2.5–4.2 (m, H-6, H-2), 5.08 (d, $J=1.8$ Hz, H-7), and 7.91 (m, aromatic protons). MS, *m/e*: 270 (M^+).

Reaction between trans-*7-chloro-8-oxo-1-azabicyclo[4.2.0]octane* (2c) *and N-phthalimidothallium.* (A). *trans*-7-Chloro-8-oxo-1-azabicyclo-[4.2.0]octane (*2c*)[2b] (0.16 g, 1 mmol) and *N*-phthalimidothallium (0.35 g, 1 mmol) were heated in DMSO (10 ml) at 150 °C for 11 h. The reaction mixture was worked up and the products were separated as described above, yielding unreacted chloro-β-lactam (*2c*) and *cis*-7-phthalimido-8-oxo-1-azabicyclo[4.2.0]-octane (*3a*) (0.011 g, 4 %).

(B). As in (A) but with 10 ml HMPA, freshly distilled in a nitrogen atmosphere, as the solvent. A reaction period of 7 h gave the β-lactam *3a* (0.015 g, 6 %).

(C). As in (A) but with 10 ml freshly distilled sulfolane as the solvent and a reaction time of 8 h. The yield of the β-lactam *3a* was 0.005 g (2 %).

Reaction between trans-*7-chloro-8-oxo-1-azabicyclo[4.2.0]octane and potassium phthalimide. trans*-7-Chloro-8-oxo-1-azabicyclo[4.2.0]octane (*2c*)[2b] (0.16 g, 1 mmol) and potassium phthalimide (0.185 g, 1 mmol) were heated in DMSO (10 ml) at 150 °C for 2 h. The usual work-up and separation procedures yielded the unreacted *trans*-chloro-β-lactam (*2c*) (0.076 g, 48 %) and traces of the *cis*-phthalimido-β-lactam *3a* (by TLC, MS).

cis-*7-Amino-8-oxo-1-azabicyclo[4.2.0]octane* (4a). *cis*-7-Phthalimido-8-oxo-1-azabicyclo-[4.2.0]octane (*3a*) (0.270 g, 1 mmol) and 95 % hydrazine (0.067 g, 2 mmol) were stirred in dry dichloromethane (25 ml) at room temperature for 24 h. The solvent was evaporated *in vacuo*. The phthalic hydrazide-amino-β-lactam salt was stirred in 0.2 N HCl (6 ml) for 1 h at room temperature and the undissolved phthalic hydrazide (0.134 g, 83 %) was filtered off and washed with water. The acidic filtrate was combined with the washings and washed with dichloromethane to remove the unreacted phthalimido-β-lactam *3a* (0.005 g, 3 %), then made basic with potassium carbonate and extracted five times with dichloromethane. The dichloromethane phase was washed with water, dried over Na_2SO_4 and the solvent was evaporated to yield the *cis*-amino-β-lactam *4a* as an oil (0.090 g, 64 %). IR, λ_{max} (film): 3385, 3310 ($-NH_2$), 1737 (>C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.1–2.1 (m, H-3, H-4, H-5), 1.87 (s, NH_2), 2.3–4.0 (m, H-2, H-6) and 4.20 (dd, $J=4.5$ Hz and 2.0 Hz, H-7). MS, *m/e*: 140 (M^+), 84.

trans-*7-Amino-8-oxo-1-azabicyclo[4.2.0]octane* (4b). The *trans*-amino-β-lactam *4b* was obtained from the corresponding *trans*-phthalimido-β-lactam *3b* in the same manner as described above. The yield was 60 %. IR, λ_{max} (film): 3375, 3300 (NH_2), 1742 (>C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.1–2.2 (m, H-3, H-4, H-5), 2.06 (s, NH_2), 2.3–4.0 (m, H-2, H-6) and 3.67 (d, $J=2$ Hz, H-7). MS, *m/e*: 140 (M^+), 84.

cis-*7-Benzamido-8-oxo-1-azabicyclo[4.2.0]-octane* (5a). To *cis*-7-amino-8-oxo-1-azabicyclo-[4.2.0]octane (*4a*) (0.17 g, 1.2 mmol) and benzoic acid (0.087 g, 1.2 mmol) in dry dichloromethane (20 ml) cooled to 0 °C was added *N,N'*-dicyclohexylcarbodiimide (0.150 g, 1.2 mmol). The reaction mixture was then stirred at room temperature overnight. The *N,N'*-dicyclohexylurea was removed by filtration and the filtrate was washed with an aqueous saturated $NaHCO_3$ solution and then with water, dried over Na_2SO_4, and the solvent was evaporated. The crude product was chromatographed on a column of silica gel, with diethyl ether as the eluent to give *cis*-7-benzamido-8-oxo-1-azabicyclo[4.2.0]octane (*5a*) (0.081 g, 53 %). M.p. 203–204 °C. (Found: C 69.2; H 6.6; N 11.2. Calc. for $C_{14}H_{16}N_2O_2$: C 68.8; H 6.6; N 11.5). IR, λ_{max} (KBr): 3310 (–NH–), 1730 (>C=O, β-lactam), 1670 (>C=O, amide) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.0–2.1 (m, H-3, H-4, H-5), 2.3–3.9 (m, H-2, H-6), 5.52 (dq, $J_{NH}=7$ Hz, $J_{H-6}=4$ Hz, $J_{H-5}=1.5$ Hz, H-7), 7.38 (m, H-3′, H-4′, H-5′), 7.83 (m, H-2′, H-6′) and 8.12 (d, $J=7$ Hz, NH). MS, *m/e*: 244 (M^+), 122, 150, 84.

trans-*7-Benzamido-8-oxo-1-azabicyclo[4.2.0]-*(5b). The *trans*-benzamido-β-lactam (*5b*) was obtained from the corresponding *trans*-amino-β-lactam *4b* in the same manner as above. The yield was 51 %. M.p. 210–211 °C. (Found: C 68.7; H 6.5; N 11.1. Calc. for $C_{14}H_{16}N_2O_2$: C 68.8; H 6.60; N 11.5). IR, ν_{max} (KBr): 3275 (>NH), 1750 (>C=O, β-lactam), 1645 (>C=O, amide) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.9–2.0 (m, H-3, H-4, H-5), 2.3–4.0 (m, H-2, H-6), 4.76 (dd, $J_{NH}=7$ Hz, $J_{H-6}=1.5$ Hz, H-7), 7.40 (m, H-3′, H-4′, H-5′), 7.86 (m, H-2′, H-6′) and 7.92 (d, $J=7$ Hz, NH). MS, *m/e*: 244 (M^+), 122, 105, 84.

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Crystal Structures of Synthetic Analgetics. VI. Normethadone Hydrochloride

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The weak analgetic normethadone crystallizes as a hydrochloride in the triclinic space group $P1$, with unit cell dimensions $a = 9.142(2)$ Å; $b = 9.438(2)$ Å; $c = 13.318(3)$ Å; $\alpha = 69.31(2)°$; $\beta = 87.82(2)°$; $\gamma = 62.27(2)°$ and two independent molecules in the asymmetric unit. The structure was determined by a combination of the heavy atom techniques and direct methods and was refined to $R = 0.039$ for 2424 observed reflections. The mean e.s.d's in bond lengths and angles are 0.006 Å and 0.4°, respectively.

The two molecules A and B are practically identical. The propylamino chain is nearly fully extended, the pertinent dihedral angle C4 – C5 – C6 – N being −165.3 and −167.1° for molecule A and B, respectively. Comparatively strong N – H···Cl hydrogen bonds of 2.93 and 2.97 Å link the organic cations to the chloride ions.

A relation between the interplanar angle and the bond angle at the bridging carbon atom is discussed for bridged diphenyl and triphenyl compounds.

Normethadone (*1a*) is a weak narcotic analgetic [1] closely related to methadone (*1b*)

$$(C_6H_5)_2C(COC_2H_5)CH_2\underset{\displaystyle R}{\underset{|}{C}}HN(CH_3)_2$$

1a, R = H; *1b*, R = CH_3.

The present X-ray analysis was undertaken as a part of a structure research program on analgetics in progress in this laboratory. Conformational investigations on biologically active compounds have to include studies with the molecules in different environments. So far, however, there has been no success in preparing single crystals of the free base of normethadone.

EXPERIMENTAL

Commercial normethadone hydrochloride was recrystallized from a solution of the compound in acetone/diethyl ether. A colourless single crystal with dimensions 0.2 mm × 0.4 mm × 0.4 mm was used for the experiments.

The crystals are triclinic and a uniform intensity distribution combined with acentric $N(Z)$-values as calculated during the structure determination, indicated space group $P1$, rather than $P\bar{1}$.

Unit cell dimensions were determined on a Syntex $P\bar{1}$ diffractometer with graphite crystal monochromated Mo$K\alpha$ radiation ($\lambda = 0.71069$ Å).

Three-dimensional intensity data were collected utilizing the $2\theta - \theta$ autocollection program with variable scan rate (4 – 8° min^{-1}) and a cut off for low intensities. The scan range was from 0.6° below $2\theta(\alpha_1)$ to 0.7° above $2\theta(\alpha_2)$ and the background was counted for 0.35 times the scan time at each end of the scan range. The intensities of three periodically measured reflections did not show any systematic variation. 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 2872 independent reflections with $\sin\theta/\lambda \leq 0.60$ were recorded; 2424 had a net count larger than $3\sigma_I$.

Corrections for Lorentz and polarization effects were carried out by ordinary methods.

All calculations were performed in a CYBER-74 computer using the programs in Ref. 2, except for the phase determination by direct methods.[3] Atomic form factors were those of Hanson *et al.*[4] for Cl, O, N, and C and of Stewart *et al.*[5] for H.

CRYSTAL DATA

Normethadone hydrochloride, $C_{20}H_{25}NO \cdot HCl$, triclinic.

Table 1. Positional and thermal parameters with their e.s.d's for molecule A. The anisotropic temperature factors are given by $\exp[-2\pi^2 (U11h^2a^{*2} + U22k^2b^{*2} + U33l^2c^{*2} + U12hka^*b^* + U13hla^*c^* + U23klb^*c^*)]$.

ATOM	X	Y	Z	U11	U22	U33	U12	U13	U23
CL	0.0000(0)	1.2000(0)	0.0000(0)	.1290(14)	.0653(9)	.0502(8)	-.0564(9)	-.0265(8)	.0128(6)
O	.4505(4)	.2566(4)	.3269(3)	.0594(21)	.0676(23)	.0754(24)	-.0409(24)	.0192(19)	-.0171(18)
N	.1374(5)	.7527(4)	.2240(3)	.0559(24)	.0365(20)	.0468(21)	-.0219(18)	.0021(17)	-.0092(17)
C1	.5727(7)	-.0824(8)	.3443(5)	.0706(40)	.0771(39)	.0862(42)	-.0275(33)	.0140(33)	-.0342(33)
C2	.4339(6)	-.0007(6)	.4013(4)	.0526(31)	.0482(29)	.0757(34)	-.0208(25)	.0129(26)	-.0247(26)
C3	.3700(6)	.1898(6)	.3732(4)	.0507(28)	.0536(28)	.0381(24)	-.0302(25)	-.0012(21)	-.0107(21)
C4	.2030(5)	.2981(5)	.4098(3)	.0509(27)	.0406(25)	.0339(22)	-.0252(22)	.0043(19)	-.0093(19)
C5	.1766(6)	.4826(6)	.3796(3)	.0586(29)	.0410(26)	.0342(23)	-.0265(23)	.0043(21)	-.0106(19)
C6	.1213(6)	.5936(6)	.2574(3)	.0669(31)	.0441(25)	.0412(25)	-.0324(24)	-.0014(22)	-.0058(20)
C7	.3144(7)	.7144(8)	.2275(4)	.0851(40)	.1046(46)	.0619(33)	-.0664(36)	-.0077(28)	.0078(31)
C8	.8522(9)	.8588(7)	.2891(5)	.1554(54)	.0534(34)	.0630(41)	-.0340(35)	.0141(38)	-.0218(31)
C9	.2238(5)	.2078(5)	.5344(4)	.0398(25)	.0369(24)	.0427(25)	-.0188(21)	.0036(19)	-.0055(20)
C10	.2690(5)	.2534(6)	.6057(3)	.0465(27)	.0480(27)	.0401(26)	-.0220(23)	.0026(21)	-.0100(22)
C11	.2983(6)	.1767(6)	.7174(4)	.0541(29)	.0589(30)	.0377(26)	-.0241(25)	.0039(21)	-.0122(23)
C12	.2684(6)	.0319(7)	.7606(4)	.0513(32)	.0716(36)	.0391(27)	-.0269(28)	.0027(22)	-.0012(25)
C13	.2243(6)	-.0241(6)	.6984(4)	.0627(32)	.0515(29)	.0508(30)	-.0311(26)	.0033(24)	-.0041(24)
C14	.2015(6)	.0626(6)	.5783(4)	.0553(29)	.0444(26)	.0463(27)	-.0272(24)	.0013(22)	-.0056(21)
C15	.0525(6)	.3111(5)	.3525(3)	.0488(28)	.0294(23)	.0374(23)	-.0223(21)	.0035(20)	-.0061(18)
C16	.0565(6)	.2853(6)	.2566(4)	.0526(28)	.0477(27)	.0512(28)	-.0259(22)	.0091(22)	-.0246(22)
C17	-.0881(7)	.3121(6)	.2220(4)	.0658(34)	.0479(30)	.0580(31)	-.0311(27)	-.0064(26)	-.0145(24)
C18	-.2350(7)	.3645(7)	.2444(5)	.0537(37)	.0591(33)	.0749(38)	-.0274(28)	-.0157(30)	-.0059(27)
C19	-.2496(6)	.3932(6)	.3395(5)	.0444(33)	.0602(33)	.0658(35)	-.0188(26)	.0021(26)	-.0084(27)
C20	-.0987(6)	.3667(6)	.3918(4)	.0508(32)	.0558(29)	.0431(26)	-.0206(25)	.0062(24)	-.0083(22)

ATOM	X	Y	Z	B	ATOM	X	Y	Z	B
H1C1	.616	-.215	.366	8.0	H2C1	.679	-.067	.360	8.0
H3C1	.535	-.321	.258	8.0	H1C2	.477	-.064	.487	6.5
H2C2	.333	-.018	.385	6.5	H1C5	.290	.474	.402	3.2
H2C5	.085	.545	.420	3.2	H1C6	-.034	.629	.237	4.2
H2C6	.195	.518	.214	4.2	HN	.048	.874	.224	4.0
H1C7	.319	.830	.203	6.0	H2C7	.370	.651	.175	6.0
H3C7	.377	.635	.307	6.0	H1C8	-.073	.889	.282	6.0
H2C8	.056	.978	.250	6.0	H3C8	.110	.797	.365	6.0
HC10	.286	.372	.574	4.0	HC11	.323	.222	.769	4.5
HC12	.282	-.031	.845	5.2	HC13	.207	-.133	.721	4.5
HC14	.167	.017	.526	4.0	HC16	.169	.245	.224	4.0
HC17	-.084	.292	.128	4.5	HC18	-.343	.388	.205	5.2
HC19	-.352	.435	.371	4.5	HC20	-.103	.389	.463	4.0

Table 2. Positional and thermal parameters with their e.s.d's for molecule B. The anisotropic temperature factors are given by $\exp[-2\pi^2(U11h^2a^{*2} + U22k^2b^{*2} + U33l^2c^{*2} + U12hka^*b^* + U13hla^*c^* + U23klb^*c^*)]$.

ATOM	X	Y	Z	U11	U22	U33	U12	U13	U23
CL	.4330(2)	.5827(2)	.5228(1)	.0723(9)	.0727(9)	.0548(8)	-.0397(8)	-.0159(7)	-.0096(7)
O	.6557(5)	.6173(5)	.9368(3)	.0659(25)	.0942(29)	.0783(27)	-.0548(24)	.0270(21)	-.0444(23)
N	.6761(5)	.4870(5)	.7246(3)	.0484(23)	.0492(23)	.0426(21)	-.0266(20)	.0010(17)	-.0152(18)
C1	.6374(12)	.8449(12)	1.0299(8)	.1308(73)	.1265(74)	.1433(79)	-.0387(59)	.0786(66)	-.0973(66)
C2	.7725(8)	.7742(9)	.9692(5)	.1119(51)	.1103(52)	.1075(48)	-.0780(45)	.0541(44)	-.0807(44)
C3	.7608(6)	.6590(6)	.9191(4)	.0516(31)	.0530(31)	.0475(28)	-.0262(27)	.0103(23)	-.0208(24)
C4	.8965(6)	.5878(6)	.8492(4)	.0471(28)	.0472(27)	.0394(25)	-.0262(23)	.0039(21)	-.0164(21)
C5	.8720(6)	.4525(6)	.8212(3)	.0463(27)	.0403(25)	.0419(25)	-.0186(22)	.0010(20)	-.0165(20)
C6	.7221(6)	.5341(6)	.7338(4)	.0570(31)	.0452(27)	.0478(27)	-.0279(24)	-.0061(23)	-.0113(22)
C7	.5954(8)	.3441(7)	.8153(5)	.0774(43)	.1167(58)	.0568(36)	-.0683(35)	.0109(31)	-.0164(39)
C8	.8214(8)	.2571(9)	.7093(5)	.0913(42)	.0620(54)	.0631(36)	-.0359(41)	.0046(38)	-.0349(35)
C9	1.0684(6)	.5001(6)	.9175(4)	.0451(28)	.0539(27)	.0359(26)	-.0237(24)	.0065(23)	-.0173(22)
C10	1.1551(6)	.3225(7)	.9762(4)	.0537(32)	.0639(31)	.0458(33)	-.0260(28)	.0062(25)	-.0213(27)
C11	1.3398(7)	.2483(7)	1.0378(5)	.0606(36)	.0772(31)	.0487(38)	-.0247(28)	-.0002(31)	-.0211(29)
C12	1.3793(9)	.3474(7)	1.0434(4)	.0548(47)	.1183(37)	.0474(32)	-.0351(37)	-.0024(32)	-.0244(27)
C13	1.2956(7)	.5221(8)	.9856(4)	.0653(41)	.1016(38)	.0584(31)	-.0526(35)	.0138(28)	-.0395(29)
C14	1.1408(6)	.5998(6)	.9229(4)	.0501(33)	.0666(31)	.0463(27)	-.0389(27)	.0048(24)	-.0201(24)
C15	.8786(6)	.7306(6)	.7419(4)	.0440(28)	.0423(31)	.0453(25)	-.0236(25)	.0048(21)	-.0193(23)
C16	.7359(6)	.6868(7)	.6969(4)	.0524(32)	.0485(36)	.0627(28)	-.0219(28)	.0026(25)	-.0217(26)
C17	.7180(7)	1.0967(8)	.5938(4)	.0643(37)	.0467(40)	.0689(32)	-.0264(32)	-.0104(27)	-.0112(29)
C18	.8473(7)	.9684(10)	.5355(5)	.0896(37)	.0630(57)	.0476(33)	-.0472(44)	-.0133(28)	-.0015(36)
C19	.9961(7)	.8105(9)	.5781(5)	.0793(42)	.0725(48)	.0491(35)	-.0457(37)	.0120(34)	-.0223(36)
C20	1.0092(7)	.6956(7)	.6813(4)	.0579(34)	.0542(33)	.0386(28)	-.0305(29)	.0011(25)	-.0138(24)

ATOM	X	Y	Z	B	ATOM	X	Y	Z	B
H1C1	.616	-.215	.366	8.0	H2C1	.679	-.067	.360	8.0
H3C1	.535	-.321	.258	8.0	H1C2	.477	-.064	.487	6.5
H2C2	.333	-.018	.385	6.5	H1C5	.290	.474	.402	3.2
H2C5	.085	.545	.420	3.2	H1C6	-.034	.629	.237	4.2
H2C6	.195	.518	.214	4.2	HN	.048	.874	.224	4.0
H1C7	.319	.830	.203	6.0	H2C7	.370	.651	.175	6.0
H3C7	.377	.635	.307	6.0	H1C8	-.073	.889	.282	6.0
H2C8	.056	.978	.250	6.0	H3C8	.110	.797	.365	6.0
HC10	.286	.372	.574	4.0	HC11	.323	.222	.769	4.5
HC12	.282	-.031	.845	5.2	HC13	.207	-.133	.721	4.5
HC14	.167	.017	.526	4.0	HC16	.169	.245	.224	4.0
HC17	-.084	.292	.128	4.5	HC18	-.343	.388	.205	5.2
HC19	-.352	.435	.371	4.5	HC20	-.103	.389	.463	4.0

$a = 9.142(2)$ Å, $b = 9.438(2)$ Å, $c = 13.318(3)$ Å, $\alpha = 69.31(2)°$, $\beta = 87.82(2)°$, $\gamma = 62.27(2)°$. $V = 940.4$ Å^3, $M = 331.9$, $Z = 2$.
$D_{obs} = 1.16$ g cm^{-3} (flotation), $D_{calc} = 1.17$ g cm^{-3}.
Space group $P1$.

STRUCTURE DETERMINATION AND REFINEMENT

The structure was determined by a combination of the heavy atom techniques and direct methods. The coordinates of the two chloride ions were established from a sharpened Patterson map. A false symmetry of the Fourier synthesis and as much as 46 non-hydrogen atoms in the asymmetric unit made the chloride ions unsuitable as a phasing model in Fourier synthesis. Application of MULTAN [3] in the ordinary way did not lead to a structure solution. However, success was achieved with the chloride ions as the fragment in a tangent-formulae refinement utilizing the 490 highest E-values (≥ 1.30). Only 17 peaks (inclusive the chloride positions) could be associated with reasonable atomic sites, but three successive fragment refinement cycles including an increasing number of atoms eventually gave an E-map which revealed all the non-hydrogen atoms. Anisotropic full-matrix least-squares refinements gave an R-factor of 0.09. Approximate positional parameters of all the 52 hydrogen atoms were calculated from stereochemical considerations. They were ascribed the isotropic temperature factors of the atoms to which they are bonded and included in the structure factor calculations. Convergence was attained at $R = 0.039$ ($R_w = 0.043$).

The final parameters are listed in Tables 1 and 2. In these tables and the subsequent discussion the two non-equivalent molecules of the asymmetric unit are labelled A and B. A complete list of the observed and calculated structure factors may be obtained from the author upon request.

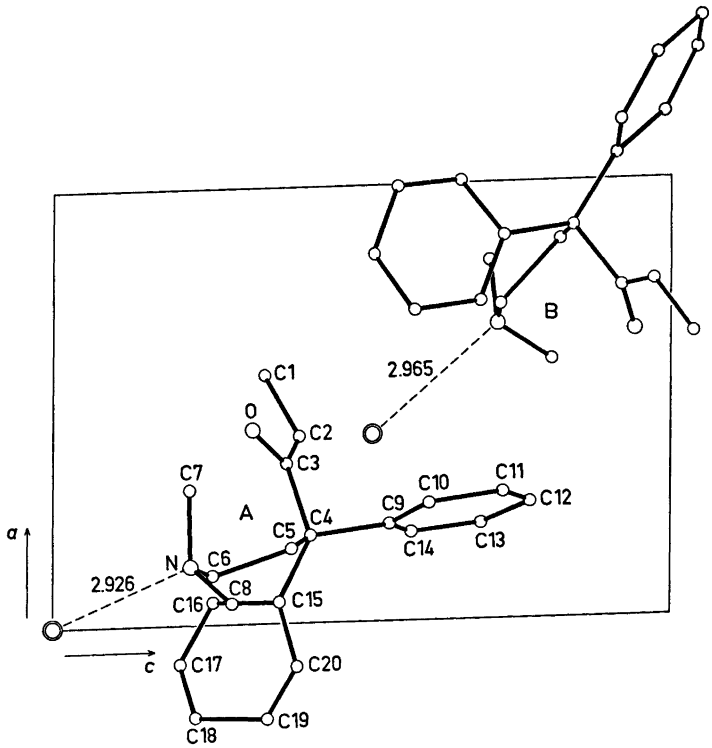

Fig. 1. The crystal structure of normethadone hydrochloride as seen when projected along the b-axis, on the ac-plane.

Table 3. Bond lengths (Å) with e.s.d's in parentheses for molecule A and B.

Molecule A		Molecule B	
DISTANCE	(A)	DISTANCE	(A)
C1 - C2	1.487(7)	C1 - C2	1.468(9)
C2 - C3	1.509(6)	C2 - C3	1.504(7)
C3 - O	1.200(5)	C3 - O	1.185(5)
C3 - C4	1.568(6)	C3 - C4	1.561(7)
C4 - C5	1.541(6)	C4 - C5	1.559(6)
C4 - C9	1.545(6)	C4 - C9	1.532(6)
C4 - C15	1.532(6)	C4 - C15	1.529(6)
C5 - C6	1.538(6)	C5 - C6	1.527(6)
N - C6	1.484(5)	N - C6	1.485(5)
N - C7	1.483(6)	N - C7	1.479(7)
N - C8	1.483(7)	N - C8	1.497(8)
C9 - C10	1.398(6)	C9 - C10	1.391(6)
C10 - C11	1.391(6)	C10 - C11	1.387(7)
C11 - C12	1.389(7)	C11 - C12	1.376(7)
C12 - C13	1.375(7)	C12 - C13	1.368(7)
C13 - C14	1.394(6)	C13 - C14	1.389(7)
C14 - C9	1.395(6)	C14 - C9	1.400(6)
C15 - C16	1.378(6)	C15 - C16	1.375(6)
C16 - C17	1.404(6)	C16 - C17	1.394(7)
C17 - C18	1.374(7)	C17 - C18	1.365(8)
C18 - C19	1.388(7)	C18 - C19	1.403(8)
C19 - C20	1.369(7)	C19 - C20	1.385(8)
C20 - C15	1.388(6)	C20 - C15	1.391(7)

Table 4. Bond angles (°) with e.s.d's in parentheses for molecule A and B.

Molecule A		Molecule B	
ANGLE	( )	ANGLE	( )
C1 - C2 - C3	115.1(4)	C1 - C2 - C3	116.0(6)
C2 - C3 - C4	119.8(4)	C2 - C3 - C4	118.3(4)
C2 - C3 - O	120.1(4)	C2 - C3 - O	120.5(5)
O - C3 - C4	120.1(4)	O - C3 - C4	121.1(4)
C3 - C4 - C5	108.6(3)	C3 - C4 - C5	108.2(4)
C3 - C4 - C9	106.9(3)	C3 - C4 - C9	107.9(4)
C3 - C4 - C15	111.4(3)	C3 - C4 - C15	111.8(4)
C5 - C4 - C9	110.3(3)	C5 - C4 - C9	109.9(4)
C5 - C4 - C15	108.1(3)	C5 - C4 - C15	107.4(3)
C9 - C4 - C15	111.5(3)	C9 - C4 - C15	111.6(3)
C4 - C5 - C6	113.5(3)	C4 - C5 - C6	113.4(4)
C5 - C6 - N	113.6(3)	C5 - C6 - N	113.2(4)
C6 - N - C7	112.3(4)	C6 - N - C7	114.2(3)
C6 - N - C8	112.6(4)	C6 - N - C8	112.3(4)
C7 - N - C8	109.6(4)	C7 - N - C8	110.1(4)
C4 - C9 - C10	122.2(4)	C4 - C9 - C10	121.7(4)
C4 - C9 - C14	119.8(4)	C4 - C9 - C14	119.9(4)
C9 - C10 - C11	120.7(4)	C9 - C10 - C11	120.8(4)
C10 - C11 - C12	121.0(4)	C10 - C11 - C12	121.2(5)
C11 - C12 - C13	118.6(4)	C11 - C12 - C13	119.3(5)
C12 - C13 - C14	120.9(4)	C12 - C13 - C14	120.6(5)
C13 - C14 - C9	120.9(4)	C13 - C14 - C9	120.4(4)
C14 - C9 - C10	118.0(4)	C14 - C9 - C10	118.4(4)
C4 - C15 - C16	122.4(4)	C4 - C15 - C16	123.8(4)
C4 - C15 - C20	119.5(4)	C4 - C15 - C20	118.3(4)
C15 - C16 - C17	120.6(4)	C15 - C16 - C17	122.0(5)
C16 - C17 - C18	119.8(5)	C16 - C17 - C18	119.2(5)
C17 - C18 - C19	119.9(5)	C17 - C18 - C19	120.8(6)
C18 - C19 - C20	119.6(5)	C18 - C19 - C20	118.3(5)
C20 - C15 - C16	117.9(4)	C20 - C15 - C16	117.7(4)

DISCUSSION

Tables 3 – 5 give the bond lengths, bond angles and torsional angles with e.s.d's as calculated from the correlation matrix. The normethadone molecule with the numbering system of the atoms is shown in Fig. 1.

Inspection of Tables 3 – 5 reveals that the molecular structure of A and B are almost completely identical. Apart from the molecular distortions at the quaternary carbon atom C4, bond distances and interbond angles do not deviate significantly from their expected values and corresponding bond lengths and angles in the two molecules do not differ by more than 0.02 Å and 2°, respectively.

Four large groups are connected by C4; C – C single bonds involving this atom are increased by 0.03 Å as compared to their normal values.[6] The corresponding C – C4 – C bond angles are in the range 107 – 112°. This lengthening of interatomic distances and large variation of "tetrahedral" bond angles are in agreement with recent structure reports on diphenylpropylamine analgetics.[7] As a result of the crowded situation around C4 this atom is displaced by 0.05 and 0.04 Å from one of the phenyl ring planes in molecule A and B, respectively.

An interesting feature of the acyclic diphenylanalgetics investigated so far is the correlation between the interplanar angle of the two phenyl rings, τ, and the bond angle θ or C9 – C4 – C15 using the present numbering. Corresponding dihedral and bond angle values of these compounds together with the values of a triphenyl derivative (DTM)[8] are given in Table 6. From this table it is obvious that the bond angle θ *increases* as the interplanar angle *decreases*.

The opening of θ is effecting a separation of the two rings to reduce non-bonded interactions between the *ortho* hydrogen atoms. Fig. 2 shows a plot of ln τ against θ and the experimental points lie fairly well on a straight line. The best line as determined by the method

Table 5. Torsional angles (°) with e.s.d's in parentheses for molecule A and B.

Molecule A

DIHEDRAL ANGLE					( )
C1	C2	C3	O	-15.8(	6)
O	C3	C4	C5	-1.9(	5)
O	C3	C4	C9	-120.9(	4)
O	C3	C4	C15	117.1(	4)
C5	C4	C9	C10	-20.8(	6)
C5	C4	C9	C14	161.7(	4)
C3	C4	C9	C13	97.9(	5)
C3	C4	C9	C14	-80.3(	5)
C3	C4	C5	C6	74.3(	4)
C9	C4	C5	C6	-168.9(	4)
C15	C4	C5	C6	-46.7(	5)
C3	C4	C15	C16	-20.9(	5)
C3	C4	C15	C20	165.3(	4)
C9	C4	C15	C16	-140.2(	4)
C5	C4	C15	C16	98.4(	4)
C4	C5	C6	N	-165.3(	4)
C5	C6	N	C7	71.2(	5)
C5	C6	N	C8	-53.1(	5)

Molecule B

DIHEDRAL ANGLE					( )
C1	C2	C3	O	-6.3(	9)
O	C3	C4	C5	-4.6(	6)
O	C3	C4	C9	-123.4(	5)
O	C3	C4	C15	113.5(	5)
C5	C4	C9	C10	-21.6(	6)
C5	C4	C9	C14	159.3(	4)
C3	C4	C9	C13	96.2(	5)
C3	C4	C9	C14	-82.9(	5)
C3	C4	C5	C6	74.9(	5)
C9	C4	C5	C6	-167.5(	4)
C15	C4	C5	C6	-45.9(	5)
C3	C4	C15	C16	-19.1(	6)
C3	C4	C15	C20	167.0(	4)
C9	C4	C15	C16	-140.0(	4)
C5	C4	C15	C16	99.5(	5)
C4	C5	C6	N	-167.1(	4)
C5	C6	N	C7	72.8(	5)
C5	C6	N	C8	-53.5(	5)

of least-squares is given by

$$\ln \tau = -0.078\theta + 12.9$$

(the r.m.s. error of the slope is 0.005). The small scattering of the experimental points in the figure may partly be due to molecular distortions as caused by crystal packing. Additionally, the non-planarity of the central carbon atom (C4) with respect to the phenyl ring planes may also affect the values.

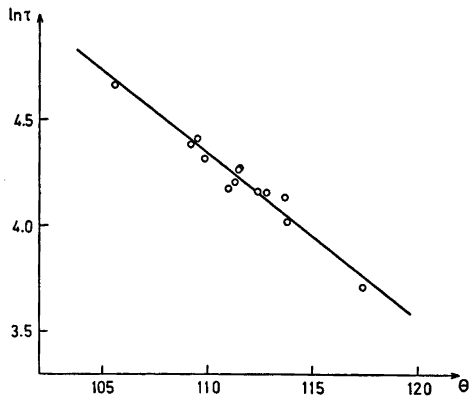

Fig. 2. A plot ln τ against θ for some diphenyl and triphenyl derivatives.

The propylamine chain has the *extended* form with the torsional angle C4–C5–C6–N of −165.3 and −167.1°, respectively. This conformation is similar to that reported for normethadone in solution [14] and corresponds closely to that usually found for the salts of analgetics.[7,11,13].

The crystal structure is shown in Fig. 2. Each normethadone molecule is connected to a chloride ion by a comparatively strong

Table 6. Interplanar (τ) and bond angles (θ) of some compounds having two or three phenyl groups at a tetrahedral carbon atom.

No.	Compound	τ(°)	θ(°)	Ref.
1	DTM [a]	106.2	105.6	8
2	Methadone	80.7	109.5	9
3	Methadone	80.6	109.2	10
4	DTM [a]	75.3	109.9	8
5	Normethadone [b]	71.1	111.6	This work
6	Normethadone [b]	70.9	111.5	This work
7	Acetylmethadol [b]	67.2	111.3	11
8	Methadol [b]	65.0	111.0	11
9	DTM [a]	64.3	112.4	8
10	Methadone [c]	63.7	112.8	12
11	Isomethadone [b]	62.5	113.7	11
12	Dextromoramide [d]	55.2	113.8	13
13	Dextromoramide	40.6	117.4	7

[a] Diphenylaminotriphenylmethane. [b] Crystal structure of the hydrochloride. [c] Crystal structure of the hydrobromide. [d] Crystal structure of the bitartrate.

N–H···Cl hydrogen bond. The present values 2.926 and 2.965 Å are far below the average value, 3.21 Å, quoted by Pimentel *et al.*[15] In spite of the two normethadone molecules A and B being nearly identical, the chloride ions have different environments. There are no other particularly short intermolecular contacts.

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Intermolecular Hydride Transfer Reactions. VI. Disproportionation Reactions of Flav-2-enes Induced by Acids

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The disproportionation reactions of flav-2-ene and closely related substances in different acids were studied. Through deuterium labelling experiments, the parent flav-2-ene was found to disproportionate faster than its 4-phenyl derivative. Fragmentation of 3-(flav-2-ene-4-yl)-2-methoxyflavane into flavylium cation and flav-2-ene was observed in perchloric and trifluoroacetic acid solutions. 4-Phenylflav-2-ene yielded the corresponding flavane on treatment with formic acid through a disproportionation sequence. Flavylium cations could be quantitatively reduced by means of formate anion. The possible mechanisms involved in these reactions are discussed.

Recently we reported that the reaction of 4-phenylflav-2-ene (*1*) with trifluoroacetic (TFA) or perchloric acids afforded *cis*-4-phenylflavane (*2*) and the 4-phenylflavylium salt (3) in almost quantitative yields.[1] The mechanism of this acid-induced disproportionation reaction involves two steps (Scheme 1). The first is protonation of the 2-flavene (*1*) to yield the carbonium ion (*4*) which acts as a hydride acceptor in the second step.

In continuation of our studies of intermolecular hydride transfer reactions, the present work is devoted to investigation of the behaviour of some flavenes and closely related substances in different acidic media.

Boiling acetic acid was found to be incapable of effecting disproportionation of the 2-flavenes *1* and *5*. The failure to observe the formation of the corresponding flavanes and flavylium acetates can be explained by supposing that the acid-induced reaction (Scheme 1) is thermodynamically unfavourable in this case due to the relatively weak acidity and strong nucleophilicity of acetic acid and its conjugate base, respectively. In an attempt to examine the reversibility of this reaction a mixture of

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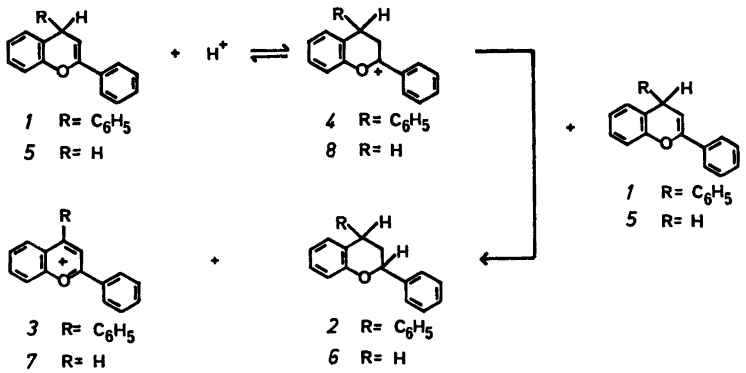


Scheme 1.

equimolar amounts of 4-phenylflavane (*2*), 4-phenylflavylium perchlorate (*3*) and potassium acetate in acetic acid solution was boiled for several hours. However, any formation of 4-phenylflavene (*1*), indicative of hydride transfer from the 4-phenylflavane (*2*) to the 4-phenylflavylium cation (*3*) followed by deprotonation of the resulting cation (*4*) by means of acetate anion, was not achieved. The disproportionation of flavenes requires protonation of the double bond with formation of a carbonium ion which can act as a hydride acceptor. Evidently, protonation of *1* takes place in boiling acetic acid since treatment of this compound with deuterioacetic acid afforded the 3-deuterio-4-phenylflav-2-ene (*1*, β-D). However, it seems likely that the exchange of the β-hydrogen of *1* can take place even though the equilibrium constant for protonation may be very small, too small to allow hydride acceptable to be important. On the other hand, this protonation-deprotonation sequence may not necessarily involve a carbonium ion sufficiently reactive to take part in a hydride transfer reaction. Thus, the failure of *1* to undergo disproportionation in acetic acid may be due to the lack of formation of a carbonium ion similar to the hydride acceptor *4* proposed to be formed in the stronger acid TFA.

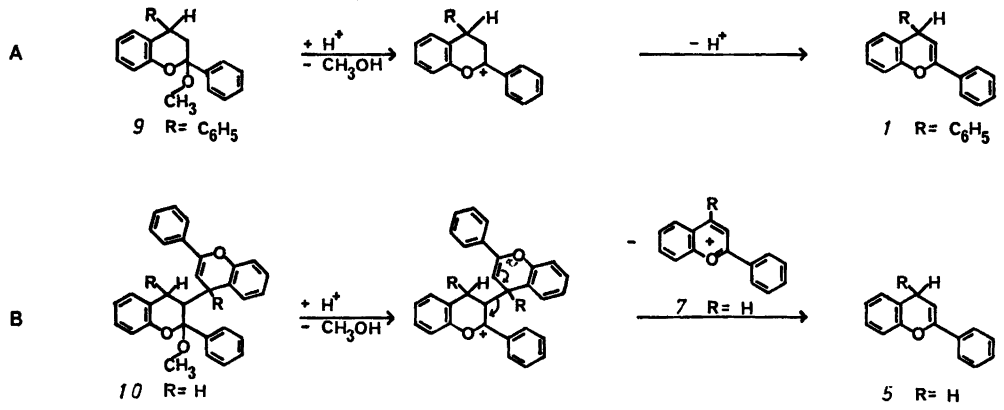
The disproportionation reaction of 3-deuterio-4-phenylflav-2-ene (*1*, β-D) in deuteriotrifluoroacetic acid afforded *cis*-3,3-dideuterio-4-phenylflavane (*2*, β,β′-D) and 3-deuterio-4-phenylflavylium salt (*3*, β-D), the latter was isolated as the perchlorate. The deuterated products *2* (β,β′-D) and *3* (β-D) were also obtained from the disproportionation of the undeuterated 4-phenylflav-2-ene (*1*) in deuteriotrifluoroacetic acid. The ^{1}H NMR spectra of the products from the latter reaction revealed no signals due to the undeuterated analogues *2* and *3*. Furthermore, only undeuterated products were obtained from the reaction of *1* (β-D) with TFA. These results indicate that in TFA, the exchange of the β-hydrogen of the 4-phenylflav-2-ene (*1*) occurs much faster than the disproportionation reaction.

The disproportionation of the parent flav-2-ene (*5*) into the flavane (*6*) and the flavylium cation (*7*) in 1:1 ratio was achieved with TFA as well as perchloric acid (Scheme 1). From the reaction carried out in deuteriotrifluoroacetic acid, a mixture of the flavylium salt (*7*) and its β-deuterio analogue (*7*, β-D) was isolated as the perchlorates. Based on the ^{1}H NMR spectrum of the salt mixture, the ratio between *7* and *7* (β-D) was estimated to be 1:1.

Assuming the hydrogen-deuterium exchange at the β-position of *1* and *5* to take place with comparable rates, the degree of deuterium incorporation in the flavylium salts offers a qualitative measure of the relative rates of the disproportionation reactions of the above flavenes in deuteriotrifluoroacetic acid solution. The formation of a mixture of the flavylium salts *7* and *7* (β-D) in a 1:1 ratio, and only the β-deuterated flavylium salt *3* (β-D) as mentioned above suggests that the γ-unsubstituted salt is formed much faster than the corresponding γ-phenyl substituted salt. It seems likely that the hydride acceptors *4* and *8* have comparable stabilities, since the positive charge at the α-position is stabilised by the same groups. With respect to the hydride donor ability, the creation of a positive charge at the γ-positions of *1* and *5* by removal of a hydride ion should be expected to occur easier with the former flavene (tertiary carbon) than with the latter (secondary carbon). From these considerations, the 4-phenylflav-2-ene (*1*) should be expected to undergo disproportionation faster than the parent flav-2-ene (*5*). However, since the opposite result was found through the deuterium labelling experiments, the rate controlling factors in these hydride transfer reactions seem to be related to steric effects exerted by the γ-substituent of *1* and *4*, as well as the presence of two available hydrogens at the γ-position of the parent flav-2-ene (*5*).

Treatment of 2-methoxy-4-phenylflavane (*9*) with perchloric acid or TFA resulted in the formation of the flavylium cation (*3*) and *cis*-4-phenylflavane (*2*). This reaction presumably occurs through the intermediacy of 4-phenylflav-2-ene (*1*) formed by acid catalysed elimination of methanol from the ketal *9* (Scheme 2A), which subsequently disproportionates according to Scheme 1. The relative yields of the products support this conclusion.

Previously, it has been reported that hydride transfer to flavylium perchlorate (*7*) in methanol affords 3-(flav-2-ene-4-yl)2-methoxyflavane (*10*).[2,3] In the present work, the reaction



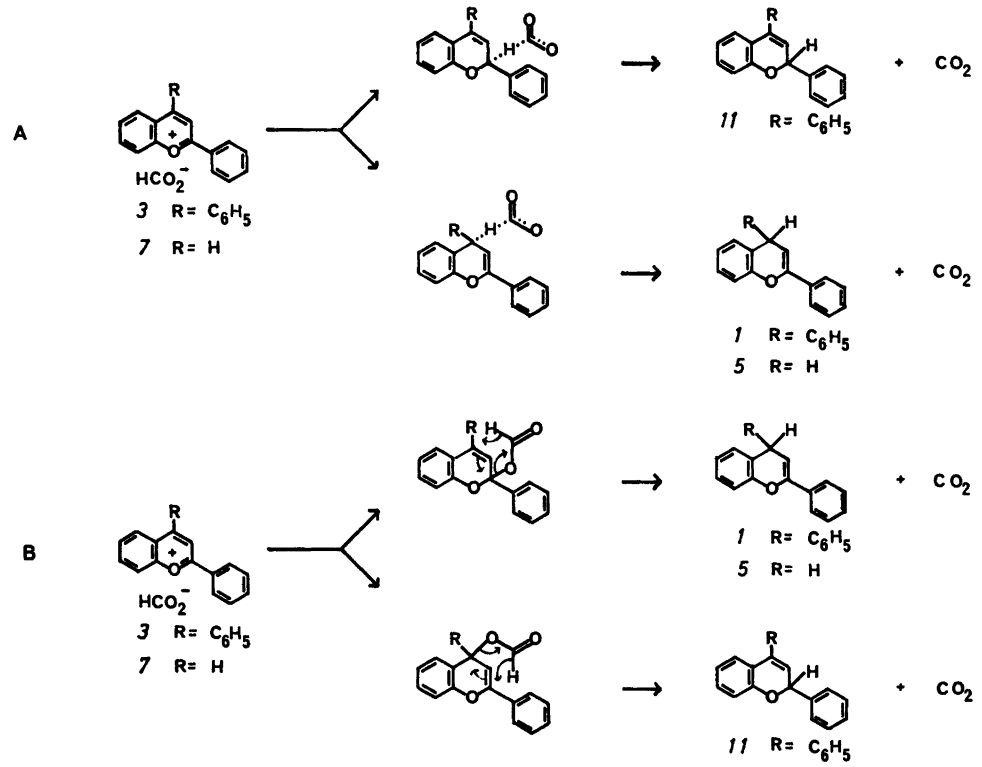
Scheme 2.

of the ketal *10* with TFA as well as perchloric acid was studied (Scheme 2B). The ^{1}H NMR spectrum of *10* in TFA showed signals due to the flavylium cation *7* and the flavane *6* in approximately 3:1 ratio. The flavylium salt was isolated as the perchlorate, while the formation of the flavane *6* was further verified by GLC. A similar result was observed with perchloric acid. The product distribution can be attributed to the fragmentation of the ketal *10* into the flavylium cation *7* and the flav-2-ene (*5*), which subsequently disproportionates according to Scheme 1. The fission of the carbon-carbon bond is the reverse of the sequence originally proposed by Reynolds and Van Allan [2] to account for the formation of *10* from flavylium perchlorate (*7*) and flav-2-ene (*5*) in methanol. It should be pointed out that similar fragmentation reactions have been reported to take place in the xanthene,[4] flavene,[5] and pyran [6] series.

Lempert-Sréter [7] has reported the formation of isobenzopyrylium formates from 2-(1-acetylpropyl)benzophenones on treatment with formic acid and the subsequent transformation of these formate salts into the corresponding isobenzochromenes. In the present work similar reduction of the flavylium cations *3* and *7* was achieved by means of formate anion. Treatment of the 4-phenyl-flavylium perchlorate (*3*) with sodium formate in boiling acetonitrile led to almost quantitative reduction of the former cation. The ^{1}H NMR spectrum of the ether soluble products revealed a 1:1 mixture of the 4-phenylflav-2-ene (*1*) and the corresponding 3-flavene (*11*). The cation of the flavylium perchlorate (*7*) was also quantitatively reduced on similar treatment; however, the reaction resulted in the formation of the flav-2-ene (*5*) only (Scheme 3). The above results made it interesting to investigate the behaviour of the flavenes (*1* and *5*) in formic acid solution.

Considering formic acid to be sufficiently strong to induce disproportionation of the flavenes, the flavanes and flavylium formates should be initially formed in a 1:1 ratio. However, subsequent decomposition of the flavylium formates into flavenes susceptible to further disproportionation-decomposition sequences would remove the oxidation products formed through the disproportionation reaction. Alternatively, formation of only flavanes may be the outcome of a direct reduction of the vinyl ether group by means of formic acid. The latter reduction path seems to be generally accepted to account for the reduction reactions of the enamine group of dihydropyridines and quinolines by means of formic acid.[8–10]

Treatment of 4-phenylflav-2-ene (*1*) with boiling formic acid led to the separation of *cis*-4-phenylflavane (*2*) in 45 % yield on cooling. The ^{1}H NMR spectrum of the residual oil, obtained after evaporation of excess formic acid, exhibited signals due to the 4-phenylflavylium cation *3*. Moreover, subsequent addition of a perchloric-acetic acid mixture led to the isolation of the 4-phenylflavylium perchlorate (*3*) in 35 % yield. These yields of *2* and *3* indicate that the formation of the flavane (*2*) takes place mainly through a disproportio-



Scheme 3.

nation sequence rather than by a direct reduction of the vinyl ether group of the flavene by means of formic acid. Furthermore, the high yield of the flavylium salt was unexpected since decomposition of flavylium formate readily took place in acetonitrile solution as mentioned above. Hence, the above experiment was repeated and after isolation of the flavane *2* and removal of excess formic acid, the residual oil, mainly consisting of 4-phenylflavylium formate (*3*) was refluxed in acetonitrile. This treatment led to the formation of a mixture of 4-phenylflav-2-ene (*1*) and the corresponding 3-flavene (*11*) in a ratio of approximately 1:1. The total yield of these flavenes is comparable to the yield of *cis*-4-phenylflavane (*2*) isolated from the formic acid solution and hence correlates well with a quantitative decomposition of the 4-phenylflavylium formate (*3*), initially formed through a disproportionation reaction of the starting material in the formic acid solution. Thus, the ease of decomposition of 4-phenylflavylium formate seems to be strongly dependent on the solvent used.

Treatment of the parent flav-2-ene (*5*) with boiling formic acid led to the formation of the flavane (*6*) in 80 % yield. No signals due to the flavylium cation *7* could be detected in the ^{1}H NMR spectrum of the residual oil after concentration of the reaction mixture. Assuming that the flavane (*6*) was formed through disproportionation-decomposition sequences, the high yield suggests that flavylium formate (*7*) undergoes decomposition much easier than the 4-phenyl substituted derivative *3* in formic acid. This may partly be attributed to the relative stabilities of these cations. The quantitative formation of 4-phenylflavylium perchlorate (*3*) and flav-2-ene (*5*) from an equimolar mixture of 4-phenylflav-2-ene (*1*) and flavylium perchlorate (*7*) shows that the former cation is more stable than the latter.

The decomposition of the flavylium formate salts discussed above can be considered to

take place through a neutral transition state by approach of the aldehydic hydrogen of the formate to the α or γ positions of the flavylium cation, followed by hydride transfer and carbon dioxide formation as shown in Scheme 3A. Alternatively, formate esters may be formed which subsequently rearrange to the flavenes with simultaneous evolution of carbon dioxide as represented in Scheme 3B. Similar mechanisms have been discussed by Stewart[11] to account for the formate reduction of the trityl cation. Stewart gave preference to the former route; however, the results from his kinetic and isotopic studies could not exclude any of these alternatives. In the present study, the reduction of the flavylium cations has not been followed kinetically. However, it should be pointed out that the rearrangement of the formate ester formed from the trityl cation requires a four-center transition state to yield triphenylmethane, while the formation of the flavenes may take place through a four-center, as well as a six-center transition state as indicated in Scheme 3B. The facile decomposition of the 4-phenylflavylium formate (*3*) in acetonitrile solution, may be attributed to the general decrease in solvation energy of anions in aprotic solvents.[12,13] A change of solvent from formic acid to acetonitrile should increase the nucleophilicity of the formate anion and hence increase the tendency towards formate ester formation. However, it seems reasonable that the hydride donor capacity (the reduction potential) of the free formate anion would increase through the same effect and consequently favour the decomposition path given in Scheme 3A. At the present stage in our investigation neither the observed solvent effects nor the product distribution seem to be conclusive with respect to the mechanism of the decomposition of the flavylium formates.

EXPERIMENTAL

The ^{1}H NMR spectra were recorded on a Varian A-60-A instrument with TMS as internal standard. A Perkin Elmer F11 instrument equipped with an OV-17 column was used for GLC analysis. The yields mentioned below were calculated according to the stoichiometry of the reactions. The compounds which were synthesized for the use as starting materials or for identification of products are listed below with their most characteristic ^{1}H NMR signals.

Flavylium perchlorate (*7*),[14] m.p. 180 °C. ^{1}H NMR in TFA: δ 8.70 (d, H_β), 9.47 (d, H_γ), $J_{\beta,\gamma} = 9.0$ Hz.

4-Phenylflavylium perchlorate (*3*),[1,15] m.p. 222 °C, ^{1}H NMR in TFA: δ 8.58 (s, H_β).

Flav-2-ene (*5*),[16] m.p. 54–55 °C. ^{1}H NMR in $CDCl_3$: δ 3.60 (d, 2 H_γ), 5.52 (t, H_β), $J_{\beta,\gamma} =$ 4.0 Hz.

4-Phenylflav-2-ene (*1*),[17] m.p. 109 °C, ^{1}H NMR in $CDCl_3$: δ 4.85 (d, H_γ), 5.60 (d, H_β), $J_{\beta,\gamma} =$ 4.0 Hz.

4-Phenylflav-3-ene (*11*),[17] m.p. 107 °C, ^{1}H NMR in $CDCl_3$: δ 5.82 (d, H_α), 5.90 (d, H_β). $J_{\alpha,\beta} = 4.0$ Hz.

Flavane (*6*), m.p. 43 °C, ^{1}H NMR in $CDCl_3$: δ 2.02 (m, H_β,$H_{\beta'}$), 2.87 (m, H_γ, $H_{\gamma'}$), 5.08 (4 lines, H_α), $|J_{\alpha,\beta} + J_{\alpha,\beta'}| = 12.0$ Hz.

cis-4-*Phenylflavane* (*2*)[1], m.p. 144 °C, ^{1}H NMR in $CDCl_3$: δ 2.30 (m, H_β, $H_{\beta'}$), 4.33 (4 lines, H_γ), $|J_{\beta,\gamma} + J_{\beta',\gamma}| = 17.6$ Hz, 5.19 (4 lines, H_α), $|J_{\alpha,\beta} + J_{\alpha,\beta'}| = 12.4$ Hz.

Reaction of the 2-flavenes (*1*) *and* (*5*) *with acetic acid.* 4-Phenylflav-2-ene (*1*) was recovered unchanged after refluxing its solution in glacial acetic acid for 72 h. *cis*-4-Phenylflavane (*2*) could not be detected by GLC analysis of the solution. Similarly no flavane (*6*) was detected in the solution of flav-2-ene (*5*) and glacial acetic acid after refluxing for 5 h.

3-Deuterio-4-phenylflav-2-ene (*1*, β-D). A solution of 4-phenylflav-2-ene (*1*) (1 g) in deuterioacetic acid (8 ml) was refluxed for 5 h. On cooling, *1* (β-D), crystallised out, m.p. 109 °C (ethanol 0.95 g, 95 % yield), ^{1}H NMR in $CDCl_3$: δ 4.85 (s, H_γ) and 6.9–7.8 (m, 14 aromatic H).

Treatment of 4-phenylflavylium perchlorate (*3*) *and potassium acetate with cis-4-phenylflavane* (*2*). A mixture of 4-phenylflavylium perchlorate (*3*) (0.19 g, 0.0005 mol), *cis*-4-phenylflavane (*2*) (0.15 g, 0.0005 mol) and anhydrous potassium acetate (0.05 g, 0.0005 mol) in glacial acetic acid (10 ml) was refluxed for 72 h. 4-Phenylflav-2-ene (*1*) could not be detected by GLC analysis of the solution.

cis-*3,3-Dideuterio-4-phenylflavane* (*2*, β,β'-D). A solution of 3-deuterio-4-phenylflav-2-ene (*1*, β-D) (0.3 g, 0.001 mol) in deuteriotrifluoracetic acid (TFA-d) (4 ml) was stirred at 20 °C overnight. The precipitated *2* (β,β'-D) m.p. 143–144 °C (0.14 g, 93 % yield) was collected by filtration. ^{1}H NMR spectrum in $CDCl_3$: δ 4.33 (s, H_γ), 5.20 (s, H_α) and 6.7–7.5 (m, 14 aromatic H).

3-Deuterio-4-phenylflavylium perchlorate (*3*, β–D). The filtrate from the above experiment was evaporated and the residue was treated with a mixture of 70 % perchloric acid and glacial acetic acid (1:10, 8 ml) followed by ether (100 ml) and kept overnight. 3-Deuterio-4-phenylflavylium perchlorate, (*3*, β-D) m.p. 222 °C (0.17 g, 85 % yield) which separated,

was collected by filtration. ^{1}H NMR spectrum in TFA: δ 7.8 – 8.4 (m, aromatic H).

Reaction of 4-phenylflav-2-ene (1) with TFA-d. The reaction of *1* with TFA-*d* carried out as described above afforded *cis*-3,3-dideuterio-4-phenylflavane (*2*, β,β'-D) in 70 % yield and 3-deuterio-4-phenylflavylium perchlorate (*3*, β-D) in 82 % yield.

Reaction of 3-deuterio-4-phenylflav-2-ene (1, β – D) with TFA. The reaction of *1* (β-D) with TFA carried out as described above afforded *cis*-4-phenylflavane (*2*), m.p. 144 °C (ethanol) in 66 % yield, and 4-phenylflavylium perchlorate (*3*) m.p. 222 °C in 70 % yield.

Disproportionation of: (a) *Flav-2-ene (5) in perchloric acid*: A solution of flav-2-ene (0.5 g, 0.0024 mol) in a mixture of 70 % perchloric and glacial acetic acids (1:10, 10 ml) was stirred at 20 °C overnight. Ether (100 ml) was added and the precipitated flavylium perchlorate (*7*) m.p. 178 – 180 °C, (0.25 g, 75 % yield) was filtered. Flavane (*6*) (0.19 g, 75 % yield) was obtained as an oil from the filtrate after washing with 10 % $NaHCO_3$, drying ($MgSO_4$) and evaporation and was identified by ^{1}H NMR.

(b) *Flav-2-ene (5) in TFA*. A solution of flav-2-ene (*5*) (0.50 g, 0.0024 mol), in TFA (8 ml) was stirred at 20 °C overnight. The ^{1}H NMR spectrum of this solution revealed the presence of the flavylium cation (*7*) and flavane (*6*) in approximately 1:1 ratio. The solution was evaporated and the residue was dissolved in a mixture of 70 % perchloric and acetic acids (1:10, 10 ml) followed by the addition of ether (100 ml). Flavylium perchlorate (*7*) m.p. 180 °C, (0.30 g, 80 % yield) which separated was filtered off. Flavane (*6*) (0.18 g, 72 % yield) was obtained from the filtrate after washing with 10 % $NaHCO_3$, drying ($MgSO_4$) and evaporation.

(c) *Flav-2-ene (5) in TFA*-d. The reaction of flav-2-ene (*5*) (0.30 g, 0.0014 mol) with TFA-*d* (3 ml) followed by treatment with a mixture of 70 % perchloric and acetic acids (1:10, 8 ml) as mentioned above led to the separation of a mixture of flavylium perchlorate (*7*) and 3-deuterioflavylium perchlorate (*7*, β-D) in 1:1 ratio (0.20 g, 90 % yield) m.p. 180 °C. The ^{1}H NMR spectrum of the mixture in TFA exhibited a singlet at δ 9.5 in between the H_γ doublet of *7*. The rest of the spectrum was identical to that of *7* except for a decrease in the intensity of the H_β doublet compared to the aromatic proton signals.

(d) *2-Methoxy-4-phenylflavane (9)*. This compound, m.p. 107 °C (methanol), was prepared from 3-(*o*-hydroxyphenyl)-3-phenylpropiophenone according to Holmberg and Axberg.[18] The reaction of *9* with 70 % perchloric acid in glacial acetic acid solution carried out as mentioned above gave 4-phenylflavylium perchlorate (*3*) (90 % yield) and *cis*-4-phenylflavane (*2*) (90 % yield). With TFA, *cis*-4-phenylflavane (*2*) separated in 90 % yield and subsequent treatment with a mixture of 70 % perchloric and acetic acids (1:10) gave 4-phenylflavylium perchlorate (*3*) in 90 % yield.

(e) *3-(Flav-2-ene-4-yl)-2-methoxyflavane (10)*. This compound, m.p. 204 °C (methanol) was prepared from flavylium perchlorate (*7*) and sodium borohydride in methanol according to VanAllan and Reynolds.[2] The ^{1}H NMR spectrum of a solution of *10* in TFA showed the characteristic signals of the flavylium cation *7*, and flavane (*6*), besides, a singlet due to a methoxy group. The band integration was consistent with a 3:1 ratio between *7* and *6*. GLC analysis confirmed the presence of the flavane (*6*). On addition of a mixture of 70 % perchloric and acetic acids (1:10, 10 ml) followed by ether (100 ml), flavylium perchlorate, m.p. 180 °C, separated out. The reaction of *10* with 70 % perchloric acid in glacial acetic acid solution carried out as mentioned for flav-2-ene afforded flavylium perchlorate (*7*) in approximately 85 % yield.

Reaction of 4-phenylflavylium perchlorate (3) with sodium formate. A solution of 4-phenylflavylium perchlorate (*3*) (0.50 g, 0.0013 mol) in acetonitrile (10 ml) was refluxed with sodium formate (0.40 g, 0.006 mol) for 10 h. Ether (100 ml) was added and the precipitate was filtered off. The crystalline residue (0.36 g, 97 % yield) obtained after evaporation of the filtrate was dissolved in deuteriochloroform. The ^{1}H NMR spectrum of this solution showed the characteristic signals of the H_β H_γ protons of *1* and the H_α, H_β protons of *11*, besides the aromatic proton signals. The integral ratio revealed a 1:1 mixture of the flavenes.

Reaction of flavylium perchlorate (7) with sodium formate. A solution of flavylium perchlorate (*7*) (1 g, 0.0033 mol) in acetonitrile (10 ml) was refluxed with sodium formate (0.70 g, 0.01 mol) for 5 h. GLC analysis of the solution showed the presence of flav-2-ene (*5*). Ether (100 ml) was added and the precipitated salts were filtered off. The filtrate after evaporation gave flav-2-ene (*5*) (0.6 g, 90 % yield) identified by ^{1}H NMR.

Reaction of 4-phenylflav-2-ene (1) with formic acid. A solution of *1* (1 g, 0.0035 mol) in anhydrous formic acid (20 ml) was refluxed for 7 h and then kept at room temperature overnight. *cis*-4-Phenylflavane (*2*), m.p. 144 °C, which separated was filtered (0.45 g, 90 % yield). The filtrate was evaporated and the residue was dissolved in deuteriochloroform. The ^{1}H NMR spectrum of this solution showed the characteristic singlet of the H_β proton of the 4-phenylflavylium cation (*3*), together with its aromatic proton multiplet. Signals due to the flavenes *1* or *11* could not be detected. On addition of a mixture of 70 % perchloric and acetic acids (1:10, 10 ml) followed by ether (100 ml), 4-phenylflavylium perchlorate (*3*), m.p. 222 °C, separated and was filtered off (0.45 g, 70 % yield).

The above experiment was repeated and after isolation of *cis*-4-phenylflavane (*2*) (0.43 g),

the filtrate was evaporated and the residue was refluxed in acetonitrile (15 ml) for 5 h. The crystalline residue (0.5 g) obtained after evaporation of the solvent was 1:1 mixture (^{1}H NMR) of 4-phenylflav-2-ene (*1*) and 4-phenylflav-3-ene (*11*).

Reaction of flav-2-ene (5) with formic acid. A solution of flav-2-ene (*5*) (0.50 g, 0.0024 mol) in anhydrous formic acid (10 ml) was refluxed for 10 h. GLC analysis showed the presence of flavane (*6*) which was obtained in 80 % yield (0.4 g) as an oil after evaporation of the solution.

Reaction of flavylium perchlorate (7) with 4-phenylflav-2-ene (1). A solution of flavylium perchlorate (*7*) (0.22 g, 0.0007 mol) and 4-phenylflav-2-ene (*1*) (0.20 g, 0.0007 mol) in acetonitrile (8 ml) was stirred at 20 °C for 2 h. On addition of ether (100 ml), 4-phenylflavylium perchlorate (*3*) (0.22 g), m.p. 222 °C, separated and was filtered off. Evaporation of the filtrate afforded flav-2-ene (0.15 g).

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Mass Spectra of Oxamides

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Mass spectra of oxamide, *N,N'*-dialkyl- and *N,N'*-diaryloxamides have been studied. Important fragmentation processes are cleavage of the C—C bond between the two carbonyl groups and hydrogen rearrangements reflecting the 1,2-dicarbonyl- and amide functionality, respectively. A specific loss of the *ortho* substituent from the molecular ions of aryl substituted oxamides is observed. Ortho effects resulting from interaction of the -NHCO moiety with a nitro or methoxy substituent are observed in the decomposition of some fragment ions.

In order to investigate the influence of the —NHCOCONH— grouping on the fragmentation pattern of oxamides, the mass spectra of oxamide and a series of *N,N'*-dialkyl- and *N,N'*-diaryloxamides are reported and discussed. Resemblance to the behaviour upon electron impact of both amides [1] and acyclic compounds with a 1,2-dicarbonyl arrangement [2,3] (α-diketones, oxalates *etc.*) is found.

OXAMIDE and *N,N'*-DIALKYLOXAMIDES

RNHCOCONHR

Symbol:	I	II	III	IV	V	VI
R:	H	CH_3	C_2H_5	C_3H_7	C_4H_9	C_5H_{11}

Symbol:	VII	VIII	IX	X
R:	C_6H_{13}	C_7H_{15}	C_8H_{17}	i-C_3H_7

Symbol:	XI	XII	XIII
R:	*sec*-C_4H_9	*tert*-C_4H_9	$C_6H_5CH_2$

Representative mass spectra are shown in Figs. 1 to 3 and the general fragmentation pattern is depicted in Scheme 1.

Both oxamide and *N,N'*-dialkyloxamides exhibit relative abundant molecular ions. In contrast, dialkyl oxalates are reported [3] to be very unstable upon ionization.

Cleavage of the bond between the two carbonyl groups is an important process, which in most cases gives rise to abundant ions, *a* (Fig. 1). Since all the oxamides investigated are symmetrical, *a* corresponds to half the molecular ion. Ion *a* decomposes by extrusion of CO or the elements of HNCO to give *b* and *c*, respectively. With increasing chain length of R, ion *b* maintains its importance, whereas the abundance of *c* (giving rise to the base peak in IV and V) rapidly decreases and is less than 2 % in IX. The reaction path leading to *b* closely corresponds to the predominant processes observed for acyclic α-diketones.[2a]

Whereas no hydrogen rearrangements are reported [2a] for α-diketones, oxamides give rise to three different types: (*i*) cleavage of the C—C bond between the two carbonyl groups with simultaneous migration of one hydrogen atom, (*ii*) McLafferty rearrangements with transfer of one or (*iii*) two hydrogen atom(s). Characteristic ions resulting from analogous McLafferty rearrangements are observed in the decomposition of the appropriate amides.[1,4]

Rearrangement (*i*) takes place preferentially for compounds with short substituent carbon chains giving rise to peaks corresponding to (*a*+H) and elimination of an alkyl isocyanate molecule. (The reversed charge distribution is to some extent also observed in the spectrum of I, $[HNCO]^+$). The predominant decomposition mode of the (*a*+H) ion is loss of CO, yielding the amine ion *d*.

The rearrangements (*ii*) and (*iii*) give rise to ions *e* and *f*, respectively. Type (*iii*) is observed in the fragmentation of IV to IX, XI and XII. It increases in importance with the

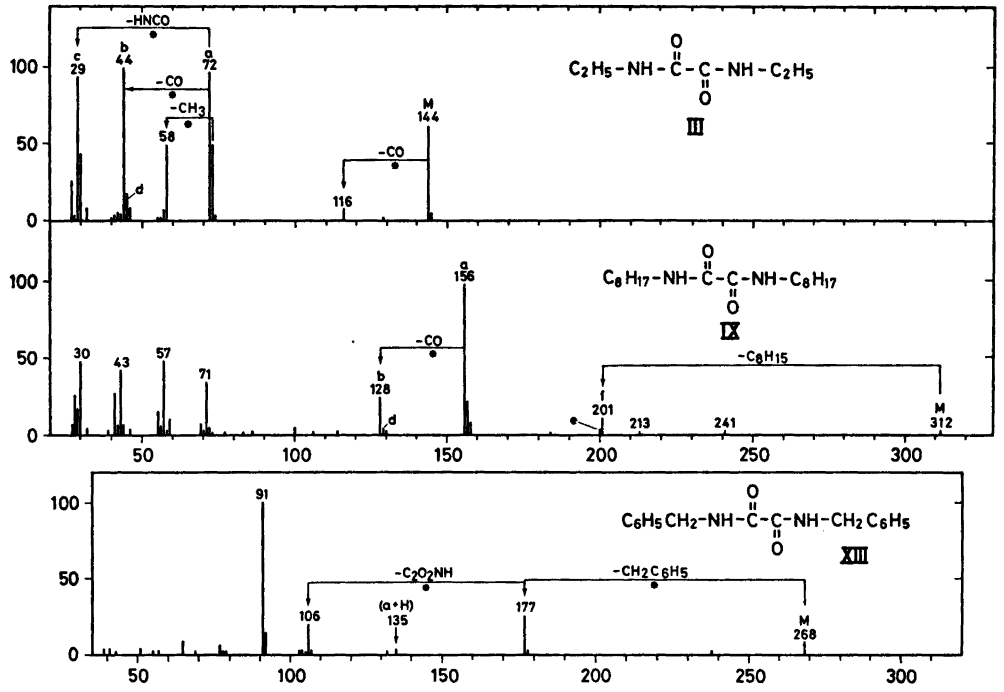


Fig. 1. Mass spectra of *N,N'*-diethyl-, *N,N'*-dioctyl- and *N,N'*-dibenzyloxamide.

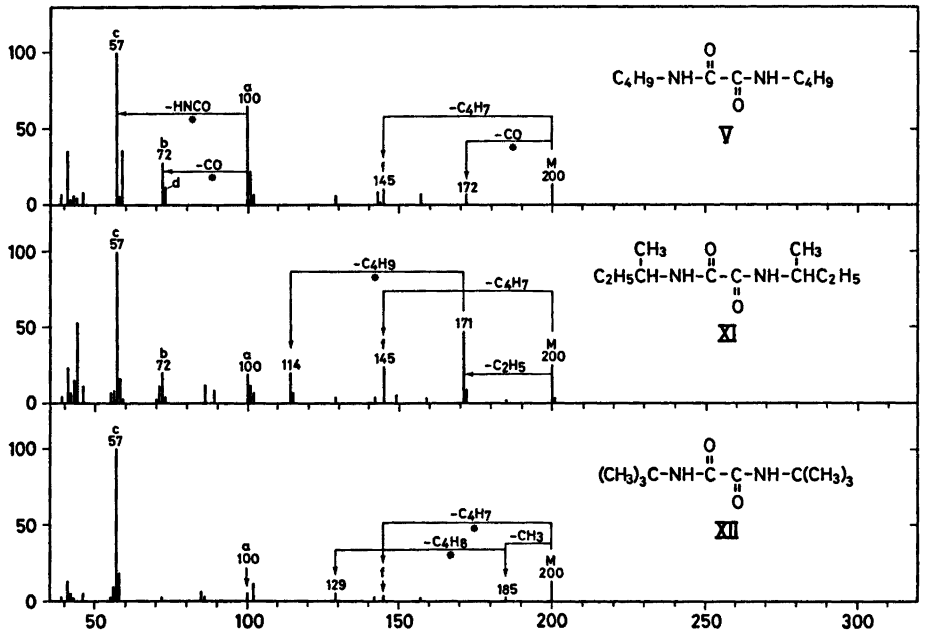
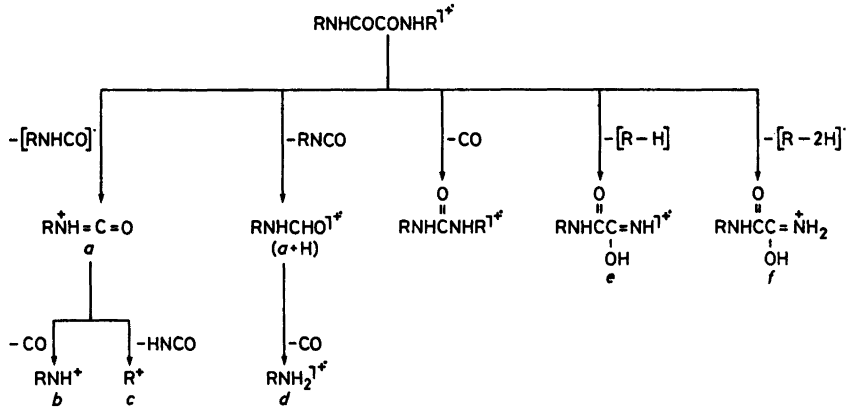


Fig. 2. Mass spectra of *N,N'*-dibutyl-, *N,N'*-di-*sec*-butyl- and *N,N'*-di-*tert*-butyloxamide showing the effect of branching at the α-carbon atom.



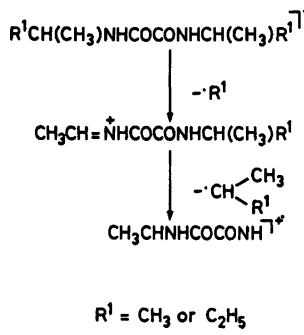
Scheme 1. (The fragmentations shown are supported by metastables).

chain length of the substituent (Figs. 1 and 2). The abundances of the ions resulting from (*ii*) are generally low.

Loss of CO from the molecular ions is a possible process for the compounds I to VII, X and XI. A corresponding extrusion is important for some of the oxalates [3] investigated, but is not observed for α-diketones.[2a]

Besides the fragmentation pattern outlined in Scheme 1, other decomposition modes gain importance, when the carbon chains are branched at the α-carbon atom (X to XII, Fig. 2). These are shown in Scheme 2 for compounds X and XI (R = *sec*-alkyl).

Further branching at the α-carbon atom in XII (R = *tert*-butyl) results in a remarkable decrease in importance of the decomposition paths shown in Scheme 1. In analogy to the fragmentation mode given in Scheme 2, a methyl group is lost from the molecular ion. However, further loss of an alkyl fragment is



$R^1 = CH_3$ or C_2H_5

Scheme 2.

in this case accompanied by a hydrogen rearrangement ($m/e = 129$, Fig. 2).

The well-known stability of the tropylium ion accounts for the unusual behaviour of XIII (R = benzyl) upon electron impact (Fig. 1).

N,N'-DIARYLOXAMIDES

R^2:	H	OCH_3	NO_2	Br
	XIV	XV: *o*-	XVIII: *o*-	XXI: *o*-
		XVI: *m*-	XIX: *m*-	XXII: *m*-
		XVII: *p*-	XX: *p*-	XXIII: *p*-

Representative spectra are shown in Fig. 3.

Loss of CO from the molecular ion of any *N,N'*-diaryloxamide is not detected. This is in contrast to the behaviour of most aliphatic oxamides and to results reported [3] for diaryl oxalates (showing abundant (60 – 100 %) $[M-CO]^+$ ions). However, the initial fragmentations connected with cleavage of the bond between the two carbonyl groups, yielding *a* and (*a*+H), are important in this series too (*cf.* Scheme 1). In the reaction path leading to (*a*+H), the reversed charge distribution is also observed, giving rise to (*a*+H) ions. This may be due to stabilization of the $[RNCO]^+$ ion by the aromatic substituent.[5] Elimination of CO from both *a* and (*a*+H), yielding *b* and *d*, respectively, normally takes place. The aromatic amine ion, *d*, is much more pronounced than the aliphatic analog, and gives rise to the base peak in the spectra of XIV to XVII,

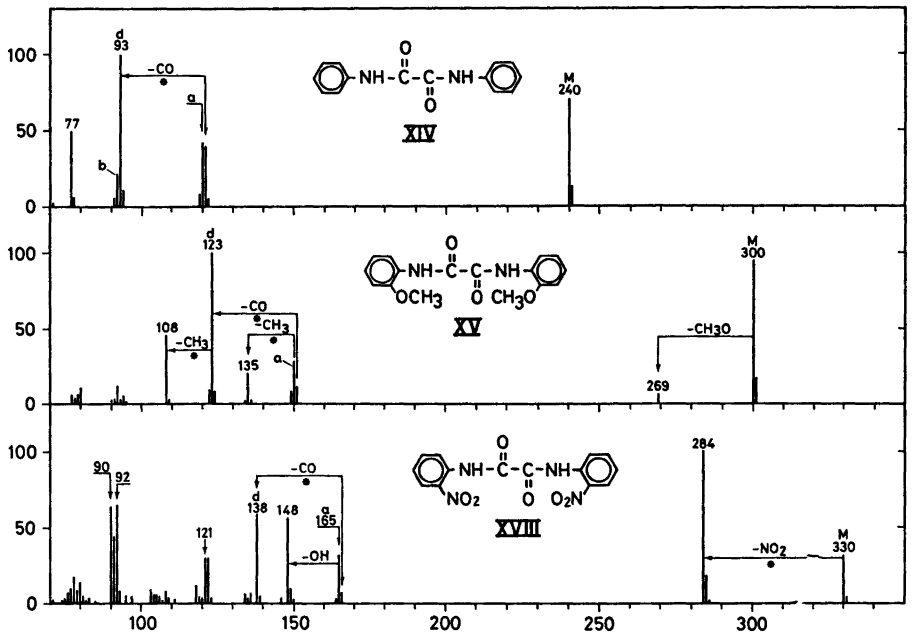
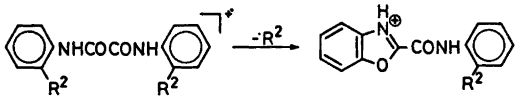


Fig. 3. Mass spectra of *N,N'*-diphenyl-, *N,N'*-di-*o*-methoxyphenyl- and *N,N'*-di-*o*-nitrophenyloxamide.

XXII and XXIII. As indicated by metastable peaks in the spectra of XV to XVII, *d* may also be formed directly from the molecular ion.

In addition decompositions specifically depending on the substituent R^2 take place. Thus, the molecular ions of the *ortho* substituted compounds eliminate $\cdot R^2$ and the following structure is proposed for the resulting ions.



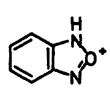
$R^2 = OCH_3$, NO_2 or Br.

The loss of a nitro or bromine radical from the molecular ions of XVIII and XIX, respectively, gives rise to the most abundant ions, whereas loss of the methoxy group is less pronounced (7 % in XV). Corresponding losses are not detected for the *meta*- and *para*-isomers.

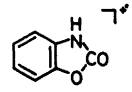
A noteworthy fragmentation process takes place for compounds with $R = NO_2$, in which cases *a* and (*a*+H) eliminate ·OH (followed by NO·) giving rise to peaks at $m/e = 148$ and 149, respectively. In the spectrum of the *ortho* isomer (XVIII, Fig. 3) the peak at $m/e = 148$ is the more abundant one, while the opposite is observed in the spectra of the two other isomers (XIX and XX). In these cases the modes of formation of the (*a*−OH) ions are rather dubious, and it is remarkable that loss of ·OH is not reported for ions corresponding to *a* formed in the fragmentation of α,α,α-trichloroacetanilides.[6] The decomposition modes of *a* derived from similarly substituted compounds in the two series are in all other respects analogous. Thus, the reported *ortho* effect involving expulsion of CO_2 (yielding $m/e = 121$) is also shown by *a* derived from XVIII. A corresponding process takes place from the (a+H) ion (yielding $m/e = 122$) and is supported by the presence of the appropriate metastable peak. However, ions with the same composition are exhibited also in the spectra of the *meta*- and *para*-isomers (XIX: 9 %, XX: 12 %). In these cases they may be formed exclusively in a two step process by successive losses of CO and O *via* the nitroaniline ion, *d* ($m/e = 138$). ($[M-O]^+$ ions are reported in the fragmentation of *m*- and *p*-nitroaniline [1]).

In correspondence with results reported for α,α,α-trichloroacetanilides, an *ortho* effect is also displayed in the decomposition of *a* in compounds with $R=OCH_3$ (XV to XVIII). Thus, the loss of a methyl group is only observed in the fragmentation of XV (Fig. 3). A corresponding process is not found for $(a+H)$.

The structure of the $[a-CO_2]^+$ and $[a-CH_3]^+$ ions resulting from interaction of the $-NHCO$ moiety with an *ortho* nitro- and methoxy substituent, respectively, have been ascribed [6] the structures shown below. Both include a five-membered ring containing at least one atom of the *ortho* substituent. Accordingly, no *ortho* effect of the bromine substituent in the decomposition of *a* is expected or observed. Loss of Br· takes place from *a* (and $(a+H)$) derived from the three isomers.



$a - CO_2^{+\cdot}$



$a - CH_3^{+\cdot}$

EXPERIMENTAL

The mass spectra were recorded on an AEI MS-902 mass spectrometer by Mrs. E. Wolff-Jensen, The H. C. Ørsted Institute, University of Copenhagen. The ion source temperature was typically 100 °C for the aliphatic and 180 °C for the aromatic substances (70 eV, direct sample insertion). The oxamides were prepared by a general method.[7]

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Ring-opening Reactions of Heterocyclic Organometallics. VIII.* On the Synthesis of Macrocyclic Alkylthiovinylacetylenes

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The reaction of 3-bromo-[11] (2,5)-thiophenophane (*7b*) with ethyllithium and ethyl iodide gives the macrocyclic alkylthiovinylacetylene, 1-ethylthio-1-cyclopentadecen-3-yne (*8*) in 45 % yield. The synthesis of *7b* is described.

We have recently shown that 2,5-dialkyl-3-thienyllithium derivatives ring-open to lithium enynethiolates, which react with alkylating agents to give alkylthiovinylacetylenes.[1,2] We were therefore interested to investigate the stability of the 3-thienyllithium derivatives with hydrocarbon chains connecting the 2- and 5-positions. Molecular models indicated that if the alkyl chain consisted of eight CH_2 groups or less, the strain energy of the cyclic enynethiolate would become too high and the thiophenophanelithium derivative should be stable. On the other hand, if the chain was made up of ten atoms or more, the ring-opening would offer the possibility for the synthesis of cyclic alkylthiovinylacetylenes, an unknown class of compounds difficultly available by other synthetic methods.

Starting from commercial cyclododecanone, *1* was prepared according to Helder.[3] The dione *1* has been cyclized by Nozaki *et al.*[4] to give *2a* in 51 % yield by the Paal-Knorr synthesis using phosphorus pentasulfide. Helder used hydrogen sulfide and hydrogen chloride in the cyclization step, and obtained an 80 % yield.[3] In our hands, despite several attempts, only a 20 % yield of *2a* was obtained together with a large amount of a nicely smelling yellow resin. However, no detectable amounts of *2b* were formed when *2a* was subjected to various bromination conditions (Br_2 in acetic acid, Br_2 in pyridine or *N*-bromosuccinimide in acetic acid). From the reaction with bromine in acetic acid a crude product was obtained, the NMR spectrum of which showed an AB quartet in the aromatic part, with a coupling constant of 3.4 Hz. This indicated the presence of an unsymmetrically 2,5-disubstituted thiophene. Helder [3] has reported rearrangements of *2a* in Friedel-Crafts *t*-butylation experiments. It was shown that *2a* gave 5-*t*-butyl-[8] (2,4)-thiophenophane together with other rearranged products upon treatment with *t*-butyl chloride and stannic chloride in carbon disulfide at room temperature. It seems therefore probable that due to the strain in the starting thiophenophane the electrophile attacks at the 2-position, giving the reactive carbonium ion *3*, which apparently can attack the 4-position in electrophilic substitution, or as is probable in our bromination be intercepted by a nucleophile such as acetate or bromide, or undergo other reactions (hydride shifts, elimination etc.).

Starting from commercial cyclodecapentanone (*4*), irradiation in petroleum ether gave crude *5*, which by oxidation with Jones' reagent [5] was converted to *6*. The crude product, which contained 75 % of *6*, was used for the next step. The reaction of *6* with hydrogen sulfide and hydrogen chloride gave only 4 % of analytically pure *7a*, while reaction with phosphorus pentasulfide gave 37 % of almost pure *7a* (20 % analytically pure). The bromination of *7a* with bromine in acetic acid was more successful than

* Part VII, see Ref. 11.

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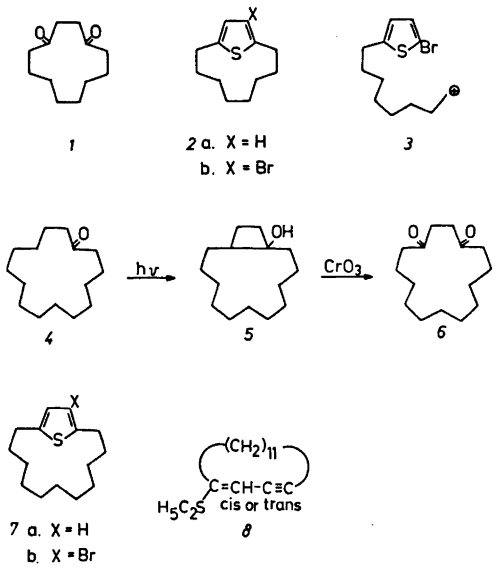
*** Taken in part from the Ph.D. thesis of T. Frejd, Lund 1975.



that of *2a*. GLC analysis of the crude product showed the presence of three components in the proportions 2:2:5 (increasing retention times). The main component was the desired *7b*, which was isolated in 25 % yield by vacuum distillation and recrystallization. No attempts were made to elucidate the structures of the other two components.

Reaction of *7b* with ethyllithium and ethyl iodide at room temperature for 4 h gave a 45 % yield of *8* and a 10 % yield of *7a* (isolated yields). The neutral ether phase from the reaction was shown to contain *7a* and *8* in the ratio of 20:80 (GLC). The structure of *8* followed from elemental analysis and spectral data. Its IR spectrum showed C≡C stretching at 2205 cm^{-1} (weak) and C=C stretching at 1575 cm^{-1}. In its ^1H NMR spectrum the resonance of the vinyl proton appeared at δ 5.48 as a broad unresolved triplet. The resonance of the thiophenic hydrogen of the isomeric 3-ethyl-[11] (2,5)-thiophenophane would occur at lower field. (*Cf.* δ 6.52 (β-H) in *7a*; the ethyl group could cause an upfield shift of 0.2 ppm.[6]) The quartet at δ 2.83 falls in the region characteristic for the $-S-CH_2$ grouping.[2] The resonances of the carbon chain protons and the methyl protons of the *S*-ethyl grouping appeared as two separate bands at δ 2.0 – 2.5 (4 H) and at 1.1 – 1.7 (21 H). A decoupled ^{13}C NMR spectrum of *8* showed the absorptions of the acetylenic carbons at δ 86.8 and 105.4, while those of the ethylenic carbons appeared at δ 114.9 and 155.7 (*cf.* Ref. 7). The low-field resonance of the ethylenic part might be assigned to the carbon attached to the sulfur atom, as in 2-methylthiophene the α-carbon resonance appears at lower field than the β-carbon resonance.[8] The resonance of C-2 in (*E*)-1-cyanopropene appears at δ 105.8 and of C-1 at δ 102.1, which also justifies the suggested assignment. The resonances of the saturated carbon atoms showed up as several lines between δ 22.2 and 43.7. It should perhaps be pointed out that the latter signal appears at an unusually low field to arise from an sp^3-hybridized carbon in an aliphatic chain.[7]

The relatively large amount (20 %) of *7a* formed in the synthesis of *8* might be due to slow ring-opening or a slow alkylation of the enynethiolate or both. The ring-opening might be slow due to some strain in the transition state leading to the lithium enynethiolate. The alkylation could be slow due to steric hindrance, as it appears from models that the sulfur atom is within a cavity of the molecule.

Our results, however, indicate that the ring-opening reaction may afford a synthetic route to macrocyclic thioenynes with more than 14 carbon atoms in the ring. Such compounds could be available starting from thiophene and then putting side-chains of suitable length and functionalities in the 2- and 5-positions. This approach to the syntheses of thiophenophanes has been extensively used by Gol'dfarb and coworkers.[9,10] We are continuing our investigation of the synthetic scope of this route to macrocyclic enynes.

EXPERIMENTAL

1,4-Cyclopentadecadione (6). A solution of 25.0 g (0.111 mol) of cyclopentadecanone (Fluka, m.p. 63 – 64 °C) in 900 ml of hexane was irradiated until the carbonyl absorption in the infrared had disappeared (48 h). This procedure was repeated 10 times, to give 250 g of a crude product which was believed to contain bicyclo[11.2.0]cyclopentadecan-1-ol (*5*). (When the solutions were more concentrated (> 5 %) with respect to cyclopentadecanone, the carbonyl absorption did not disappear even after irradiation for 100 h.) The crude product (250 g) was oxidized with Jones' reagent, which gave 216 g of a colourless oil which showed a

strong carbonyl absorption in the infrared (1720 cm^{-1}), but no hydroxylic band. Combined GLC-MS (column OV 1, 3 %, 150 – 300 °C, 10 °C/min) showed mainly two components in the proportions 1:3, with $m/e = 138$ and 238. The most abundant compound was evidently the title compound (calc. for $C_{15}H_{26}O_2 = 238$). The crude product was used without purification.

*[11] (2,5)-Thiophenophane (*7a*).* (a) A solution of 37.0 g (0.155 mol) of crude *6* in 1 l of ethanol (99.5 %) was cooled to 0 °C and hydrogen sulfide and hydrogen chloride were bubbled through the solution for 8 h (*cf.* Ref. 3). The temperature was kept below + 5 °C, and subsequently most of the solvent was evaporated. The residue was taken up in hexane, washed with water and dried. Evaporation of the solvent gave 30.4 g of a yellow oil which was dissolved in hexane and chromatographed on a silica gel column. Thus a fraction weighing 7.4 g was obtained, which gave 1.5 g (4.1 %) of the title compound in the cold (– 25 °C). Recrystallization from acetonitrile (– 25 °C) afforded pure *7a*, m.p. 51 – 53 °C. NMR (CCl_4): δ 6.52 (s, 2 H, β-H), 2.6 – 2.9 (m, 4 H, $-CH_2-$), 0.6 – 1.8 (18 H). [Found: C 76.17; H 10.25; S 13.56. Calc. for $C_{15}H_{24}S$ (236.42): C 76.21; H 10.23; S 13.56].

(b) A mixture of 150 g (0.629 mol) of *6* and 100 g (0.450 mol) of P_2S_5 was heated at 80 °C for 4 h under nitrogen (*cf.* Ref. 4). The reaction mixture was cooled and extracted with hexane, whereupon the combined organic extracts were washed with 2 N aq. NaOH, and water, and then dried. The solution was filtered through silica gel and the solvent was evaporated to give 55.2 g (37 %) of almost pure title compound. Crystallization from acetonitrile afforded 30.0 g (20 %) of *7a* with the same properties as mentioned above.

*Attempted synthesis of 3-bromo-[8] (2,5)-thiophenophane (*2b*).* Samples of 1.2 g (6.2 mmol) of *2a* [4] were treated as follows:

(a) With bromine (1.0 g, 6.3 mmol) in 25 ml of acetic acid at room temperature for 30 min. After the usual work-up (hexane extraction), 1.4 g of a crude product was obtained. NMR (CCl_4) showed an AB quartet: δ 6.83 (d) and 6.62 (d); ($J = 3.4$ Hz), and several absorptions in the aliphatic region.

(b) With *N*-bromosuccinimide (1.1 g, 6.2 mmol) in 25 ml of acetic acid. A yellow crude product containing several components according to GLC (column OV 1, 3 %, 100 – 300 °C, 15 °C/ min) was obtained.

(c) With bromine (1.0 g, 6.3 mmol) in 10 ml of pyridine. A yellow resin that did not give a reproducible gas chromatogram was obtained.

*3-Bromo-[11] (2,5)-thiophenophane (*7b*).* To a solution of 15.0 g (0.0634 mol) of *7a* in 500 ml of acetic acid, 10.4 g (0.065 mol) of bromine in 50 ml of acetic acid was added dropwise at room temperature. The mixture was stirred for 2 h, neutralized with $NaHCO_3$ and extracted with ether. Drying and evaporation yielded 17.5 g of a crude product containing three components (2:2:5) according to GLC (column OV 1, 3 %, 200 – 300 °C, 10 °C/min). Distillation of 15.0 g of the crude product gave 5.2 g (30 %) of the title compound, b.p. (5×10^{-3} mmHg) 140 – 144 °C, which crystallized. Recrystallization from *N*,*N*-dimethylformamide in the cold (– 25 °C) gave 4.3 g of pure *7b*, m.p. 45 – 46 °C. NMR ($CDCl_3$): δ 6.57 (s, 1 H, 4-H), 2.60 – 2.95 (m, 4 H, $-CH_2-$), 0.60 – 1.85 (m, 18 H). [Found: C 56.6; H 7.39; S 10.3. Calc. for $C_{15}H_{23}BrS$ (315.32): C 57.14; H 7.35; S 10.17].

*1-Ethylthio-1-cyclopentadecen-3-yne (*8*).* Nitrogen gas was supplied for 30 min to the pre-dried, hot (110 °C) apparatus, consisting of a three-necked round-bottomed flask fitted with a condenser (drying tube, $CaCl_2$), stirrer, dropping funnel and a neck for the gas inlet. The dropping funnel was also supplied with nitrogen gas. To 2.00 g (6.34 mmol) of *7b* in 25 ml of anhydrous ether, 8.0 ml (6.4 mmol) of 0.80 M ethereal ethyllithium was added at room temperature. After 10 min, 5.0 g (32 mmol) of ethyl iodide was added and the mixture stirred for 4 h. The reaction mixture was hydrolyzed with water and the aqueous phase was extracted three times with ether. The combined ether phases were washed with water and dried over magnesium sulfate. Evaporation of the ether gave 1.55 g of crude product, which combined GLC-MS analysis (column OV 1, 3 %, 150 – 250 °C, 15 °C/min) showed to consist of *7a* ($m/e = 236$; calc. for $C_{15}H_{24}S = 236$) and of *8* ($m/e = 264$; calc. for $C_{17}H_{28}S = 264$) in the proportions of 1:4. The two components were separated and isolated by preparative TLC (silica gel, 1 mm, hexane), which gave 0.15 g (10 %) of *7a* (R_F 0.48 – 0.55) and 0.75 g (45 %) of the title compound (R_F 0.10 – 0.39). IR: C≡C 2205 cm^{-1} (weak), C=C 1575 cm^{-1}. ^1H NMR (CCl_4): δ 5.48 (bt, 1 H, 2-H), 2.83 (q, 2 H, $S-CH_2-$), 2.0 – 2.5 (4 H, alkyl), 1.1 – 1.7 (21 H). $J_{SCH_2-CH_3} = 7$ Hz. ^{13}C NMR (acetone, decoupled spectrum): δ 155.7 (1-C), 114.9 (2-C), 86.8 and 105.4 (3-C and 4-C), 22.2 – 43.7 (5-C – 15-C), 43.7 ($S-CH_2-$), 22.2 ($-CH_3$). [Found: C 76.4; H 10.5; S 11.9. Calc. for $C_{17}H_{28}S$ (264.48): C 77.20; H 10.67; S 12.12].

GLC analyses were performed with a Perkin-Elmer 900 gas chromatograph. Mass spectra were recorded on an LKB-9000 mass spectrometer with an ionization energy of 70 eV. ^1H NMR spectra were recorded on a Varian A-60 NMR spectrometer and ^{13}C NMR spectra on a Jeol FX-60 NMR spectrometer. IR spectra were recorded on a Perkin-Elmer 257 grating infrared spectrometer.

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Metabolism of 1-^{3}H-Ethanol by Isolated Liver Cells. Time-course of the Transfer of Tritium from *R,S*-1-^{3}H-Ethanol to Lactate and β-Hydroxybutyrate

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Parenchymal cells isolated from the liver of 24 h fasted rats were incubated with 65 mM *R,S-1*-^{3}H-ethanol plus 3 mM pyruvate as substrates in the absence and presence of 1.7 mM 4-methylpyrazole. Metabolite levels and the time-course of the transfer of tritium from ethanol to lactate and β-hydroxybutyrate was measured during the first 15 min of ethanol metabolism.

The time-course of the loss of tritium from 2-^{3}H-L-lactate and 3-^{3}H-β-D-hydroxybutyrate in experiments identical to the above-mentioned was estimated.

A GLC method for the isolation of lactate and β-hydroxybutyrate and the preparation of 2-^{3}H-L-lactate and 3-^{3}H-β-D-hydroxybutyrate is described.

The incorporation rate of tritium from ethanol into lactate and β-hydroxybutyrate decreased with time. Addition of 4-methylpyrazole decreased the incorporation rate roughly proportional to the decrease in ethanol and acetaldehyde metabolism. The observed incorporation rates of tritium to lactate were corrected for the detritiation rates measured in experiments with 2-^{3}H-L-lactate and 3-^{3}H-β-D-hydroxybutyrate as substrates.

The rate of extramitochondrial acetaldehyde oxidation was calculated from the corrected initial rates of incorporation of tritium into lactate to $0-0.4$ μmol min^{-1} (ml of cells)$^{-1}$.

Acetaldehyde is the first product of the oxidation of ethanol by liver tissue. Ethanol may be oxidized in the cytosol by alcohol dehydrogenase,[1]* and when present in high concentrations [2] also in the peroxisomes by catalase [3] or in the endoplasmatic reticulum by MEOS.[4] The intracellular production of acetaldehyde must therefore be localized to these subcellular structures.

The oxidation of acetaldehyde in the liver apparently proceeds quite efficiently as only a minor fraction of the ethanol metabolized is released from the liver as acetaldehyde and as no accumulation of acetaldehyde occurs during ethanol metabolism [5]. It may be inferred from distribution studies and kinetic properties of aldehyde dehydrogenases in liver tissue [6–9] that the intracellular localization of acetaldehyde oxidation might be microsomal, cytoplasmic, or mitochondrial.

The intracellular localization of acetaldehyde oxidation was suggested as mainly mitochondrial by Lindroos *et al.*[10,11]

The evidence for this suggestion was, however, rather indirect and moreover the liver was exposed to unphysiologically high concentration of acetaldehyde. Recently, reports have appeared concerning the intracellular localization of acetaldehyde oxidation during ethanol metabolism at low concentrations (< 10 mM).[12–14] These reports indicate a mainly mitochondrial oxidation of acetaldehyde.

The present work was undertaken in an attempt to estimate the amount of acetaldehyde oxidized in the extramitochondrial cell compartment at physiological concentrations of acetaldehyde, *i.e.* during ethanol metabolism.

R,S-1-^{3}H-Ethanol was used as a substrate for ethanol oxidation by isolated rat liver

* *Enzymes:* Alcohol dehydrogenase (EC 1.1.1.1); aldehyde dehydrogenase (EC 1.2.1.3); catalase (EC 1.11.1.6); glutamate dehydrogenase (EC 1.4.1.3); β-hydroxybutyrate dehydrogenase (EC 1.1.1.30); lactate dehydrogenase (EC 1.1.1.27).

parenchymal cells. The method applied allows measurement of the time-course of transfer of tritium from ethanol *via* NAD^+ to lactate and β-hydroxybutyrate: The *S*-tritium atom of *R,S*-1-^{3}H-ethanol is retained at C-1 in acetaldehyde formed by ethanol oxidation, catalyzed by any of the three enzymes mentioned above, whereas the *R*-tritium atom is transferred to NAD^+ in the reaction catalyzed by alcohol dehydrogenase only. Further oxidation of acetaldehyde to acetate results in the transfer of tritium to the 4-A position of NAD^+. As alcohol dehydrogenase can be inhibited competitively with ethanol by 4-methylpyrazole,[15] it is possible to avoid the formation of 4-^{3}H-NADH in the reaction catalyzed by this enzyme, the only source of 4-^{3}H-NADH being acetaldehyde formed by oxidation of ethanol, catalyzed by enzymes other than alcohol dehydrogenase. The intracellular localization of 4-^{3}H-NADH formation is then identical to the localization of the acetaldehyde oxidation.

Supposing that the extra- and intramitochondrial 4-^{3}H-NADH is equilibrated isotopically with lactate and with β-hydroxybutyrate *via* the lactate dehydrogenase and the β-hydroxybutyrate dehydrogenase reaction, respectively, information about the intracellular localization of acetaldehyde oxidation should arise from a time-course study of the transfer of tritium from ethanol to lactate and β-hydroxybutyrate. However, such time-course studies can be used quantitatively only if all tritium formed by ethanol and acetaldehyde oxidation is transferred to the two substrates mentioned, and as this is not the case, a correction for the 'tritium loss' should be applied. In the present study the time-course for the tritium loss from added 2-^{3}H-L-lactate and 3-^{3}H-β-D-hydroxybutyrate have been used to correct the labelling pattern obtained.

EXPERIMENTAL

Materials.

[^{14}C]Lactate was obtained from NEN Chemicals GmbH (Frankfurt/Main, Germany) and [^{14}C]β-hydroxybutyrate from the Radiochemical Center (Amersham, England). The purity of these compounds was determined by the GLC procedure described in 'Methods'.

R,S-1-^{3}H-Ethanol (specific activity 100 mCi/mmol) was purchased from NEN Chemicals GmbH (Frankfurt/Main, Germany) or from ICN Pharmaceuticals, Inc. (Cleveland, Ohio, U.S.A.). The content of water-exchangeable tritium was determined by extraction with chloroform.[16] The *R,S*-1-^{3}H-ethanol used in the experiments contained less than 5 % of water-exchangeable radioactivity. One sample obtained from NEN Chemicals contained 80 % of water-exchangeable radioactivity.

Enzymes and coenzymes were from Boehringer Mannheim GmbH (Mannheim, Germany) except that collagenase II from Worthington Biochemical Corporation (Freehold, New Jersey, U.S.A.) was used in some experiments. Hexamethyldisilazane and trichloromethylsilane were from Pierce (Rockford, Illinois, U.S.A.) and DEAE-Sephadex A 25 from Pharmacia Fine Chemicals (Uppsala, Sweden). Serum albumin from Armour Chemical Co. (England) was treated with charcoal to remove fatty acids.[17] 4-Methylpyrazole was synthesized and kindly provided by Dr. S. E. Hansen (this laboratory). The purity of this compound was checked by GLC and IR spectrometry. Other chemicals used were of analytical grade.

Methods.

Isolated liver parenchymal cells were prepared from 24 h fasted female Wistar rats of 200 g weight as previously described,[18] except for the omission of hyaluronidase in the perfusion fluid.

Cells were incubated at 37 °C in 15 ml Erlenmeyer flasks with atmospheric air as the gas phase. CO_2 was absorbed in KOH placed in a center well. The incubation buffer was Hanks' buffer [19] containing 25 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM phosphate, 8 mM glucose, 1.2 mM $CaCl_2$ and 1 % serum albumin. The cell concentration was 80 – 120 µl of tightly packed cells (corresponding to 130 – 200 mg of liver tissue [2] in a total volume of about 2.3 ml. The cells were incubated 5 min with 3 mM pyruvate and 1.7 mM 4-methylpyrazole (when added) prior to the ethanol addition. The experiments were terminated at the times indicated by perchloric acid precipitation of a sample (2 ml) of the incubation mixture. The final concentration of perchloric acid was 0.7 M.

Results are expressed per ml of cells. The volume of the cells was measured after centrifugation to constant volume.

Metabolite levels. Lactate,[20] pyruvate,[21] β-hydroxybutyrate,[22] and acetoacetate [23] were measured by the enzymatic procedures indicated. Acetaldehyde was determined with yeast alcohol dehydrogenase.[24] The concentration of these metabolites was determined in separate experiments run parallel to the experiments with tritium-labelled ethanol as a substrate.

Specific activity of lactate and β-hydroxybutyrate. The neutralized supernatant from the

perchloric acid precipitation was lyophilyzed and redissolved in dry pyridine (0.50 ml). Solutions of [^{14}C]lactate and of [^{14}C]β-hydroxybutyrate were added as internal standards and the trimethylsilyl esters of lactate and β-hydroxybutyrate prepared by addition of hexamethyldisilazane (100 μl) and trimethylchlorosilane (50 μl). A 25 μl sample was injected into a gas chromatograph (Hewlett-Packard 7620A) equipped with a beam-splitting device. The conditions for the chromatography were: 1.6 m × 6 mm o.d. glass column packed with 2 % E. 350 diatomite M-AW-DMCS, 90 – 100 mesh (JJ's Chromatography Ltd., Norfold, England), oven temperature 90 °C, injection port temperature 200 °C, detector temperature 260 °C, and nitrogen flow rate 30 ml/min. Retention times for lactate and β-hydroxybutyrate were 200 and 370 s, respectively.

The eluates corresponding to the lactate and the β-hydroxybutyrate peaks were collected over 60 and 90 s, respectively, in either small cold traps or in water. The recovery by the chromatography was about 50 %. Samples were counted in a Packard 2425 scintillation spectrometer with Instagel as a scintillation liquid.

Preparation of specifically labelled substrates. 2-^{3}H-Lactate (specific activity 10 μCi/μmol) was prepared by incubation of *R,S*-1-^{3}H-ethanol, NAD^+, and pyruvate together with alcohol dehydrogenase and lactate dehydrogenase. The incubation mixture was applied to a column of DEAE-Sephadex A 25 equilibrated with 25 mM ammonium acetate. The column was washed with 25 mM ammonium acetate and lactate eluted with 100 mM ammonium acetate. The radiochemical purity of the product was determined to larger than 85 % by the GLC-procedure described above.

3-^{3}H-D-β-Hydroxybutyrate (specific activity 40 μCi/μmol) was prepared by reduction of acetoacetate by 4-A,B-^{3}H-NADH in the presence of β-hydroxybutyrate dehydrogenase, and was purified by thin layer chromatography on cellulose plates with butan-2-ol; (2 N NH_3, H_2O) (80:20, v/v) as a solvent. The radiochemical purity was determined by the GLC procedure described above as larger than 80 %.

4-A,B-^{3}H-NADH was prepared by reduction of 4-^{3}H-NAD^+ by *R,S*-1-^{3}H-ethanol in the presence of alcohol dehydrogenase and semicarbazide.[25] Purification was achieved on a DEAE-Sephadex A 25 column as described for the preparation of 2-^{3}H-lactate. NADH was eluted with 500 mM ammonium acetate.

4-^{3}H-NAD^+ was prepared by oxidation of 4-A-^{3}H-NADH with α-oxoglutarate and NH_4^+ in the presence of glutamate dehydrogenase. The reaction mixture was ultrafiltrated and lyophilized before conversion of the 4-^{3}H-NAD^+ to 4-A,B-^{3}H-NADH.

4-A-^{3}H-NADH was prepared by reduction of NAD^+ with *R,S*-1-^{3}H-ethanol in the presence of alcohol dehydrogenase and semicarbazide. NADH was purified as described above for 4-A,B-^{3}H-NADH.

RESULTS

Metabolite levels. All results on metabolite concentrations appear in Table 1. The lactate production, the metabolism of pyruvate to metabolites other than lactate, and the ketone body production have been calculated from the measurements of metabolite levels.

The 15 min values in the absence and presence of 4-methylpyrazole as regards the lactate to pyruvate and β-hydroxybutyrate to acetoacetate ratios and the level of acetaldehyde, agree with previously reported results.[5,27,28]

Lactate and pyruvate. As judged from the lactate to pyruvate ratios the results of Table 1 show that the cells do not attain a steady-state with regard to the redox state of the cytoplasmic NAD-couple within the first 15 min after addition of ethanol. This may seem at variance with previously reported results [13,26] and is probably due to the high initial concentration of pyruvate in the present experiments. The production of lactate is high within the first 5 min after ethanol addition and declines with the level of pyruvate in the 5 – 15 min period after ethanol addition (at the end of the 15 min incubation period, the pyruvate added was nearly used up by the cells). Concomitant with the decrease in the lactate production from 5 – 15 min after ethanol addition, a very striking increase in the lactate to pyruvate ratio is observed (Table 1). The presence of 4-methylpyrazole does not affect the metabolism of pyruvate by pathways other than that catalyzed by lactate dehydrogenase.

β-Hydroxybutyrate and acetoacetate. The β-hydroxybutyrate to acetoacetate ratio shows a significant change within the first 5 min after ethanol addition if compared with the ratio in the presence of 4-methylpyrazole. A very abrupt increase in ketone body production is observed from 3 to 5 min after ethanol addition.

Acetaldehyde. The concentration of acetaldehyde in the incubation medium is almost constant within 15 min of incubation and about 100 μM (Table 1). The fact that the concentration of acetaldehyde is unaffected by the rate of acetaldehyde production as re-

Table 1. Metabolic variables as a function of incubation time. Figures are means ± S.E. of six experiments, except where noted. Comparison of results in the absence and presence of 4-methylpyrazole was carried out by the paired-data *t*-test and * indicates P values <0.05. In the last three lines of the table, average rates of lactate production, pyruvate metabolism minus lactate production and ketone body formation are calculated in μmol min^{-1} (ml of cells)$^{-1}$ in the time intervals indicated (*i.e.* 1 – 3, 3 – 5 and 5 – 15 min).

	− 4-Methylpyrazole				+ 4-Methylpyrazole			
Incubation time, min	1	3	5	15	1	3	5	15
[Acetaldehyde] μM	100 ± 13	105 ± 20	115 ± 17	81 ± 10	92 ± 5	117 ± 14	114 ± 16	111 ± 19
[β-Hydroxybutyrate] μM	196 ± 37	232 ± 58	304 ± 60	599 ± 87	191 ± 28	190 ± 26	240 ± 51	225 ± 26*
[Acetoacetate] μM	280 ± 42	327 ± 50	502 ± 53	395 ± 30	244 ± 67	367 ± 65	547 ± 80	728 ± 93*
[β-Hydroxybutyrate]/ [Acetoacetate]	0.75 ± 0.11	0.73 ± 0.11	0.63 ± 0.11	1.49 ± 0.13	0.96 ± 0.17	0.58 ± 0.09*	0.44 ± 0.05*	0.33 ± 0.03*
[Lactate] mM	0.59 ± 0.04	0.95 ± 0.05	1.29 ± 0.09	1.49 ± 0.08	0.51 ± 0.06	0.57 ± 0.05*	0.60 ± 0.06*	0.82 ± 0.08*
[Pyruvate] mM	1.61 ± 0.19	1.03 ± 0.22	0.53 ± 0.21	0.04 ± 0.01[a]	1.56 ± 0.24	1.32 ± 0.21*	1.06 ± 0.26*	0.53 ± 0.14*
[Lactate]/[Pyruvate]	0.39 ± 0.06	1.22 ± 0.32	8.10 ± 3.44	47.5 ± 6.5[a]	0.37 ± 0.08	0.51 ± 0.11*	0.77 ± 0.20*	2.15 ± 0.44*
Δ Lactate		4.76 ± 0.37	4.29 ± 0.78	0.57 ± 0.41		0.36 ± 0.42*	0.80 ± 0.36*	0.46 ± 0.16
Δ Pyruvate − Δ Lactate		− 2.54 ± 0.80	− 2.13 ± 0.59	− 0.95 ± 0.19		− 3.18 ± 0.41	− 2.18 ± 1.15	− 0.30 ± 0.14
Δ Ketone bodies		1.01 ± 0.38	3.18 ± 0.59	0.40 ± 0.14		1.04 ± 0.43	3.32 ± 0.47	0.30 ± 0.14

[a] In two experiments the concentration of pyruvate was below 5 μM. These experiments have been omitted in this value.

Fig. 1. Incorporation of tritium into lactate from *R,S*-1-^{3}H-ethanol. Triangles are the values actually measured and circles these values as corrected for the detritiation of 2-^{3}H-L-lactate (see 'Discussion'). Open and closed symbols represent values in the absence and presence of 4-methylpyrazole (1.7 mM), respectively. Ethanol was added at time zero. Average values of three experiments.

flected in the independency of 4-methylpyrazole addition is at present not understood. The acetaldehyde concentration in the presence of ethanol agrees closely with the levels obtained in the intact rat liver during ethanol metabolism.[5]

Labelling pattern of lactate and β-hydroxybutyrate. The incorporation of tritium from ethanol into lactate (Fig. 1) and the specific activity of lactate increases with time. The specific activity of lactate relative to that of ethanol after 15 min of incubation is about 0.13, in accordance with previously reported values.[16,29] The labelling rate of lactate apparently declines with the fall in lactate production and the concomitant decrease in the concentration of pyruvate (*cf.* Table 1). In the presence of 4-methylpyrazole, the transfer of label from ethanol to lactate is about 6 times smaller than in the absence of inhibitor, which corresponds roughly to the decrease in the [^{3}H]NADH production due to the lower rate of ethanol and acetaldehyde oxidation.

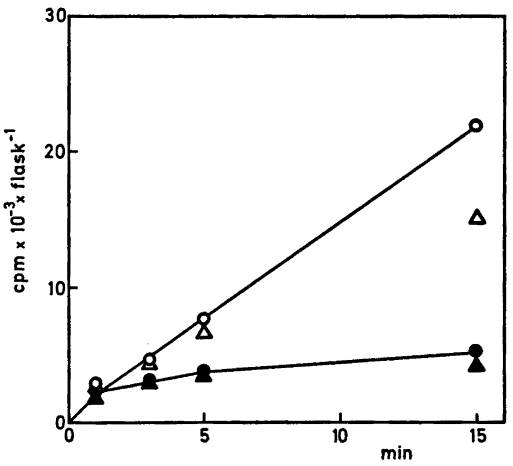


Fig. 2. Incorporation of tritium into β-hydroxybutyrate from *R,S*-1-^{3}H-ethanol. Triangles are the values actually measured and circles these values as corrected for the detritiation of 3-^{3}H-β-D-hydroxybutyrate (see 'Discussion'). Open and closed symbols represent values in the absence and presence of 4-methylpyrazole (1.7 mM), respectively. Ethanol was added at time zero. Average values of three experiments.

The incorporation of tritium from ethanol into β-hydroxybutyrate is shown in Fig. 2.

The relative specific activity of β-hydroxybutyrate of 0.015 obtained in the present work is in accordance with values reported previously.[30] The extensive labelling of β-hydroxybutyrate reported by us [31] probably was due to insufficient methods for the isolation of β-hydroxybutyrate.

The detritiation of lactate and of β-hydroxybutyrate was measured with 2-^{3}H-lactate and 3-^{3}H-β-D-hydroxybutyrate plus ethanol as substrates in experiments identical to those applying *R,S*-1-^{3}H-ethanol as a substrate (Figs. 3 and 4). The results have been used to correct the results of Figs. 1 and 2 for the detritiation of lactate and β-hydroxybutyrate, respectively, to obtain a measure of the true [^{3}H]-NADH production in the cytosol (see 'Discussion').

The correction was carried out by assuming a linear detritiation of both the initial amount of labelled substrate present and of the tritium incorporation in each of the three time intervals measured, *e.g.* for the labelling of lactate: The fractional detritiation of lactate in the time interval from t_1 to t_2 is denoted x, the observed labelling at t_1 is denoted α, the observed labelling at t_2 is denoted $\alpha + \Delta\alpha$ and the amount of label incorporated into lactate in the absence of any detritiation in the time interval from t_1 to t_2 is denoted β. The detritiation from t_1 to t_2 of α is αx and of the increase in labelling from t_1 to t_2 it is $0.5\beta x$, resulting in a total detritiation of $\alpha x + 0.5\beta x$. The observed increase in labelling from t_1 to t_2 equals the true increase in labelling from t_1 to t_2 minus the detritiation in this time interval: $\Delta\alpha = \beta - (\alpha x + 0.5\beta x)$ or $\beta = (\Delta\alpha + \alpha x)/(1 - 0.5x)$.

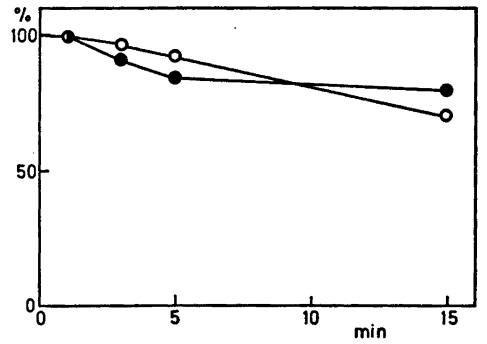


Fig. 3. Detritiation of 2-^{3}H-L-lactate by isolated liver cells. Ethanol (65 mM) and 2-^{3}H-L-lactate (27 000 cpm) were added at time zero. ○, −4-methylpyrazole; ●, +1.7 mM 4-methylpyrazole. Average values of two experiments.

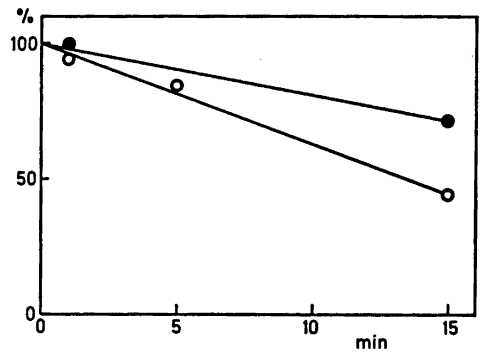


Fig. 4. Detritiation of 3-^{3}H-β-D-hydroxybutyrate by isolated liver cells. Ethanol (65 mM) and 3-^{3}H-β-D-hydroxybutyrate (46 000 cpm) were added at zero time. ○, −4-methylpyrazole; ●, +1.7 mM 4-methylpyrazole. One experiment.

The corrected labelling pattern of lactate is shown in Fig. 1 (○,●) and that of β-hydroxybutyrate in Fig. 2 (○,●). In the experiments with 2-^{3}H-lactate as a substrate, no labelling of β-hydroxybutyrate could be detected. Similarly, no labelling of lactate could be detected with 3-^{3}H-β-D-hydroxybutyrate as a substrate.

The tritium content of both lactate and β-hydroxybutyrate after 1 min of ethanol oxidation appeared independent of the presence of 4-methylpyrazole (Figs. 1 and 2). However, in parallel experiments it has been shown that ethanol oxidation, catalyzed by alcohol dehydrogenase becomes maximally inhibited by 1.7 mM 4-methylpyrazole already 15 s after addition of the inhibitor to a cell suspension (unpublished results).

DISCUSSION

Metabolite levels. The production of ketone bodies shows a very abrupt increase 3 to 5 min after addition of ethanol (Table 1) and corresponds in this time interval to 6.5 (μmol C-2 units) min^{-1} ml^{-1} and is independent of the presence of 4-methylpyrazole. Ketone bodies may be formed from pyruvate, acetaldehyde [6] or fatty acids. In the absence of 4-methylpyrazole, the ketone bodies produced from 3 to 5 min after ethanol addition, might be derived from acetaldehyde and pyruvate (*cf.* Table 1), whereas in the presence of 4-methylpyrazole, about 3 μmol min^{-1} of ketone bodies must be formed from other substrates, *e.g.* by an increase in the β-oxidation pathway. An inhibition of the utilization of ketone bodies is not a likely explanation of the increase in the level of ketone bodies, as the capacity of liver tissue to metabolize ketone bodies is very limited.[32]

Labelling pattern. The specific activity of lactate after 15 min was about 0.13 (with 4-methylpyrazole only 0.06) relative to the specific activity of ethanol. The deviation from the theoretical value of 0.5 may be due to a cytosolic NADH production from substrates other than ethanol and/or to a rapid exchange reaction of the cytosolic NADH-pool with water, *e.g.* caused by a rapid exchange of malate between the cytosolic and the mitochondrial cell compartment.[33] A possible compartmentation between NADH formed in the reaction catalyzed by alcohol dehydrogenase and the cytosolic NADH-pool [29,30,34] might also contribute to the low specific activity of lactate in the steady-state situation.

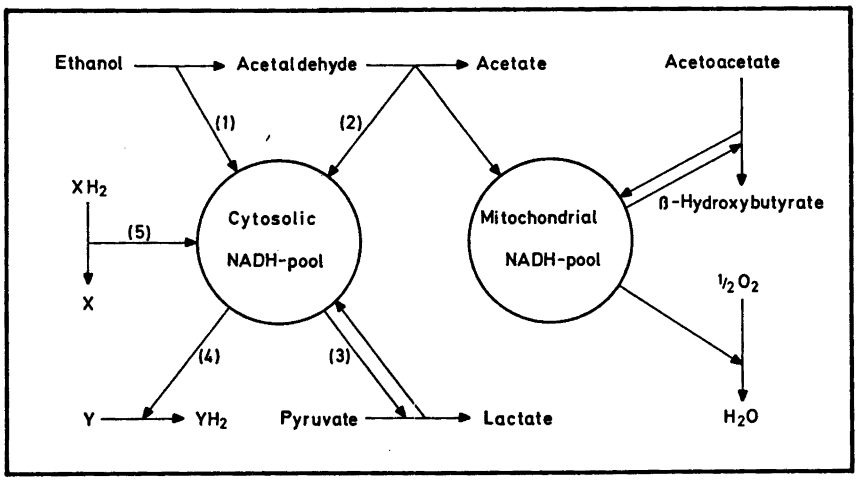
The specific activity of β-hydroxybutyrate is only about 0.02 relative to the specific activity of ethanol. This very low labelling of β-hydroxybutyrate may be due to an extensive transfer of tritium from mitochondria [^{3}H]NADH to water [35,36] and/or to a lack of isotopic equilibration between the mitochondrial NADH-pool and β-hydroxybutyrate.

The intracellular localization of acetaldehyde oxidation. The results of the present experiments allow calculation of the cytosolic oxidation of acetaldehyde from the measurements of the initial incorporation of tritium into lactate.

Cytosolic [^{3}H]NADH is formed from *R,S*-1-^{3}H-ethanol in the reaction catalyzed by alcohol dehydrogenase (reaction 1 of Scheme 1) and by the oxidation of that part of the acetaldehyde, which is oxidized extramitochondrially (reaction 2 of Scheme 1).

The total amount of [^{3}H]NADH formed by the cytosolic oxidation of ethanol and acetaldehyde equals the total amount of tritium actually measured in lactate plus the amount of tritium, which is deposited in substrates other than lactate (*cf.* Scheme 1). The significance of the last mentioned pathway is assumed to be measured by the rate of detritiation of 2-^{3}H-L-lactate. This assumption is valid only if lactate/pyruvate equilibrates rapidly with the cytoplasmic NADH/NAD^+ pool as compared to other redox couples sharing the same NADH/NAD^+ pool, and if no significant recirculation of tritium to ethanol takes place. Recirculation of tritium to ethanol probably will be insignificant during the 15 min of incubation, as the major labelled products of reaction 4 (Scheme 1) will be water and glucose.[12,16]

The total radioactivity measured in lactate plus the radioactivity removed by reaction 4 (Scheme 1) (as determined by the detritiation of 2-^{3}H-lactate) is then a measure of the production of cytosolic [^{3}H]NADH by reaction 1 and 2 (Scheme 1). The rate of reaction 1 is known from measurements of the rate of ethanol oxidation and the rate of reaction 2 can therefore be calculated.



Scheme 1. Schematic flow-diagram for the C-1 hydrogens of ethanol.

In the presence of 4-methylpyrazole, the rate of tritium transfer as corrected for the detritiation of lactate was determined to 2926 cpm min^{-1} $flask^{-1}$ (Fig. 1). Given the specific activity of the *R*- or *S*-tritium atom of ethanol of 140 cpm $nmol^{-1}$ each and the amount of cells per flask of 92 µl, the above-mentioned rate of tritium transfer to lactate corresponds to a cytosolic [^{3}H]NADH production of 0.23 µmol min^{-1} ml^{-1}.

The rate of NAD^{3}H production in the cytosol will depend partly on the isotope effects on alcohol dehydrogenase and aldehyde dehydrogenase and partly on the degree of inhibition of alcohol dehydrogenase by 4-methylpyrazole. The isotope effect on the reaction catalyzed by aldehyde dehydrogenases is unknown, but assuming a value between 1 and 0.6 (the isotope effect on the alcohol dehydrogenase, see below), the oxidation rate of acetaldehyde in the cytosol will be in the range 0.23 – 0.38 µmol min^{-1} ml^{-1} if complete inhibition of alcohol dehydrogenase is obtained by 4-methylpyrazole.

The degree of inhibition of alcohol dehydrogenase in the present experiments probably is 95 % or higher.[2,28]

The rate of ethanol oxidation by alcohol dehydrogenase under the present experimental conditions was determined to 3.18 ± 0.19 µmol min^{-1} ml^{-1} ($n = 5$), and thus 95 % inhibition of the enzyme results in a calculated rate of extramitochondrial acetaldehyde oxidation of about 0.13 – 0.28 µmol min^{-1} ml^{-1}, assuming isotope effects of 0.6 and 1 – 0.6, respectively, in the reactions catalyzed by alcohol dehydrogenase [16,37,38] and aldehyde dehydrogenase.

In the absence of 4-methylpyrazole, the rate of tritium transfer as corrected for the detritiation of lactate was determined to 19 366 cpm min^{-1} ml^{-1} (Fig. 2), corresponding to a cytosolic [^{3}H]NADH production of 1.5 µmol min^{-1} ml^{-1}. The rate of [^{3}H]NADH production from the reaction catalyzed by alcohol dehydrogenase equals the rate of ethanol oxidation (3.2 µmol min^{-1} ml^{-1}, see above) corrected for the isotope effect in this reaction (0.5 – 0.6),[16,37,38] *i.e.* 1.6 – 1.9 µmol min^{-1} ml^{-1}. The figures 1.5 and 1.6 – 1.9 µmol min^{-1} ml^{-1} seems to be different, indicating that some [^{3}H]NADH from the reaction catalyzed by alcohol dehydrogenase escapes equilibrium with the cytosolic NADH pool, a finding which is compatible with the results obtained by Curstedt.[29]

We therefore conclude, that under the experimental conditions applied the oxidation rate of acetaldehyde in the cytosol is in the range from 0 to 0.4 µmol min^{-1} (ml of cells)$^{-1}$. This result is reconcilable with the distribution studies of Marjanen,[7] who found that about 20 % of the low-K_m aldehyde dehydrogenase activity was confined to the cytosol in rat liver.

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Diels-Alder Reactions of Thiophene Oxides Generated *in situ*

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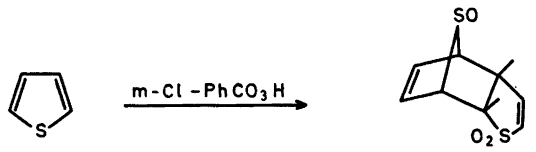
Dedicated to Professor Karl Myrbäck on his 75th birthday

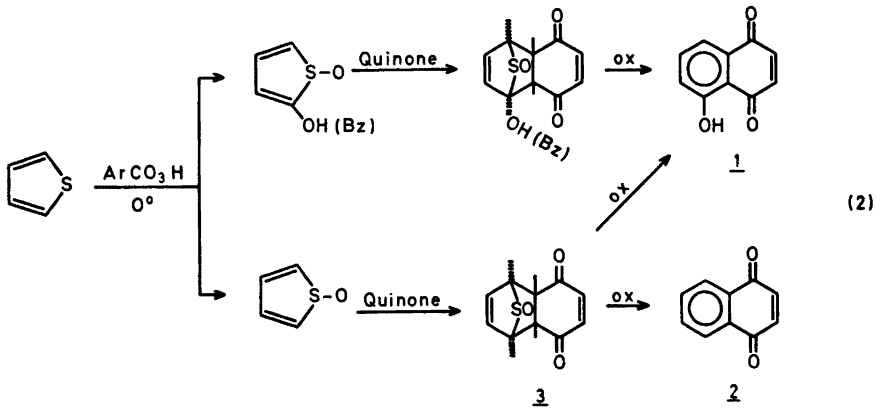
Oxidation of thiophenes with *m*-Cl-perbenzoic acid in the presence of quinones gives sulfoxide-bridged adducts and naphtho- or anthraquinones. Benzoquinone and thiophene give unexpectedly juglone in a yield of *ca.* 20 % among other products. Thiophene sulfoxide is a very unstable and reactive intermediate.

By engaging the lone pairs of the thiophene sulfur in bond formation, the aromatic stabilization is destroyed and the double bonds receive more diene character. This is borne out in the properties of thiophene sulfoxide [1] and thiophene sulfone [2] which are unstable and have not been prepared in a pure state. They dimerize in a Diels-Alder fashion (1) and have thus both ene and diene properties.[3] A few stable substituted thiophene sulfones have been prepared.[4] It therefore seemed reasonable to anticipate the formation of Diels-Alder adducts when the sulfoxide was generated in presence of suitable enes.

Thiophene was treated with two equivalents of *m*-chloroperbenzoic acid in methylene chloride at 0 °C for 48 h in the presence of phenylacetylene, ethyl propiolate, or acrylonitrile, but none of the expected cyclic derivatives were obtained. A reaction occurred with quinones but it took a somewhat unexpected course. The major product from benzoquinone, thiophene, and *m*-chloroperbenzoic acid (1:1:2.4) was juglone *1* (21 %), naphthoquinone *2*, and traces of a compound thought to be acylated juglone. The presence of the adduct *3* was proved spectroscopically in the crude product. It was isolated in a pure condition (*ca.* 20 %) by using chloroform as the reaction medium. When thiophene was oxidized separately at 0 °C overnight and benzoquinone then added, no adduct or naphthoquinones could be detected, which shows that thiophene sulfoxide is shortlived and reactive even at 0 °C. There is a competition between *S*- and *C*-hydroxylation. The latter occurs at an early stage of the reaction since naphthoquinone is stable to the peracid under the reaction conditions. *3* decomposed slowly on standing, forming *2* as one of the products, but no juglone *1* could be detected by TLC. Refluxing *3* in chloroform in the presence of *m*-chlorobenzoic acid did not produce juglone, *i.e.*, it is not formed *via* a Pummerer rearrangement but it was slowly formed together with naphthoquinone (2:1) by the action of peracid on *3* at 0 °C. Alternatively, juglone could arise from the Diels-Alder reaction of 2-hydroxy (or benzoyloxy) thiophene oxide. 2,5-Dimethylthiophene gives a stable sulfone,[4] which was found to react very slowly with benzoquinone at room temp. Refluxing in chloroform overnight was necessary to bring about the formation of *6*. When 2,5-dimethylthiophene was oxidized at 0 °C in the presence of benzoquinone, the adduct *4a* was obtained in a yield of 33 % but neither 2,5-dimethylthiophene sulfone nor *6* could be detected in the crude reaction mixture, *i.e.*, the thiophene sulfoxides are much more reactive enophiles than are the sulfones and furthermore, the naphthoquinones produced are not formed by initial oxidation of the thiophene to the sulfone followed by Diels-Alder addition and splitting off sulfur dioxide. *6* was stable in the presence of trifluoroacetic acid but decomposed in contact with silica and in basic solution forming *5a* as one of the products. Prolonged refluxing of *6* in chloro-



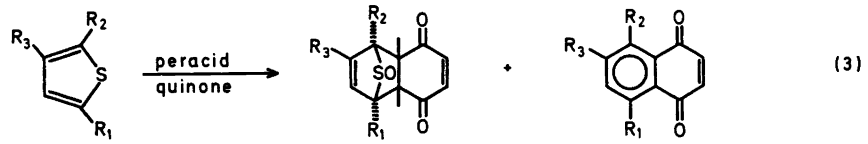
(1)



(2)

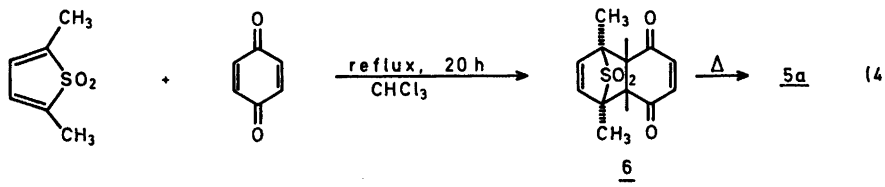
form gave *5a*, whereas under these conditions *4a* is practically not affected. After 5 h reflux of *4a* in toluene only *ca.* 18 % of *4a* decomposed. Thus the sulfone bridge is considerably more unstable than the sulfoxide bridge, a circumstance that was noted earlier.[3,6] The possibility remains that the naphthoquinones are formed *via* an oxidation of the sulfoxide bridge to a sulfone function. The most plausible routes to the naphthoquinones are depicted in (2).

From toluquinone a mixture (crude yield *ca.* 30 %) was obtained of 2-methylnaphthoquinone and its 5- and/or 8-hydroxylated derivatives which were difficult to separate.



(3)

4a, *5a* $R_1=R_2=CH_3$; $R_3=H$
4b, *5b* $R_1=CH_3$; $R_2=R_3=H$
4c, *5c* $R_1=R_2=H$; $R_3=CH_3$
5d $R_1=CH_3$; $R_2=OH$; $R_3=H$
5e $R_1=H$; $R_2=OH$; $R_3=CH_3$
4f, *5f* $R_1=R_2=Cl$; $R_3=H$
5g $R_1=R_2=Ph$; $R_3=H$



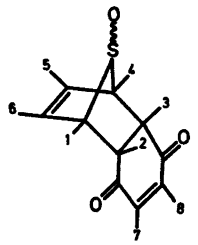
(4)

Addition to the methyl side of toluquinone was not observed.

A series of substituted thiophenes were oxidized in the presence of benzoquinone as dienophile. 2-Carbomethoxy-, 2,4-diphenyl, and 2-phenylthiophene gave no adducts, but small amounts of the corresponding naphthoquinones could be isolated from 2,5-diphenyl- and 2,5-dichlorothiophene. The oxidation of thiophenes with electron withdrawing groups is slow. Better yield of the adducts (*4a–c*) was obtained from the methylated thiophenes.

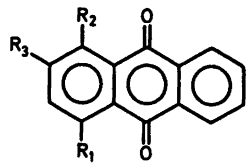
Following the principle of maximal π-orbital in the transition state of the diene reaction, the adduct was given the configuration depicted in (3). This is in agreement with the NMR data for *3*. In similar systems,[5] $J_{1,2(exo)}$ 3–4 Hz and $J_{1,2(endo)}$ ~0 Hz. The configuration of the SO group is unknown. Only one isomer was obtained except in the reactions with 3-methylthiophene, where the NMR spectrum of the crude product indicated the presence of two isomeric products (~10:1), of which only the most abundant one was isolated in a pure condition.

Naphthoquinone, thiophene, and peracid gave anthraquinone, *7a* (25 or 58 %, based upon recovered naphthoquinone), but no 1-hydroxyanthraquinone could be isolated. The methyl substituted thiophenes gave anthraquinones, *7b–7c*, in a total yield of *ca.* 10–20 %, and also some hydroxylated derivatives. Considerable amounts of adducts with the sulfoxide bridge retained were also formed (NMR), but they decomposed slowly to intractable material and were not isolated in a pure state.



3

$J_{1,2}$ = 4.0 Hz; $J_{2,5} \sim J_{2,6} \sim J_{2,7} \sim 0$ Hz
$J_{2,3}$ = 8.7 Hz; $J_{1,3} \sim J_{1,4} \sim 0$ Hz
$J_{1,5}$ = 0.9 Hz
$J_{1,6}$ = 4.8 Hz
$J_{5,6}$ = 10.2 Hz



7a $R_1 = R_2 = R_3 = H$
7b $R_1 = R_3 = H$; $R_2 = CH_3$
7c $R_1 = R_2 = H$; $R_3 = CH_3$

EXPERIMENTAL

Juglone, 1, naphthoquinone, 2, and adduct 3. A mixture of thiophene (0.84 g, 10 mmol), benzoquinone (1.08 g, 10 mmol), and *m*-chloroperbenzoic acid (5.0 g, *ca.* 81 %, 25 mmol) in methylene chloride (35 ml) was stirred for 2 days at 0 °C. The precipitated *m*-chlorobenzoic acid was filtered off, the solution washed with aqueous sodium bicarbonate, dried over sodium sulfate, and evaporated *in vacuo*. Ethanol (4 ml) was added and the solution was left standing in the freezer (–15 °C) overnight. *1* (320 mg) precipitated, contaminated with *2* (*ca.* 10 %). A second crop of crystals (180 mg) could be obtained by partial evaporation of the filtrate consisting of *1* (*ca.* 40 %), *2* (40 %), and benzoquinone (20 %, NMR). The yields of juglone and naphthoquinone were 21 and 7 %, respectively. The identity of juglone was verified by comparison with an authentic sample. The NMR spectrum of the crude reaction product showed the presence of the adduct *3*. Preparative TLC of a sample of the crude product gave also a small fraction which according to the IR spectrum appeared to be *m*-chlorobenzoyl juglone. It was not investigated further.

Preparation of the adducts, 3, 4b, and 4c. Chloroform was used as solvent for the thiophene oxidation described above. The same work-up method was followed. Acetonitrile (1 ml) and then ether (12 ml) were added to the crude product obtained after evaporation of the chloroform. *3* (0.44 g, 21 %) precipitated as dark-brown crystals after standing overnight in the freezer. *3* was purified by dissolving in acetonitrile (2 ml) and precipitation with ether, m.p. 129 °C, brown crystals. (Found: C 57.69; H 3.97. Calc. for $C_{10}H_8SO_3$: C 57.69; H 3.84). NMR ($CDCl_3$): δ 3.99 (2 H, distorted t), 4.31 (2 H, m), 6.20 (2 H, distorted t), 6.79 (2 H, s). Evaporation of the ether filtrate and chromatography of the remainder on silica gave 0.2 g of a mixture of *1* and *2* in the quinone fraction.

4b (24 %) m.p. 133–135 °C, and *4c* (18 %) m.p. 124–126 °C, were prepared from 2-methylthiophene and 3-methylthiophene, respectively, according to the same procedure. (Found for *4b*: C 59.35; H 4.55. Found for *4c*: C 58.78; H 4.60. Calc. for $C_{11}H_{10}SO_3$: C 59.45;

H 4.50). NMR ($CDCl_3$), *4b*: δ 1.75 (3 H, s,) 3.5 (1 H, d), 4.1 (2 H, m), 6.16 (2 H, m), 6.73 (2 H, s). NMR ($CDCl_3$), *4c*: δ 1.74 (3 H, d, $J = 1.6$ Hz), 3.9 (2 H, m), 4.15 (2 H, m), 5.77 (1 H, d, t, $J = 1.6$ and 4.3 Hz), 6.78 (2 H, s).

Evaporation of the ether filtrate and chromatography on silica ($CHCl_3$ – light petroleum, 1:1) gave a crude quinone fraction, *ca.* 0.3 – 0.4 g. 2-Methylthiophene gave 5-methylnaphthoquinone, *5b*, (90 mg), m.p. 119 – 122 °C (lit.[7] 121 – 122 °C) and 5-methyl-8-hydroxynaphthoquinone, *5d*, (40 mg), red-yellow needles, m.p. 160 – 163 °C, NMR ($CDCl_3$), *5d*: δ 2.61 (3 H, s), 7.15 and 7.43 (2 H, AB-system, $J_{AB} = 8.6$ Hz). 3-Methylthiophene gave 6-methylnaphthoquinone, *5c*, (170 mg), m.p. 86 – 88 °C (lit.[8] 90 – 91 °C) and 5-hydroxy-6-methylnaphthoquinone, *5e* (30 mg), m.p. 107 – 109 °C; UV (EtOH), *5e*: λ_{max} 219, 252, 434, ε 4.20, 4.13, 3.62; NMR ($CDCl_3$), *5e*: δ 2.36 (3 H, s), 6.94 (2 H, s), 7.54 (2 H, s); mol.weight 188 (MS).

The yields of the naphthoquinone derivatives were raised somewhat when the reaction was performed in chloroform, but the adducts were more difficult to isolate.

Preparation of adduct 4a. *m*-Cl-Perbenzoic acid (3.5 g, 80 %) were added to 2,5-dimethylthiophene (2.3 g) and benzoquinone (2.15 g) in methylene chloride (50 ml) at −10 °C. After 2 h at 0 °C a further portion of the peracid (3.5 g) was added and the mixture was stirred at 0 °C for 36 h. The precipitated *m*-Cl-benzoic acid was filtered off and the chloroform solution extracted twice with sodium bicarbonate solution, dried, and evaporated *in vacuo*. Addition of ethanol (5 ml) and carbon tetrachloride (10 ml) gave *5a* (1.3 g) on cooling. Evaporation of the filtrate and preparative TLC of the remainder (SiO_2, $CHCl_3$) gave another 0.2 g of *4a*. The total yield was 33 %. *4a* was recrystallized from acetonitrile adding some ether to the solution, m.p. 139 °C. (Found: C 61.03; H 5.34. Calc. for $C_{12}H_{12}O_3S$: C 61.00; H 5.12). NMR ($CDCl_3$): δ 1.67 (6 H, s), 3.58 (2 H, s), 6.04 (2 H, s), 6.67 (2 H, s). The fast-going TLC fraction gave 100 mg of the naphthoquinone *5a*, m.p. 121 – 122 °C (lit.[9] 124 – 125 °C).

Preparation of 4f *and* 5f. A mixture of 2,5-dichlorothiophene (1.5 g, 10 mmol), benzoquinone (1.1 g, 10 mmol), and *m*-chloroperbenzoic acid (4.6 g, 80 %, 23 mmol) in methylene chloride (40 ml) was kept at room temp. for 2 days and then refluxed for 6 h. After the usual work-up, a mixture of *4f* (40 %) and *5f* (60 %) (0.2 g) precipitated from the ethanolic solution (5 ml) in the freezer. *5f* dissolved in carbon tetrachloride and was recrystallized from ethanol, yellow needles, m.p. 168 – 169 °C (lit.[10] 173 – 174 °C). *4f*, which is sparely soluble in carbon tetrachloride, was recrystallized from acetonitrile, yellow cubes, dec. 135 °C. (Found: C 43.88; H 2.45. Calc. for $C_{10}H_6O_3SCl_2$: C 43.34; H 2.17). NMR ($CDCl_3$): δ 4.00 (2 H, s), 6.36 (2 H, s), 6.84 (2 H, s).

5,8-Diphenylnaphthoquinone 5g was obtained in a yield of 8 % using the preceding method (reflux 2 h) and preparative TLC of the crude reaction mixture from the work-up, m.p. 142 – 143 °C (lit.[11] 141 – 142 °C).

Anthraquinone 7a. Naphthoquinone (1.58 g, 10 mmol), thiophene (0.85 g, 10 mmol), and *m*-chloroperbenzoic acid (5.5 g, 80 %, 28 mmol) in methylene chloride were stirred for 2 days at 0 °C. After the usual work-up, methanol (20 ml) was added to the remainder of the evaporated chloroform extract and from the cooled solution 0.6 g *7a*, m.p. 284 °C, precipitated, identical with an authentic specimen. Naphthoquinone (0.7 g) was isolated by preparative TLC from the evaporated filtrate.

1-Methyl anthraquinone 7b. A stirred mixture of naphthoquinone (1.58 g, 10 mmol), 1-methylthiophene (1.0 g, 10 mmol), and *m*-chloroperbenzoic acid (4.6 g, 80 %, 23 mmol) in methylene chloride (50 ml) at 0 °C, 2 days, gave after usual work-up a crude product (the NMR spectrum showed the presence of considerable amounts of the adduct which was not isolated in a pure condition), which was chromatographed on silica ($CHCl_3$ – light petroleum, 2:1). The quinone fraction (0.39 g) contained *7b* (*ca.* 70 %), naphthoquinone (*ca.* 30 %), and traces of a hydroxylated anthraquinone. *7b* was recrystallized from ethanol, m.p. 170 – 172 °C (lit.[12] 171 – 173 °C).

2-Methylanthraquinone 7c was obtained in a similar manner from 3-methylthiophene. The chromatographed quinone fraction (0.52 g from 3-methylthiophene (1.0 g), naphthoquinone (1.6 g) and perbenzoic acid (4 g)), contained *ca.* 65 % *7c*, 35 % naphthoquinone, and traces of a hydroxylated anthraquinone. Recrystallization from ethanol gave pure *7c*, m.p. 177 – 179 °C (lit.[13] 178 – 179 °C).

2,5-Dimethylthiophene sulfone [4] was prepared by oxidation of 2,5-dimethyl thiophene (5 g) with *m*-chloroperbenzoic acid (26 g ~ 70 %, *ca.* 2.3 eqv.) in methylene chloride (100 ml) at 0 °C for 48 h. The suspension was filtered and the filtrate washed with aqueous sodium bicarbonate. The solvent was evaporated. The product which solidified in the refrigerator could be recrystallized from methanol giving 2.2 g of pure sulfone. NMR ($CDCl_3$): δ 2.10 (6 H, d, $J = 0.8$ Hz), 6.31 (2 H, q, $J = 0.8$ Hz).

The Diels-Alder adduct 6. 2,5-Dimethylthiophene sulfone (1.1 g) and benzoquinone (1.0 g) were refluxed overnight in chloroform (20 ml). The solvent was evaporated and cyclohexane – carbon tetrachloride (2:1, 10 ml) was added and the black precipitate filtered off. Evaporation and recrystallization from ethanol (5 ml) gave the adduct *6* as yellow crystals, m.p. 59 – 60 °C, 0.8 g. NMR ($CDCl_3$): δ 1.82 (6 H, s), 3.52 (2 H, s), 5.82 (2 H, s), 6.72 (2 H, s). Evaporation of the solvent gave a yellow oil of impure adduct, contaminated by some benzoquinone and traces of 5,8-dimethylnaphtho-

quinone. The highest peaks in the MS correspond to $M-SO_2$ and $M-H_2SO_2$ (equal to naphthoquinone).

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Preparation and Reactions of 5*H*-Indeno[1,2-*c*]-pyridazine Derivatives

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3-Methoxy-5-methyl-5*H*-indeno[1,2-*c*]pyridazine *5a* and its 6,9-dimethylated derivative *5b* have been prepared. Alkylation in neutral solution takes place at N^1 and at N^2, respectively. In basic solution, however, electrophilic attack takes place at position 5 as illustrated by methylation (methyl iodide) and by hydroxylation (oxygen) of *5a* to give *5c* and *5d*, respectively.

Only a single derivative of the indenopyridazine (*1*, $n=1$) has been described,[1] and no chemistry of the ring system has been reported. With a few modifications, the preparation of the ring system *1*, $n=1$, follows that utilized for the preparation of the two similar ring systems, *1*, $n=2$ and 3 (*cf.* Experimental).[2,3] Chlorination of the pyridazinones *3a* and *3b* with phosphorus oxychloride to give the chloropyridazines *4a* and *4b* is very facile, in contrast to the general conditions obtaining for the preparation of chloropyridazines [2-4] and to a similar reaction in the indenopyridine series.[5]

The acidity [6] of the C(5)-hydrogen gives rise to a reactive nucleophilic center when the compound is dissolved in, *e.g.*, sodium methoxide as indicated by the formation of an intense blue colour and by the reactions of the anion of *5a* with methyl iodide or with oxygen to give the 5-methylated and 5-hydroxylated compounds *5c* and *5d*, respectively.

Methylation of *5a* in *neutral* solution takes place at N^1 to give the pyridazinium iodide *6*, apparently, as judged by ^{1}H NMR spectroscopy, without attack at position 2, *cf.* the corresponding reaction of 3-methoxy-6-phenylpyridazine (ratio of quaternization at position

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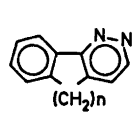
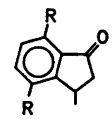


1



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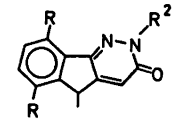
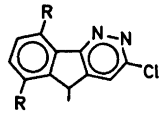
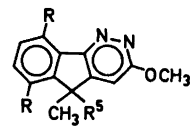
2b R = CH_3



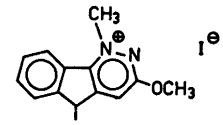
3a R = R^2 = H
3b R = CH_3; R^2 = H
3c R = R^2 = CH_3
3d R = H; R^2 = CH_3



4a R = H
4b R = CH_3



5a R = R^5 = H
5b R = CH_3; R^5 = H
5c R = H; R^5 = CH_3
5d R = H; R^5 = OH



6

1 to position 2 = 78/22).[8] In both cases it may be assumed that the phenyl and the pyridazine rings are approximately coplanar,[8,9] but in the former (compound *5a*) the five-membered ring displaces the hydrogen at position 9 so as to render position 1 less hindered. Substitution of H^9 by methyl as in *5b* results in quaternization at N^2; compound *3c* was isolated,[8] see Experimental.

Prolonged heating of the indenopyridazine *5a* in basic monodeuteriomethanol gave the 4,5-dideuterated compound.[10]

An improved synthesis of 3,4,7-trimethylindanone *2b* is given.[11]

EXPERIMENTAL

^1H NMR spectra were recorded on a Varian A-60 instrument using $CDCl_3$ as a solvent where not otherwise indicated. Boiling points and melting points are uncorrected.

3,4,7-Trimethylindanone 2b.[11] Crotonic acid (65 g) in *p*-xylene (190 ml) was added to a suspension of aluminium chloride (117 g) in *p*-xylene (100 ml) (10–15 °C, 30 min). The mixture was allowed to stand overnight at room temperature (20 °C) and then poured onto ice. Work up [12] included a crude distillation (up to 132 °C/0.2 mmHg), a recrystallization from ligroin (200 ml, 80–110 °C), and finally from a mixture of ethanol, water, and acetic acid (55 ml of each) to give 3-(2,5-dimethylphenyl)-butyric acid [11] (79 g (60 %), m.p. 106–111 °C, lit.[11] 111–112 °C). An attempt to prepare the acid by the method given for the synthesis of 3-phenylbutyric acid [12] led only to an inhomogeneous product. Conversion of the acid to the acid chloride [13] and cyclisation by addition of aluminium chloride (one mol, *cf.* Ref. 13) to a molar solution of the acid chloride in benzene at 10–20 °C and subsequent reaction at 20 °C for 30 min gave after work-up [13] the spontaneously crystallizing ketone *2b* (yield 73 %, b.p. 92 °C/0.5 mmHg; 30–32 °C after one recrystallization from petroleum ether; lit.[11] 32–33 °C).

3-Methoxy-5H-indeno[1,2-c]pyridazine 5a was prepared [2,3] from the ketone *2a* [13] by lithiation with freshly prepared lithium amide in liquid ammonia, alkylation with bromoacetic acid in ether,[2] cyclization of the crude mixture of diastereomeric keto acids with hydrazine in ethanol [4] and bromination [4] at 80 °C to give hydrobromide of *3a* (40.9 g from 172 g of ketone). Two recrystallizations from a mixture of ethanol (300 ml) and water (150 ml) gave the pyridazinone *3a*. Yield 18.4 g (8.2 %), m.p. 230–234 °C.

The pyridazinone (*3a*, 5.48 g) was rapidly (*ca.* 1 min) heated to reflux in phosphorus oxychloride (50 ml) and refluxed for 20 s, cooled by applying vacuum, and the phosphorus oxychloride removed *in vacuo*. Addition of ice, neutralization with aqueous ammonia, extraction with chloroform, the extract washed with water, treated with Norite, dried, concentrated *in vacuo*, and the product crystallized from ethanol (20 ml) at 0 °C gave crystals (3.6 g). Recrystallizations from toluene (25 ml, treatment with Norite) and from ethanol (15 ml) gave colourless crystals of *4a*. Yield 2.38 g (40 %), m.p. 140–144 °C, depending on the rate of heating.

The chloropyridazine (*4a*, 1.25 g) was methoxylated in a solution of sodium methoxide (680 g of sodium and 10 ml of methanol) in an evacuated ampoule and kept at 80 °C for 20 h. Addition of water, four extractions with chloroform, the combined extracts washed once with water and once with 10 % aqueous acetic acid, dried, and concentrated *in vacuo* gave an oil. The oil was dissolved in ligroin (80/110 °C, 20 ml), treated with Norite at 50 °C, filtered, cooled to 20 °C without inducing crystallization, decanted from a red oil, and allowed to crystallize at −80 °C to give colourless crystals. Yield 780 mg (64 %); m.p. 87–90 °C). Further crystallizations from ligroin as above gave *5a*, m.p. 89–91 °C. (Found: C 73.09; H 5.73; N 13.18. Calc. for $C_{13}H_{12}N_2O$: C 73.57; H 5.70; N 13.20). ^1H NMR, δ 1.46 (3 H, d, J 7.5 Hz); 3.88 (H^5, broadened quartet, J *ca.* 7.5 Hz; 4.09 (methoxyl); 6.95 (H^4, d, J 1 Hz); *ca.* 7.35 (3 H, m); between 7.91 and 8.16 (1 H, m).

The homolog *5b* was prepared by the same procedure (above). Recrystallization, finally from ethanol at −80 °C, gave colourless crystals, m.p. 103–104 °C. (Found: C 75.00; H 6.68; N 11.61. Calc. for $C_{15}H_{16}N_2O$: C 74.97; H 6.71; N 11.66). ^1H NMR, δ 1.39 (3 H, d, J 7.5 Hz); 2.32 (methyl, at position 6? *cf.* toluene, δ 2.32); 2.83 (methyl, at position 9?); 3 81 (H^5, broadened quartet, J *ca.* 7.5 Hz); 4.12 (methoxy); 6.82 (H^4, d, J 1 Hz); 7.02 (H^7 and H^8, nearly an A_2-system; a suspected coupling, J 7.5 Hz, was confirmed on a Bruker instrument at 90 MHz).

N-Methylation. The indenopyridazine (*5a*, 97 mg) was dissolved in chloroform (0.3 ml) and methyl iodide (0.3 ml) and kept at *ca.* 22 °C for 27 h. The crystals were washed with chloroform to give the crude quaternary salt *6*. Yield 141 mg (86 %) m.p. (destr., evolution of gas) between 189–191 °C and 194–195 °C, depending on rate of heating. Recrystallization from acetic acid for analysis. (Found: C 47.12; H 4.26; N 7.76. Calc. for $C_{14}H_{15}N_2OI$: C 47.48; H 4.27; N 7.91). ^1H NMR, in deuteriochloroform/trifluoroacetic acid 1/1, δ 1.71 (3 H, d, J 7.5 Hz); 4.20 (methoxy);[8] 4.83 (*N*-methyl); 7.54 to 8.34 (4 H, m).

^1H NMR inspection of the crude product from a similar experiment after removal of solvents *in vacuo* revealed no signals attributable to isomers of *6* or to pyridazinones such as *3d*.

The same procedure applied to the sterically hindered compound *5b* gave approximately 40 % conversion to *3c*. Quaternization in acetonitrile [8] for 3 days of 80 °C gave according to ^{1}H NMR analysis only *3c* besides some unidentified products. Recrystallizations from ligroin 80/110 °C and from ethanol at −80 °C gave colourless crystals, m.p. 132−133 °C. (Found: C 74.49; H 6.84; N 11.37. Calc. for $C_{15}H_{16}N_2O$: C 74.97; H 6.71; N 11.66). ^{1}H NMR, δ 1.45 (3 H, d, J 7.5 Hz); 2.38 and 2.64 (s, two methyl groups); 3.85 (s, *N*-methyl); 3.94 (broadened and partly hidden quartet, J *ca.* 7 Hz); 6.84 (d, J 1 Hz); 7.07 (2H apparently, a singlet).

C-*Methylation.* A solution of the indenopyridazine *5a* (220 mg) in ethanol (1.8 ml) and methyl iodide (1.4 ml) was added *in vacuo* to an ethanolic solution of sodium ethoxide (20 ml, from 1.0 g of sodium). The exothermic reaction was completed within 2 min, and the deep blue colour had faded. Addition of water and extractions with chloroform gave brown crystals (257 mg). Recrystallizations from equal volumes of toluene and ligroin 80/100 °C gave colourless crystals of *5c*, m.p. 107−109 °C. (Found: C 73.99; H 6.19; N 12.33. Calc. for $C_{14}H_{14}N_2O$: C 74.31; H 6.24; N 12.38). ^{1}H NMR, δ 1.48 (6 H, s); 4.14 (methoxy); 6.89 (H^4, s); *ca.* 7.40 (3 H, m), 7.95 to 8.32 (H^9, m).

Oxidation. Air was led through a solution of *5a* (426 mg, crude) in ethanolic sodium ethoxide (from 200 mg of sodium and 10 ml of ethanol) for 2 h. The blue colour disappeared. Addition of water to the reddish solution and extractions with chloroform and a recrystallization from toluene (5 ml) gave *5d*. Yield 221 mg (48 %), m.p. 177−185 °C. Recrystallization from toluene and from ethanol gave colourless crystals, m.p. 196−197 °C. (Found: C 68.25; H 5.27; N 12.24. Calc. for $C_{13}H_{12}N_2O_2$: C 68.41; H 5.30; N 12.27). ^{1}H NMR, δ (dimethyl sulfoxide-d_6): 1.65 (3 H, s); 4.10 (s, methoxy); 6.08 (s, hydroxy, exchangeable with CH_3OD); 7.36 (H^4, s); 7.45−8.17 (4 H, m). IR (KBr): 3300 cm^{-1} (OH).

Deuteration. Sodium methoxide in monodeuteriomethanol (from 170 mg of sodium and 5 ml of monodeuteriomethanol) was added to the indenopyridazine *5a* (200 mg) in an evacuated ampoule and kept at 80 °C for 3 days. Addition of water and extraction with chloroform gave crude 3-methoxy-4,5-dideuterio-5-methylindenopyridazine. ^{1}H NMR, δ 1.46 (3 H, s); 4.09 (methoxy); 6.95 (weak singlet, indicating that exchange was not complete) and multiplets as for *5a*, above.

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Palladium(II) Catalyzed Aromatic Acetoxylation. VII.* Preparative Scale Acetoxylation by Potassium Peroxydisulfate with Palladium(II) as a Catalyst

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The palladium(II) catalyzed acetoxylation of aromatic substrates can be achieved by using potassium peroxydisulfate as the oxidant. Preparative runs on a number of substrates show that the reaction is remarkably clean and that monosubstituted arenes with normally *ortho,para*-directing substituents give *meta* acetoxy isomer to a high extent. Side-chain acetoxylation can be favored for suitable substrates by adding excess acetate ion. The reaction appears to be subject to autoinhibition by the aryl acetates formed.

Previous work on the palladium(II) catalyzed acetoxylation of aromatic substrates has shown that nuclear acetoxylation is favored by the presence of a co-oxidant, such as dichromate,[1] nitrate,[2,3] or peroxydisulfate.[4] Moreover, nuclear acetoxylation of simple benzene derivatives displays a certain degree of selectivity for *meta* substitution,[3–6] in some cases as high as 80 – 90 %. The *meta* selectivity is most pronounced when oxygen itself serves as the co-oxidant,[5,6] but can be retained also with others if a complexing amine of suitable steric requirements is added.[4] 2,2′-Bipyridine has been found to function well in this respect.[3,4]

Semi-micro scale experiments with the system potassium peroxydisulfate/acetic acid in the presence of catalytic amounts of 2,2′-bipyridine-palladium(II) complex have shown [4] that aromatic substrates can be acetoxylated in the ring as well as the α position in a remarkably clean reaction. We now present additional results for this reaction, mainly with the aim of demonstrating its synthetic scope and utility.

RESULTS

The synthetic runs were performed on a 0.25 mol scale with palladium(II) acetate and 2,2′-bipyridine added in catalytic amounts. Potassium peroxydisulfate was employed in slight excess relative to substrate. The heterogeneous reaction mixture was stirred vigorously for 4 h at reflux temperature, after which period the peroxydisulfate had been consumed. Inadequate stirring caused palladium metal to precipitate and thus the catalytic effect to cease rapidly. Control experiments without any catalyst added gave no product at all. In the absence of peroxydisulfate the reaction was very slow, as noted previously,[5,6] only traces of product being obtained after 4 h.

Table 1 shows typical results for a number of aromatic compounds which undergo exclusively or predominantly nuclear acetoxylation, whereas Table 2 gives results for substrates which are acetoxylated to some extent, predominantly or only in the α position of the side-chain. The α substitution reaction is favored by the addition of sodium acetate, as has been found previously,[6,7] and hence the reactions in Table 2 have been run with 0.1 M sodium acetate present.

Inspection of the tables shows that the reaction is indeed catalytic in palladium(II) (yield

* Part VI, see Ref. 4.
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Table 1. Acetoxylation of aromatic compounds by potassium peroxydisulfate with Pd(II) as a catalyst.[a]

Compound (Exp. No.)	Acetoxy derivatives Yield,[b] % A	B	C	D	Isomer distribution *ortho*[d]	*meta*[e]	*para*	Other products (Yield,[c] %)
Benzene (1)	700	28	18	–	–	–	–	Unknowns (3)
Fluorobenzene (2)[f]	400	16	12	–	20	42	38	
Chlorobenzene (3)	940	38	26	82	7	51	42	Dichlorobenzene (trace)
Bromobenzene (4)	860	34	20	–	8	55	37	Dibromobenzene (trace)
Benzotrifluoride (5)[g]	138	6	3	–	Trace	73	27	Unknowns (5)
Acetophenone (6)	400	16	11	81	1	77	22	
Phenyl acetate (7)	605	24	14	–	25	42	33	
Anisole (8)[h]	640	26	15	–	2	58	40	Phenol (2)
Methyl benzoate (9)	575	23	12	–	4	62	34	
Diphenylmethane (10)[i]	748	30	25	68	3	60	37	
t-Butylbenzene (11)	550	22	15	–	Trace	56	44	
Biphenyl (12)	775	31	24	–	3	52	45	
2,2-Dimethylindan (13)[j]	750	30	22	–	–	–	–	2,2-Dimethylindanone (1)
Indan (14)	190	8	0[k]	–	34	66		
Naphthalene (15)	395	16	9	–	22	78		
Thiophene (16)	–	–	–	–	–	–	–	Polymer only
Toluene (17)	630	25	30	65	6	59	36	See also Table 2

[a] Reaction conditions, see text. [b] A: GLC yield based on the amount of Pd(II) used; B: GLC yield based on initial amount of substrate; C: Isolated yield based on initial amount of substrate: D: Yield based on substrate consumed. [c] Yield according to B. [d] α isomer in exp. 15 and 4- in exp. 14. [e] β isomer in exp. 15 and 5- in exp. 14. [f] Reaction period 6 h. [g] Reaction period 8 h. [h] Nuclear acetate 76, phenyl acetoxymethyl ether 24 %. [i] Nuclear acetate 69, α isomer 31 %. [j] [NaOAc] 0.1 M; acetoxy derivatives: 1-acetoxy-2,2-dimethylindan, nuclear acetate (trace). [k] Could not be isolated.

Table 2. Acetoxylation of some methylbenzenes by potassium peroxydisulfate with Pd(II) as a catalyst.[a]

Compound (Exp. No.)	Acetoxy derivatives Yield,[b] % A	B	C	D	Isomer distribution α	Nuclear	Other products (Yield,[c] %)
Toluene (17)[d]	630	25	20	65	10	90[e]	Benzaldehyde
o-Xylene (18)[d]	580	23	15	74	39	61[f]	1,2,4-Trimethylbenzene (+) 3,4-dimethylbenzyl acetate (2)
p-Xylene (19)	470	19	11		86	14	Trimethylbenzenes (5) *p*-Methylbenzaldehyde (5)
Mesitylene (20)	406	16	11		99	Trace	3,5-Dimethylbenzaldehyde (2)
Durene (21)	775	31	19		100	–	Hexamethylbenzene (trace)
Hexamethylbenzene (22)	890	36	29	–	100	–	

[a] Reaction conditions, see text, [NaOAc] 0.1 M. [b] A: GLC yield based on Pd(II); B: GLC yield based on initial amount of substrate; C: Isolated yield based on initial amount of substrate; D: Yield based on substrate consumed. [c] Yield according to B. [d] No sodium acetate present. [e] Isomer distribution: *ortho* 6, *meta* 59, *para* 36. [f] 3,4-Dimethylphenyl acetate.

A) but that the degree of conversion "in one pass", to borrow a phrase from heterogeneous catalysis terminology (yields B and C), is fairly low. On the other hand, yields based on the amount of substrate consumed (D) are high, between 60 and 80 %. We made several attempts to increase the degree of conversion by increasing the peroxydisulfate to substrate ratio but it turned out that the reaction ceased after 4 h, independently of the ratio employed. A control experiment in which a mixture of chlorophenyl acetates was added from the beginning of a reaction aiming at the acetoxylation of chlorobenzene, demonstrated that very little additional chlorophenyl acetate had been formed during the usual reaction period of 4 h. Hence it can be concluded that the reaction is gradually inhibited by the products formed.

DISCUSSION

The acetoxylation of aromatic compounds can be achieved by various oxidative substitution processes, where the oxidant is either the anode [8] or a suitable metal ion,[9] such as Pb(IV), Mn(III), Co(III), Ce(IV), Pd(II), and Ag(II).[10] Of the metal ion promoted reactions, only the two last-mentioned combine the desirable features of being catalytic, giving a reasonably clean reaction, and being selective for ring positions in the presence of C–H bonds in the α position. The palladium(II) reaction has the additional bonus of giving a highly unusual ring substitution pattern, with the *meta* acetoxy compounds as the predominant isomer (Table 1). From the synthetic point of view, this reaction therefore commands great interest as a possibility of modifying substitution patterns in aromatic substitution, *e.g.*, by changing the nature of the amine ligand. We are presently working on this and other aspects of Pd(II) catalyzed acetoxylation.

EXPERIMENTAL

Materials. See Refs. 3–6, 11, and 12.

Acetoxylation procedure. A mixture of substrate (0.25 mol), potassium peroxydisulfate (0.30 mol), palladium(II) acetate (0.01 mol) and 2,2′-bipyridine (0.005 mol) in glacial acetic acid (1000 ml) was stirred vigorously for 4 h at reflux temperature (in exps. 2 and 5, 6 and 8 h, respectively). The reaction mixture was worked up by addition of water (500 ml) and extraction by dichloromethane (500 ml). The water/acetic acid layer was extracted with two additional portions of dichloromethane of 150 ml each. The combined extracts were washed with water (2 × 500 ml), and sodium bicarbonate solution (100 ml) and finally dried with magnesium sulfate. After filtration and evaporation the residue was distilled in a Claisen flask. In some cases where GLC analysis indicated the presence of by-products, the crude product was distilled in a concentric tube column (Fischer Spaltrohr-System) of *ca.* 40 theoretical plates. In experiment 22 the crude product mixture, containing hexamethylbenzene and pentamethylbenzyl acetate, was washed with ligroin (b.p. 40–60 °C) to remove hexamethylbenzene, whereafter the acetate was recrystallized from ethanol.

Analyses. Unless otherwise noted, yields and isomer distributions were determined using a Varian 1400 gas chromatograph, equipped with a disc integrator (2 m × 3 mm neopentylglycol succinate, 5 % on Chromosorb W or 2 m × 3 mm Apiezon L, 10 % on Chromosorb W column). The yields were calculated using an internal standard calibrated against authentic samples. Used standards: In exp. 1, phenol; exps. 2–11, 13, 14, 15, 17, biphenyl; exp. 12, β-naphthyl acetate; exps. 18, 20, 22, 2,4,5-trimethylbenzyl acetate; exp. 19, *m-t*-butylphenyl acetate; exp. 21, 3,5-dimethylbenzyl acetate. The identification of products was based on GLC/MS comparison (LKB 9000 system) with authentic samples.

1-Acetoxy-2,2-dimethylindan was identified by its ^{1}H NMR spectrum: δ 1.12 [6 H, s, $C(CH_3)_2$], 2.06 (3 H, s, $OCOCH_3$), 2.7–2.9 (2 H, m, methylene protons), 5.8 (1 H, s, methine proton), 7.1–7.4 (4 H, m, aromatic protons). The products in exp. 2 (fluorophenyl acetates) and 17 (cresyl acetates and benzyl acetate) were analyzed as the corresponding hydroxy compounds. The *meta*/*para* ratio in exp. 9 was determined by ^{1}H NMR spectroscopy using a lanthanide shift technique.

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Short Communications

The Distribution of the D-Galactose Residues in Guaran and Locust Bean Gum

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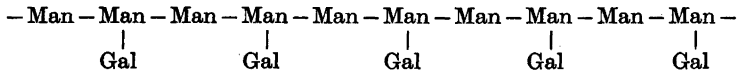
The galactomannans from the seeds of leguminous plants are composed of chains of (1→4)-linked β-D-mannopyranose residues, some of which are substituted at O-6 with an α-D-galactopyranosyl group.[1] The ratio of D-galactose to D-mannose in these polysaccharides varies with their origin, and in two of the most important, guaran and locust bean gum, it is about 1:2 and 1:4, respectively.[1]

X-Ray diffraction spectra of stretched films of guaran were interpreted many years ago [2] as indicating that the D-galactose residues were arranged along the chains in an alternating manner, as shown in (*1*). On the other hand, studies of the digestion of locust bean gum by (1→4)-β-D-mannanase [3] indicated that, whereas rather more than half of the D-galactose residues occupied isolated (non-contiguous) positions in the chains, the remainder was present in block-type structures, as shown in (*2*). The ability of locust bean gum to increase the strength of agar [4] and carrageenan [5] gels has been interpreted [6] as indicating the presence of blocks of contiguous, unsubstituted D-mannose residues, as in (*2*).

We recently studied the distribution of α-D-galactopyranosyl groups in guaran by a method based upon periodate oxidation.[7] It had previously been observed that the periodate oxidation of guaran, containing 36 % D-galactose residues, virtually stopped when about 58 % of the D-mannose residues had been oxidised.[8] For locust bean gum, containing 19 % D-galactose, we have now determined the corresponding value to be 80 %. Anomalous oxidation-limits are also observed for other polysaccharides, such as sodium alginate.[9,10] This effect was traced to the formation of inter-residue hemiacetal linkages between the aldehyde groups of oxidised residues and the hydroxyl groups of adjacent, unoxidised residues. When there is a free hydroxyl at C-6 in the oxidised residue, formation of intra-residue hemiacetals competes with the formation of inter-residue hemiacetals; protection of adjacent residues against periodate oxidation is then incomplete, and they are ultimately fully oxidised. When, however, OH-6 in an oxidised residue is blocked by substitution, the inter-residue hemiacetals are so stable that adjacent units become virtually resistant to subsequent attack by periodate.[8–10]

It follows that, for a galactomannan with an alternating structure (*1*), or any other structure in which all the D-galactose residues



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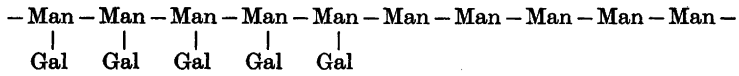

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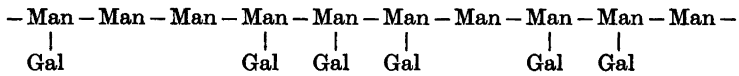

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Table 1. Methylation analysis of periodate oxidised–borohydride reduced locust bean gum. The experimental conditions have been repeated.[7,8] Components (area, of peak, relative to 2,3,6-Man = 1.00) on GLC, column SP 1000.

Time of oxidation	1,4-Eryt [b]	1-Eryt [b]	2,3-Man
0 min [a]	–	–	0.37
30 min	0.35	0.16	0.37
90 min	0.24	0.26	0.42
3 h	0.33	0.37	0.43
5 h	0.33	0.45	0.43
24 h	0.43	0.62	0.42
30 h	0.50	0.68	0.45
48 h	0.95	0.67	0.45

[a] In the analysis of unoxidised polysaccharide 2,3,4,6-tetra-*O*-methyl-D-galactose (20 %) was also detected. [b] These figures are inaccurate because the volatility of acetylated tetritol ethers leads to considerable losses during the evaporation of solutions.[7]

are isolated, only unbranched D-mannose residues would be completely protected from oxidation. A methylation analysis of the oxidised and borohydride-reduced [8,10] polysaccharide should therefore give 2,3,6-tri-*O*-methyl-D-mannose as the only hexose derivative in the hydrolysate. On the other hand, for a galactomannan in which all the D-galactose residues are contiguous, as in (*2*), it would be mostly 6-*O*-substituted D-mannose residues that were protected, and methylation analysis should give, almost exclusively, 2,3-di-*O*-methyl-D-mannose as the only hexose derivative.

For a perfectly random distribution of D-galactose residues, and an equal rate of oxidation of unsubstituted and substituted D-mannose residues, the ratio between 2,3-di-*O*-methyl- and 2,3,6-tri-*O*-methyl-D-mannose should be the same as in the methylation analysis of the original galactomannan. In fact, there is an indication [7] that the unsubstituted residues are oxidised somewhat faster than the substituted residues, so that the ratio should be a little higher for the oxidised than for the original polysaccharide.

For guaran, the above ratio for the oxidised material was approximately 2:1, on a molar basis, compared to 1.6:1 as observed for the starting material.[7] For locust bean gum it increased from 0.37, for the starting material, to a constant value of about 0.45 (Table 1).

It is evident, therefore, that simple, alternating structures (*1*) and simple, block structures (*2*) are excluded for both polysaccharides. The results are fully consistent with a near-random arrangement such as (*3*), which is the simplest interpretation of the present data. However, they are not in conflict with the kind of structure proposed by Courtois and Le Dizet [3] for locust bean gum, in which sequences such as (*1*) and (*2*) are both present, in a ratio (based upon D-mannose) of approximately 2:1.

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Debromination of 1,2-Dibromides with Sodium Dithionite *

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In connection with a current investigation of sulfinates,[1] we have found that sodium dithionite can act as a debromination agent.[2]

Treatment of *meso*-α,α′-dibromostilbene with an excess of anhydrous sodium dithionite in refluxing *N*,*N*-dimethylformamide afforded *trans*-stilbene almost quantitatively. The re-

* Kempe, T. and Norin, T., presented in part at "Organikerdagarna", Stockholm, June 1972.

Table 1. Methylation analysis of periodate oxidised–borohydride reduced locust bean gum. The experimental conditions have been repeated.[7,8] Components (area, of peak, relative to 2,3,6-Man = 1.00) on GLC, column SP 1000.

Time of oxidation	1,4-Eryt [b]	1-Eryt [b]	2,3-Man
0 min [a]	–	–	0.37
30 min	0.35	0.16	0.37
90 min	0.24	0.26	0.42
3 h	0.33	0.37	0.43
5 h	0.33	0.45	0.43
24 h	0.43	0.62	0.42
30 h	0.50	0.68	0.45
48 h	0.95	0.67	0.45

[a] In the analysis of unoxidised polysaccharide 2,3,4,6-tetra-*O*-methyl-D-galactose (20 %) was also detected. [b] These figures are inaccurate because the volatility of acetylated tetritol ethers leads to considerable losses during the evaporation of solutions.[7]

are isolated, only unbranched D-mannose residues would be completely protected from oxidation. A methylation analysis of the oxidised and borohydride-reduced [8,10] polysaccharide should therefore give 2,3,6-tri-*O*-methyl-D-mannose as the only hexose derivative in the hydrolysate. On the other hand, for a galactomannan in which all the D-galactose residues are contiguous, as in (*2*), it would be mostly 6-*O*-substituted D-mannose residues that were protected, and methylation analysis should give, almost exclusively, 2,3-di-*O*-methyl-D-mannose as the only hexose derivative.

For a perfectly random distribution of D-galactose residues, and an equal rate of oxidation of unsubstituted and substituted D-mannose residues, the ratio between 2,3-di-*O*-methyl- and 2,3,6-tri-*O*-methyl-D-mannose should be the same as in the methylation analysis of the original galactomannan. In fact, there is an indication [7] that the unsubstituted residues are oxidised somewhat faster than the substituted residues, so that the ratio should be a little higher for the oxidised than for the original polysaccharide.

For guaran, the above ratio for the oxidised material was approximately 2:1, on a molar basis, compared to 1.6:1 as observed for the starting material.[7] For locust bean gum it increased from 0.37, for the starting material, to a constant value of about 0.45 (Table 1).

It is evident, therefore, that simple, alternating structures (*1*) and simple, block structures (*2*) are excluded for both polysaccharides. The results are fully consistent with a near-random arrangement such as (*3*), which is the simplest interpretation of the present data. However, they are not in conflict with the kind of structure proposed by Courtois and Le Dizet [3] for locust bean gum, in which sequences such as (*1*) and (*2*) are both present, in a ratio (based upon D-mannose) of approximately 2:1.

Acknowledgements. Free samples of guaran and locust bean gum were kindly supplied by Meyhall Chemical AG, Kreuzlingen, Switzerland. This work was supported by the Swedish Natural Science Research Council.

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Treatment of *meso*-α,α′-dibromostilbene with an excess of anhydrous sodium dithionite in refluxing *N*,*N*-dimethylformamide afforded *trans*-stilbene almost quantitatively. The re-

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action appeared to be fast and was complete in about 15 min. Similar treatment of *trans*-1,2-dibromocyclohexane gave cyclohexene, which distilled during the debromination. The formation of cyclohexene was complete after 30 min with an overall 60 % yield.

The reaction was not stereospecific as shown by an investigation of the debromination of *meso*- and *racemic*-2,3-dibromobutanes. The butenes thus formed were collected and analysed by GLC. Both the *meso*- and *racemic*-2,3-dibromobutanes gave a mixture of *cis*- and *trans*-2-butenes in the ratio 1:1 in about 65 % of the theoretical yield.

Reduction reactions involving sodium dithionite can formally be considered to proceed *via* nucleophilic attack of the dithionite anion followed by cleavage of the sulfur-sulfur bond to sulfur dioxide and an intermediate sulfinate [3-7] or *via* electron transfers by sulfur dioxide radical anions ($SO_2^{\cdot-}$).[8-12]

The results obtained in the debromination reaction may be explained by assuming a nucleophilic attack of the dithionite anion *via* a common carbocation intermediate to give a mixture of *threo*-2-bromo-1-methylpropanesulfinate and *erythro*-2-bromo-1-methylpropanesulfinate. The sulfinates should then decompose in a stereospecific manner to *cis*- and *trans*-2-butene, bromide ion and sulfur dioxide, respectively, according to a known reaction.[1]

Available data, however, do not exclude a mechanism involving a nucleophilic attack of the sulfur dioxide radical anion ($SO_2^{\cdot-}$) on an intermediate carbocation from the vicinal dibromides. An alternative mechanism would be a one-electron transfer reaction, which lacks high stereospecificity.[13] Electrochemical reductions of vicinal dibromides, however, proceed in a stereospecific manner,[14] which may be due to experimental conditions.

Experimental. Debromination of meso-α,α'-*dibromostilbene. meso*-α,α'-Dibromostilbene (13.6 g, 0.04 mol) and sodium dithionite (8.7 g, 0.05 mol) were dissolved in *N*,*N*-dimethylformamide (100 ml). The reaction mixture was heated for 15 min at 140 – 145 °C and then poured into water (1000 ml). The stilbene precipitate was filtered off. Recrystallization from ethanol yielded *trans*-stilbene (6.5 g, yield 90 %), identified by comparison with an authentic sample (m.p. and mixed m.p. 124 °C).

Debromination of trans-*1,2-dibromocyclohexane. trans*-1,2-Dibromocyclohexane (6.0 g, 0.025 mol) and sodium dithionite (8.7 g, 0.05 mol) were dissolved in *N*,*N*-dimethylformamide (50 ml). The reaction mixture was heated for 1 h at 140 – 145 °C. The cyclohexene thus formed was continuously distilled from the reaction mixture (1,2 g, b.p. 83 °C/760 mmHg, yield 60 %) and characterized by comparison with an authentic sample.

Debromination of meso- *and* racemic-*2,3-dibromobutane.* To a stirred mixture of sodium dithionite (17.5 g, 0.1 mol) in *N*,*N*-dimethylformamide (200 ml) at 140 – 145 °C was added *meso*- or *racemic*-2,3-dibromobutane (10.8 g, 0.05 mol) over 10 min. The gaseous butenes were formed immediately and collected in about 60 – 65 % yield in a graduated cylinder *via* a washing bottle containing 2 M aqueous sodium hydroxide. Both the *meso*- and *racemic*-2,3-dibromobutanes gave a mixture of *cis*- and *trans*-2-butenes in the ratio 1:1 as shown by GLC analysis (column, 4.5 m × 3 mm, packed with 15 % dimethylsulfolane on Gas Chrom. RZ 60/80 mesh, relative retention times *trans*- and *cis*-2-butene 1.00:1.08, column temperature 30 °C).

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1,4-Dihydroxy-2-methoxy-6-methylbenzene, a Metabolite of *Penicillium baarnense*

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The mould *Penicillium baarnense* produces as secondary metabolites among other substances orsellinic acid, penicillic acid and barnol.[1] The relative amounts of these substances are highly dependent on the composition of the culture substrate. When Czapek-Dox medium is used, only trace amounts of barnol are formed whereas orsellinic acid and penicillic acid are produced in substantial amounts. The reverse situation is at hand, when the organism is grown on Raulin-Thom medium. When investigating this phenomenon the formation of a phenolic compound was observed irrespective of culture substrate used. The phenolic compound appeared as the first phenolic substance excreted into the culture fluid when the organism was grown on Raulin-Thom medium. However, the compound was reutilized by the organism at the time of barnol production. As the compound could be on the pathway of barnol synthesis it seemed important to establish its structure.

The organism was grown on Czapek-Dox medium in a fermentation tank. The phenolic compound was isolated from the culture filtrate by extraction with organic solvent. The purification procedure involved washing with aqueous hydrogen carbonate, sublimation and recrystallization from carbon tetrachloride, m.p. 130–131 °C, ν_{max} (KBr) 3320, 1610 cm^{-1}, λ_{max}(MeOH) 208 (12 660), 288.5 (3918) nm. The NMR spectrum ($CDCl_3$) showed resonances due to one aromatic methyl group (δ 2.12, 3 H, s), one methoxy group (δ 6.10, 3 H, s) and the signals of two aromatic protons (δ 6.36 dd, J 2.0 Hz). Resonance that could be assigned to two phenolic hydroxy groups (δ 5.27, 2 H, s) were also visible. The mass spectrum had a large molecular ion peak (% relative intensities), *m/e* 154 (100) and further abundant fragments were found at *m/e* 139 (79) and *m/e* 111 (51).

The spectroscopic data indicated the structure shown in the figure. This structure was confirmed by comparison with a synthetic specimen. The preparation of 1,4-dihydroxy-2-methoxy-6-methylbenzene was performed by reduction with sodium dithionite of the corresponding quinone, the synthesis of which was recently reported.[2] It has been demonstrated that the quinone is an intermediate in the biosynthesis of penicillic acid in *Penicillium cyclopium*.[2,3] The presence of its dihydro derivative in *P. baarnense* has presumably no relation to the barnol formation but to the penicillic acid synthesis in this organism. Apparently, when *P. baarnense* is grown on Raulin-Thom medium the organism develops an alternative route for the degradation of 1,4-dihydroxy-2-methoxy-6-methylbenzene which does not lead to penicillic acid.

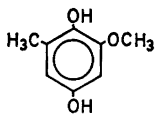

Fig. 1. 1,4-Dihydroxy-2-methoxy-6-methylbenzene.

Experimental. Infrared spectra of KBr discs were recorded on a Perkin-Elmer model 257 spectrometer and ultraviolet spectra on a Beckman DK-2. The NMR spectra ($CDCl_3$) were recorded on a Varian Anaspect EM 360 instrument with TMS as internal standard and the mass spectrum on a LKB instrument type 9000 using the direct inlet system.

Penicillium baarnense v. Bayma, CBS 315.59 was grown in 5 l of Czapek-Dox medium ($NaNO_3$, 2.0 g; KH_2PO_4, 1.0 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; yeast extract, 1.0 g; glucose, 40 g; distilled water to 1000 ml) using a New Brunswick tank fermentor (300 rpm, aeration 4000 ml/min) at 27 °C. The fermentor was inoculated with culture from two 500 ml shake flasks pregrown for 3 days on the same medium. After 4 days the mycelium was filtered off and washed with water. After acidification with HCl the filtrate was extracted with ether. The acid substances were removed from the ether phase by washing with aqueous $NaHCO_3$ followed by distilled water. The ether solution was dried over anhydrous Na_2SO_4 and then evaporated to dryness in a rotary evaporator. The semisolid residue was sublimed at 12 mmHg and 100 °C and the white sublimate repeatedly recrystallized from CCl_4, yield 100 mg.

Acknowledgement. This work was supported by grants from the Swedish Natural Science Research Council.

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Synthesis of [2.2](3,6)-Phenanthrenophanediene

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Several large ring compounds of fused benzene, thiophene and furan rings have been synthesized. Dopper and Wynberg [1] have suggested and defined the name circulenes for a special class of such compounds. Coronene or [6]circulene is a well-known member of this class. Like coronene,[2] tetraoxo[8]circulene [3] and some thia-[7]circulenes [4] seem to be planar molecules. A non-planar circulene is also known; corannulene or [5]circulene is bowl-shaped because of the short periphery.[5] On the other hand, [7]circulene and [8]circulene should have long peripheries and thus be expected to form saddle-shaped molecules.

We now report an attempted synthesis of [8]-circulene by photochemical ring closures of [2.2.2.2]paracyclophanetetraenes which can be conveniently prepared by Wittig reactions.[6]

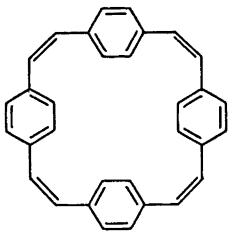
Results and discussion. The photolysis of [2.2.2.2]paracyclophanetetraene *1* in the presence of iodine and air gave no [8]circulene *2*. Instead, products from the oxidation of double bonds were observed.

Iodo-substituents in [2.2.2.2]paracyclophanetetranes might favour photochemical ring closures under an inert atmosphere to avoid photo-oxidation of strained double bonds. Such a cyclophane *3* was prepared by a Wittig reaction between terephthalaldehyde and the bis-triphenylphosphonium salt from α,α'-dibromo-2-iodo-*p*-xylene (Scheme 1).

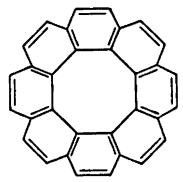
Photolysis of di-iodo[2.2.2.2]paracyclophanetetraene *3* under nitrogen gave a 15 % yield of [2.2](3,6)phenanthrenophanediene *4*. The cyclophane *4* has four internal hydrogens which show a large chemical shift δ 9.46 whereas the outer protons show somewhat smaller chemical shifts than the same protons in phenanthrene.[7] This could be due to a small paramagnetic ring current over the entire molecule as in [4n]annulenes [8] indicating a relatively planar structure with π-electron delocalisation.

However, the UV spectrum of cyclophane *4* is very similar to that of phenanthrene, and the molar extinction coefficient is twice as large which may indicate that the two phenanthrene parts of [2.2](3,6)phenanthrenophanediene *4* are almost perpendicular *5* with no significant π-electron interaction as inspection of simple molecular models of the compound shows.

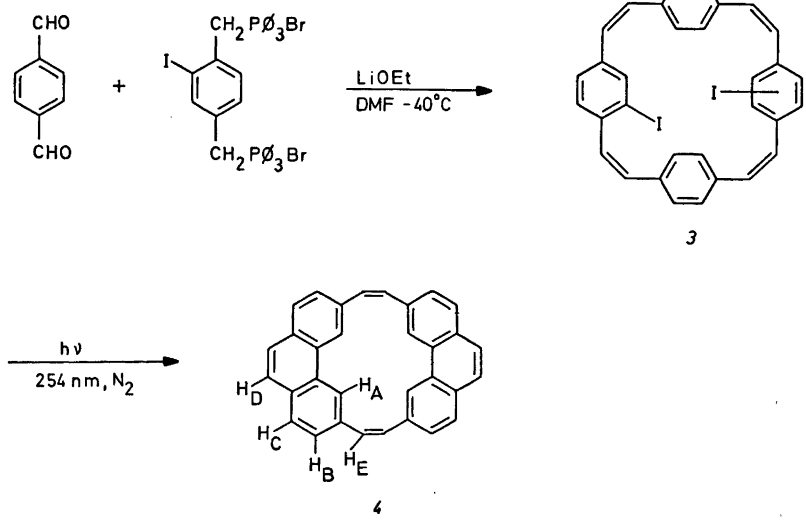
[2.2](3,6)Phenanthrenophanediene *4* was irradiated under various conditions. In the absence of air the compound is relatively stable and decomposes slowly. In the presence of air



1



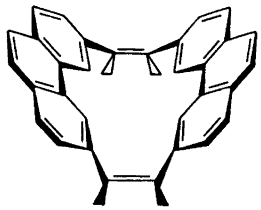
2



3

4

Scheme 1.



5

the compound decomposes to give a complex mixture of aromatic aldehydes, showing the ease of photo-oxidation of strained double bonds. No [8]circulene *2* has yet been observed among the reaction products.

Clearly photo-oxidation must be suppressed in order to prepare [8]circulenes by photolysis of suitable [2.2.2.2]cyclophanetetraenes. One possibility is to photocyclize tetraiodo[2.2.2.2]-paracyclophanetetraene under an inert atmosphere, another interesting possibility is to photocyclize [2.2.2.2]cyclophanetetraenes with two or more thiophene or furan rings to obtain hetero[8]circulenes with less steric strain in reaction intermediates and products. These routes to [8]circulenes are now being tried.

Experimental. Mass spectra were recorded on an AEI MS 902, UV spectra on a Beckman DK 2, IR spectra on a Beckman IR 9 and NMR spectra on a Bruker WH 270. Photochemical experiments were run in a Rayonet reactor RPR-100 with low pressure mercury lamps (254 nm).

*Bistriphenylphosphonium salt of α,α'-dibromo-2-iodo-*p*-xylene.* 2-Iodo-*p*-xylene (0.1 mol), recrystallized *N*-bromosuccinimide (0.22 mol) and dibenzoyl peroxide (0.1 g) were refluxed in dry redistilled carbon tetrachloride (500 ml) for 24 h. The hot solution was filtered to remove succinimide. α,α'-Dibromo-2-iodo-*p*-xylene precipitated from the cooled filtrate (10.2 g, 26 %, m.p. 114–16 °C). The crude product was refluxed in dry dimethyl formamide (200 ml) with triphenylphosphine (57.8 g) overnight. The white crystals formed were collected, washed with ethanol and dried in a vacuum at 110 °C. The yield was 90 %. (Found: C 57.7; H 4.12; Br 17.6; I 13.2; P 6.65. Calc. for $C_{44}H_{37}Br_2IP_2$: C 57.9; H 4.10; Br 17.5; I 13.9; P 6.60).

4,20(21)-Di-iodo[2.2.2.2]paracyclophanetetraene 3. Terephthalaldehyde (10 mmol) and the bistriphenylphosphonium salt from α,α'-dibromo-2-iodo-*p*-xylene (10 mmol) were stirred in dry dimethylformamide (500 ml) under nitrogen. The mixture was kept at −40 °C in a thermostated bath. A freshly prepared solution of lithium ethoxide in ethanol (*ca.* 0.3 M) was added dropwise to allow for consumption of the coloured ylid between successive additions. The addition required 10 h. When no colour change was observed on addition of the base the reaction mixture was diluted with water (*ca.* 500 ml) and extracted with diethyl ether (3 × 500 ml). The combined ether solution was washed with water several times, dried over sodium sulfate and the solvent distilled off. The yellow residue was chromatographed on silica gel with dichloromethane as eluent. The first yellow fraction gave yellow needles (0.33 g, 10 %) melting at 221–224 °C after recrystallisation from carbon tetrachloride. (Found: C 58.3; H 3.36; I 38.4. Calc. for $C_{32}H_{22}I_2$: C 58.2; H 3.35; I 38.4). IR: 1585 cm^{-1}(m), 1418 (s), 1028 (s), 895 (s), 840 (s). UV: 302 nm, log $\varepsilon = 4.71$. MS: *m/e* 660 (M^+, 100 %), 532 (8), 406 (50), 405 (12), 404 (10). NMR: δ 7.75 (~1, broad s, protons adjacent to iodine), 7.29–7.27 (2, m, protons *meta* and *para* to iodine), 7.18–7.13 (4, m, aromatic protons), 6.53–6.37 (4, m, olefinic protons).

A second yellow fraction from the chromatography gave a yellow residue (< 1 %). The mass spectrum was consistent with a tri-iodo-[2.2.2.2.2.2]paracyclophanehexaene (*m/e* 990, M^+, 45 %).

[2.2](3,6)Phenanthrenophanediene 4. Cyclophane *3* (0.1 mmol, 66 mg) was dissolved in benzene (150 ml, spectroscopic grade) and nitrogen was bubbled through the solution for 1 h. The water-cooled solution was then irradiated for 3 h. The solvent was distilled off and the residue purified by chromatography on silica gel with carbon tetrachloride as eluent. The second fraction gave almost white crystals of [2.2](3,6)phenanthrenophanediene *4* (6 mg, 15 %, m.p. 321–323 °C) after recrystallization from ethanol. (Abs. mass: Found 404.154 ± 0.003. Calc. for $C_{32}H_{20}$ 404.156). IR: 1603 cm^{-1} (m), 1235 (m), 1150 (m), 910 (s), 878 (s), 850 (s), 839 (s), 740 (s) and 657 (s). UV: 250 nm, log $\varepsilon = 5.11$. NMR: δ 9.45 (4, broad s, H_A), 7.71 (4, d, H_C), 7.58 (4, s, H_D), 7.41 (4, broad d, H_B, $J_{BC} = 8.0$ Hz) and 7.23 (4, s, H_E). MS: *m/e* singly charged ions; 405 (35), 404 (100), 403 (8), 402 (12), 401 (8), 400 (10), 399 (5), 398 (8), 387 (9), 374 (6), doubly charged ions; 202.5 (6), 202 (22), 201.5 (10), 201 (20), 200.5 (10), 200 (20), 199.5 (7), 199 (15), 193.5 (12), 187 (14).

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Preparation and Relative Configurations of Some 2,2′-Spirobiindanes

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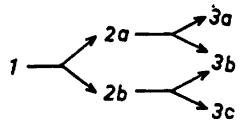
Stepwise reduction of 2,2′-spirobi[2*H*-indene]-1,1′(3*H*,3′*H*)-dione (*1*) according to Scheme 1 gives two diastereomeric monohydroxy ketones (*2a,b*) and three diastereomeric dihydroxy compounds (*3a,b,c*); formulae, see Fig. 1 and Table 1.



Scheme 1.

The monohydroxy ketone (*2b*) and the dihydroxy compound (*3b*) were previously prepared [1] by reduction of *1* with lithium tri-*t*-butoxyaluminium hydride [$LiAl(O\text{-}t\text{-}Bu)_3H$] and lithium aluminium hydride, respectively, in tetrahydrofuran (THF).

In continuation of these experiments, *1* was reduced with excess of $LiAl(O\text{-}t\text{-}Bu)_3H$ in THF. The product was a mixture of the epimeric dihydroxy compounds (*3a*) and (*3b*) of which *3a* was isolated in 60 – 65 % yield.

Reduction of *2b* with $LiAl(O\text{-}t\text{-}Bu)_3H$ in THF afforded a mixture of preferentially *3b* and *3c*.

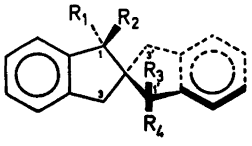


Fig. 1.

Table 1. ^1H NMR data [a] for some 2,2′-spirobiindane derivatives in DMSO-d_6 solution.

Signal	δ [b]	δ_{AB} [c]	J_{AB}/Hz [c]
4,[2] $R^1=R^2=R^3=R^4=H$			
CH_2	2.89		
5,[2] $R^1,R^2=$ $=O$; $R^3=R^4=H$			
H(1′)=H(3′)	3.08	0.39	15.5
H(3)	3.18		
6, $R^1=R^2=H$; $R^3,R^4=H,OH$			
H(1) [d]	2.85	*f*	*f*
H(1′) (H)	4.78	0.60	6.0
(OH)	5.38		
H(3′) [d]	2.86	*f*	*f*
H(3)	2.93	0.83	16.0
2a, $R^1,R^2=$ $=O$; $R^3,R^4=OH,H$			
(H1′) (H)	5.01	0.42	6.5
(OH)	5.43		
H(3′)	3.12	0.44	16.0
H(3)	3.23	0.08	17.9
2b, $R^1,R^2=$ $=O$; $R^3,R^4=H,OH$			
H(1′) (H)	5.33	0.45	6.3
(OH)	5.78		
H(3′)	3.03	0.32	15.4
H(3)	3.21	0.92	17.3
3a, $R^1,R^2=H,OH$; $R^3,R^4=OH,H$			
H(1)=H(1′) (H)	4.99	0.43	4.2
(OH)	5.42		
H(3)=H(3′)	2.73	0.62	15.5
3b, $R^1,R^2=H,OH$; $R^3,R^4=H,OH$			
H(1) [e] (H)	4.59	0.55	6.5
(OH)	5.14		
H(1′) [e] (H)	5.27	0.48	6.5
(OH)	4.79		
H(3)	3.00	0.90	16.0
H(3′)	2.77	*f*	*f*
3c, $R^1,R^2=OH,H$; $R^3,R^4=H,OH$			
H(1)=H(1′) (H)	5.01	0.46	6.2
(OH)	5.47		
H(3)=H(3′)	2.79	0.81	15.6

[a] The spectra were recorded at 60 MHz except for compound *2a* which was recorded at 100 MHz. TMS was used as internal reference. [b] For the methylene groups the quoted values are the arithmetic means of the chemical shifts of the A and B protons. [c] δ_{AB} and J_{AB} are the differences in chemical shift between protons A and B, and the corresponding coupling constants, respectively. [d,e] Assignments may be reversed. [f] Not calculated because of lack of fine structure.

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Preparation and Relative Configurations of Some 2,2′-Spirobiindanes

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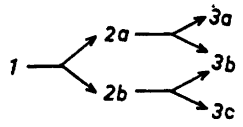
Stepwise reduction of 2,2′-spirobi[2*H*-indene]-1,1′(3*H*,3′*H*)-dione (*1*) according to Scheme 1 gives two diastereomeric monohydroxy ketones (*2a,b*) and three diastereomeric dihydroxy compounds (*3a,b,c*); formulae, see Fig. 1 and Table 1.



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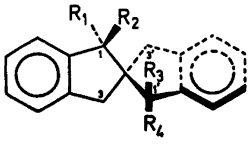
Reduction of *2b* with $LiAl(O\text{-}t\text{-}Bu)_3H$ in THF afforded a mixture of preferentially *3b* and *3c*.



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H(3)=H(3′)	2.79	0.81	15.6

[a] The spectra were recorded at 60 MHz except for compound *2a* which was recorded at 100 MHz. TMS was used as internal reference. [b] For the methylene groups the quoted values are the arithmetic means of the chemical shifts of the A and B protons. [c] δ_{AB} and J_{AB} are the differences in chemical shift between protons A and B, and the corresponding coupling constants, respectively. [d,e] Assignments may be reversed. [f] Not calculated because of lack of fine structure.

The yield of *3c* was 65–70 %. The NMR spectra of *3b* and *3c* demonstrated that these two dihydroxy compounds were identical with those previously obtained by reduction of the ω-camphanic ester of *2b* with $LiAlH_4$ in THF.[1]

The dihydroxy compounds (*3a,b,c*) were characterized as their diacetates.

Partial reoxidation of the dihydroxy compounds by means of manganese dioxide demonstrated that *3a* and *3c* gave only a single monohydroxy ketone *2a* and *2b*, respectively, while *3b* afforded a mixture of *2a* and *2b*. *3a* could be differentiated from *3c* since *3a* formed an *O,O*-isopropylidene derivative on treatment with 2,2-dimethoxypropane and a catalytic amount of *p*-toluenesulfonic acid.

The relative configurations of the monohydroxy ketones (*2a,b*) and the dihydroxy compounds (*3a,b,c*) could be established by studying the ^1H NMR splitting pattern of the AB systems exhibited by the protons at the 1,1′ and 3,3′ positions, respectively, in compounds *2a–6*, (see Fig. 1 and Table 1).

The relative assignments of the secondary alcohol groups and the methylene groups, as well as the specification of the H and OH signals in the monohydroxy and dihydroxy compounds, were performed by deuteration of the hydroxyl groups.

The assignments of the monohydroxy ketones (*2a,b*) and the dihydroxy compounds (*3a,b,c*) were based mainly on the variation in the δ_{AB} parameters of the H(3) and H(3′) methylene groups as a result of the different configurations of the secondary alcohol groups H(1′) and H(1), respectively.

The chemical oxidations of *3a,b,c*, the formation of an *O,O*-isopropylidene derivative of *3a* and the NMR assigments according to Table 1 were consistent with the correlations depicted in Scheme 1. The isolation of *2b* and *3a* after reduction of *1* with deficiency and excess of $LiAl(O\text{-}t\text{-}Bu)_3H$, respectively, was, however, incompatible with Scheme 1. The observation that *2a* could easily be rearranged to *2b* under alkaline conditions as well as the detection of small amounts of *2a* in the monoreduction of *1* with $LiAl(O\text{-}t\text{-}Bu)_3H$ suggested that *2a* was the primary reduction product which epimerized to *2b* under the alkaline conditions used in the work-up procedure.

Experimental. NMR spectra were recorded on Varian A-60 and XL-100 spectrometers. IR spectra were recorded on a Perkin-Elmer model 521 spectrophotometer. Elemental analyses were performed by Novo Industry A/S, Copenhagen.

cis,cis-*1,1′,3,3′-Tetrahydro-2,2′-spirobi*[2H-*indene*]-*1,1′-diol* (*3a*). A solution of *1* (3.72 g, 15 mmol) in THF (150 ml, distilled over $LiAlH_4$) was added to a stirred mixture of $LiAl(O\text{-}t\text{-}Bu)_3H$ (15.27 g, ≥ 90 %, Fluka) and THF (50 ml) at room temperature. The mixture was refluxed with stirring for 3 h. Ethyl acetate (15 ml) was added to the warm reaction mixture. After the exothermic reaction had subsided, the mixture was cooled and H_2O (20 ml) added. Next day, the inorganic precipitate was filtered with suction and washed with warm $CHCl_3$. The combined filtrates were evaporated and the crystalline residue was recrystallized from benzene (220 ml) to give *3a* (2.47 g, 65 %), m.p. 235.5–237.5 °C. Anal.: $C_{17}H_{16}O_2$: C, H. Evaporation of the filtrate from *3a* afforded two fractions, the first of which contained mainly *3b* (0.42 g).

trans,trans-*Isomer* (*3c*). The hydroxy ketone *2b* (1.0 g, 4 mmol) was added to a mixture of $LiAl(O\text{-}t\text{-}Bu)_3H$ (4.07 g) and THF (54 ml). The mixture was stirred and refluxed for 3 h and then decomposed by addition of ethyl acetate (4 ml) and H_2O (5 ml). After work-up as described for *3a*, the product was recrystallized from benzene to give *3c* (0.71 g, 70 %), m.p. 154.5–155.5 °C. Anal.: $C_{17}H_{16}O_2$: C, H. Evaporation of the filtrate from *3c* afforded a crystalline residue which was shown by means of NMR and TLC to contain mainly *3b*.

Diacetyl derivatives of 3a,b,c. A mixture of the dihydroxy compound (1 mmol), acetic anhydride (10 mmol) and pyridine (2 ml) was left at room temperature for 2 days. The reaction mixture was concentrated *in vacuo* and the residue dissolved in benzene (5–10 ml). The benzene solution was washed successively with HCl (3 N), Na_2CO_3 (5 %) and saturated NaCl solution. After drying and evaporation of the solution to dryness, the residue was recrystallized from benzene-light petroleum to give the pure diacetate in 70–90 % yield. Diacetate of *3a*. M.p. 202–203.5 °C. Anal.: $C_{21}H_{20}O_4$: C, H. ^1H NMR (60 MHz, $CDCl_3$): δ 7.66–7.17 (8 H, m), 6.28 (2 H, s), 2.84 (4 H, dd, *J* 15 Hz), 1.94 (6 H, s). Diacetate of *3b*. M.p. 143–144 °C. Anal.: $C_{21}H_{20}O_4$: C, H. ^1H NMR (60 MHz, $CDCl_3$): δ 7.51–7.18 (8 H, m), 6.49 (1 H, s), 5.92 (1 H, s), 3.19 (2 H, dd, *J* 16 Hz), 2.97 (2 H, s), 2.06 (3 H, s), 1.94 (3 H, s). Diacetate of *3c*. M.p. 156–157.5 °C. Anal.: $C_{21}H_{20}O_4$: C, H. ^1H NMR (60 MHz, $CDCl_3$): δ 7.43–7.17 (8 H, m), 6.23 (2 H, s), 3.12 (4 H, dd, *J* 16 Hz), 1.85 (6 H, s).

O,O-*Isopropylidene derivative of 3a*. A mixture of *3a* (0.2 g), *p*-toluenesulfonic acid (0.02 g, dried at 60–70 °C), and 2,2-dimethoxypropane (4 ml) was left at room temperature for 45 h. $CHCl_3$ (10 ml) was added to the reaction mixture and the organic phase was washed successively with H_2O, $KHCO_3$ (5 %) and saturated NaCl solution. After drying ($CaSO_4$), the solution was evaporated and the residue (0.24 g) recrystallized from light petroleum to give the pure compound (0.13 g), m.p. 109–110.6 °C. Anal.: $C_{20}H_{20}O_2$: C, H. ^1H NMR (60 MHz, $CDCl_3$): δ 7.60–7.16 (8 H, m), 4.94 (2 H, s), 2.95 (4 H, dd, *J* 15.8 Hz), 1.48 (6 H, s). IR (CCl_4): 1228 cm^{-1} (C–O–C).

cis-*1′-Hydroxy-1′,3′-dihydro-2,2′-spirobi*-[2H-*inden*]-*1*(3H)-*one* (*2a*). A mixture of *3a* (1.01 g, 4 mmol) and $CHCl_3$ (80 ml), dried

over $CaCl_2$ and then distilled, was refluxed with stirring. When the mixture was homogeneous, MnO_2 [3] (1.6 g) was added in one lot. Stirring under reflux was continued for 1 h. The warm mixture was filtered with suction through celite. Evaporation of $CHCl_3$ from the filtrate afforded a crystalline residue. The combined residues (3.5 g) from the oxidation of a total of 3.47 g of *3a* were recrystallized from benzene to give unchanged *3a* (2.19 g) and a crystalline residue (1.20 g), which was shown by TLC to be a mixture of the spirodiketone (*1*) and *2a*. This fraction was chromatographed on silica gel (24 × 2 cm) using benzene – $CHCl_3$ as eluent. Evaporation of the first fractions of eluate (85 ml) gave the spirodiketone *1* (0.14 g). A middle fraction (100 ml) contained a mixture of *1* and *2a*. Evaporation of the following fractions (140 ml) afforded mainly *2a* (0.75 g). Recrystallization of this fraction from ethyl acetate – light petroleum (1:3) gave pure *2a* (0.55 g); m.p. 121 – 122.5 °C. (Found: C 81.48; H 5.59. Calc. for $C_{17}H_{14}O_2$: C 81.58; H 5.64). IR (CCl_4): 1724 cm^{-1} (C=O).

Rearrangement of 2a. A mixture of *2a* (0.032 g, 0.125 mmol), ethanol (1.25 ml, 96 %), H_2O (0.75 ml) and aqueous KOH (0.5 ml, 0.5 N) was refluxed for 1 min. The reaction mixture which contained a precipitate was diluted with H_2O (5 ml) and then cooled. The yield of *2b* was 0.0265 g (84 %); m.p. 196.5 – 199.5 °C.

The product was identical with the monoreduction product (*2b*) of *1* [1] (TLC and mixed m.p.).

1,1',3,3'-Tetrahydro-2,2'-spirobi[2H-*indene*]-*1-ol* (*6*). A mixture of $NaBH_4$ (0.12 g, 3 mmol), ethanol (7 ml, 96 %) and the spiroketone (*5*) [2] (0.47 g, 2 mmol) was stirred at room temperature for 2 h. After cooling in ice, cold HCl (3 ml, 2 N) was gradually added followed by $CHCl_3$. The organic phase was washed with water and saturated NaCl solution. After drying ($CaSO_4$), the $CHCl_3$ solution was evaporated, and the residue recrystallized from light petroleum to give *6* (0.40 g, 84 %), m.p. 70.2 – 73.4 °C. (Found: C 86.05; H 6.78. Calc. for $C_{17}H_{16}O$: C 86.40; H 6.83). IR(CCl_4): 3500 cm^{-1} (hydrogen bonded OH), 3660 cm^{-1} (free OH).

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3. Mancera, O., Rosenkranz, G. and Sondheimer, F. *J. Chem. Soc.* (1953) 2189.

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The Molar Absorptivity of Horseradish Peroxidase

P.-I. OHLSSON and K.-G. PAUL

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The absorption coefficient for the Soret band of HRP* has been given by various authors as $\varepsilon_{mM} = 91 - 107$, and this uncertainty has hampered kinetic and stoichiometric analyses.[1,2] HRP has also been stated not to obey Beer's law.[3] The deviations were attributed to aggregations but no experimental support was given. We have redeterminated the light absorption coefficients of HRP and present them together with some comments on storage and handling of HRP.

Materials and methods. HRP A2 (pI 3.9) and C2(pI 8.8) were prepared as described [4] and found to be homogeneous in disc electrophoresis and isofocussing. Samples of HRP were also purchased from Boehringer, Mannheim (grade I), Worthington Biochemical Corp., New Jersey (code HPOFF), Sigma Chemical Company, St. Louis (type IV), and Nutritional Biochemicals, Cleveland (catalogue No. 100963). The commercial preparations were dissolved in phosphate buffer pH 7.0, dialyzed overnight against distilled water, and centrifuged for 30 min at 48 000 *g*.

HRP concentrations were based on pyridine hemochromogen determinations. ε for the α-band of this heme derivative has been given as 34.7 [5] and 33.9 [6] mM^{-1} cm^{-1}, $\Delta\varepsilon$(α-band – minimum at ~540 nm) as 24.6 [5] and 24.5 [6] mM^{-1} cm^{-1}, and the ratio $A_{\alpha\text{-band}}/A_{min}$ as 3.46 – 3.50 [5] and 3.60 [6] The values 33.9 and 24.5 give identical results when used to assay unknown solutions whereas 34.7 and 24.6 do not. For this reason, and because of the higher ratio and the complete absence of a plateau at 610 – 640 nm in the hemochromogen spectrum in the recent study [6] we have used the value $\Delta\varepsilon$ = 24.5 mM^{-1} cm^{-1} in the present investigation.

Cytochrome *c* (Sigma, type VI) was reduced with dithionite in 50 mM sodium phosphate, pH 7.0, and filtered through Sephadex G-25 medium. $\Delta\varepsilon_{550\ red-ox} = 19.6$ [7] mM^{-1} cm^{-1}.

Citric acid "nach Sørensen" (Merck, Darmstadt) was used. Pyridine (Mallinckrodt) was distilled over KOH. Water was distilled twice in all-glass vessels. Buffers, of ionic strength 0.1 unless otherwise stated, were filtered through Sartorius 0.2 μ membrane filters immediately before use. Ammonium sulfate *p.a.* (Merck, Darmstadt) was used for storage of HRP solutions.

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over $CaCl_2$ and then distilled, was refluxed with stirring. When the mixture was homogeneous, MnO_2 [3] (1.6 g) was added in one lot. Stirring under reflux was continued for 1 h. The warm mixture was filtered with suction through celite. Evaporation of $CHCl_3$ from the filtrate afforded a crystalline residue. The combined residues (3.5 g) from the oxidation of a total of 3.47 g of *3a* were recrystallized from benzene to give unchanged *3a* (2.19 g) and a crystalline residue (1.20 g), which was shown by TLC to be a mixture of the spirodiketone (*1*) and *2a*. This fraction was chromatographed on silica gel (24 × 2 cm) using benzene – $CHCl_3$ as eluent. Evaporation of the first fractions of eluate (85 ml) gave the spirodiketone *1* (0.14 g). A middle fraction (100 ml) contained a mixture of *1* and *2a*. Evaporation of the following fractions (140 ml) afforded mainly *2a* (0.75 g). Recrystallization of this fraction from ethyl acetate – light petroleum (1:3) gave pure *2a* (0.55 g); m.p. 121 – 122.5 °C. (Found: C 81.48; H 5.59. Calc. for $C_{17}H_{14}O_2$: C 81.58; H 5.64). IR (CCl_4): 1724 cm^{-1} (C=O).

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Citric acid "nach Sørensen" (Merck, Darmstadt) was used. Pyridine (Mallinckrodt) was distilled over KOH. Water was distilled twice in all-glass vessels. Buffers, of ionic strength 0.1 unless otherwise stated, were filtered through Sartorius 0.2 μ membrane filters immediately before use. Ammonium sulfate *p.a.* (Merck, Darmstadt) was used for storage of HRP solutions.

* HRP, horseradish peroxidase (E. C. 1. 11. 1. 7, donor:H_2O_2 oxidoreductase). RZ, $A_{402.5}/A_{280}$.

Table 1. ε_{mM} for horseradish peroxidase A2 and C2 in 100 mM sodium phosphate, pH 7.0. Mean ± S.D.

HRP A2 nm	ε_{mM}	HRP C2 nm	ε_{mM}
280	26.0[a] ±0.6	280	30.1[b] ±0.6
310[c]	12.9	313[c]	13.1
402.5	109.1[a] ±1.2	402.5	102.1[b] ±1.4
459[c]	9.9	454[c]	9.4
500	11.1	500	11.2
604[c]	2.2	607[c]	2.0
636	3.2	642	3.2

[a] $n=6$, one preparation of HRP A2. [b] $n=10$, four preparations of HRP C2. [c] Minima.

Spectra were registered with a Beckman Acta III spectrophotometer, calibrated against a holmium oxide filter. Linearity in absorbance *vs.* concentration was checked by means of dilutions of a sodium dichromate solution in 5 mM H_2SO_4 for the range A_{257} 0.0085 – 1.620 (four values $A<0.1$).[8] HRP samples to pyridine hemochromogen and absorbance measurements were taken with the same pipette. All spectra were registered at 25 °C.

Results. Freshly prepared [4] HRP A2 and C2 give RZ 4.15 – 4.25 and 3.45 – 3.55; the values decrease by ~2 % within a few days, and the initial RZ cannot be restored by rechromatography. The ratio $A_{\alpha\text{-band}}/A_{min}$ of their pyridine hemochromogen spectra remains, however, at 3.65 – 3.70. The values in Table 1 were obtained on material stored for weeks in saturated ammonium sulfate at +2 °C and then dissolved in, and dialyzed against 100 mM phosphate buffer, pH 7.0. The use of TRIS-HCl at pH 7.0 or citrate at pH 4.5 did not significantly change the absorption coefficients. ε and RZ were independent of the concentration of HRP at ≤ 20 μM; higher concentrations were not tried. These results oppose those reported in Ref. 3, and HRP follows Beer's law (Table 2).

The value $\varepsilon_{402.5}=102.1$ mM^{-1} cm^{-1} implies a further change in the direction wanted by Dunford.[9] A solution of H_2O_2 was found to be 3.92 mM when standardized against Fe(II) cytochrome *c*, using $\Delta\varepsilon_{550}$ (reduced-oxidized) = 19.6 mM^{-1} cm^{-1} and a trace of HRP as catalyst, and 3.93 mM when used to titrate Fe(III) HRP C2 to compound I. Thus $\varepsilon_{402.5}=102.1$ mM^{-1} cm^{-1} is in reasonable agreement with two other numerical values.

The spectra of HRP A2 and C2 differ significantly in ε_{280} and $\varepsilon_{402.5}$ and also in the positions of the minima at 310/313 nm and the maxima at 636/642 nm. The positional difference in red is interesting since this part of the spectrum reflects the binding of the iron rather than the status in the porphyrin ring.[10] $E_{m,pH\,7}$ is −212 mV and −265 mV for these preparations of HRP A2 and C2.[11]

Table 2. Absence of concentration effect on the molar absorption coefficients of horseradish peroxidase C2. Sodium phosphate, μ 0.1, pH 6.96, 25 °C.

HRP C2 μM	$A_{402.5}$	ε_{mM}
0.20	0.020	100.0
0.50	0.052	104.0
0.99	0.106	107.1
1.64	0.167	101.8
3.26	0.335	102.8
6.39	0.654	102.3
12.30	1.270	103.3
17.79	1.859	104.5

RZ of the four commercial preparations ranged from 2.75 to 3.15 at ≤ 20 μ M in sodium phosphate, pH 7.0, increasing slightly with concentration. $\varepsilon_{402.5}$ was found to be 94, 97, 99, and 102 mM^{-1} cm^{-1} at pH 7, the three lower values being given by the lyophilized preparations. Variations in ε_{280} and RZ between preparation from the same manufacturer were sometimes noticed. Preparations with low RZ gave for some reason less reproducibility in both $\varepsilon_{402.5}$ and ε_{280} at < 3 μM concentrations in the citrate buffer. Otherwise these preparations also followed Beer's law.

HRP A2 and C2 in saturated ammonium sulfate solution can be kept for months at +2 °C and for a few days at room temperature without losses of activity or changes in spectrum. On the other hand we have occasionally found lyophilization or freezing (−18 °C) of buffered solutions to cause losses of activity and changes in spectrum, in agreement with published results on freezing.[12]

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Morpholine and Thiophene in Glow Discharge Reactions

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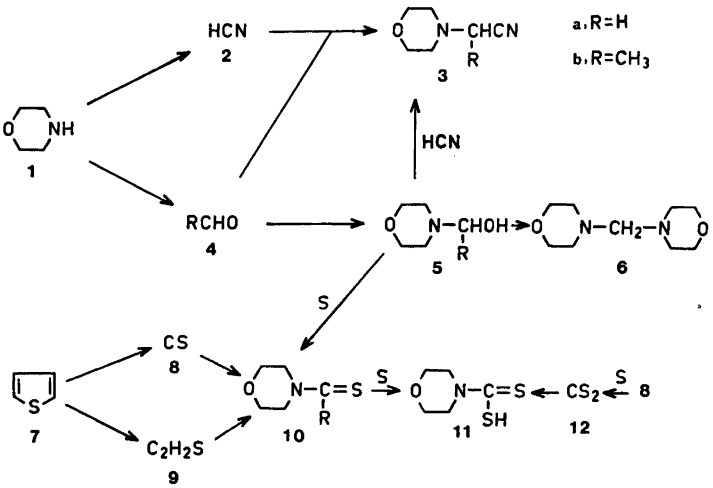
Organic molecules undergo a number of transformations in high frequency glow discharge reactions.[1] Ring-opening reactions of *N*-heterocyclic compounds often yield products in which the original *N*-heteroatom has become part of a nitrile group.[1] We have reexamined [2] the behaviour of morpholine under glow discharge conditions (27 MHz) using the apparatus shown in Fig. 1, and have confirmed morpholinoacetonitrile (*3a*) and dimorpholinomethane (*6*) as major products. A third product was identified as α-morpholinopropionitrile (*3b*) whereas formation of β-morpholinopropionitrile has previously been reported.[2] The products obtained in the glow discharge reactions are explained by intermediate formation of formaldehyde, acetaldehyde and HCN which react further with other molecules of morpholine. The respective product paths were readily demonstrated in solution reactions. Formation of the acetonitrile *3a* has previously been explained by dehydrogenation in the plasma zone of β-morpholinoethylamine; the latter was assumed to be an intermediate reaction product.[2] Such an explanation fails to account for the formation of the propionitrile *3b*.

In the further study morpholine and its reaction products were admixed with the reactive intermediates from plasma reactions of thiophene by feeding morpholine and thiophene together [ratio 5:1 (v/v)] into the glow discharge zone. A solid was identified as the morpholine salt of morpholinocarbodithioic acid (*11*); the other major products were *N*-thioformylmorpholine (*10a*) [3] and *N*-thioacetylmorpholine (*10b*) [4] besides the morpholine derivatives *3* and *6*.

The major solid products from thiophene alone were polymeric, whereas the volatile products were identified as S, H_2S and CS. Carbon monosulfide and thioketene may be the initial products in the reactions of thiophene and will react further with morpholine to yield the thioacyl derivatives *10*. Thioketene is highly reactive. It can be formed by pyrolysis of *tert*-butyl ethynyl sulfide but is polymerised above −80 °C.[5] Photolysis of 1,2,3-thiadiazole has also been shown to yield thioketene and the isomeric ethynylthiol; photodecomposition of thioketene yields carbon mono-



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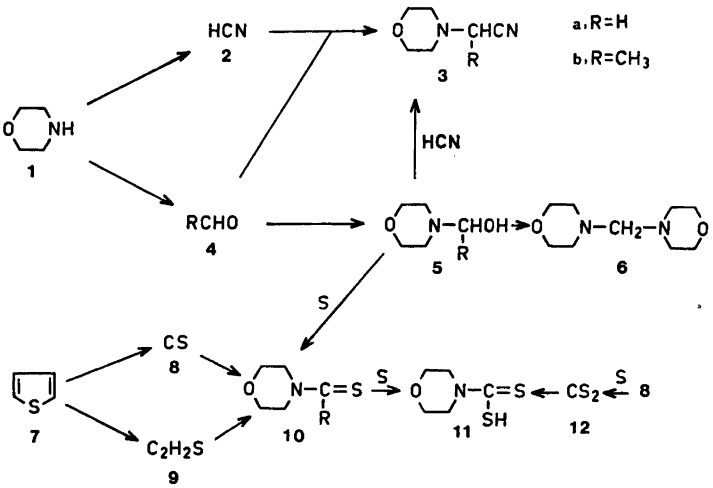

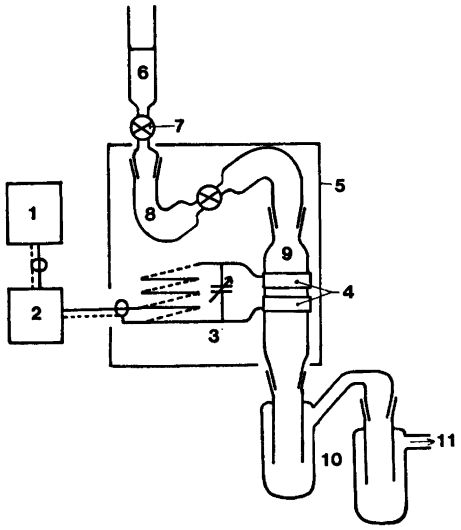

Fig. 1. Apparatus for high frequency glow discharge reactions. For explanations, see text.

sulfide.[6] The major route to the thioacy derivatives *10*, however, probably involves reactions between sulfur radicals formed from thiophene and the intermediate morpholino-adduct *5*, since it has been found that sulfur, formaldehyde and primary and secondary amines react under relatively mild conditions to yield thioformamides.[3] Sulfur is a major product in the plasma reaction of thiophene; in the case of benzo[*b*]thiophene, phenylacetylene and sulfur were the only products isolated.[7] Furthermore, sulfur is known to react with thioformamides to yield the corresponding dithioic acid.[8] By analogy a similar reaction may contribute to the formation of the dithioic acid *11* from *10a* although the major route to this compound is probably the reaction between morpholine and CS_2. It is tempting to speculate that CS_2 in part is formed from thiophene-2-thiol after an initial sulfur radical insertion reaction of thiophene.

Experimental. The glass vessels are made of Pyrex glass except for the reaction tube (9) which is of quartz glass (l. 30 cm, i.d. 3.2 cm). The energy for the discharge is provided by a radio frequency generator (1) (Sommerkamp FT DX 505) operated at 27 MHz with a wattmeter (2) (Thruline model 43) and a tuned circuit. The latter was made from a coil of silver coated 6 mm copper tube (l. 5 cm, d. 5 cm) and a variable capacitor (3) which are coupled to 2 silver bands (4, thick. 1 mm, w. 18 mm) 1 cm apart surrounding the reaction tube (9). The reaction vessels are enclosed in an oven (5) controlled by a thermostat in order to prevent condensation near the plasma zone, and to secure rapid evaporation of the reactants which are fed slowly into the evaporation tube (8) from the reservoir (6) through a capillary valve (7).

The oven was kept at 70 °C and the oil pump (11) was operated at 0.2 mmHg. The distillation rates of morpholine in two separate experiments were 280 and 490 g/kWh using the effects 78 and 100 W, respectively; the product yields were: *3a*, 35 and 30 g/kWh; *3b*, 5 and 10; *6*, 80 and 70.

In another set of two experiments under the same temperature and pressure conditions a mixture of morpholine and thiophene [5:1 (v/v)] were distilled at the rates 115 and 255 g/kWh with effects of 42 and 51 W, respectively; the product yields were: *10a*, 15 and 10 g/kWh; *10b*, 1 and 1; *3a*, 10 and 5; *3b*, 5 and 5; *6*, 25 and 15.

The products were collected in the cooling traps (10) and analysed by GC with a Hewlett-Packard 5700A gas chromatograph on an OV-17 glass column (i.d. 2 mm, l. 2.4 m) with the oven programmed at 80 – 250 °C (4 °C/min) using a flame ionisation detector. Preparative separations were carried out with a Varian 711 gas chromatograph on an OV – 17 aluminium column (9 mm, 9 m) with the oven programmed at 50 – 200 °C (6 °C/min). The identities of the products were established by spectroscopy (MS, NMR) and by comparisons with authentic specimens.

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Synthesis of Penicillin Analogs, (—)-Methyl 6-*epi*-6-Bromobisnorpenicillanate and (±)-Methyl 6-*epi*-6-Bromopenicillanate*

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The [(4-methoxycarbonylthiazolidino)carbonyldihalomethyl]phenylmercury compounds *2a,b* and *5a,b* have been synthesized. When heated in refluxing bromobenzene they form halogenated penicillin analogs. (−)-Methyl 6-*epi*-6-bromobisnorpenicillinate (*3a*) and methyl 6-*epi*-6-bromopenicillinates (*6a,b*) have been prepared and isolated.

As the result of a search for new synthetic methods for β-lactam syntheses that would allow the preparation of penicillin analogs modified in the thiazolidine part, two methods have been developed by Åkermark *et al.*, namely photocyclization of α-oxoamides [1] and thermal decomposition and ring closure of organic mercury compounds.[2-4]

This communication reports the syntheses of (−)-methyl 6-*epi*-6-bromobisnorpenicillanate (*3a*) and (±) methyl 6-*epi*-6-bromopenicillanate (*6a*) by the decomposition of the mercury compounds *2a* and *5a*, respectively. The synthesis of (+)-methyl 6-*epi*-6-bromopenicillanate (*6b*) has been reported as a preliminary communication.[5]

Preparation and isolation of the mercury compounds 2 a, b *and* 5 a, b. A 35−40 % yield of *2a* was obtained when *1a* and phenylmercury chloride were consecutively added to *t*-BuOK in THF at −75 °C.[3] The yield of the dichloro analog *2b* was only about 10 % by this procedure, whereas each of the compounds *5a* and *5b* was isolated in 80 % yield.

The racemic phenylmercury compound *5a* crystallized nicely when treated with diethyl ether. This was not the case with the optically active compounds *2a, b* and *5b*. Attempts to crystallize these from common organic solvents or to extract them selectively from the crude reaction mixtures resulted in decomposition. However, they were successfully purified on a silica gel column at −10 to −20 °C.

Thermal decomposition of the mercury compounds 2a, b and 5a, b. The mercury compounds *2a, b* and *5a, b* decomposed almost completely on refluxing in bromobenzene for 2−3 h. The crude reaction products all exhibited IR absorptions at 1780−1785 cm^{-1}, characteristic of the β-lactam carbonyl of the halogenated penicillin analogs *3a, b* and *6a, b*.[6] From IR data the yields were estimated to be 10−35 %. However, due to decomposition during

* Strained Heterocyclic Compounds. 6. Part 5. Åkermark, B., Byström, S., Florin, E., Johansson, N.-G. and Lagerlund, I. *Acta Chem. Scand. B 28* (1974) 375.

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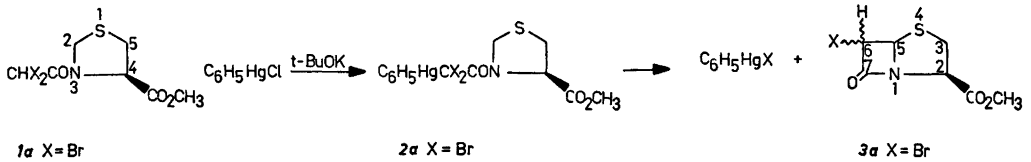
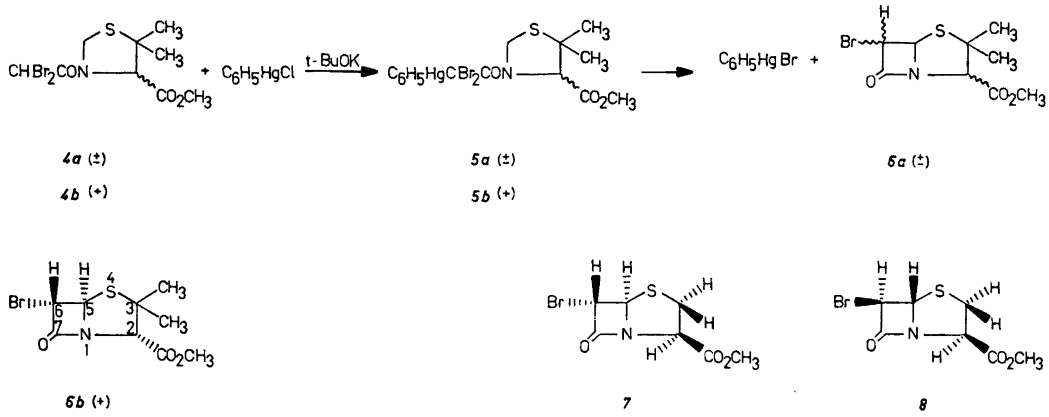


1a X=Br *2a* X=Br *3a* X=Br

1b X=Cl *2b* X=Cl *3b* X=Cl



4a (±)
4b (+)
5a (±)
5b (+)
6a (±)
6b (+)
7
8

the isolation process, the yields of the pure compounds *3a* and *6a, b* were lower (4 – 9 %). Compound *3a* completely decomposed during the isolation process.

Phenylmercury halides (35 – 90 %) were isolated from the decomposition products of each of the compounds *2a, b* and *5a, b*. The dihaloacetamides *1a, b* and *4a, b* (IR 1660 cm^{-1}), were also produced during the decomposition of the corresponding mercury compounds. In addition, unknown substances with IR absorptions at 1660 – 1680 cm^{-1} were formed in minor amounts.

Configurations of the bromo-β-lactams 3a *and* 6a, b. (+)-Methyl 6-*epi*-6-bromopenicillanate *6b*, formed from *5b* is identical with an authentic sample prepared from 6-aminopenicillanic acid [6] (m.p., mixed m.p., $[\alpha]_D$, IR, ^1H NMR and mass spectra). The ^1H NMR and mass spectra of the racemic compound *6a* are also identical with those of the authentic sample. The 6-CH NMR coupling constant is 1.4 Hz, characteristic of a 5-CH and 6-CH *trans*-relationship.[3,7–9] The mass spectrometric fragmentations of *6a, b* are analogous to those of methyl 6-*epi*-6-chloropenicillinate [10] and other penicillin derivatives.[10–12]

The mass spectrum of compound *3a* is analogous to that of *6a, b*. A difference between the spectra is a strong peak at $m/e = 158$ ($C_6H_8NO_2S$) for *3a*, the equivalent of which is lacking for *6a, b*.

The ^1H NMR coupling constants for 5-CH and 6-CH of the bromo-β-lactam *3a* are 1.4 Hz, indicating a *trans*-arrangement.[3,7–9] Compound *3a* may be assigned either of the configurations *7* or *8*. It is not possible to make a definite choice between these two configurations on the bases of available data. However, *8* is the favoured configuration, since a *trans*-relationship was established between 2-CH and 5-CH in the formation of *6a* and *6b* and it is reasonable to assume that the formation of *3a* is analogous.

Discussion of the thermal decomposition of the mercury compounds 2a, b *and* 5a, b. The formation of β-lactams and phenylmercury halides by the thermal decomposition of the compounds *2a, b* and *5a, b*, probably occurs *via* carbenoid intermediates.[3] However, the solvent has a great effect upon the course of the decomposition reaction and the hetero-atoms of the thiazolidine ring also influence the reaction.

The mercury compound *2a* was decomposed in different solvents. Decompositions in chloro-, bromo- and iodobenzene produced β-lactams (IR 1785 cm^{-1}) whereas no β-lactam was observed when *2a* was decomposed in dimethoxyethane, *o*-xylene, methyl benzoate, or 1-bromoheptane.

Besides the solvent, the sulfur atom and possibly also the ester group exert an influence on the decomposition of the dichloro compound *2b*. When heated in refluxing bromobenzene, *2b* decomposed almost completely within 2 h, which is the same time as that required for the decomposition of the dibromo analog *2a*. By contrast, the decomposition of (piperidinocarbonyldichloromethyl)phenylmercury is about ten times slower than the decomposition of the corresponding dibromo compound [2,3] and the bromochloro compound.[4]

The sulfur atom is a good ligand for mercury [13] and forms ylides with carbenes.[14–16] Possibly, the ester group also could influence the decomposition reaction by binding to a carbenoid intermediate. Unstable oxygen ylides have been suggested as intermediates in the reaction between carboalkoxycarbenes and ketones or ethers,[17–20] and for the reaction between dichlorocarbene and carboxylic acids.[21]

For comparison the decomposition reactions of halomethylphenylmercury compounds may be mentioned. Seyferth and coworkers [22,23] studied the decomposition of (bromodichloromethyl)phenylmercury in the presence of olefins. They postulated that free or very weakly complexed dichlorocarbene was first formed and then added to the olefins. One of the arguments put forward by them in support of this postulate was the indifference of the reaction to polar or non-polar solvents. However, reactions between trihalomethylphenylmercury compounds and compounds containing atoms with lone-pair electrons gave complex mixtures of reaction products, and are not believed to involve free dihalocarbenes.[24]

EXPERIMENTAL

Melting points are uncorrected. The IR spectra of liquids were determined neat and those of solids in KBr. ^{1}H NMR spectra were recorded in $CDCl_3$/TMS with a Varian A-60 instrument. Mass spectra were recorded with a LKB 9000 instrument (70 eV). Unless otherwise stated column chromatography was performed with silica gel (Merck 0.05–0.2 mm), using increasing amounts of distilled ether in distilled light petroleum as eluent. THF was purified as described previously.[3]

(−)-3-(Dibromoacetyl)thiazolidine-4-carboxylic acid methyl ester (1a). Dibromoacetylchloride [2] (57.6 g) in dry ether (100 ml) was slowly added to a vigorously stirred ice-cold solution of (−)-thiazolidine-4-carboxylic acid methyl ester [25] (35.7 g) and triethylamine (24.5 g) in dry ether (750 ml). After the addition was complete, the precipitate formed was removed by filtration and washed with chloroform. The chloroform solution and the ether filtrate solution were each washed with water, combined and dried (Na_2SO_4). Evaporation of the solvent and recrystallization of the residue from toluene gave (−)-3-(dibromoacetyl)thiazolidine-4-carboxylic acid methyl ester (*1a*) (46 g, 55 %) m.p. 127–129 °C. $[\alpha]_D^{25}$ −102 (*c*, 1.12, $CHCl_3$). (Found: C 24.4; H 2.26; Br 45.88. Calc. for $C_7H_9NO_3SBr_2$: C 24.23; H 2.61; Br 46.05.) ^{1}H NMR: δ 3.2–3.4 (m, 5-CH), 3.8 (s, CH_3), 4.82 (broad d, *J* 7 Hz, 2-CH), 5.0–5.2 (m, 4-CH), 6.2 (s, $CHBr_2$, the chemical shift is dependent on the concentration). IR: 1750, 1660 C=O.

(−)-3-(Dichloroacetyl)thiazolidine-4-carboxylic acid methyl ester (1b). Prepared in the same way as *1a* (yield 78 %), m.p. 94–96 °C. $[\alpha]_D^{25}$ −132 (*c*, 1.1, $CHCl_3$). (Found: C 32.71; H 3.63; Cl 27.36. Calc. for $C_7H_9NO_3SCl_2$: C 32.57; H 3.51; Cl 27.47.) ^{1}H NMR: δ 3.2–3.4 (m, 5-CH), 3.78 (s, CH_3), 4.82 (broad d, *J* 5 Hz, 2-CH), 4.95–5.15 (m, 4-CH), 6.3 (s, $CHCl_2$, the chemical shift is dependent on the concentration). IR: 1750, 1670 C=O.

(±)-3-(Dibromoacetyl)5,5-dimethylthiazolidine-4-carboxylic acid methyl ester (4a). (±)-5,5-Dimethylthiazolidine-4-carboxylic acid [26] was suspended in dry ether and vigorously stirred and treated with diazomethane. The crude ester product had a m.p. of 25–30 °C and was spectroscopically pure (IR, ^{1}H NMR). It was used without further purification.

Dibromoacetylchloride (19.2 g) in dry ether (100 ml) was slowly added to a vigorously stirred ice-cold solution of (±)-5,5-dimethylthiazolidine-4-carboxylic acid methyl ester (14.2 g) and triethylamine (8.2 g) in dry ether (400 ml). After the addition was complete the precipitate was filtered off and washed with ether. The filtrate and the ether washed solutions were combined, washed with water and dried (Na_2SO_4). Evaporation of the solvent and purification by chromatography on a silica gel column yielded (±)-3-(dibromoacetyl)-5,5-dimethylthiazolidine-4-carboxylic acid methyl ester (*4a*). (22 g, 72 %) m.p. 78–80 °C. (Found: C 29.02; H 3.48; Br 42.47. Calc. for $C_9H_{13}NO_3SBr_2$: C 28.82; H 3.49; Br 42.61.) ^{1}H NMR: δ 1.46, 1.60 (two s, *gem* CH_3), 3.76 (s, CO_2CH_3), 4.55 (s, 4-CH), 4.95 (s, 2-CH), 6.2 (s, $CHBr_2$, the chemical shift is dependent on the concentration). IR: 1745, 1660 C=O.

(+)-3-(Dibromoacetyl)5,5-dimethylthiazolidine-4-carboxylic acid methyl ester (4b). This was prepared from dibromoacetylchloride (3.8 g) and (+)-5,5-dimethylthiazolidine-4-carboxylic acid methyl ester [26,27] (3.4 g) in the same way as *4a* (6.9 g, 94 %) m.p. 72–73 °C. $[\alpha]_D^{25}$ +55 (*c*, 1.0, $CHCl_3$). (Found: C 29.01; H 3.57; Br 42.41. Calc. for $C_9H_{13}NO_3SBr_2$: C 28.82; H 3.49; Br 42.61.) ^{1}H NMR: Identical to that of *8a*. IR: 1750, 1660 C=O.

(−)-[(4-Methoxycarbonylthiazolidino)carbonyldibromomethyl]phenylmercury (2a). The glass equipment was dried and the reaction was performed in an atmosphere of purified nitrogen.

t-BuOK [3] (23 ml of a 0.95 M THF solution) in THF (225 ml) was stirred with a magnetic stirrer and cooled to −75 °C. (−)-3-(Dibromoacetyl)thiazolidine-4-carboxylic acid methyl ester *1a* (7.6 g) in THF (75 ml) was added over 10 min, immediately followed by the addition of phenylmercury chloride (6.8 g) in THF (100 ml) also over 10 min. The solution was stirred at −75 °C for an additional 30 min,

after which the temperature was raised to +10 °C. The solvent was removed as described previously [3] and the crude product was chromatographed on a silica gel column, maintained at −20 °C by circulating cold ethanol in a jacket. (−)-[(4-Methoxycarbonylthiazolidino)-carbonyldibromomethyl]phenylmercury (*2a*) was obtained (5.2 g, 38 %) m.p. 45−50 °C. $[\alpha]_D^{25}$ −80° (*c*, 1.0, benzene). (Found: C 25.13; H 2.47; Br 25.72; Hg 32.05. Calc. for $C_{13}H_{13}NO_3SBr_2Hg$: C 25.03; H 2.10; Br 25.62; Hg 32.16.) ^{1}H NMR: δ 3.1−3.3 (m, 5-CH), 3.70 (s, CH_3), 4.78 (broad d, *J* 10 Hz, 2-CH), 5.1−5.4 (m, 4-CH), 7.29 (s, arom.). IR: 1750, 1610 C=O.

(−)-3-(Dibromoacetyl)thiazolidine-4-carboxylic acid methyl ester (*1a*) (1.6 g, 21 %) was recovered.

(−)-[(4-Methoxycarbonylthiazolidino)carbonyldichloromethyl]phenylmercury (*2b*). This compound was synthesized and isolated by a method similar to that used for *2a*. Yield 9 %, m.p. 40−50 °. $[\alpha]_D^{25}$ −50° (*c*, 0.8, benzene). (Found: C 28.35; H 2.50; Cl 12.92; Hg 36.88. Calc. for $C_{13}H_{13}NO_3SCl_2Hg$: C 29.19; H 2.45; Cl 13.36; Hg 37.51.) IR: 1750, 1620 C=O.

(−)-3-(Dichloroacetyl)thiazolidine-4-carboxylic acid methyl ester (*1b*) was recovered.

(±)-[(Methoxycarbonyl-5,5-dimethylthiazolidino)carbonyldibromomethyl]-phenylmercury (*5a*). This compound was synthesized by a method similar to that used for *2a*. When the crude product was treated with dry ether at room temperature *5a* crystallized (81 %), m.p. 125 °C. (Found: C 27.42; H 2.72; Br 24.58; Hg 31.12. Calc. for $C_{15}H_{17}NO_3SBr_2Hg$: C 27.64; H 2.63; Br 24.52; Hg 30.78.) ^{1}H NMR: δ 1.46, 1.62 (two s, *gem* CH_3), 3.76 (s, CO_2CH_3), 4.53 (broad s, 4-CH), 5.3 (broad d, *J* 13 Hz, 2-CH), 7.28 (s, arom.). IR: 1750, 1600 C=O.

(+)-[(4-Methoxycarbonyl-5,5-dimethylthiazolidino)carbonyldibromomethyl]-phenylmercury (*5b*). This compound was synthesized and isolated by a method similar to that used for *2a* (yield 78 %), m.p. 118−119 °C. $[\alpha]_D^{25}$ +75° (*c*, 0.8 benzene). (Found: C 27.65; H 2.73; Br 24.39; Hg 31.02. Calc. for $C_{15}H_{17}NO_3SBr_2Hg$: C 27.64; H 2.63; Br 24.52; Hg 30.78.) ^{1}H NMR: identical to that of *5a*. IR: 1740, 1600 C=O.

Thermal decomposition of (−)-[(methoxycarbonylthiazolidino)-carbonyldibromomethyl]-phenylmercury (*2a*). The compound *2a* (4.5 g) was heated in refluxing, freshly distilled bromobenzene (700 ml) for 2 h. The solvent was removed on a rotary evaporator using a high-vacuum pump, chloroform was added to the residue and the insoluble phenylmercury bromide (1.4 g, 55 %) was removed by filtration. (Found: Br 22.34. Calc. for C_6H_5BrHg: Br 22.34.) An IR spectrum of the remaining material indicated that it contained about 10 % of the starting material (1610 cm^{-1}) and 15-25 % of a halo-β-lactam (1785 cm^{-1}).[6] Hydrogen sulfide was bubbled through the chloroform solution for 2 min to destroy the unreacted starting material, which would otherwise contaminate the β-lactam containing fractions obtained in a later stage of purification. The chloroform was evaporated and the residue was chromatographed on a silica gel column cooled to −10 °C. The β-lactam fraction (0.56 g) had IR carbonyl absorptions at 1785, 1750 and 1660. The relative intensities of these indicated the presence of approximately 60 % of the halo-β-lactam (about 20 % yield from the starting material). When this fraction was sublimated in a gradient-heated tube (45 °C, 10^{-3} mmHg) for a 35−40 h period only 75 mg of *7a* was obtained. The remainder of the material showed IR absorptions only at 1750 and 1660 cm^{-1}.

The chromatographic separation could also be successfully performed on a short column of thin-layer-grade silica gel [28] at room temperature. Separations on cooled column of aluminia (Schuckardt, neutral) or Florisil gave less satisfactory results.

(−)-Methyl 6-epi-6-bromobisnorpenicillanate (*3a*). (75 mg, 4 %). This was recrystallized from redistilled light petroleum. Colorless crystals, m.p. 57−60 °C. $[\alpha]_D^{25} = -160°$ (*c*, 1.1, $CHCl_3$). A fresh clear $CHCl_3$-solution turned yellowish after half an hour at room temperature. The ^{1}H NMR spectrum indicated an initially small peak at δ 2.15, which was observed to increase with time. (Found: C 31.63; H 3.12; Br 29.84. Calc. for $C_7H_8NO_3SBr$: C 31.59; H 3.03; Br 30.03.) ^{1}H NMR: δ 3.47 (d, *J* 1.4 Hz, 3-CHa), 3.55 (s, 3-CHb), 3.78 (s, CH_3), 4.72 (d, *J* 1.4 Hz, 6-CH), 5.0−5.2 (m, 2-CH), 5.20 (d, *J* 1.4 Hz, 5-CH). IR: 1780, 1730 C=O. MS, *m/e* (%): 265, 267 (M^+, 16); 158 ($C_5H_8NO_2S$, 100), 151, 153 (C_3H_4SBr, 50); 146 ($C_5H_8NO_2S$, 100); 86 ($C_4H_6O_2$ and C_3H_4NS, 100).

(−)-3-(Dibromoacetyl)thiazolidine-4-carboxylic acid methyl ester (*1a*) (0.50 g, 20 %) was also isolated.

Thermal decomposition 2a *in different solvents.* 15-20 mg of *2a* was dissolved in 15−25 ml of solvent and heated. IR spectra (neat) were recorded of the decomposition products. Chlorobenzene: 132 °C, 10 h. IR: 1785 (m), 1745 (s), 1680−1660 (s), 1610 (m). Iodobenzene: 150 °C 4 h. IR: 1785 (w), 1745 (s), 1680−1660 (s), 1610 (m). Dimethoxyethane: 85 °C 8 h. IR: 1745 (s), 1680−1660 (m), 1610 (m). *o*-Xylene: 144 °C 5 h. IR: 1745 (s), 1680−1660 (s), 1610 (w). Methyl benzoate: 150 °C 2 h. IR: 1745 (s), 1680−1660 (m), 1610 (m). 1-Bromoheptane: 150 °C, 1 h. IR: 1745 (s), 1680−1660 (s), 1610 (w).

Thermal decomposition of (−)-[(4-methoxycarbonylthiazolidino)carbonyldichloromethyl]-phenylmercury (*2b*). The compound *2b* (3.00 g) was subjected to a decomposition process similar to that for *2a* (2 h). The bromobenzene solution rapidly turned black. Only a small amount of phenylmercury chloride was formed (0.60 g, 35 %). An IR spectrum of the crude product showed carbonyl absorptions at 1785,

1735, 1635, and 1625. Their relative intensities were 1.0, 1.8, 1.9, and 0.3, respectively. Attempts to isolate *3b* on a cooled column gave a small quantity of material (0.34 g) which had a weak IR absorption at 1785 and decomposed on sublimation.

Thermal decomposition of (±)-[(4-methoxycarbonyl-5,5-dimethylthiazolidino)carbonyldibromomethyl]phenylmercury (5a) The compound *5a* (4.0 g) was decomposed by a process similar to that used for *2a* but the reaction time was prolonged to 3.5 h. The phenylmercury bromide formed (1.9 g, 88 %) was removed by filtration. (Found: Br 22.96. Calc. for C_6H_5Br: Br 22.34.) Chromatography of the remaining material on a cooled silica gel column gave a 0.4 g fraction that had IR absorptions at 1790, 1750 and 1660 with the relative intensities of 1.0, 1.0 and 0.4, respectively. Sublimation of this fraction yielded (±)-methyl 6-*epi*-6-bromopenicillanate (*6a*) (0.15 g, 9 %) m.p. 89–91 °C. (Found: C 36.95; H 4.24; Br 27.12. Calc. for $C_9H_{12}NO_3SBr$: C 36.75; H 4.11; Br 27.17.) ^1H NMR: δ 1.46, 1.62 (two s, *gem* CH_3), 3.76 (s, CO_2CH_3), 4.56 (s, 2-CH), 4.79–4.95 (d, J 1.4 Hz, 6-CH, the chemical shift is dependent on concentration), 5.42 (d, J 1.2 Hz, 5-CH). IR: 1785, 1735 C=O. MS, *m/e* (%): 295, 293 (M^+, 23); 181, 179 (C_6H_8SBr, 28); 174 ($C_7H_{12}NO_2S$, 100); 114 ($C_6H_{10}O_2$ and C_5H_8NS, 29).

Thermal decomposition of (+)-[(4-methoxycarbonyl-5,5-dimethylthiazolidino)carbonyldibromomethyl]phenylmercury (5b). The compound *5b* (2.15 g) was decomposed by a process similar to that used for *5a*. The phenylmercury bromide formed (0.80 g, 70 %) was removed by filtration. Chromatography of the remaining material on a cooled silica gel column, sublimation and recrystallization from light petroleum gave (+)-methyl 6-*epi*-6-bromopenicillanate (*6b*) (0.8 g, 9 %), m.p. 45–47 °C. $[\alpha]_D^{25}$ +185° (*c*, 0.4, acetone). ^1H NMR: δ 1.46, 1.62 (two s, *gem* CH_3), 3.76 (s, CO_2CH_3), 4.56 (s, 2-CH), 4.79-4.93 (d, J, 1.4 Hz, 6-CH, the chemical shift is dependent on concentration), 5.42 (d, J 1.2 Hz, 5-CH). IR: 1785, 1750 C=O. MS, *m/e* (%): 295, 293 (M^+, 23); 181, 179 (C_6H_8SBr, 28); 174 ($C_7H_{12}NO_2S$, 100); 114 ($C_6H_{10}O_2$ and C_5H_8NS, 33).

Synthesis of (+)-methyl 6-epi-6-bromopenicillanate from 6-APA (cf. Ref. 29). 6-Aminopenicillanic acid (2.15 g) was dissolved in a mixture of distilled water (25 ml), methanol (70 ml) and concentrated hydrobromic acid (48 %, 5 ml) and cooled to 0–5 °C. Sodium nitrite (1.5 g) was added in one portion. After 45 min. at 0–5 °C, chloroform (150 ml) and water (100 ml) were added. The phases were vigorously stirred, separated and the chloroform phase was washed with cool water (50 ml) and dried ($MgSO_4$). The crude product was treated with diazomethane in dry ether to give crude (+)-methyl 6-*epi*-6-bromopenicillanate (2.1 g, 70 %). This was further purified by recrystallization from light petroleum, m.p. 46–47 °C $[\alpha]_D^{25}$ +185° (*c*, 1.0, acetone). ^1H NMR, IR and MS were identical to those of *6b* obtained from *5b*.

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Strained Heterocyclic Compounds 9.* Synthesis of 2-Hydroxy-β-lactams and Oxazolidin-4-ones by Photocyclization of 2-Oxoamides

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The 6-hydroxypenam system (*12*) has been synthesized by photolysis of (−)-3-(oxophenylacetyl)thiazolidine-4-carboxylic acid ethyl ester (*11*). 7-Hydroxy-8-oxo-1-azabicyclo[4.2.0]octanes (*2,7*) are similarly formed from oxophenylacetylpiperidine (*1*) and 2-oxopropionylpiperidine (*6*). 2,3-Dihydroxysuccinylpiperidines (*5, 10*) were also isolated from the photolysis of *1* and *6*. The major photoproducts were, however, fused oxazolidin-4-ones, tetrahydro-5*H*-oxazolo[3,2,-*a*]pyridin-3(2*H*)-ones (*3,4,8,9*) and two tetrahydro-3-oxo-7a*H*-thiazolo[2,3-*b*]oxazoles (*13,14*).

As part of a program for synthesis of penicillin analogs we have studied the photocyclizations of 2-oxoamides. In analogy to the formation of hydroxycyclobutanones from 2-oxoketones,[1–5] the amides gave hydroxy-β-lactams, but the yields were low (5−10 %). Unexpectedly, oxazolidin-4-ones were formed as major products. The results have been reported as a preliminary communication.[6]

Recently, Henery-Logan and Chen [7] confirmed the utility of this photocyclization approach when they obtained the 6-hydroxypenam compounds *16 a,b,c* by ultraviolet irradiation of the corresponding 2-oxoamides *15 a,b,c*.

Synthesis of 2-oxoamides. Condensation of oxophenylacetylchloride and the appropriate amines gave the oxophenylacetylamides *1* and *11*. Ethoxyoxoacetylpiperidine and methylmagnesium bromide at −80 °C gave 2-oxopropionylpiperidine (*6*). 2-Oxoamides can also be made by coupling of the acid and an amine with carbodiimide.[7]

Photoproducts. The compounds *1*, *6* and *11* in dry benzene were irradiated by 3500 Å ultraviolet light for 19−40 h. Chromatography over silica gel and recrystallization gave the 7-hydroxy-1-azabicyclo[4.2.0]-octan-8-ones *2* and *7* in 8 % and 5 % yield, respectively, and (−)-6-hydroxy-7-oxo-6-phenyl-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid ethyl ester (*12*) in 8 % yield. The major products were the 6,7,8,8a-tetrahydro-5*H*-oxazolo[3,2-*a*]pyridin 3(2*H*)-ones *3* (21 %), *4* (15 %), *8* (25 %), and *9* (20 %) and (−)-tetrahydro-3-oxo-2-phenyl-7a*H*-thiazolo[2,3-*b*]oxazole-5-carboxylic acid ethyl esters (*13, 14*; 13 and 9 %). The 2,3-dihydroxysuccinylpiperidines *5* and *10* were also isolated (15 and 6 %), respectively).

From the photolysis of *1* an additional compound *A* (5 %; m.p. 97−100 °C) was isolated. Its ^{1}H NMR spectrum indicated that it contained phenyl groups and a rearranged piperidine moiety in the proportions 2:1, but it was not further characterized.

Spectroscopic data. The photolysis products were identified by their ^{1}H NMR, IR and MS data. The structure of compound *4* has been confirmed by an X-ray crystallographic analysis.[8] The main mass spectrometric fragments of compounds *2* and *4* were determined by high resolution mass spectrometry, and the frag-

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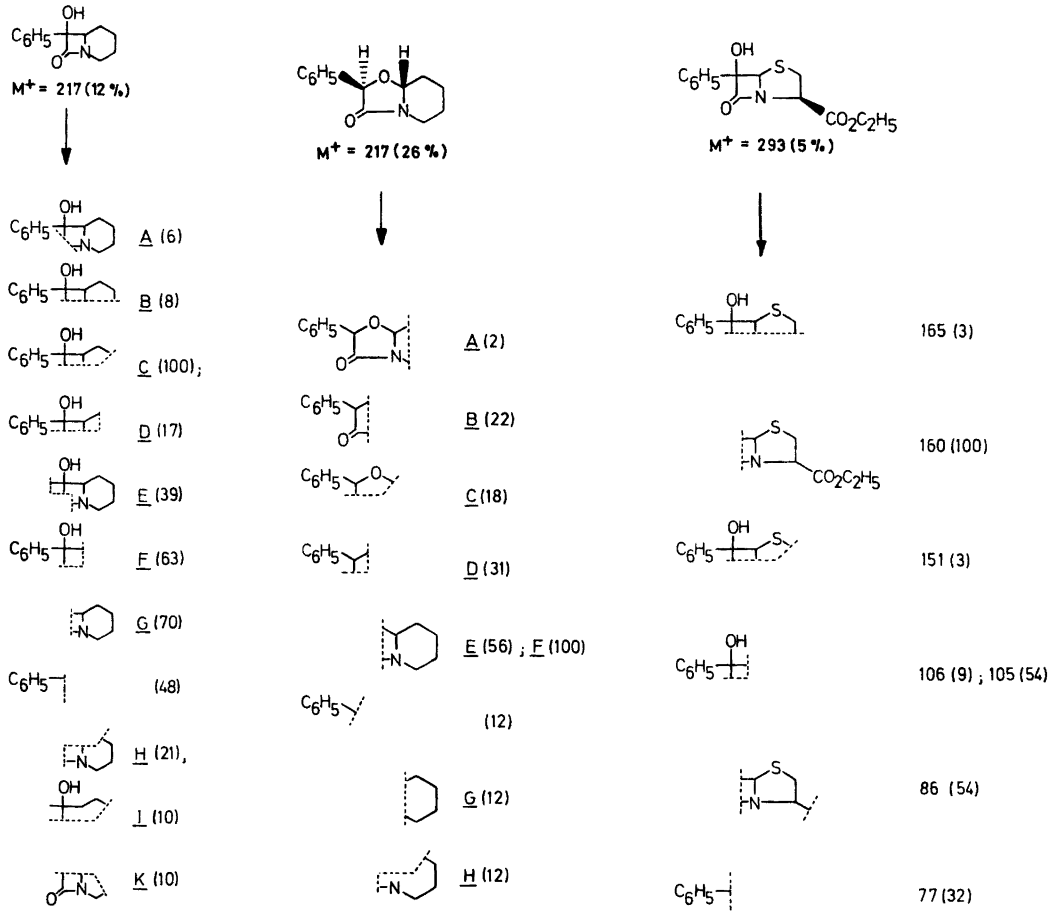


Schemes 1–3. MS fragmentation of compound *2*, *4*, and *12*.

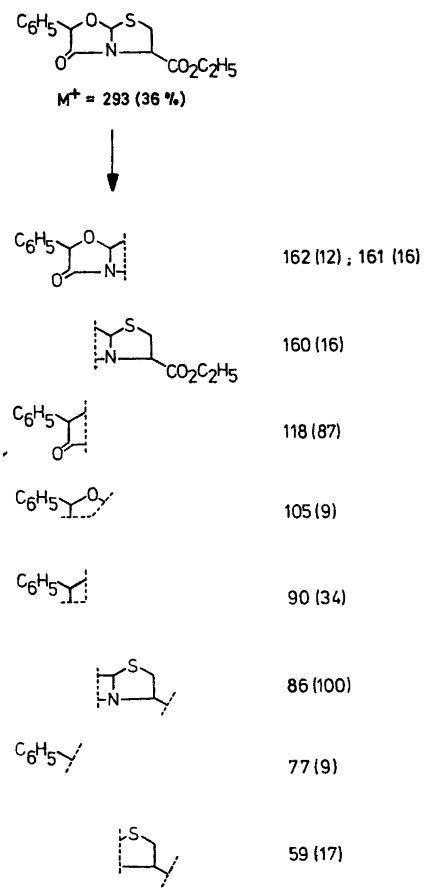
mentation patterns are illustrated in Schemes 1 and 2, respectively. The mass spectrometric fragmentations of compound *12* are depicted in Scheme 3 and of compound *13* in Scheme 4.

β-Lactams 2, 7 *and* 12. The β-lactam IR carbonyl absorptions for solids *2* and *7* are as low as 1725 and 1720 cm^{-1}, respectively, due to intermolecular hydrogen bonding. In dioxan solutions the absorptions are raised to 1750 and 1745 cm^{-1}. The carbonyl absorption of the β-lactam *12* is similarly raised from 1750 cm^{-1} in the solid state to 1770 cm^{-1} in dioxan. The carbonyl absorption of the ester group is not affected.

NMR signals were assigned to the *geminal* 2-CHα- and 2-CHβ-protons in *2* and *7* by consideration of the diamagnetic anisotropic effects of the β-lactam carbonyl groups,[9,10] and by comparison with the almost identical ^{1}H NMR signals of compounds *17a,b*, *18* and *19a,b*.[9,11]

^{1}H NMR signals were assigned to compound *12* after comparison between the ^{1}H NMR spectra of compound *12* and methyl 6-bromobisnorpenicillanate,[12] bisnorpenicillin V,[13] and other penicillins.[14]

The mass spectrometric fragmentation patterns of *2* (Scheme 1) and *7* are compatible with the suggested structures and are analogous to the fragmentations of unfused β-lactams [14] and penicillins.[14–17] Compound *12* (Scheme 3) behaves in the same principal manner as other penicillins,[14–17] with the thiazoline ion ($m/e = 160$) as a major characteristic peak.



Scheme 4. MS fragmentation of compound *13*.

Oxazolidin-4-ones 3, 4, 8, 9, 13 and 14. Oxazolidin-4-ones fused with a piperidine ring have previously been obtained by oxidation of the oxazolidine rings in Garrya alkaloids.[18,19]

Their IR carbonyl absorptions occur at 1700 – 1705 cm^{-1}. The carbonyl absorptions of *3*, *4*, *8* and *9* are similarly at 1695 – 1710 cm^{-1}. The oxazolidinones *13* and *14*, fused to a thiazolidine moiety, absorb at 1735 – 1740 cm^{-1}.

The high resolution mass spectrum of compound *4* is shown in Scheme 2. The mass spectra of *8*, *9*, *13* (Scheme 4) and *14* are analogous.

The *trans*-configuration of compound *4* has been established by X-ray crystallography.[8] Consequently, the isomeric compound *3* possesses *cis*-configuration. The NMR chemical shift of the 2-C hydrogen atom of the *trans*-compound *4* is at lower field (δ 5.13) than the corresponding shift of the *cis*-isomer *3* (δ 5.08).

This relative resonance of the 2-C protons is analogous to shifts observed for stereoisomers of thiazolidines[20] and 1,3-dioxolans.[21–23] In thiazolidines the 4-C proton resonance occurred at lower field when *cis* to an alkyl substituent at 2-C, compared to compounds in which the 4-C proton was *cis* to a hydrogen at 2-C.[20]

The 2-C acetal proton of 1,3-dioxolans has also been shown to be deshielded by *cis*-alkyl substituents at 3-C or 4-C.[21–23]

In the same way, the stereochemistry assigned to the isomers *8* and *9* is deduced from the relative NMR shifts of their 2-C protons. The 2-C proton of the *trans*-compound *9* absorbs at lower field (δ 4.28) than the corresponding proton of the *cis*-compound *8* (δ 4.17).

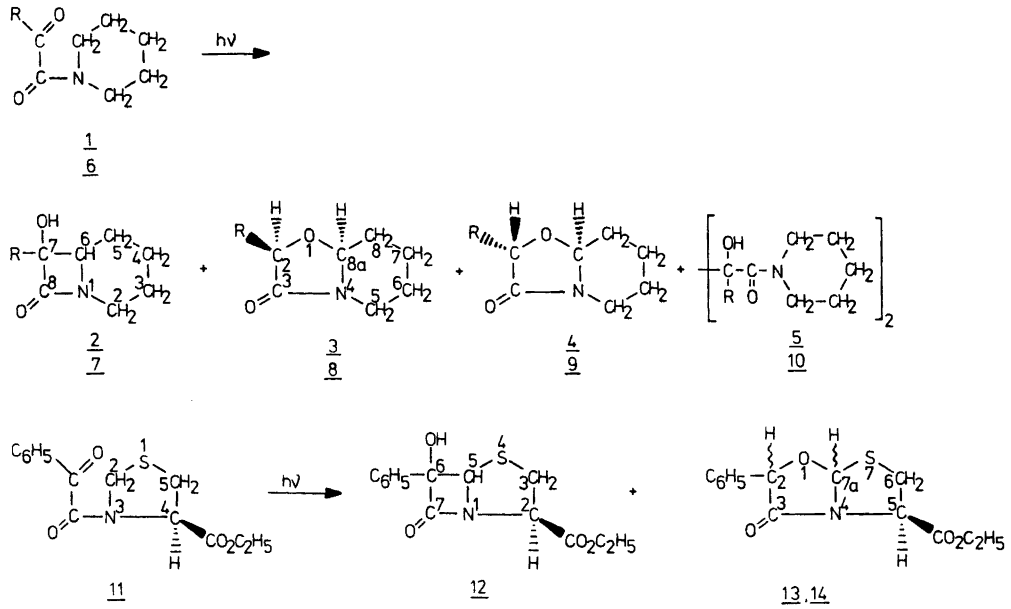
Pinacol formation. The mass spectra of *5* and *10* are indicative of pinacol structures. The molecular ion peaks are small (< 1 %) but peaks corresponding to the loss of one piperidine carbonyl moiety are prominent. The NMR spectrum of *5* exhibits absorptions of the aromatic protons (δ 6.84 – 7.11), the hydroxyl proton (δ 7.40, s) and the piperidine moiety (δ 3.25 – 3.55 and 0.8 – 1.6) in the proportion 5:1:10. The NMR spectrum of *10* is analogous.

No dimer was isolated from the photoproducts of *11*. It might have been formed in small amounts (< 5 %) but escaped detection since the photolysis mixture of *11* was more difficult to separate than those of *1* and *6*.

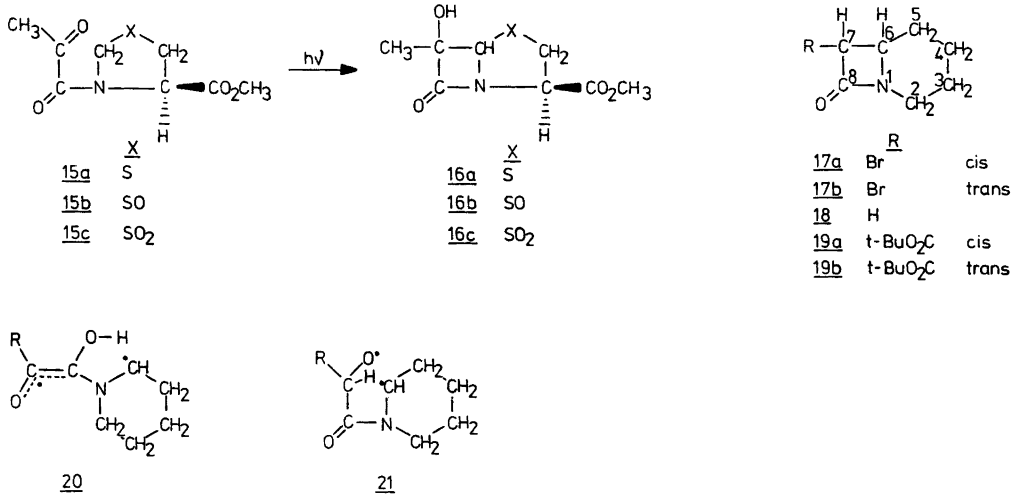
DISCUSSION

On photolysis of the α-oxoamides *1*, *6* and *11* hydroxy-β-lactams were formed. Although the formation of this type of compounds (hydroxy cyclobutanones) is a major photoreaction of 1,2-diketones,[1–5] only 5 – 10 % yields of hydroxy-β-lactams were obtained from α-oxoamides. In analogy to the cyclization of octane-4,5-dione and decane-5,6-dione [1] the reaction would be expected to yield two stereoisomeric cyclic products. However, only one isomer was isolated in each case (*2*, *7* and *12*). It is possible that small amounts of the other stereoisomeric hydroxy-β-lactams were formed but escaped detection.

The major photoproducts from the α-oxoamides *1*, *6* and *11* were the oxazolidinones *3*, *4*, *8*, *9*, *13*, and *14*. This type of photocyclization



Scheme 5. *1 – 5*, R = C_6H_5; *6 – 10*, R = CH_3.



	X
15a	S
15b	SO
15c	SO_2

	X
16a	S
16b	SO
16c	SO_2

	R	
17a	Br	cis
17b	Br	trans
18	H	
19a	t-BuO_2C	cis
19b	t-BuO_2C	trans

does not appear to have any precedents in the photochemistry of α-oxoesters or ketones.[24] A reasonable hypothesis is that some hydrogen abstraction occurs *via* a 5-membered transition state to yield an intermediate diradical *20*, which would cyclize either to an unstable hydroxyaziridine or an oxazolidinone. The intermediate *21* is also possible but hydrogen abstraction by the carbon of an excited carbonyl function has little precedent.

Finally, pinacols could be isolated from the photolysis of the oxoamides *1* and *6*. Analogous products have been isolated from photolysis of alkyl 2-oxopropionates and a number of other α-oxocarbonyl compounds [25–31] when the reactions were performed in solvents that were

good hydrogen donors. Since benzene is a poor donor, the α-oxoamides *1* and *6* probably serve themselves as hydrogen donors.

EXPERIMENTAL

Melting points were determined on a micro hot stage and are uncorrected. When recording infrared spectra solids were measured in KBr discs and liquids as films between NaCl discs. IR spectra were also recorded on dioxan solutions. The data are given in cm^{-1}. Proton magnetic resonance spectra were recorded on a Varian A-60 instrument. The chemical shifts are given as δ-values relative to TMS as an internal standard. Mass spectra were recorded on an LKB-9000 instrument and high-resolution mass spectra on an SM-1, Atlas-MAT-Bremen instrument (Ionization energy 70 eV). A Rayonet photochemical Reactor No. 100 equipped with 3500 Å lamps (24 W) was used for photolysis. Column chromatography was performed over silica gel (Kebo 0.15–0.30 mm) using increasing amounts of diethyl ether in light petroleum (b.p. 40–60 °C) as eluent.

TLC was performed on silica gel G (Merck) and on silica gel PF-254 (Merck) plates using mixtures of diethyl ether and light petroleum as eluent. Spots were located by UV illumination and by iodine vapor or by spraying the plates with chromic acid solutions.

1-(Oxophenylacetyl)piperidine (*1*). Oxophenylacetic acid (15 g) in 50 ml of dry diethyl ether was slowly added to freshly distilled thionylchloride (13 g) and pyridine (8.7 g) in 150 ml of dry, ice-cold diethyl ether. The precipitated pyridine hydrochloride was removed by filtration and the solution was slowly added to piperidine (18.7 g) in 150 ml of dry, ice-cold diethyl ether. The precipitate was filtered off, washed with ether and the combined ether solutions were washed with 0.2 N HCl and H_2O. The solvent was evaporated to give 1-(oxophenylacetyl)piperidine (14.7 g, 68 %) m.p. 107–108 °C. (Found: C 72.03; H 6.94; O 14.03. Calc. for $C_{13}H_{15}NO_2$: C 71.87; H 6.96; O 14.73.) ^1H NMR ($CDCl_3$): δ 1.50–1.70 (m, 3-CH_2, 4-CH_2, 5-CH_2); 3.23 (t, J=5 Hz, 2-CH, 6-CH); 3.65 (t, J=5 Hz, 2-CH, 6-CH); 7.4–7.6 (m, arom.); 7.8–8.0 (m, arom). MS, *m/e* (%): 217 (M^+, 6); 112 ($M-C_6H_5CO$, 100).

IR, KBr (dioxan): 1665 (1675), 1635, (1635) C=O. TLC (ether-light petroleum, 80–20) R_F=0.50.

Ethoxyoxoacetylpiperidine. Ethoxyoxoacetylchloride [32] (130 g) was added dropwise to piperidine (170 g) in 1500 ml of ice-cold diethyl ether. The precipitate was filtered off and the ether solution was washed with 0.5 N HCl, 0.5 N $NaHCO_3$ and H_2O. Distillation in vacuum gave ethoxyoxoacetylpiperidine (121 g, 68 %) b.p. 158 °C/12 mmHg. Lit. b.p.[33] 158–159 °C/11 mmHg.

N-(2-Oxopropionyl)piperidine (*6*). Methylmagnesium bromide (prepared from 13 Mg and 100 g CH_3Br) in 600 ml of dry diethyl ether was vigourously stirred at −80 °C and ethoxyoxoacetylpiperidine (50 g) in 200 ml of dry ether was added over a period of 3 h. The temperature was raised to −20 °C and kept at −20 °C for 70 min, after which 2 N HCl (250 ml) was slowly added. Chromatography on silica gel and distillation gave *N*-(2-oxopropionyl)piperidine (*6*) (29 g, 70 %) b.p. 138 °C/15 mm. (Found: C 61.79; H 8.38; O 20.78. Calc. for $C_8H_{13}NO_2$: C 61.91; H 8.44; O 20.62.) ^1H NMR ($CDCl_3$): 1.50–1.65 (m, 3-CH_2, 4-CH_2, 5-CH_2); 2.35 (s, CH_3); 3.22–3.60 (m, 2-CH_2, 6-CH_2). MS, *m/e* (%): 155 (M^+, 7); 112 ($M-CH_3CO$, 100). IR, neat (dioxan): 1710 (1710), 1640 (1640) C=O. TLC (diethyl ether): R_F=0.60.

(−)-Thiazolidine-4-carboxylic acid ethyl ester. Prepared in the same way as the corresponding methyl ester,[34] b.p. 91 °C/1 mmHg.

(−)-3-(Oxophenylacetyl)thiazolidine-4-carboxylic acid ethyl ester (*11*). Oxophenylacetylchloride, prepared from the acid (15 g, see above), in 125 ml of dry diethyl ether was slowly added to thiazolidine-4-carboxylic acid ethyl ester (17.8 g) and pyridine (8.7 g) in 200 ml of dry, ice-cold diethyl ether. The reaction mixture was worked up as described for *1*. Chromatography on silica gel gave (−)-3-(oxophenylacetyl)thiazolidine-4-carboxylic acid ethyl ester (24.8 g, 85 %), as a viscous liquid. $[\alpha]_D^{20}$ −80° (*c*, 0.64, $CHCl_3$). (Found: C 57.28; H 5.33; O 21.97. Calc. for $C_{14}H_{15}NO_4S$: C 57.32; H 5.15; O 21.82.) ^1H NMR ($CDCl_3$): δ 0.95–1.4 (m, C-CH_3); 3.23–3.38 (2 d, 5-CH_2); 3.9–4.4 (m, O-CH_2); 4.5 (d, J=4.5 Hz, 2-CH); 4.75 (d, J=2 Hz, 2-CH); 4.95–5.25 (m, 4-CH); 7.40–7.55 and 7.95–8.15 (m, arom.). IR, KBr (dioxan): 1735 (1735), 1670 (1675), 1640 (1645). C=O. TLC (diethyl ether-light petroleum, 60-40): R_F=0.42.

Photolysis of N-(oxophenylacetyl)piperidine (*1*). *N*-(Oxophenylacetyl)piperidine (*1*) (8.2 g) was dissolved in benzene (200 ml, distilled from Na) in a 4×50 cm pyrex tube. The solution was saturated with N_2 and the stoppered tube was irradiated with 3500 Å light for 19 h. Column chromatography over silica gel (400 g) gave a total recovery of 6.7 g and recrystallization from diethyl ether-light petroleum gave the compounds *2*–*5*. 1–2 % of the starting material was also recovered.

7-Hydroxy-7-phenyl-1-azabicyclo[4.2.0]octan-8-one (*2*). (0.6 g, 8 %) m.p. 148–150 °C. (Found: C 71.14; H 6.94; N 6.84; O 15.21. Calc. for $C_{13}H_{15}NO_2$: C 71.87; H 6.96; N 6.45; O 14.73.) MS, *m/e* (Letters refer to mass fragments in Scheme 1):

Found: 189.1156 (*A*). Calc. for $C_{12}H_{15}NO$: 189.1154.
Found: 146.0729 (*B*). Calc. for $C_{10}H_{10}O$: 146.0732.
Found: 133.0645 (*C*). Calc. for C_8H_9O: 133.0653.
Found: 120.0569 (*D*). Calc. for C_8H_8O: 120.0575.
Found: 112.0760 (*E*). Calc. for $C_6H_{10}NO$: 112.0762.
Found: 105.0335 (*F*). Calc. for C_7H_5O: 105.0340.
Found: 84.0809 (*G*). Calc. for $C_5H_{10}N$: 84.0813.
Found: 56.0514 (*H*). Calc. for C_3H_6N: 56.0500.
Found: 56.0268 (*I*). Calc. for C_3H_4O: 56.0262.
Found: 56.0150 (*K*). Calc. for C_2H_2NO: 56.0136.

^{1}H NMR ($CDCl_3$): δ 1.2–1.7 (m, 3-CH_2, 4-CH_2, 5-CH_2); 2.75 (m, 2-CH_α); 3.58 (dd, $J=4$ and 10 Hz, 6-CH); 3.80 (broad dd, $J=4$ and 13 Hz, 2-CH_β); 5.28 (s, OH); 7.32 (s, arom). IR, KBr (dioxan): 3215 (3340) OH; 1725 (1750) C=O. TLC (ether-light petroleum, 80–20): $R_F=0.28$.

*6,7,8,8aα-Tetrahydro-2β-phenyl-5*H*-oxazolo-[3,2-a]pyridin-3(2*H*)-one* (*3*) (1.7 g, 21 %) m.p. 83–84 °C. (Found: C 71.69; H 7.02; N 6.61; O 14.69. Calc. for $C_{13}H_{15}NO_2$: C 71.87; H 6.96; N 6.45; O 14.73.)
MS, *m/e* (%): 217 (M^+, 17); 161 (2); 118 (14); 105 (16); 90 (27); 84 (53); 83 (100); 77 (11); 55 (25). ^{1}H NMR ($CDCl_3$): δ 1.18–2.10 (m, 5-CH_2, 6-CH_2, 7-CH_2); 2.65 (m, 5-CH_α); 3.98 (broad dd, $J=3.5$ and 14 Hz); 4.85 (broad m, 8a-CH); 5.08 (s, 2-CH); 7.3 (m, arom). IR, KBr (dioxan): 1695 (1700) C=O. TLC (ether-light petroleum, 80–20): $R_F=0.28$.

*6,7,8,8aα-Tetrahydro-2α-phenyl-5*H*-oxazolo-[3,2-a]pyridin-3-(2*H*)-one* (*4*). (1.2 g, 15 %) m.p. 78–80 °C. (Found: C 71.84; H 6.68; N 6.54; O 14.76. Calc. for $C_{13}H_{15}NO_2$: C 71.87; H 6.96; N 6.45; O 14.73.)
MS, *m/e* (Letters refer to mass fragments in Scheme 2):

Found: 161.0470 (*A*). Calc. for $C_9H_7NO_2$: 161.0477.
Found: 118.0413 (*B*). Calc. for C_8H_6O: 118.0419.
Found: 105.0332 (*C*). Calc. for C_7H_5O: 105.0340.
Found: 90.0461 (*D*). Calc. for C_7H_6: 90.0469.
Found: 84.0820 (*E*). Calc. for $C_5H_{10}N$: 84.0813.
Found: 83.0736 (*F*). Calc. for C_5H_9N: 83.0735.
Found: 55.0528 (*G*). Calc. for C_4H_7: 55.0547.
Found: 55.0398 (*H*). Calc. for C_3H_5N: 55.0421.

^{1}N NMR ($CDCl_3$): 1.15–2.10 (m, 5-CH_2, 6-CH_2, 7-CH_2); 2.65 (m, 5-CH_α); 4.01 (broad dd, $J=$ 4 and 13 Hz, 5-CH_β); 5.08 (broad m, 8a-CH); 5.13 (d, $J=1.8$ Hz, 2-CH); 7.3 (m, arom). IR, KBr (dioxan): 1695 (1700) C=O. TLC (ether-light petroleum, 80–20): $R_F=0.41$.

2,3-Dihydroxy-2,3-diphenylsuccinylpiperidine (*5*). (1.2 g, 15 %) m.p. 163–166 °C. (Found: C 71.35; H 7.03; N 6.82; O 15.32. Calc. for $C_{26}H_{33}N_3O_4$: C 71.53; H 7.39; N 6.42; O 14.66.) ^{1}H NMR ($CDCl_3$): δ 0.8–1.6 (m, CH_2-CH_2-CH_2); 3.25–3.55 (m, CH_2-N-CH_2); 6.84–7.11 (m, arom.); 7.40 (s, OH). MS *m/e* (%): 436 (M^+, 1); 324 (36); 219 (100); 112 (74); 105 (77); 86 (95). IR, KBr (dioxan): 3220 (3260) OH; 1590 (1600) C=O. TLC (ether–light petroleum, 80–20): $R_F=0.70$.

Photolysis of N-(2-oxopropionyl)piperidine (*6*). *N*-(2-Oxopropionyl)piperidine (*6*) (10.0 g) in benzene (150 ml, distilled from NaH) was irradiated with 3500 Å light for 27 h in the same fashion as for compound *1*. Column chromatography over silica gel (550 g) gave a total recovery of 8.3 g. The compounds *7–10* were identified. 1.4 g (15 %) of the starting material was also recovered.

7-Hydroxy-7-methyl-1-azabicyclo[4.2.0]octan-8-one (*7*). The crude material (0.4 g, 5 %) was rechromatographed over silica gel, m.p. 138–139 °C. (Found: C 61.13; H 8.49; O 21.01. Calc. for $C_8H_{13}NO_2$: C 61.91; H 8.44; O 20.62.) MS, *m/e* (%): 155 (M^+, 1); 127 (12); 112 (25); 84 (32); 71 (100); 58 (10); 56 (19); 55 (11). ^{1}H NMR ($CDCl_3$): δ 1.2–1.9 (m, 3-CH_2, 4-CH_2, 5-CH_2); 1.29 (s, CH_3); 2.85 (m, 2-CH_α); 3.52 (broad, 6-CH); 3.82 (broad, 2-CH_β); 4.92 (s, OH). IR, KBr (dioxan): 3250 (3400) OH; 1720 (1745) C=O. TLC (diethyl ether): $R_F=$ 0.35.

*6,7,8,8aα-Tetrahydro-2β-methyl-5*H*-oxazolo-[3,2-a]pyridin-3(2*H*)-one* (*8*). (2.5 g, 25 %) colorless liquid. n_D^{20} 1.4853. (Found: C 61.90; H 8.51; N 8.97. Calc. for $C_8H_{13}NO_2$: C 61.91; H 8.44; N 9.03.) MS, *m/e* (%): 155 (M^+, 25); 154 (45); 153 (18); 127 (17); 116 (17); 100 (37); 99 (48); 98 (26); 84 (100); 83 (44); 82 (25). ^{1}H NMR ($CDCl_3$): δ 1.2–2.2 (m, 5-CH_2, 6-CH_2, 7-CH_2); 1.25 (d, $J=6$ Hz, CH_3); 2.75 (m, 5-CH_α); 3.85 (broad dd, $J=2.5$ and 11 Hz, 5-CH_β); 4.17 (m, $J=6$ Hz, 2-CH); 4.87 (broad m, 8a-CH). IR, neat (dioxan): 1710 (1710) C=O. TLC (diethyl ether): $R_F=0.47$.

*6,7,8,8aα-Tetrahydro-2α-methyl-5*H*-oxazolo-[3,2-a]pyridin-3(2*H*)-one* (*9*). (*Ca.* 2 g, 20 %) colorless liquid. n_D^{20} 1.4931. (Found: C 61.60; H 8.29. Calc. for $C_8H_{13}NO_2$: C 61.91; H 8.44.) MS, *m/e* (%): 155 (M^+, 28); 154 (72); 153 (46); 127 (29); 116 (35); 100 (52); 99 (75); 98 (43); 84 (33); 83 (75); 82 (39). ^{1}H NMR ($CDCl_3$): δ 1.2–2.2 (m, 5-CH_2, 6-CH_2, 7-CH_2); 1.38 (d, $J=6$ Hz, CH_3); 2.75 (m, 5-CH_α); 4.07 (broad dd, $J=3$ and 11 Hz, 5-CH_β); 4.28 (m, $J=6$ Hz, 2-CH); 4.87 (broad m, 8a-CH). IR, neat (dioxan): 1710 (1710) C=O. TLC (diethyl ether): $R_F=0.43$.

2,3-Dihydroxy-2,3-dimethylsuccinylpiperidine (*10*). (0.6 g, 6 %). Recrystallized from diethyl ether-light petroleum, m.p. 129–131 °C. (Found: C 61.03; H 8.79; O 21.23. Calc. for $C_{16}H_{28}N_2O_4$: C 61.51; H 9.03; O 20.49.) ^{1}H NMR ($CDCl_3$): δ 1.43 (s, CH_3); 1.60 (broad s, CH_2-CH_2-CH_2); 3.5 and 4.05 (two broad s, CH_2-N-CH_2); 6.3 (s, OH). MS, *m/e* (%) 312 (M^+, 1); 201 (12); 200 (98); 157 (37); 113 (12); 112 (100); 86 (50). IR, KBr (Dioxan): 3260 (3300) OH; 1585 (1590) C=O. TLC (diethyl ether): $R_F=$ 0.75.

Photolysis of (–)-3-(oxophenylacetyl)thiazolidine-4-carboxylic acid ethyl ester (*11*). 3-(Oxo-

phenylacetyl)thiazolidine-4-carboxylic acid ethyl ester (*11*) (11.0 g) in benzene (250 ml, dried over Na) was irradiated at 3500 Å for 40 h in the same way as for compound *1*. Column chromatography over silica gel gave a total recovery of 7.8 g. The compounds *12* – *14* were identified and recrystallized from diethyl ether-light petroleum. 2.0 g (18 %) of the starting material was also recovered.

(–)-6-Hydroxy-7-oxo-6-phenyl-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid ethyl ester (*12*). (0.7 g, 8 %) m.p. 109 – 111 °C. $[\alpha]_D^{20}$ – 305° (*c*, 0.67, $CHCl_3$). (Found: C 57.24; H 5.18; N 4.89; O 21.93. Calc. for $C_{14}H_{15}NO_4S$: C 57.32; H 5.15; N 4.78; O 21.82.) ^1H NMR ($CDCl_3$): δ 1.15 – 1.4 (C-CH_3); 3.05 – 3.35 (m, 3-CH_2); 4.0 – 4.3 (O-CH_2); 4.3 – 4.6 (broad, OH); 4.85 – 5.05 (m, 2-CH); 5.31 (s, 5-CH) 7.16 – 7.40 (m, arom.). MS, *m/e* (%): 293 (M^+, 5); 294 (M + 1, 0.17 × 5. Calc. for M + 1: 0.167 × 5); 295 (M + 2, 0.06 × 5. Calc. for M + 2: 0.063 × 5). IR, KBr (dioxan): 3400 (3600 – 3300) OH; 1735 (1735), 1750 (1770) C=O. TLC (diethyl ether-light petroleum, 60 – 40): R_F = 0.42.

*(–)-Tetrahydro-3-oxo-2-phenyl-7a*H*-thiazolo[2,3-*b*]oxazole-5-carboxylic acid ethyl ester* (*13*). (1.3 g, 13 %) m.p. 54 – 57 °C. $[\alpha]_D^{20}$ – 145° (*c*, 0.75, $CHCl_3$). (Found: C 57.52; H 5.11; N 4.87; O 21.75. Calc. for $C_{14}H_{15}NO_4S$: C 57.32; H 5.15; N 4.78; O 21.82.) ^1H NMR ($CDCl_3$): δ 1.2 (t, J = 7 Hz, C-CH_3); 3.25 and 3.33 (two d, J = 0.8 Hz and J = 4.0 Hz, respectively, 6-CH_2); 4.14 (q, J = 7 Hz, O-CH_2); 5.12 – 5.28 (m, 5-CH); 5.31 (s, 2-CH); 7.11 (s, 7a-CH); 7.25 – 7.40 (m, arom.). MS, *m/e* (%): 293 (M^+, 36); 294 (M + 1, 0.18 × 36. Calc. for M + 1: 0.16 × 36); 295 (M + 2, 0.07 × 36. Calc. for M + 2: 0.063 × 36). IR, KBr (dioxan): 1735 (1740), 1740 (1740) C=O. TLC (diethyl ether-light petroleum, 60 – 40): R_F = 0.61.

*(–)-Tetrahydro-3-oxo-2-phenyl-7a*H*-thiazolo[2,3-*b*]oxazole-5-carboxylic acid ethyl ester* (*14*). (0.9 g, 9 %) m.p. 103 – 106 °C. $[\alpha]_D^{20}$ – 100° (*c*, 0.68, $CHCl_3$). (Found: C 57.44; H 5.17; N 4.88; O 21.83. Calc. for $C_{14}H_{15}NO_4S$: C 57.32; H 5.15; N 4.78; O 21.82.) ^1H NMR ($CDCl_3$): δ 1.3 (t, C-CH_3); 3.11 and 3.18 (two d, J = 4.2 Hz and J = 0.9 Hz, respectively, 6-CH); 4.17 (q, O-CH_2); 5.11 – 5.31 (m, 5-CH); 5.43 (s, 2-CH); 7.0 (s, 7a-CH); 7.2 – 7.4 (m, arom.). MS, *m/e* (%): 293 (M^+, 34); 294 (M + 1, 0.17 × 34. Calc. for M + 1: 0.16 × 34); 295 (M + 2, 0.07 × 34. Calc. for M + 2: 0.063 × 34); 162 (8); 161 (9); 160 (6); 118 (47); 105 (8); 90 (28); 86 (100). IR, KBr (dioxan): 1735 (1735) C=O. TLC (diethyl ether-light petroleum, 60 – 40): R_F = 0.58.

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The Synthetic Utility of Heteroaromatic Azido Compounds. III. Preparation of Some Furo-, Thieno- and Selenolo[3,2-*b*]pyrroles

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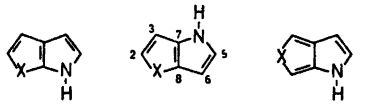
Some 3-azido-2-vinyl derivatives of furan, thiophene, and selenophene have been used for preparation of [3,2-*b*]-fused pyrrole systems by thermal decomposition. One of the resulting derivatives, namely 5-nitroacetylthieno-[3,2-*b*]pyrrole could be transformed to the corresponding unsubstituted thieno[3,2-*b*]pyrrole by alkaline treatment at high temperature. Attempts to obtain the unsubstituted furo- and selenolo-fused systems by this method failed.

The ^1H and ^{13}C NMR parameters for the thieno[3,2-*b*]pyrrole are presented.

Systems consisting of a pyrrole nucleus fused to a furan, thiophene, or selenophene ring are of interest since they are isosteric with indoles, which gives them a potential pharmacological importance. Research in this field has mainly been centered on preparation of analogues of naturally occurring indole derivatives. This is especially the case for the thieno-pyrrole systems; Snyder and his group have prepared a large variety of compounds, perhaps culminating in the synthesis of the thiophene analogues of tryptophan and tryptamine.[1]



Scheme 1.

There are three different *b*-fused isomers (A—C) for each unsubstituted heterocyclic analogue of indole (see Scheme 1).

In the furan series none of the parent compounds is known, and only a few simple derivatives have been described.[2,3] In the thiophene series two of the parent compounds, A and B, are known. Both have been prepared by decarboxylation of the corresponding 5-carboxy derivatives.[4,5] Thieno[3,2-*b*]pyrrole (B) has also been prepared by sodium borohydride reduction of 2*H*-thieno[3,2-*b*]pyrrol-3-one.[6] However, since Hemetsberger *et al.*[3] recently have described a new, easy way to generate 5-carboxy derivatives in these series, the former method seems to be the one of choice. A great number of derivatives of thiophene systems has been reported in the literature. In the selenophene series none of the parent compounds is known and only a few derivatives have been described.[7]

SYNTHESIS

This paper describes a general route to 5-acyl derivatives of furo-, thieno-, and selenolo[3,2-*b*]-pyrroles by thermal cyclization of suitably substituted 3-azidofurans, -thiophenes, and -selenophenes. This type of reaction has been used earlier in the benzene series for preparation of substituted indoles [8] and can mechanistically be regarded as an insertion of the initially generated nitrene into a C—H bond. The azido compounds used as starting materials in this study were 3-azido-2-formylfuran, -thiophene, and -selenophene.[9] The issue at hand was then to convert the formyl function into the necessary vinylic moiety. In principle, this could be done either by Wittig olefin synthesis or by condensation with compounds containing an active methyl or methylene group. The former

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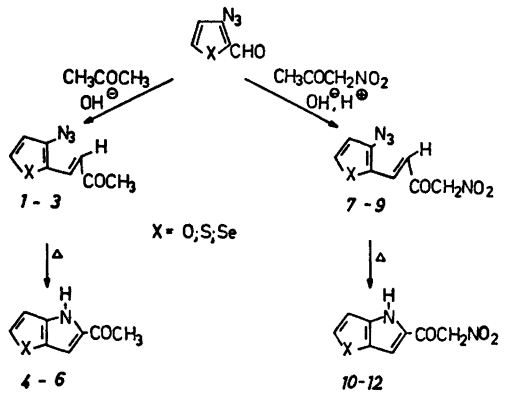


Scheme 2.

method has been shown in this laboratory [10] to be inapplicable in these azido systems, and thus the second method was adopted.

When the azido aldehydes were treated with excess acetone in 2 % sodium hydroxide solution (see Scheme 2), the expected vinylic compounds *1 – 3* were formed in good yields (77 – 92 %). If stronger alkaline solutions were used (> 5 %), the reaction mixtures grew dark and a slow evolution of gas, indicating destruction of the azido function, took place.

These vinylic compounds were then heated in xylene at temperatures of 120 – 130 °C (see Scheme 2) until the evolution of nitrogen gas had ceased. Thus *4 – 6* were obtained in good to excellent yields (69 – 90 %).

Dornow *et al.*[11] have shown that nitroacetone in alkaline medium reacts with aromatic formyl groups to give α,β-unsaturated carbonyl compounds, and that it is the methyl group which participates in the condensation. In order to obtain any significant yields from the reaction of nitroacetone with the azido aldehydes used in this study we found it necessary to modify Dornow's procedure. Thus a much larger amount of alkaline water solution had to be used, probably due to the low solubility of the azido aldehydes in water. We also found it more advantageous to isolate the sodium salt of the product prior to the acidification. Under these conditions (see Scheme 2) the vinylic compounds *7 – 9* were obtained in good yields (65 – 72 %). These vinylic compounds were then heated in an inert solvent under the same conditions as described above for compounds *1 – 3* (see Scheme 2). The heating was continued until no asymmetric N_3-stretching absorption was evident from the IR spectra of the reaction mixture. This gave *10 – 12* in moderate to good yields (54 – 78 %). In the cyclization of the furan compound *7* the reaction was accompanied by extensive tar formation. This could perhaps be due to a sensitivity of the furan ring to the acidic methylene group in the side-chain, at these temperatures.

Russian workers [12] have shown that 2-acetylindole can easily be isomerized to the 3-acetyl compound by the action of various acids (trifluoroacetic acid, polyphosphonic acid). When *5* was subjected to treatment by these acids, either the starting material was recovered unchanged or only resinous material could be isolated, depending on the reaction conditions. No sign of isomerization was evident in this case.

In a futile effort to reduce the carbonyl function of *11* by the Wolff-Kishner method, it was found by VPC that one of the products was unsubstituted thieno[3,2-*b*]pyrrole (*13*). The procedure was then modified and when *11* was treated with potassium hydroxide in diethylene glycol at 190 – 195 °C, under nitrogen, for one hour, this gave *13* as the major product in a yield of 51 %. This reaction sequence then offers an alternative to Snyder's two previously reported methods for preparing this compound.[4,6] Deswarte [13] and Pearson *et al.*[14] have shown that ω-nitroacetophenone under neutral and alkaline conditions is cleaved to benzoic acid and nitromethane or their anions. In order to demonstrate if this cleavage also occurred under our conditions, ω-nitroacetophenone was treated in the same way as *11*. After acidification, an almost quantitative yield of benzoic acid was obtained and no benzene could be detected by VPC. Since it is known from the literature [15] that at least 2-carboxyindoles can decarboxylate under the influence of alkali, it seems plausible that the reaction of *11* with potassium hydroxide at high temperatures could take place *via* the carboxylate anion. However, it should be pointed out that 5-carboxythieno-[3,2-*b*]pyrrole could not be isolated from these reactions. When *10* and *12* were subjected to the same conditions as described above, tars were formed. Neither the unsubstituted systems nor the 5-carboxy derivatives could be isolated from or detected in these tars.

The results presented above and the availability of a number of different carbonyl compounds capable of reacting in a similar manner shows this to be a useful method for obtaining 5-acyl derivatives in these series. This route could prove to be of synthetic value for the preparation of furan, thiophene, and selenophene analogues of naturally occurring indole derivatives.[16]

SPECTRAL DATA

The IR spectra of the azido compounds prepared in this work showed N_3-asymmetric stretching absorptions in the region of *ca.* 2110 – 2140 cm^{-1}. The fused pyrroles showed characteristic absorptions in the region of *ca.* 3250 – 3400 cm^{-1} due to NH-stretching vibrations.

The ^1H NMR data for most of the compounds are given in the experimental part. The assignments of different bands to the various protons have been based on knowledge of shifts and coupling constants in furans, thiophenes, selenophenes, and indoles, and on comparison with literature data for previously prepared compounds in these series.[17,18] The ^1H NMR spectra for the vinylic compounds *1 – 3* and *7 – 9* showed vicinal coupling constants of the magnitude 15.5 – 16.6 Hz indicating an exclusive *trans* configuration for these compounds.

As far as we know, no ^1H NMR spectral data for the unsubstituted thieno[3,2-*b*]pyrrole (*13*) have been hitherto reported. Therefore the spectrum of this compound, in carbon tetrachloride, was analyzed as a 5-spin system in an iterative mode utilizing an extended version of the QCPE program UEAITR (No. 188).[19] The results of this treatment are as follows: δ 6.84 (H-2), 6.48 (H-3), 7.32 (H-4), 6.46 (H-5), 6.22 (H-6). $J_{23} = 5.35$ Hz; $J_{25} = 1.40$ Hz; $J_{36} = 0.70$ Hz; $J_{45} = 2.55$ Hz; $J_{46} = 1.80$ Hz; $J_{56} = 3.00$ Hz.

We have also determined the ^{13}C NMR parameters for thieno[3,2-*b*]pyrrole (*13*). This was done with deuteriochloroform as solvent and deuterium as internal lock signal. The shifts were obtained from the proton decoupled spectre using TMS as internal standard. The assignments of shifts and coupling constants were made as described below.

The proton decoupled spectra showed six distinct signals. Two of these, at 121.9 and 138.6 ppm, were of low intensity indicating that they arise from the quaternary, bridging carbon atoms (C-7 and C-8, see Scheme 1). However, it is not evident which one is which since it is difficult to estimate the size of the mesomeric and inductive effects of the heteroatoms at these positions. Since the undecoupled spectrum showed signs of long-range couplings between carbon atoms and the nitrogen proton, and since we felt it safe to assume that such couplings could only occur to a significant extent with the carbon atoms in the pyrrole part of the molecule, we tried to eliminate these couplings in order to facilitate the assignments of the remaining four peaks. Addition of piperidine to a dioxan solution of pyrroles gives a rapid hydrogen exchange which effectively eliminates the couplings between the α- and β-protons and the nitrogen proton.[20] In order to ascertain if this was also the case for thieno-[3,2-*b*]pyrrole (*13*) we added a few drops of piperidine to a solution in deuteriochloroform. The ^1H NMR spectra showed a complete decoupling of the nitrogen proton couplings as did also the ^{13}C NMR spectra.

This result clearly indicated that the signals at δ 101.3 and 123.0 correspond to the peripheral carbon atoms in the pyrrole ring while those at δ 111.2 and 123.7 correspond to those in the thiophene ring. Comparison with literature data for the direct carbon-hydrogen couplings in pyrrole ($J_{C\alpha H\alpha}$ 184 Hz, $J_{C\beta H\beta}$ 170 Hz)[21] gives the assignments of carbons 5 and 6 (see Scheme 1). Further proof for this can be found from the fact that a small downfield shift (~0.2 ppm) was observed for the peak assigned to carbon 5 when piperidine was added to the sample. A similar but larger effect was also observed in the ^1H NMR spectra for the 5-proton when piperidine was added. It seems that the closeness to the centre where exchange is taking place gives rise to a downfield shift. The assignment of carbons 2 and 3 (see Scheme 1) was made by comparison with literature data for direct couplings and long-range couplings for thiophene compounds.[22] The ^{13}C parameters found were thus: δ 123.7 (C-2), 111.2 (C-3), 123.0 (C-5), 101.3 (C-6), 121.9 [C-7 (or C-8)], 138.6 [C-8 (or C-7)]. $J_{C_2H_2}$ 185 Hz, $J_{C_2H_3}$ 6.8 Hz, $J_{C_3H_3}$ 170 Hz, $J_{C_3H_2}$ 4.6 Hz, $J_{C_3H_5 \text{ or } H_6}$

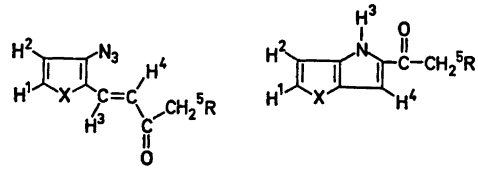
0.7 Hz, $J_{C_5H_5}$ 185 Hz, $J_{C_5H_4}$ 4.1 Hz, $J_{C_5H_5}$ 8.4 Hz, $J_{C_6H_6}$ 176 Hz, $J_{C_6H_4}$ 5.9 Hz, $J_{C_6H_5}$ 8.2 Hz, $J_{C_6H_2 \text{ or } H_3}$ 1.4 Hz.

It is interesting to note that the α-carbon in the thiophene part of the molecule resonates at lower field than the β-carbon. This is the opposite of what has been found for thiophene itself.[22] A similar but smaller reversing effect has also been demonstrated in the ^{13}C NMR spectra of benzo[*b*]thiophene.[23]

Another point of interest is that there are long-range couplings between the two rings. Thus carbon 3 couples with one of the protons in the pyrrole moiety and carbon 6 couples with one of the thiophene protons. Some pertinent information about which protons these carbons are coupled to could probably be obtained from deuteration experiments. However, due to the lability of the thieno[3,2-*b*]-pyrrole system, no efforts in this direction have been made. Similar long-range couplings have been observed in other systems.[24]

EXPERIMENTAL

The ^1H NMR spectra were obtained with a Varian A-60 high resolution spectrometer and a Jeol MH-100 spectrometer. The ^1H NMR data given below refers to the following protons:



X = O, S, Se
R = H, NO_2

The ^{13}C NMR spectra were recorded on a Jeol FX-60 spectrometer. The IR spectra were recorded on a Perkin-Elmer spectrometer model 257. The gas chromatograph used was a Perkin-Elmer 900 analytical instrument (OV-25, 3 %, Chrom Q, 3 m; BDS, 10 %, Chrom W., 2 m). Mass spectra were obtained with an LKB 9000 mass spectrometer. Elementary analyses were carried out at the Analytical Department of the Chemical Institute and by Dornis and Kolbe, Mikroanalytisches Laboratorium, Mülheim/Ruhr.

trans-*4-(3-Azido-2-furyl)-3-buten-2-one* (*1*). To a solution of 1.5 g of sodium hydroxide in 75 ml of water, 6.7 g (0.049 mol) of 3-azido-2-formylfuran [9] in 30 ml of acetone was added. The resulting heterogeneous mixture was stirred at room temperature for one hour and then cooled in ice. The resulting precipitate was filtered off and recrystallized from methanol, giving 7.5 g (85 %) of the product with m.p. ~105 °C (decomp.). IR spectrum (KBr): N_3= 2120 cm^{-1}. NMR spectrum ($CDCl_3$): δ 7.46 (H-1), 6.49 (H-2), 7.22 (H-3), 6.52 (H-4), 2.30 (H-5); $J_{1,2}$ 2.2 Hz, $J_{3,4}$ 15.9 Hz. [Found: C 54.2; H 4.03; N 24.3; m.wt. 177. Calc. for $C_8H_7N_3O_2$: C 54.2; H 3.99; N 23.7; m.wt. 177.18].

trans-*4-(3-Azido-2-thienyl)-3-buten-2-one* (*2*) was prepared as described above for *1* from 2.0 g of sodium hydroxide, 100 ml of water, 10.0 g (0.0654 mol) of 3-azido-2-formylthiophene [9] and 35 ml of acetone. This gave after recrystallization from methanol 11.0 g (92 %) of the product, m.p. ~105 °C (decomp.). IR spectrum (KBr): N_3= 2120 cm^{-1}. NMR spectrum ($CDCl_3$): δ 7.42 (H-1), 6.95 (H-2), 7.58 (H-3), 6.42 (H-4), 2.30 (H-5); $J_{1,2}$ 5.6 Hz, $J_{2,3}$ 0.8 Hz, $J_{3,4}$ 16.6 Hz. [Found: C 49.5; H 3.65; S 16.5; m.wt. 193. Calc. for $C_8H_7N_3OS$: C 49.7; H 3.66; S 16.6; m.wt. 193.24].

trans-*4-(3-Azido-2-selenyl)-3-buten-2-one* (*3*) was prepared as described above for *1* from 1.0 g of sodium hydroxide, 50 ml of water, 6.0 g (0.030 mol) of 3-azido-2-formylselenophene [9] and 25 ml of acetone. This gave after recrystallization from methanol 5.5 g (77 %) of the product, m.p. 87.0 – 90.0 °C (decomp.). IR spectrum (KBr): N_3= 2110 cm^{-1}. NMR spectrum ($CDCl_3$): δ 8.04 (H-1), 7.23 (H-2), 7.64 (H-3), 6.25 (H-4), 2.87 (H-5); $J_{1,2}$ 5.9 Hz, $J_{3,4}$ 16.0 Hz. [Found: C 40.1; H 2.94; Se 32.9; m.wt. 241. Calc. for $C_8H_7N_3OSe$: C 40.0; H 2.94; Se 32.9; m.wt. 240.14].

5-Acetylfuro[3,2-b]pyrrole (*4*). 5.2 g (0.029 mol) of 4-(3-azido-2-furyl)-3-buten-2-one (*1*) was dissolved in 175 ml of xylene. The mixture was heated at 120 – 130 °C with stirring and kept at this temperature until the evolution of nitrogen gas had ceased (30 – 45 min). The solution was then allowed to attain room temperature, whereupon the solvent was removed by evaporation. The dark residue was filtered off and repeatedly washed with low-boiling petroleum ether. Charcoal treatment and recrystallization from toluene gave 3.0 g (69 %) of the product, m.p. 184.5 – 187.0 °C. IR spectrum (KBr): NH = 3280 cm^{-1}, CO = 1630 cm^{-1}. NMR spectrum (DMSO-d_6): δ 7.82 (H-1), 6.62 (H-2), 11.7 (H-3), 6.98 (H-4), 2.45 (H-5); $J_{1,2}$ 2.2 Hz, $J_{2,4}$ 0.8 Hz, $J_{3,4}$ 1.7 Hz. [Found: C 64.8; H 4.78; N 9.27; m.wt. 149. Calc. for $C_8H_7NO_2$: C 64.4; H 4.74; N 9.39; m.wt. 149.16].

5-Acetylthieno[3,2-b]pyrrole (*5*) was prepared as described above for *4* from 7.5 g (0.039 mol) of 4-(3-azido-2-thienyl)-3-buten-2-one (*2*) in 200 ml of xylene. Recrystallization from toluene gave 5.8 g (90 %) of the product, m.p. 160.5 – 163.0 °C. IR spectrum (KBr): NH = 3295 cm^{-1}, CO = 1630 cm^{-1}. NMR spectrum (DMSO-d_6): δ 7.59 (H-1), 7.02 (H-2), 11.9 (H-3), 7.30 (H-4), 2.45 (H-5); $J_{1,2}$ 5.4 Hz, $J_{2,4}$ 0.8 Hz, $J_{3,4}$ 2.2

Hz. [Found: C 58.2; H 4.24; S 19.2; m.wt. 165. Calc. for C_8H_7NOS: C 58.2; H 4.28; S 19.4; m.wt. 165.22].

5-Acetylselenolo[3,2-b]pyrrole (6) was prepared as described above for *4* from 4.2 g (0.017 mol) of 4-(3-azido-2-selenyl)-3-buten-2-one (*3*) in 125 ml of xylene. Charcoal treatment and recrystallization from toluene gave 3.0 g (82 %) of the product, m.p. 167.0 – 168.0 °C. IR spectrum (KBr): NH = 3250 cm^{-1}, CO = 1625 cm^{-1}. NMR spectrum (DMSO-d_6): δ 8.04 (H-1), 7.27 (H-2), 11.9 (H-3), 7.29 (H-4), 2.30 (H-5); $J_{1,2}$ 6.0 Hz, $J_{3,4}$ 0.7 Hz. [Found: C 45.4; H 3.36; Se 37.2; m.wt. 213. Calc. for C_8H_7NOSe: C 45.3; H 3.33; Se 37.2; m.wt. 212.12].

trans-*4-(3-Azido-2-furyl)-1-nitro-3-buten-2-one (7)*. 10.0 g (0.0730 mol) of 3-azido-2-formylfuran [9] and 10.0 g (0.0971 mol) of nitroacetone [19] were dispersed in 400 ml of 5 % sodium hydroxide solution. The heterogeneous mixture was stirred at room temperature for 24 h and during this time a voluminous, yellow precipitate, consisting of the sodium salt of the product, was formed. This precipitate was filtered off and as fast as possible added with stirring to 300 ml of ice water containing 14 ml of conc. hydrochloric acid. The stirring was then continued for 30 min, whereupon the now distinctly orange precipitate was filtered off and allowed to dry. This orange substance was treated with 25 ml of methanol at a temperature of 35 – 40 °C for 5 min, with stirring. The colour of the solid material changed during this operation from orange to yellow. The mixture was cooled in ice and the solid material filtered off. This gave 11.2 g (70 %) of the product. An analytical sample which was obtained by recrystallization from ethanol showed an IR spectrum identical to that of the crude product; m.p. 81.5 – 83.0 °C. IR spectrum (KBr): N_3 = 2120 cm^{-1}, CO = 1665 cm^{-1}. NMR spectrum (DMSO-d_6): δ 7.97 (H-1), 6.70 (H-2), 7.35 (H-3), 6.51 (H-4), 6.05 (H-5); $J_{1,2}$ 2.0 Hz, $J_{3,4}$ 15.5 Hz. [Found: C 43.4; H 2.75; N 25.2; m.wt. 222. Calc. for $C_8H_6N_4O_4$: C 43.2; H 2.73; N 25.2; m.wt. 222.18].

trans-*4-(3-Azido-2-thienyl)-1-nitro-3-propen-2-one (8)* was prepared as described above for *7* from 13.0 g (0.0850 mol) of 3-azido-2-formylthiophene,[9] 9.7 g (0.094 mol) of nitroacetone [19] and 800 ml of 5 % sodium hydroxide solution. This gave after treatment with warm methanol 14.6 g (72 %) of the product. An analytical sample which was obtained by recrystallization from ethanol showed an IR spectrum identical to that of the crude product; m.p. ~105 °C (decomp.). IR spectrum (KBr): N_3 = 2140 cm^{-1}, CO = 1675 cm^{-1}. NMR spectrum (DMSO-d_6): δ 7.89 (H-1), 7.26 (H-2), 7.65 (H-3), 6.57 (H-4), 6.02 (H-5); $J_{1,2}$ 5.3 Hz, $J_{3,4}$ 15.7 Hz. [Found: C 40.1; H 2.66; S 13.5; m.wt. 238. Calc. for $C_8H_6N_4O_3S$: C 40.3; H 2.54; S 13.5; m.wt. 238.24].

trans-*4-(3-Azido-2-selenyl)-1-nitro-3-propen-2-one (9)* was prepared as described above for *7* from 2.5 g (0.013 mol) of 3-azido-2-formylselenophene,[9] 1.5 g (0.015 mol) of nitroacetone [19] and 160 ml of 5 % sodium hydroxide solution. However, the reaction time in this case was 48 h. After treatment with warm methanol, 2,7 g of the crude product was obtained. Recrystallization from ethanol gave 2.4 g (65 %) of the product; m.p. ~105 °C (decomp.). IR spectrum (KBr): N_3 = 2105 cm^{-1}, CO = 1685 cm^{-1}. NMR spectrum (DMSO-d_6): δ 8.48 (H-1), 7.51 (H-2), 7.71 (H-3), 6.52 (H-4), 5.96 (H-5); $J_{1,2}$ 6.0 Hz, $J_{3,4}$ 15.6 Hz. [Found: C 33.7; H 2.29; Se 27.8; m.wt. 286. Calc. for $C_8H_6N_4OSe$: C 33.7; H 2.13; Se 27.7; m.wt. 285.14].

5-Nitroacetylfuro[3,2-b]pyrrole (10). 11.2 g (0.0505 mol) of *trans*-4-(3-azido-2-furyl)-1-nitro-3-buten-2-one (*7*) was dissolved in 350 ml of xylene. The mixture was heated at 120 – 125 °C with stirring until its IR spectrum (film) showed no more asymmetric N_3-stretching absorption. The solution was then allowed to attain room temperature, whereupon the solvent was removed by evaporation. The dark residue was filtered off and repeatedly washed with low-boiling petroleum ether. The solid was then transferred to a Soxhlet apparatus and continuously extracted with 350 ml of methanol for 5 days. Evaporation of the solvent gave 5.3 g (54 %) of the product. An analytical sample, obtained by recrystallization from methanol, showed an IR spectrum identical with that of the crude product, m.p. 202.5 – 205.0 °C (decomp.). IR spectrum (KBr): NH = 3290 cm^{-1}, CO = 1640 cm^{-1}. NMR spectrum (DMSO-d_6): δ 7.94 (H-1), 6.68 (H-2), 12.0 (H-3), 7.15 (H-4), 6.12 (H-5); $J_{1,2}$ 2.2 Hz, $J_{2,4}$ 0.9 Hz, $J_{3,4}$ 1.5 Hz. [Found: C 49.1; H 3.14; O 33.1; m.wt. 194. Calc. for $C_8H_6N_2O_4$: C 49.5; H 3.12; O 33.0; m.wt. 194.16].

5-Nitroacetylthieno[3,2-b]pyrrole (11) was prepared as described above for *10* from 10.2 g (0.0429 mol) of *trans*-4-(3-azido-2-thienyl)-1-nitro-3-propen-2-one (*8*) in 500 ml of xylene. In this case, however, the crude product, obtained after evaporation of xylene and thorough washing with petroleum ether, was treated with 600 ml of boiling methanol and filtered while hot. The filtrate was evaporated to dryness to give 7.0 g (78 %) of the product. An analytical sample, obtained by recrystallization from methanol, showed an IR spectrum identical with that of the crude product, m.p. 171.5 – 174.5 °C (decomp.). IR spectrum (KBr): NH = 3320 cm^{-1}, CO = 1645 cm^{-1}. NMR spectrum (DMSO-d_6): δ 7.70 (H-1), 7.06 (H-2), 12.4 (H-3), 7.47 (H-4), 6.20 (H-5); $J_{1,2}$ 5.3 Hz, $J_{2,4}$ 0.6 Hz, $J_{3,4}$ 1.5 Hz. [Found: C 45.5; H 2.91; S 15.1; m.wt. 210. Calc. for $C_8H_6N_2O_3S$: C 45.7; H 2.88; S 15.3; m.wt. 210.22].

5-Nitroacetylselenolo[3,2-b]pyrrole (12) was prepared as described above for *10* from 3.5 g (0.012 mol) of *trans*-4-(3-azido-2-selenyl)-1-nitro-3-propen-2-one (*9*) in 150 ml of xylene. In this case, however, the crude product, obtained after evaporation of xylene and thorough

washing with petroleum ether, was treated with 500 ml of boiling methanol and charcoal and filtered while hot. The filtrate was evaporated to dryness, yielding 2.3 g (74 %) of the product. An analytical sample, obtained by recrystallization from methanol, showed an IR spectrum identical with that of the crude product, m.p. 177.0 – 181.0 °C (decomp.). IR spectrum (KBr): NH = 3320 cm^{-1}, CO = 1640 cm^{-1}. NMR spectrum (DMSO-d_6): δ 8.15 (H-1), 7.25 (H-2), 12.4 (H-3), 7.40 (H-4), 6.15 (H-5); $J_{1,2}$ 5.7 Hz. [Found: C 37.4; H 2.40; Se 30.7; m.wt. 258. Calc. for $C_6H_5N_2O_2Se$: C 37.4; H 2.36; Se 30.7; m.wt. 257.12].

*Thieno[3,2-*b*]pyrrole (13).* 2.5 g (0.012 mol) of 5-nitroacetylthieno[3,2-*b*]pyrrole (*11*) and 2.8 g of potassium hydroxide were dispersed in 50 ml of diethylene glycol. The mixture was heated to 190 – 195 °C with stirring and nitrogen inlet, and kept at this temperature for one hour. After attaining room temperature, the black solution was poured into 200 ml of water and the resulting mixture extracted four times with 75 ml of ether. The combined ether phases were washed once with 40 ml of water and dried over magnesium sulfate. Evaporation of the solvent gave 1.1 g of an oil which was immediately transferred to a sublimation apparatus. At 60 – 80 °C/1.2 mmHg, 0.75 g (51 %) of product condensed on the water-cooled sublimation finger; m.p. 24 – 26 °C (lit. value:[6] 25 – 28 °C). IR spectrum (film): NH = 3400 cm^{-1}. NMR spectrum (CCl_4): see text.

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Formation of Aromatic Compounds from Carbohydrates. Part III.* Reaction of D-Glucose and D-Fructose in Slightly Acidic, Aqueous Solution

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The reaction of D-fructose in aqueous solutions of pH 4.5 at 96 or 160 C° yielded 5-(hydroxymethyl)-2-furaldehyde (*1*), 5,5′-oxy-dimethylene-bis(2-furaldehyde) (*2*), 5-(acetoxymethyl)-2-furaldehyde (*3*), 1-(2-furyl)-2-hydroxyethanone (*4*), 1,2-benzenediol (*5*), 1,2,3-benzenetriol (*6*), 6,7-dihydroxy-1(3*H*)-isobenzofuranone (*7*), 3-methyl-1,2,benzenediol (*8*), 4-methyl-1,2-benzenediol (*9*), 4-methyl-1,2,3-benzenetriol (*10*), 3-hydroxy-6-hydroxymethyl-2-methyl-4*H*-1-benzopyran-4-one (*11*), 1-(3,4-dihydroxy-6-methylphenyl)-2-hydroxyethanone (*12*), 3,4-dihydroxybenzaldehyde (*13*), and 2-methylbenzofuran-5,6-diol (*14*). The reaction of D-glucose at pH 4.5 and 96 °C gave the same compounds but in lower yields. Compounds *7*, *11*, *12*, and *14* as well as the synthesised 4,5-dihydroxy isomer of *7* seem to be new compounds. The ^{1}H NMR-spectra of *o*-dihydroxy- and *o*-dimethoxy-1(3*H*)-isobenzofuranones are discussed.

Acid-catalyzed dehydration reactions of D-fructose and D-glucose in aqueous solution have been extensively studied [1–4] (for a recent summary, see Ref. 1), primarily with respect to nonenzymatic browning and the formation of flavours in foods. 5-(Hydroxymethyl)-2-furaldehyde (*1*), 1-(2-furyl)-2-hydroxyethanone (*4*), levulinic acid and formic acid are long-known major products of these reactions. More recently Shaw *et al.*[3,4] also identified 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, 2-acetyl-3-hydroxyfuran (isomaltol), 5-methyl-2-furaldehyde, and 3,4,5-trihydroxy-3,5-hexadiene-2-one (acetylformoin) as minor products from acid treatment of D-fructose.

We recently isolated a series of cyclic degradation products, including many catechols and chromones, from the treatment of hexuronic acids and pentoses in slightly acidic, aqueous solution.[5,6] In the present investigation, we have studied the possible formation of phenolic compounds by the similar treatment of D-fructose and D-glucose.

RESULTS AND DISCUSSION

The ethyl acetate soluble part of the reaction mixture from treatment of D-glucose and D-fructose yielded the compounds given in Scheme 1 after chromatographic fractionation. These included the furan derivatives *1*, *2* and *4* previously obtained from hexoses [1,2] and the phenols *5*, *6* and *8* resulting from hexuronic acids under similar conditions.[5] The yields and chromatographic properties of the compounds are given in Table 1. It was shown that none of these compounds originated from solvents or chromatographic material or were present as impurities in the original carbohydrates.

Compounds *1*, *3*, *5*, *6*, *8*, *9*, *10*, and *13* were identical (IR, MS, NMR) with authentic samples and the identification of compounds *2* and *4* was based on NMR, MS and a comparison of their melting points with those previously reported.[2]

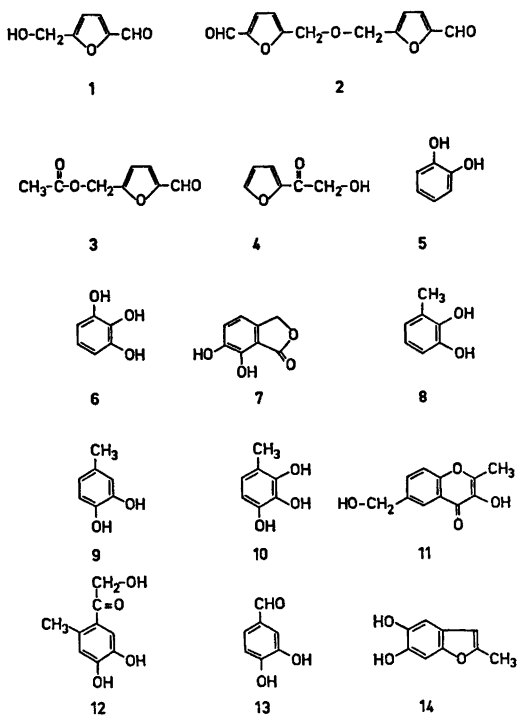
Elemental analysis of compound *7* corresponded to $C_8H_6O_4$. The NMR data of *7* and its dimethyl ether indicated an isobenzofuranone structure containing two hydroxyl groups and two adjacent aromatic protons but did

* Part II. See Ref. 15.

Table 1. Yields and chromatographic properties of compounds isolated after treatment of D-fructose and D-glucose at pH 4.5.

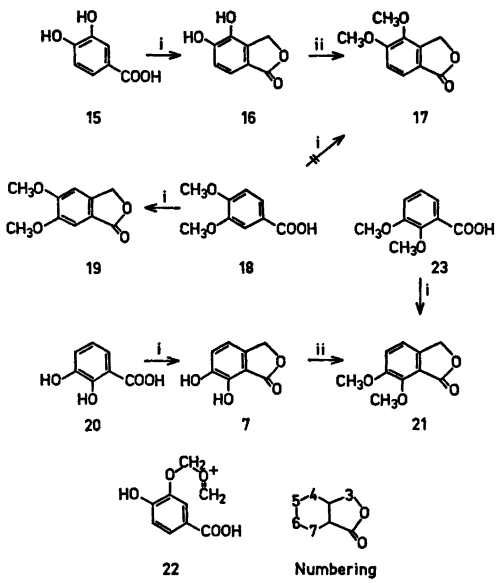
Compound	Yield %				Colour	
	Exp. A	Exp. B	Exp. C	R_C[c]	Spray A	Spray B
1	2.60	1.40	3.40	0.88	orange	–
2	0.01	0.02	0.09	1.88	orange	–
3	0.04	0.01	0.06	2.00	orange	–
4	0.04	0.01	0.07	1.58	brownish yellow	–
5	[a]	[a]	0.02	1.00	gray	bluish gray
6	[a]	[a]	0.03	0.40	grayish green	black
7	0.03	0.01	0.13	0.71	grayish yellow	blue
8	[a]	[a]	0.02	1.42	grayish red	black
9	[a]	[a]	0.01	1.04	grayish brown	grayish green
10	[a]	[a]	0.01	0.60	brown	bluish black
11	[a]	[a]	0.02	0.30[b]	–	violet
12	[a]	[a]	[a]	0.30	–	blue
13	[a]	[a]	[a]	0.52	orange	bluish green
14	[a]	[a]	0.02	0.98	greenish yellow	pale green

[a] Trace amounts. [b] Tailing. [c] Mobilities relative to compound *5*.



not distinguish between the 4,5-, 6,7- and 4,7-dihydroxy derivatives. The two former isomers (*16* and *7*, respectively) were synthesised from the 3,4- and 2,3-dihydroxybenzoic acids (*15* and *20*, respectively; see Scheme 2). The synthetic sample of *7* was identical in all respects (IR, NMR and MS) with that obtained in experiments A, B and C. The dimethyl ether of 7 was identical with the previously known compound meconin (*21*),[7] synthesised from 2,3-dimethoxybenzoic acid (*23*). In the syntheses of the isobenzofuranones (Scheme 2), we found it notable that reaction of 3,4-dihyhroxybenzoic acid (*15*) with formaldehyde in concentrated hydrochloric acid gave the 4,5-dihydroxy derivative (*16*), while 3,4-dimethoxybenzoic acid (*18*) gave the 5,6-dimethoxy derivative (*19*). The formation of *16* from *15* might be explained by primary attack on the *meta*-hydroxyl group by the protonated, dehydrated dimer of formaldehyde to give the intermediate *22*. Similar attack on the *para*-hydroxyl group is disfavoured by resonance involving the carboxyl group. Although the yield is modest, the synthesis of *17* from *15* via *16* is much simpler than the previously reported procedure.[8]

The NMR spectral data for the isobenzofuranones are collected in Table 2. All assignments of the protons were confirmed by appropriate spin decoupling experiments. The $|J|$ values (0.2 – 0.3 Hz) referring to a methoxyl group and its *ortho* proton are as expected.[9] As to the $|J|$ values involving an aromatic proton and those of the ring methylene group, the position in the benzene ring may be arranged in the order 4 > 6 > 7 > 5. This is con-



i: HCHO in conc. HCl ; ii: CH_2N_2 in CH_3-OH

sistent with the order $o > p > m$ for the corresponding toluene constants.[10] The order 7 > 5 may be explained by the more extended, zigzag-like path between a methylene proton and that in position 7.[10] Apparently, only the coupling to proton 4 has been observed previously.[11]

The mass spectrum of compound *11* was very similar to those of 3-hydroxy-2-methylchromones;[6] a molecular ion and losses of 28, 29, 55, and 71 mass units (m.u.). The elemental composition (MS) corresponded to $C_{11}H_{10}O_4$, indicating a hydroxymethyl or methoxy derivative of 3-hydroxy-2-methyl-chromone. The presence of a hydroxymethyl group was supported by the NMR spectra of the monomethyl ether and of the diacetate. The aromatic proton (H-5) in the latter spectrum showed up at δ 8.19. A comparison between this shift and that of H-5 in 2-methylchromone (δ 8.15) previously reported,[12] and our value [6] (δ 8.08) for the diacetate of 3,8-dihydroxy-2-methylchromone, indicated the structure 3-hydroxy-6-hydroxymethyl-2-methyl-4*H*-1-benzopyran-4-one, (3-hydroxy-6-hydroxymethyl-2-methylchromone) or its 7-hydroxymethyl-isomer. The former structure was assigned, however, based upon the small $|J|$-value (2.2 Hz) for H-5.

The elemental composition (MS) of compound *12* corresponded to $C_9H_{10}O_4$ and methylation gave a dimethyl ether. In the MS of compound *12*, the loss of 31 m.u. from the molecular ion (*m/e* 182) to give an intense peak at *m/e* 151 indicated the presence of a hydroxyacetyl group as in compound *4*. The NMR of compound *12* showed a broad three-proton singlet at δ 2.41, a two-proton singlet at δ 4,63 and two one-proton singlets at δ 6.67 (broad) and at δ 7.18. The high δ-values for the methyl group and one of the aromatic protons indicated

Table 2. ^{1}H NMR spectral data for 1(3*H*)-isobenzofuranones.[a]

Positions (s)	*16* in CD_3OD	*17* in $CDCl_3$	*19* in $CDCl_3$	*7* in CD_3OD	*21* in $CDCl_3$
δ values					
3	5.24	5.31	5.22	5.20	5.18
4		*3.95*	6.91	6.81	7.07
5		*3.96*	*3.98*	7.14	7.25
6	6.96	7.08	*3.94*		*3.91*
7	7.25	7.62	7.31		*4.09*
Coupling constants [J(Hz)]					
3,4			0.8	0.8	0.9
3,5				0.1	0.1
3,6	0.4	0.5			
3,7	0.1	0.3	0.3		
4,5			*0.2 – 0.3*	8.0	8.4
4,7			0.3		
5,6		*0.2 – 0.3*			*0.2 – 0.3*
6,7	8.2	8.2	*0.2 – 0.3*		

[a] Data for methoxyl groups are italicized; data for hydroxyl groups have been omitted.

ortho-position to the carbonyl group, and since no coupling between the aromatic protons was found, the structure 1-(3,4-dihydroxy-6-methylphenyl)-2-hydroxyethanone was assigned to compound *12*. For comparison, 1-(3,4-dihydroxy-6-methylphenyl)-ethanone was synthesised and found to have a very similar NMR spectrum as compound *12* (see EXPERIMENTAL). The elemental composition (MS) of compound *14* corresponded to $C_9H_8O_3$. The MS of compound *14* showed a very similar fragmentation pattern including a doubly charged ion (*m/e* 81.5 for *14*) to that of 2-methyl-benzofuran.[13] Methylation gave a dimethyl ether, established by NMR as 5,6-dimethoxy-2-methyl-benzofuran (see EXPERIMENTAL). The chemical shift and coupling constants of proton H-3 are in accordance with those reported for 2-methyl-benzofurans.[14]

To our knowledge compounds *7*, *11*, *12*, and *14*, isolated in the present investigation, as well as the synthesised compound *16*, are not previously reported.

The present results show that hexoses are degraded to various phenolic compounds, which might be active intermediates in the colour formation by heat treatment or storing of foods or solutions of fructose, glucose or sucrose under slightly acidic conditions. Naturally, the formation of these compounds, although formed to a much lower extent than the phenols from the corresponding treatment of pentoses and hexuronic acids,[5] might under more drastic treatments also introduce other non-wanted properties apart from discoloration to the products. The degradation products of hexoses described above differ markedly from those of pentoses and hexuronic acid,[5,6] and also from those of D-glucose produced in alkali.[15] Only compounds *5*, *9* and *13* were identified from both acid and alkaline treatments of this monosaccharide.

EXPERIMENTAL

Melting points are corrected. Concentrations were carried out at reduced pressure below 40 °C. TLC was performed on silica gel HF_{254} (Merck) with 9:1 chloroform/acetic acid as solvent. Silicic acid (100 mesh Mallinckrodt) and Sephadex LH-20 were used for column chromatography. TLC plates were studied in UV light before treatment with (A) *p*-anisidine hydrochloride (B) iron(III) chloride, (C) diazotised sulfanilic acid or (D) 25 % sulfuric acid (and heating) as spray reagents. The sublimations (or distillation) were done at 0.5 mmHg in an electrically heated tube. NMR spectra were recorded at 60 or 100 MHz (Perkin Elmer R 12 and Varian HA 100 D, respectively) and chemical shifts are given in δ units (d, dd, m, and s denote doublet, double doublet, multiplet, and singlet, respectively). Mass spectra were recorded on a Varian CH-7 and high resolution mass spectra on a Varian SM 1 instrument.

Experiment A. D-Fructose (400 g) in 0.3 M acetate buffer of pH 4.5 (2.0 l) was heated at 96 °C for 48 h with a stream of nitrogen bubbling through the solution. The cooled brown solution was continuously extracted for 2 days with ethyl acetate (2 × 1.3 l) The ethyl acetate extract (27.8 g) was fractionated on a Sephadex LH-20 column (4.7 × 110 cm). Six main fractions were collected by elution with water, and a final one using ethanol as solvent. In fraction I (3.36 g) no aromatic compounds detected; II (13.63 g) compound *1–4*; III (0.8 g) compounds *5–7* and *11*; IV (0.56 g) compounds *8–10*; V (0.78 g) compounds *12* and *13*; VI (0.57 g) compound *14*; and VII (1.91 g) unidentified strongly coloured products.

Repeated chromatography of fractions II–IV were made on silicic acid columns with 9:1 dichloromethane-acetone and of fractions V and VI with 8:2 dichloromethane-acetone. The individual compounds were sometimes further purified by sublimation (distillation). For yields and chromatographic properties of pure compounds see Table 1.

Experiment B. D-Glucose (400 g) was treated (as in Exp. A) and the ethyl acetate fraction (20.1 g) obtained was fractionated as in Exp. A (see Table 1).

Experiment C. D-Fructose (72 g) in acetate buffer of pH 4.5 (2.7 l) was treated in a stainless autoclave at 160 °C for 4 h and the ethyl acetate extract (10.2 g) obtained was fractionated as in Exp. A (see Table 1).

Characterization and identification of compounds *2–4*, *7*, *10–12* and *14*

Compound 2. Crystallization from light petroleum (b.p. 60–80 °C), m.p. 112–113 °C (lit. value[2] 112 °C) MS *m/e* (%): 234 (M^+, 3), 206(15), 125(9), 110(44), 109(100), 95(16), 82(36), 81(76), 53(56), 52(16), 51(13), 41(16). ^{1}H NMR, δ (100 MHz, $CDCl_3$): 4.63 (CH_2, s), 6.56 (H-4, d, $|J|$ 3.4 Hz), 7.20 (H-3, d, $|J|$ 3.4 Hz) and 9.63 (–CHO, s). Long range couplings (0.1–0.5 Hz) could also be detected between CHO and H-3, CHO and H_4, CH_2 and H-4 and between CH_2 and H-3.

Compound 3. Identical with acetylated (Ac_2O/Pyr) *1* (MS, NMR, IR). It could not be detected, however, from treatment of *1* in an acetate buffer as in Exp. A.

Compound 4. Crystals after sublimation m.p. 81.5–82.5 °C (lit. values 83–85 °C [2] and 79 °C [16]). MS *m/e* (%): 126 (M^+, 18), 96(9), 95(100), 67(7), 39(38), ^1H NMR, δ (100 MHz, $CDCl_3$): 4.74 (CH_2, s), 6.59 (H-4, dd, $|J|$ 1.8 and 3.5 Hz), 7.30 (H-3, dd, $|J|$ 0.8 and 3.5 Hz) and 7.63 (H-5, dd, $|J|$ 0.8 and 1.8 Hz).

Compound 7. Identical (m.p., mixed m.p., NMR, IR) with 6,7-dihydroxy-1(3*H*)-isobenzofuranone (yield 10 % after purification on a silicic acid column; eluant 4:1 dichloromethane-acetone) from 2,3-dihydroxybenzoic acid (*20*), in the same way as previously reported for the synthesis [7] of 6,7-dimethoxy-1(3*H*)-isobenzofuranone (*21*). Crystallization from ethanol, m.p. 221–222 °C (dec.). Found C 57.9; H 36. Calc. for $C_8H_6O_4$: C 57.8; H 3.6. MS, *m/e* (%. 166 (M^+, 90), 137(100), 120(46), 92(21), 81(28): 64(18), 63(19), 53(22), 52(18), 51(23). IR, ν max (KBr): 1720 (broad), 1615, 1520, 1450, 1405, 1310, (broad), 1280, 1220, 1095, 1000, 915.

Acetylation (Ac_2O/Pyr.) of compound *7* yielded the diacetate. Crystals after storage, m.p. 150.5–153.0 °C. MS, *m/e* (%): 250 (M^+, 1), 208(12), 166(55), 137(28), 97(16), 95(12), 85(13), 83(18), 81(15), 71(23), 69(25), 67(12), 57(43), 55(38), 43(100). ^1H NMR, δ (100 MHz, $CDCl_3$): 2.33 (3 H, s), 2.40 (3 H, s), 5.25 (2 H, broad s), 7.31 (1 H, d, $|J|$ 8.0 Hz) and 7.48 (1 H, d, $|J|$ 8.0 Hz).

Compound 10. Identical (m.p., mixed m.p., NMR, IR) with 4-methyl-1,2,3-benzenetriol, synthesised by reduction [17] of 2,3,4-trihydroxybenzaldehyde (prepared from 1,2,3-benzenetriol [18]). Recrystallization from ethanol, m.p. 142–143 °C (lit. value [17] 140–141 °C). ^1H NMR, δ (60 MHz, CD_3OD): 2.09 (3 H, broad s), 6.19 (1 H, d, $|J|$ 8.4 Hz) and 6.44 (1 H, d, $|J|$ 8.4 Hz).

Compound 11. Crystals after sublimation, m.p. 198–202.5 °C. MS, *m/e* (%): 206(M^+, 100), 205(31), 178(10), 177(53), 160(9), 151(16), 135(12), 105(14), 77(16), 44(17), 43(29). Found: *m/e* 206.0593. Calc for $C_{11}H_{10}O_4$: *m/e* 206.0579. Methylation (CH_2N_2) of compound *11* yielded the monomethyl ether (amorphous). MS, *m/e* (%): 220 (M^+, 27), 205(12), 151(11), 150(21), 149(24), 129(16), 111(19), 43(100). Peaks between *m/e* 43 and *m/e* 100 are omitted. ^1H NMR, δ (60 MHz, $CDCl_3$): 2.43 (3 H, s), 3.86 (3 H, s), 4.71 (2 H, s), 7.35 (1 H, d, $|J|$ 8 Hz), 7.67 (1 H, dd, $|J|$ 8 and 2 Hz) and 8.14 (1 H, d, $|J|$ 2 Hz).

Acetylation (Ac_2O/Pyr.) of compound *11* yielded the diacetate. Crystals after storage, m.p. 108–115 °C. MS, *m/e* (%): 290 (M^+, 2), 249(11), 248(70), 206(25), 205(20), 189(22), 188(24), 160(26), 133(11), 77(12), 43(100). ^1H NMR, δ (100 MHz, $CDCl_3$): 2.11 (3 H, s), 2.37 (6 H, s), 5.18 (2 H, s), 7.43 (1 H, d, $|J|$ 8.5 Hz), 7.65 (1 H, dd, $|J|$ 8.5 and 2.2 Hz) and 8.19 (1 H, d, $|J|$ 2.2 Hz).

Compound 12 (amorphous). MS, *m/e* (%): 182 (M^+, 10), 152(19), 151(100), 123(31), 77(17), 51(12), Found: *m/e* 182.0592. Calc. for $C_9H_{10}O_4$: *m/e* 182.0579 and found: *m/e* 151.0405. Calc. for $C_8H_7O_3$: *m/e* 151.0395. ^1H NMR δ (60 MHz, CD_3OD): 2.41 (3 H, broad s), 4.63 (2 H, s), 6.67 (1 H, broad s), and 7.18 (1 H, s).

Methylation (CH_2N_2) of compound *12* yielded the dimethyl ether (amorphous). MS, *m/e* (%): 210 (M^+, 21), 180(26), 179(100), 165(43), 151(50), 149(14), 138(17), 137(34), 122(10), 121(17), 108(10), 107(10), 93(14), 91(22), 78(10), 77(14), 65(20). ^1H NMR, δ (60 MHz, $CDCl_3$): 2.58 (3 H, broad s), 3.87 (3 H, s), 3.91 (3 H, s), 4.69 (2 H, s), 6.70 (1 H, broad s) and 7.11 (1 H, s). For comparison, 1-(3,4-dihydroxy-6-methylphenyl)-ethanone was synthesised by the same procedure as by the synthesis of 2,3-dihydroxy-acetophenone,[19] starting with 4-methyl-1,2-benzenediol. After purification on a silicic acid column using 9:1 dichloromethane-acetone as solvent, a yield of 10 % was obtained. Crystallization from aqueous ethanol, m.p. 170.5–171.0 °C (lit. value [20] 169 °C. MS, *m/e* (%): 167(10), 166(M^+, 50), 152(21), 151(100), 123(42), 87(21), 51(18), 43(16). ^1H NMR, δ (100 MHz, CD_3OD): 2.38 (3 H, broad s), 2.47 (3 H, s), 6.64 (1 H, broad s) and 7.32 (1 H, s).

Compound 14. Crystals after sublimation, m.p. 146–151 °C MS, *m/e* (%): 164 (M^+, 99), 163(100), 147(8), 136(6), 135(6), 118(6), 81.5(2), 77(10), 69(16), 65(10), 63(13), 43(16). Found: *m/e* 164.0470. Calc. for $C_9H_8O_3$: *m/e* 164.0473. ^1H NMR, δ (60 MHz CD_3OD): 2.30 (3 H, broad s), 6.15 (1 H, broad s) and 6.83 (2 H, broad s). IR, ν_{max} (KBr): 1610 (broad), 1490, 1475, 1350, 1320, 1265, 1200, 1165, 1145, 1095, 930, 880, 845, 810.

Methylation (CH_2N_2) of compound *14* yielded the dimethyl ether (amorphous). MS, *m/e* (%): 193(15), 192(M^+, 100), 191(14), 178(27), 177(63), 163(34), 149(23), 147(13), 135(13), 134(15), 131(14), 121(45), 106(23), 103(14), 91(14), 77(25), 69(25), 65(13), 63(15), 43(33). ^1H NMR, δ (100 MHz, $CDCl_3$): 2.42 (CH_3, d, $|J|$ 1.1 Hz), 3.91 (2 OCH_3, broad s), 6.26 (H-3, broad dd, $|J|$ 1.1 and 0.9 Hz), 6.94 (H-4, broad s), 7.0 (H-7, broad d, $|J|$ 0.9 Hz).

Synthesis of compounds *16*, *17*, *19* and *21*.

Compound 16. Synthesized from 3,4-dihydroxybenzoic acid (*15*) in the same way as *7* from 2,3-dihydroxybenzoic acid. Pure *16* was obtained in 17 % yield. Crystallization from water, m.p. 243.5–246 °C (dec.) Found: C 57.7; H 3.6. Calc. for $C_8H_6O_4$: C 57.8; H 3.6. MS, *m/e* (%): 166 (M^+, 64), 165(18), 137(100), 109(24), 108(11), 81(24), 63(17), 53(19), 52(16), 51(19). IR ν_{max} (KBr): 1720 (broad), 1625, 1610, 1530, 1410, 1300, 1240, 1090, 920.

Compound 17. Prepared by methylation (CH_2N_2) of *16*, m.p. 120–121.5 °C (lit. value[6] 122.5 °C).

Compound 19. Prepared in analogy with a previous procedure[21] from 3,4-dimethoxybenzoic acid (*18*), m.p. 154–156 °C (lit. value[21] 155 °C).

Compound 21. Prepared by a previous procedure[7] from 2,3-dimethoxybenzoic acid (*23*), as well as by methylation (CH_2N_2) of *7*, m.p. 99.5–100.5 °C (lit. value,[7,21] 101–103 °C). MS was in accordance with that previously reported.[22]

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Periodate Oxidation of Phenols. XX.* Reactions of 5-Substituted 2,4-Cyclohexadienones with Dienophiles

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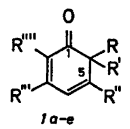
2,4-Cyclohexadienones carrying a 5-substituent (CH_3 or OCH_3) do not dimerize, but undergo Diels-Alder addition to 2,3-dimethyl-*p*-benzoquinone, dimethyl acetylenedicarboxylate, maleic anhydride and bromomaleic anhydride. They failed, however, to add to the methyl, dimethyl and dichloro derivatives of maleic anhydride, *trans*-1,2-dicyanoethylene, tetracyanoethylene, and cyclopentadiene. The reasons for the resistance of the 5-substituted 2,4-cyclohexadienones to dimerization are discussed.

In a preceding paper,[1] the preparation of 2,4-cyclohexadienones carrying a small substituent (CH_3 or OCH_3) in the 5-position, such as compounds *1a-e*, was reported. These dienones were stable as monomers even when heated at 110 °C and thus differed from the analogues lacking the 5-substituent which readily dimerize by Diels-Alder addition in a stereospecific and regiospecific manner.[2] Further investigation of the ability of 5-substituted 2,4-cyclohexadienones to undergo Diels-Alder reactions therefore seemed of interest, and the present study deals with the behaviour of compounds *1a—e* towards a number of dienophiles.



1a-e

a R=OH; R',R'',R'''=CH_3; R''''=H
b R=OCH_3; R',R'',R''''=CH_3; R'''=H
c R=OC_2H_5; R',R''=CH_3; R''',R''''=H
d R,R''=OCH_3; R'=CH_3; R''',R''''=H
e R,R',R''=OCH_3; R''',R''''=H

RESULTS

6-Ethoxy-5,6-dimethyl-2,4-cyclohexadienone (*1c*) reacted with 2,3-dimethyl-*p*-benzoquinone in refluxing toluene to give a fair yield of the Diels-Alder adduct *2c*. A small amount of this adduct, in addition to other products, had also been obtained on oxidation of 2,3-dimethylphenol with sodium periodate in water/ethanol.[1] It has been shown earlier[3] that 2-methylphenols with free 3-positions, when oxidized with periodate in the presence of *p*-benzoquinones, yield *o*-quinol-*p*-quinone adducts similar to *2c* in addition to the *o*-quinol dimers. The formation of *2c* then indicates that the diene activity of the 5-substituted *o*-quinol ether *1c* is comparable to that of *o*-quinols carrying a hydrogen atom in position 5.

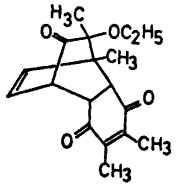
Diels-Alder additions of 2,4-cyclohexadienones to acetylenedicarboxylic esters, as well as to maleic anhydride, have been reported.[4] As illustrated by the formation of *3e* from the *o*-quinone ketal *1e*, as well as the production of the adducts *4a—e* from compounds *1a—e*, these reactions are not inhibited by the presence of a 5-substituent in the dienone. (The steric arrangement of the geminal bridge substituents indicated in adducts *2c* and *4a—d* has not been investigated but has been assumed to be analogous to that found for other *o*-quinol-*p*-quinone adducts[3] and for 2,4-cyclohexadienone dimers.[5—9])

Neither methylmaleic anhydride nor dimethylmaleic anhydride, which are comparatively

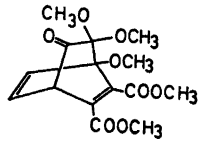
* Part XIX, see Ref. 1.

weak dienophiles, [10] added to the 5-substituted cyclohexadienones. However, bromomaleic anhydride, which has about the same dienophilic reactivity as maleic anhydride,[10] added to *1e* to give *7e*, whereas dichloromaleic anhydride, expected to be a strong dienophile, was unreactive.

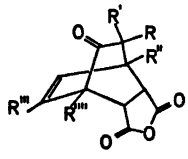
trans-1,2-Dicyanoethylene, a dienophile which, in its additions to cyclopentadiene or 9,10-dimethylanthracene, is about 50 times less reactive than maleic anhydride,[11] did not react with *1e*, and the highly reactive tetracyanoethylene (rate constants about 500 times larger than with maleic anhydride [11]) also failed to add to *1e*.



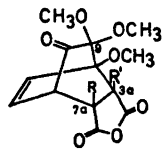
2c



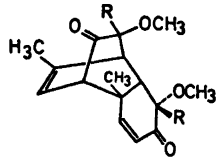
3e



4a–e



(*5e* R=CH_3, R'=H)
(*6e* R=R'=CH_3)
7e R=Br, R'=H
(*8e* R=R'=Cl)



9 R=OCH_3
10 R=CH_3

Cyclopentadiene has been reported to add as dienophile to a number of 5-unsubstituted 2,4-cyclohexadienones [4] but proved to be unreactive when heated together with the methoxy analogue of *1c* [1] or with *1d*.

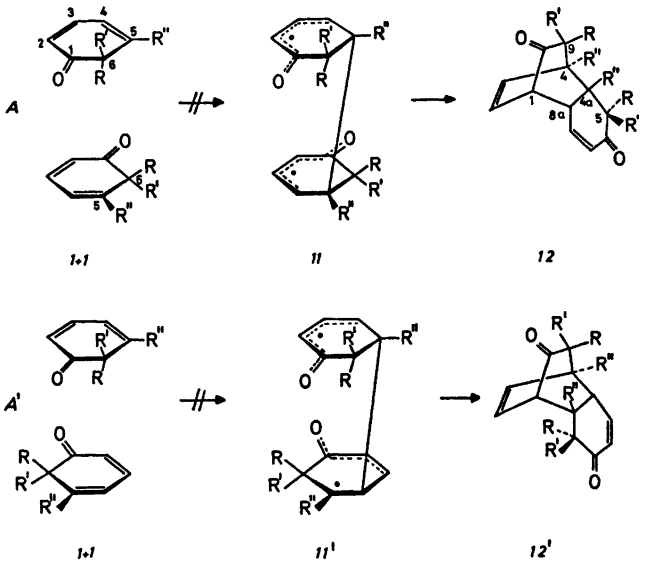
DISCUSSION

Addition of methylmaleic anhydride to the 5-substituted 2,4-cyclohexadienones did not take place although it seemed to be sterically possible. Reaction with *1e*, for instance, could be expected to give adduct *5e* with the methyl substituent in position 7a; dimers 9 [2] and 10,[5] with CH_3 in a similar position, are known. Since bromomaleic anhydride reacted to give adduct *7e*, and methyl and bromine are of comparable size, the different behaviour of the two monosubstituted maleic anhydrides can be assumed to be due mainly to the opposite electronic effects of the two substituents.

Apparently, the steric effect of the 5-substituent of the dienes is strong enough to hinder the approach of comparatively weak dienophiles, such as methylmaleic anhydride, *trans*-1,2-dicyanoethylene and cyclopentadiene. This effect is overcome by stronger dienophiles with comparable steric requirements, such as 2,3-dimethyl-*p*-benzoquinone, maleic anhydride and bromomaleic anhydride. However, dienophiles with a fully substituted double bond (dimethylmaleic anhydride, dichloromaleic anhydride, tetracyanoethylene) fail to react, because the 3a-substituent of the hypothetical adducts as illustrated by formulae *6e* and *8e* would be in steric opposition to the 9-substituent *anti* to the ethylenic bond.

The latter type of steric interaction is also responsible for the failure of the 5-substituted 2,4-cyclohexadienones to dimerize.[1,4] Inspection of Dreiding models of the (nonexisting) dimers of type *12* (Scheme 1 *A*) indicates severe steric opposition between groups R(9) and R''(4a), as well as between groups R(5) and R''(4).

The stereochemical and structural orientations of the monomers presented in Scheme 1 *A* are those established for the dimers of 5-unsubstituted 2,4-cyclohexadienones.[5–9,12] It would seem, however, that an orientation of the monomers as depicted in Scheme 1 *A'* might result in dimerization, since no critical steric interaction would be involved. The fact that dimerizations of type A' do not take place – even if there is no 5-substituent (R'') – may be understood from recent mechanistic considerations. According to Epiotis,[13] perturbation theory applied to Diels-Alder reactions between dienes and dienophiles carrying substituents of similar electronic properties led to false predictions of the regioselectivity, if a fully concerted mechanism was assumed. The orientations found experimentally were consistent, however, with a highly unsymmetrical,



Scheme 1.

biradical-like transition state. In a theoretical study of three typical Diels-Alder reactions, Dewar, Griffin and Kirschner [14] similarly concluded that the transition states of these reactions were "very unsymmetrical, corresponding to biradicaloid structures." The transition states of reactions *A* and *A'* (Scheme 1) are, for simplicity, given as fully developed biradicals. As indicated by their hybrid structures, *11* is more stable than *11'*. This is in harmony with the fact that 5-unsubstituted monomers always give dimers of type *12* (R″=H), whereas isomers of type *12'* have not been observed,[5-9] and also with the fact that the 5-substituted analogues fail to dimerize according to *A'* although the latter reaction, contrary to reaction *A*, would seem sterically possible.

EXPERIMENTAL

UV spectra were run in ethanol on a Cary model 15, and IR spectra were recorded in KBr on a Beckman 9A instrument, unless otherwise stated. UV data are given as λ_{max} values in nm, with log ε values in parentheses, IR data as ν_{max} values in cm^{-1}. ^1H NMR spectra were taken in $CDCl_3$ on a Varian A-60 spectrometer. Analytical and preparative TLC, as well as column chromatography, were performed on silica gel with benzene/ethyl acetate (4:1) as mobile phase.

10-Ethoxy-1,4,4a,8a-tetrahydro-1,6,7,10-tetramethyl-1,4-ethanonaphthalene-5,8,9-trione (2c). A solution of 6-ethoxy-5,6-dimethyl-2,4-cyclohexadienone (*1c*)[1] (350 mg=2 mmol) and 2,3-dimethyl-*p*-benzoquinone (400 mg=3 mmol) in toluene (15 ml) was refluxed for 15 h. (After a heating period of 3 h, TLC indicated the presence of some unchanged *1c*.) The solvent was removed and the remaining solid recrystallized from ethyl acetate to give *2c* (410 mg, 65 %), identical by m.p. (160–161 °C), mixed m.p. and IR spectrum with the same compound obtained according to Ref. 1.

Dimethyl 1,7,7-trimethoxy-8-oxobicyclo[2.2.2]octa-2,5-diene-2,3-dicarboxylate (3e). A solution of 5,6,6-trimethoxy-2,4-cyclohexadienone (*1e*)[1] (500 mg=2.7 mmol) and dimethyl acetylenedicarboxylate (700 mg=5.5 mmol) in toluene (25 ml) was refluxed for 8 h. Column chromatography of the product obtained after removal of the solvent gave (1) unchanged acetylenedicarboxylate; (2) compound *3e*, 640 mg (71 %) of colourless crystals of m.p. 112–113 °C after recrystallization from isopropyl ether; (3) 85 mg (17 %) of unchanged *1e*. Compound *3e* was further characterized as follows. (Found: C 55.44; H 5.59. Calc. for $C_{15}H_{18}O_8$: C 55.21; H 5.56). UV sh 240 (3.42), sh 305 (2.40). IR 1730 (CO), 1718 (α,β-unsat. ester), 1655 and 1602 (C=C). NMR δ 3.46, 3.53, 3.69, 3.78, 3.88 (3 H each, 5 OCH_3). H-6, H-5 and H-4 give rise to an ABX pattern: 4.48 (dd, 1 H, H-4), 6.52 (dd, 1 H, H-5), 6.82 (dd, 1 H, H-6); $J_{4,5}$=6 Hz, $J_{4,6}$=2 Hz, $J_{5,6}$=8Hz.

Adducts of 2,4-cyclohexadienones 1a–e *with maleic anhydride (general procedure).* A solution

of the cyclohexadienone[1] (2–5 mmol) and a twofold excess of maleic anhydride in toluene (10–25 ml) was refluxed for 6 h. (After a reaction time of 3 h, some unchanged dienone was still present according to TLC.) The reaction mixture was evaporated under vacuum. Excess maleic anhydride was removed from the resulting solid by sublimation and subsequent extraction of the residue with boiling ether. The remaining crude adduct was purified by recrystallization. Yields given below refer to recrystallized product.

3a,4,7,7a-Tetrahydro-9-hydroxy-4,6,9-trimethyl-4,7-ethanoisobenzofuran-1,3,8-trione (4a). From 6-hydroxy-3,5,6-trimethyl-2,4-cyclohexadienone (*1a*) and maleic anhydride in 70 % yield; m.p. 182–183 °C (ethyl acetate-hexane). (Found: C 62.57; H 5.67. Calc. for $C_{13}H_{14}O_5$: C 62.39; H 5.64). UV 205 (3.61), 308 (2.11) (*β,γ*-enone system). IR ($CHCl_3$) 1738 s (CO), 1780 s and 1860 m (five-membered cyclic anhydride), 3430 (OH).

3a,4,7,7a-Tetrahydro-9-methoxy-4,7,9-trimethyl-4,7-ethanoisobenzofuran-1,3,8-trione (4b). From 6-methoxy-2,5,6-trimethyl-2,4-cyclohexadienone (*1b*) and maleic anhydride in 90 % yield, m.p. 150–152 °C (isopropyl ether). (Found: C 63.84; H 6.20; OCH_3 11.93. Calc. for $C_{14}H_{16}O_5$: C 63.63; H 6.10; OCH_3 11.74).

9-Ethoxy-3a,4,7,7a-tetrahydro-4,9-dimethyl-4,7-ethanoisobenzofuran-1,3,8-trione (4c). From 6-ethoxy-5,6-dimethyl-2,4-cyclohexadienone (*1c*) and maleic anhydride in 82 % yield; m.p. 115–116 °C (isopropyl ether). (Found: C 63.77; H 6.09. Calc. for $C_{14}H_{16}O_5$: C 63.63; H 6.10). IR ($CHCl_3$) 1730 s, 1780 s, 1855 m.

3a,4,7,7a-Tetrahydro-4,9-dimethoxy-9-methyl-4,7-ethanoisobenzofuran-1,3,8-trione (4d). From 5,6-dimethoxy-6-methyl-2,4-cyclohexadienone (*1d*) and maleic anhydride in 80 % yield; m.p. 164–165 °C (benzene-hexane). (Found: C 58.80; H 5.17; OCH_3 23.09. Calc. for $C_{13}H_{14}O_6$: C 58.64; H 5.30; OCH_3 23.31). IR 1730 s, 1801 s, 1872 m.

3a,4,7,7a-Tetrahydro-4,9,9-trimethoxy-4,7-ethanoisobenzofuran-1,3,8-trione (4e). From 5,6,6-trimethoxy-2,4-cyclohexadienone (*1e*) and maleic anhydride in 74 % yield; m.p. 150–151 °C (benzene-hexane). (Found: C 55.33; H 4.82; OCH_3 32.68. Calc. for $C_{13}H_{14}O_7$: C 55.32; H 5.00; OCH_3 32.98). IR ($CHCl_3$) 1746 s, 1786 s, 1870 m.

7a-Bromo-3a,4,7,7a-tetrahydro-4,9,9-trimethoxy-4,7-ethanoisobenzofuran-1,3,8-trione (7e). From *1e* and bromomaleic anhydride as described above for the maleic anhydride adducts, but reaction time 75 h. (After 24 h, some unchanged *1e* was detected by TLC.) Compound *7e* was separated from excess bromomaleic anhydride by column chromatography. Yield, 77 %; m.p. 124–125 °C (isopropyl ether). (Found: C 43.22; H 3.71; Br 22.08. Calc. for $C_{13}H_{13}O_7Br$: C 43.23; H 3.63; Br 22.12). UV 320 (1.97). IR 1745 s, 1803 s, 1870 m. NMR δ 3.57 (s, 6 H, 2 OCH_3), 3.83 (broadened s, overlapping the upfield doublet due to H-7, about 3.5 H, OCH_3); the downfield doublet of H-7 is found at 3.92; 4.45 (s, 1 H, H-3a). The signals of H-5 and H-6 constitute the AB part of an ABX spectrum (X = H-7): 6.21 (dd, 1 H, H-6), 6.49 (dd, 1 H, H-5). $J_{6,7} = 6$ Hz, $J_{5,7} = 1.5$ Hz, $J_{5,6} = 8.5$ Hz.

Unsuccessful attempts to add methylmaleic anhydride, dimethylmaleic anhydride, dichloromaleic anhydride, *trans*-1,2-dicyanoethylene, tetracyanoethylene and cyclopentadiene to *1a-e* in toluene solution at room temperature, as well as at reflux temperature, were made using prolonged reaction times.

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X-Ray Structure Determination of Three Polycyclic *cis*-Azoalkanes; Consequences of the Gradual Introduction of Four-membered Rings

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The crystal structures of I, 2,3-diazabicyco-[2.2.2]-oct-2-ene; II, 7,8-diazatricyclo[4.2.2.$0^{2,5}$]-dec-7-ene; and III, 7,8-diazapentacyclo-[4.2.2.$0^{2,5}$.$0^{3,9}$.$0^{4,10}$]-dec-7-ene have been determined at −165 °C by X-ray methods using 417, 924, and 933 reflections, respectively, collected on a counter diffractometer. The crystal data are as follows:

I. Orthorhombic, space group $P2_12_12$, a = 7.930(5) Å; b = 6.580(8) Å; c = 5.594(5) Å.

II. Monoclinic, space group $P2_1/m$, a = 5.894(1) Å; b = 10.038(3) Å; c = 6.577(1) Å; β = 113.83(2)°.

III. Monoclinic, space group $P2_1/n$, a = 9.249(2) Å; b = 6.408(2) Å; c = 10.294(2) Å; β = 103.25(2)°.

The structures were refined to conventional R-factors of 0.046(I), 0.042(II), and 0.059(III); the standard deviations in bond lengths and angles involving nitrogen and carbon atoms are in the ranges 0.001 − 0.004 Å and 0.1 − 0.2°, respectively.

The effect of the introduction of four-membered rings into the 2,3-diazabicyclo-[2.2.2]oct-2-ene structure on the molecular geometry is discussed and correlated with photoelectron spectroscopy measurements.

Although aromatic azo compounds have been of great chemical and industrial importance for the past hundred years, serious interest in the azo functionality of alkanes is a relatively recent development. In particular the availability of a wide range of *cis*-azoalkanes [1–3] has stimulated a host of experimental and theoretical studies.

Several of the physically oriented studies have been supplemented by semi-empirical calculations.[4–6] Lacking detailed structural information for *cis*-azoalkanes workers have derived molecular geometries by extrapolating data from the few *trans*-azo structures available,[7] from a single heavily substituted five-membered ring case,[8] and from azobenzenes.[9] Alternatively geometry/energy optimizations based on the CNDO-MO-SCF approximation have been performed.[6,10]

Given the general interest in *cis*-azoalkanes and the present lack of structural data for this important functional group, we have undertaken an X-ray analysis of the three polycyclic derivatives (see Fig. 1):

I. 2,3-diazabicyclo[2.2.2]oct-2-ene
II. 7,8-diazatricyclo[4.2.2.$0^{2,5}$]dec-7-ene
III. 7,8-diazapentacyclo[4.2.2.$0^{2,5}$.$0^{3,9}$.$0^{4,10}$]-dec-7-ene

EXPERIMENTAL

Compounds I and II were prepared from the corresponding semicarbazides by the cupric halide oxidation method.[3] The precursor for II-semicarbazide has been described.[11] Compound III was derived from the corresponding *N*-phenyl urazole polycycle.[12]

Crystals formed by sublimation (I) or recrystallization from hexane (II, III) were cut to approximately cubic forms with dimensions 0.2 − 0.4 mm along the edges. The X-ray experiments were performed on a SYNTEX P$\bar{1}$ four-circle diffractometer using Mo$K\alpha$ radiation (graphite monochromator). The temperature at the crystal site was kept at −165 °C using an Enraf-Nonius cooling device modified by H. Hope. Cell dimensions were determined

from diffractometer measurements on 15 general reflections; the results are given together with space group data below.

Intensity data were collected using the ω scan technique for reflections up to a $\sin\theta/\lambda$ value of 0.7 Å^{-1} with scan speeds of $3-6°$ min^{-1} depending on the peak intensity. Scan ranges were 1.5° for compounds I and II, and 1.3° for compound III. Background counts were taken for 0.35 times the scan time ± 1.5, ± 1.0 and ± 1.0° off the peak position, respectively, for the three compounds. Three standard reflections were measured after every 100 reflections; for the three runs of data collection they were stable within 5, 3 and 5 %. No corrections were made for these fluctuations. The estimate of the standard deviation of the intensity was based on counting statistics with an additional term of 2 % of the net intensity. Reflections with intensity larger than $2.5\sigma(I)$ were regarded as observed and used for the structure determination. The data sets were corrected for Lorentz and polarization effects, no absorption or secondary extinction corrections were carried out. The numbers of observed reflections were 417 for compound I, 924 for compound II and 933 for compound III.

Descriptions of the computer programs applied for the structure analyses are given in Refs. 13 and 14. In the full matrix least-squares program the quantity minimized was $\sum w\Delta F^2$ where w is the inverse of the variance of the observed structure factor. Atomic form factors used were those of Doyle and Turner [15] for nitrogen and carbon and of Stewart, Davidson and Simpson [16] for hydrogen atoms.

CRYSTAL DATA

Compound I. 2,3-Diazabicyclo[2.2.2]oct-2-ene, $C_8H_{10}N_2$. Orthorhombic, $a=7.930(5)$ Å; $b=6.580(8)$ Å; $c=5.594(5)$ Å; ($t=-165$ °C); $V=291.9$ Å^3; $M=110.1$; $F(000)=120$; $Z=2$. $D_{calc}=1.261$ g cm^{-3}. Absent reflections: ($h00$) for h odd; ($0k0$) for k odd. Space group $P2_12_12$.

Compound II. 7,8-Diazatricyclo[4.2.2.0^{2,5}]-

Table 1. Fractional atomic coordinates and thermal parameters with estimated standard deviations. The anisotropic temperature factor is given by $\exp -2\pi^2(U_{11}a^{*2}h^2+U_{22}b^{*2}k^2+U_{33}c^{*2}l^2+U_{12}a^*b^*hk+U_{13}a^*c^*hl+U_{23}b^*c^*kl)$.

Compound I	x	y	z	U_{11} (Å^2)	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
N1	.0545(2)	.0692(3)	.5577(3)	.0269(8)	.0302(8)	.0253(7)	.0009(7)	-.0018(6)	-.0012(7)
C5	.1794(2)	-.0427(3)	.1792(4)	.0185(7)	.0312(10)	.0272(8)	.0025(7)	.0032(8)	.0037(10)
C6	.1066(2)	.1393(3)	.3171(4)	.0215(7)	.0241(8)	.0270(9)	-.0028(7)	-.0009(8)	.0002(9)
C7	-.0529(2)	.2174(3)	.1917(4)	.0265(9)	.0244(9)	.0287(8)	.0051(8)	-.0013(9)	.0020(9)
H1C5	.293(3)	-.079(3)	.247(3)	1.9(5)					
H2C5	.198(2)	.005(3)	.004(4)	1.2(5)					
HC6	.187(3)	.244(3)	.339(4)	2.3(5)					
H7C7	-.020(2)	.259(3)	.027(3)	1.4(4)					
H2C7	-.099(3)	.333(4)	.282(4)	2.6(5)					
Compound II									
N1	.0309(2)	.3125(1)	.0836(2)	.0209(4)	.0313(5)	.0284(4)	.0030(3)	.0117(3)	.0057(3)
C5	.3781(2)	.3263(1)	-.0258(2)	.0232(4)	.0340(5)	.0219(4)	.0006(4)	.0109(3)	.0063(4)
C6	.2795(2)	.3751(1)	.1442(2)	.0225(4)	.0195(4)	.0270(5)	.0026(3)	.0108(3)	.0023(3)
C7	.4372(2)	.3270(1)	.3807(2)	.0268(4)	.0227(5)	.0212(4)	.0004(3)	.0102(3)	-.0021(3)
C10	.7241(2)	.3267(1)	.4641(2)	.0266(5)	.0297(5)	.0287(5)	-.0040(4)	.0023(4)	-.0019(4)
H1C5	.544(3)	.362(1)	.012(2)	2.7(3)					
H2C5	.269(3)	.361(2)	-.173(3)	3.1(3)					
HC6	.250(2)	.471(1)	.133(2)	2.1(2)					
HC7	.375(2)	.370(1)	.490(2)	2.8(3)					
H1C10	.802(3)	.367(2)	.609(3)	4.1(4)					
H2C10	.790(3)	.370(2)	.361(3)	3.8(3)					
Compound III									
N1	.3344(2)	.7503(4)	.3766(2)	.0184(10)	.0253(13)	.0263(12)	-.0006(10)	.0118(9)	-.0024(11)
N2	.2666(2)	.7808(4)	.2583(2)	.0165(10)	.0280(13)	.0279(13)	-.0017(10)	.0070(9)	-.0008(11)
C3	.3599(2)	.7982(5)	.1614(2)	.0156(12)	.0350(18)	.0171(12)	-.0035(12)	.0024(9)	.0014(12)
C4	.4751(3)	.6236(4)	.1631(3)	.0282(16)	.0246(16)	.0237(15)	-.0060(13)	.0124(11)	-.0026(13)
C5	.5582(3)	.5858(5)	.3083(3)	.0249(16)	.0214(15)	.0263(15)	.0051(12)	.0158(12)	.0007(12)
C6	.4964(2)	.7374(5)	.4001(2)	.0180(11)	.0249(15)	.0158(11)	.0026(12)	.0059(9)	.0003(11)
C7	.5757(3)	.9139(5)	.3414(2)	.0154(13)	.0277(15)	.0171(13)	-.0010(12)	.0032(10)	-.0014(12)
C8	.4919(3)	.9516(4)	.1956(2)	.0142(14)	.0217(15)	.0235(14)	-.0004(11)	.0062(10)	.0013(11)
C9	.5925(3)	.7882(5)	.1466(2)	.9194(12)	.0290(17)	.0157(13)	-.0021(12)	.0077(10)	-.0007(11)
C10	.6771(2)	.7513(5)	.2934(2)	.0156(11)	.0281(16)	.0200(12)	.0017(13)	.0066(9)	.0012(13)
HC3	.298(3)	.823(5)	.074(3)	2.3(7)					
HC4	.446(3)	.509(5)	.104(3)	2.8(7)					
HC5	.580(3)	.445(5)	.338(3)	2.4(7)					
HC6	.533(3)	.703(4)	.494(3)	1.3(6)					
HC7	.611(3)	1.031(5)	.395(3)	1.9(7)					
HC8	.476(3)	1.097(4)	.167(3)	1.4(6)					
HC9	.632(3)	.809(4)	.069(3)	2.1(6)					
HC10	.783(3)	.739(5)	.332(3)	2.6(6)					

Table 2. Molecular dimensions with estimated standard deviations. The numbering of the atoms may be found in Fig. 1.

	Bond length (Å)	Corrected bond length (Å)		Bond angle (°)
Compound I				
N1–N2	1.255(4)	1.260	N2–N1–C6	114.7(1)
N1–C6	1.482(3)	1.487	N1–C6–C5	108.5(2)
C6–C5	1.537(3)	1.542	N1–C6–C7	106.8(2)
C5–C4	1.527(3)	1.533	C5–C6–C7	109.9(2)
C6–C7	1.535(3)	1.539	C6–C5–C4	108.5(2)
			C6–C7–C8	108.1(2)
C–H is in the range: 0.95–1.04 Å.				
Compound II				
N1–N2	1.255(2)	1.259	N2–N1–C6	114.9(1)
N1–C6	1.493(1)	1.497	N1–C6–C5	106.9(1)
C6–C5	1.534(2)	1.540	N1–C6–C7	105.4(1)
C6–C7	1.532(2)	1.538	C5–C6–C7	112.7(1)
C5–C4	1.532(3)	1.537	C6–C5–C4	108.6(1)
C7–C8	1.547(2)	1.553	C6–C7–C8	108.4(1)
C7–C10	1.552(2)	1.556	C6–C7–C10	119.2(1)
C10–C9	1.540(3)	1.545	C7–C10–C9	90.1(1)
			C8–C7–C10	89.9(1)
C–H is in the range: 0.97–1.02 Å.				
Compound III				
N1–N2	1.246(4)	1.251	N1–N2–C3	115.4(2)
N1–C6	1.464(4)	1.468	N2–N1–C6	115.9(2)
N2–C3	1.461(4)	1.464	N2–C3–C4	116.8(3)
C3–C8	1.544(4)	1.549	N2–C3–C8	117.0(3)
C3–C4	1.542(5)	1.548	C4–C3–C8	87.2(2)
C6–C7	1.543(5)	1.547	N1–C6–C5	116.2(3)
C6–C5	1.550(4)	1.555	N1–C6–C7	117.0(3)
C4–C5	1.530(5)	1.536	C5–C6–C7	87.0(2)
C4–C9	1.550(5)	1.554	C3–C4–C5	108.5(2)
C5–C10	1.560(5)	1.564	C3–C4–C9	90.3(2)
C7–C8	1.537(5)	1.543	C5–C4–C9	90.3(2)
C7–C10	1.555(5)	1.560	C6–C5–C4	108.6(3)
C8–C9	1.558(5)	1.563	C6–C5–C10	90.0(2)
C9–C10	1.546(4)	1.553	C4–C5–C10	90.3(2)
			C6–C7–C8	108.4(2)
			C6–C7–C10	90.4(2)
C–H is in the range: 0.94–0.98 Å.			C8–C7–C10	90.3(2)
			C3–C8–C7	108.5(2)
			C3–C8–C9	89.9(2)
			C7–C8–C9	90.1(2)
			C4–C9–C8	86.4(2)
			C4–C9–C10	90.1(2)
			C8–C9–C10	89.8(2)
			C5–C10–C7	86.2(2)
			C5–C10–C9	89.3(2)
			C7–C10–C9	89.9(2)

Table 2. Continued.

Dihedral angles (°). The angles are positive in a righthand screw.

Compound I			
C6–N1–N2–C3	1.5(4)	N2–N1–C6–C5	–59.3(3)
N2–N1–C6–C7	59.2(3)	N1–C6–C5–C7	51.5(2)
C7–C6–C5–C4	–65.0(2)	N1–C6–C7–C8	–59.2(2)
C5–C6–C7–C8	58.4(2)	C6–C5–C4–C3	5.3(2)
Compound II			
N2–N1–C6–C5	59.4(1)	N2–N1–C6–C7	–60.8(1)
N1–C6–C5–C4	–55.5(1)	C7–C6–C5–C4	59.9(1)
N1–C6–C7–C10	157.0(1)	N1–C6–C7–C8	56.5(1)
C5–C6–C7–C10	40.8(1)	C5–C6–C7–C8	–59.8(1)
C6–C7–C10–C9	–111.1(1)	C6–C7–C8–C9	120.8(1)
Compound III			
C6–N1–N2–C3	0.2(4)		
N1–N2–C3–C8	–50.6(4)	N1–N2–C3–C4	50.7(4)
N2–N1–C6–C7	50.1(4)	N2–N1–C6–C5	–50.6(4)
N2–C3–C8–C9	137.5(3)	N2–C3–C8–C7	47.4(4)
N1–C6–C7–C10	–136.9(2)	N1–C6–C7–C8	–46.5(3)
N2–C3–C4–C9	–137.8(3)	N2–C3–C4–C5	–47.4(4)
N1–C6–C5–C10	137.6(3)	N1–C6–C5–C4	47.3(3)
C4–C3–C8–C7	–71.4(3)	C4–C3–C8–C9	18.7(2)
C5–C6–C7–C8	71.4(3)	C5–C6–C7–C10	–19.0(2)
C8–C3–C4–C5	71.6(3)	C8–C3–C4–C9	–18.8(2)
C7–C6–C5–C4	–71.4(3)	C7–C6–C5–C10	18.9(2)
C10–C9–C4–C3	108.5(2)	C8–C9–C4–C3	18.7(2)
C9–C10–C5–C6	108.7(2)	C7–C10–C5–C6	–18.8(2)
C3–C8–C9–C10	108.7(2)	C6–C7–C10–C5	18.8(2)
C6–C7–C10–C9	108.2(2)	C3–C8–C9–C4	–18.7(2)
C4–C9–C10–C7	–86.2(2)	C8–C9–C10–C5	86.5(2)

dec-7-ene, $C_8H_{12}N_2$. Monoclinic, $a = 5.894(1)$ Å; $b = 10.038(3)$ Å; $c = 6.577(1)$ Å; $\beta = 113.83(2)°$; ($t = -165$ °C); $V = 365.0$ Å^3; $M = 136.1$; $F(000) = 148$; $Z = 2$. $D_{calc} = 1.286$ g cm^{-3}. Absent reflections: ($0k0$) for k odd. Space group $P2_1/m$

Compound III. 7,8-Diazapentacyclo-[4.2.2.$0^{2,5}$.$0^{3,9}$.$0^{4,10}$]-dec-7-ene(diazabasketene), $C_8H_8N_2$. Monoclinic, $a = 9.249(2)$ Å; $b = 6.408(2)$ Å; $c = 10.294(2)$ Å; $\beta = 103.25(2)°$. ($t = -165$ °C); $V = 593.9$ Å^3; $M = 132.1$; $F(000) = 280$; $Z = 4$. $D_{calc} = 1.477$ g cm^{-3}. Absent reflections: ($h0l$) for $h+l$ odd, ($0k0$) for k odd. Space group $P2_1/n$.

STRUCTURE DETERMINATIONS

The structures were determined by the use of the program assembly MULTAN [25] and refined by Fourier and least-squares techniques. Anisotropic temperature factor parameters were introduced for nitrogen and carbon atoms; for hydrogen atoms positional parameters (calculated from stereo-chemical considerations) and isotropic thermal parameters were refined. In order to avoid the systematic errors in ΔF for the low angle data [17] the final least-squares refinement cycles included only observations with $\sin\theta/\lambda > 0.3$ with the refinement of only the nitrogen and carbon parameters.

The refinement converged to conventional R-factors of 0.046(I), 0.042(II) and 0.059(III) including all observed reflections. The corresponding weighted R-values were 0.038(I), 0.051(II) and 0.056(III). The standard deviations of an observation of unit weight, $[\sum w\Delta F^2/(m-n)]^{\frac{1}{2}}$ were 1.58(I), 2.43(II) and 1.46(III) and the overdetermination ratios in the final refinements 10.0(I), 18.2(II) and 9.1(III).

Final atomic parameters are listed in Table 1. The experimental data may be obtained from the authors upon request.

A rigid-body analysis showed that the thermal motion of the molecules to a good approximation could be interpreted in terms of

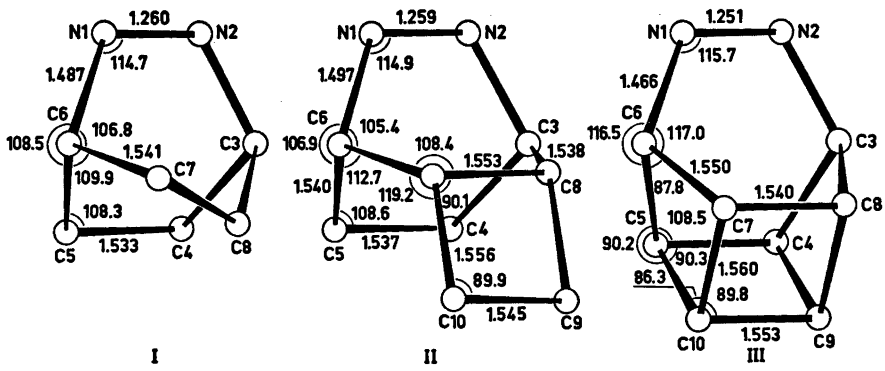


Fig. 1. Mean bond lengths and angles of chemically equivalent parameters. (In order to maintain the analogy between the compounds crystallographically equivalent atoms are given different numbers.

translational and librational oscillations (r.m.s.-ΔU were found equal to 0.0009 Å² (I); 0.0006 Å² (II) and 0.0012 Å² (III)). The bond lengths used during the discussion are those corrected for libration.[18]

Bond lengths and angles are listed in Table 2, dihedral angles involving the heavy atoms are also given. Estimated standard deviations are calculated from the variance-covariance matrix.

DESCRIPTIONS OF THE STRUCTURES

For all the three compounds the intermolecular forces in the crystals are of the van der Waals type. The shorter distances between molecules are mainly compatible with normal hydrogen-hydrogen contacts.

The molecular geometry may be seen from Fig. 1 where the atomic numbering is indicated. The bond lengths and angles given in the figure are mean values of those being chemically equivalent. Generally, when comparing distances and angles, differences of less than 0.01 Å and 0.5° may not be significant.

Compound I, 2,3-diazabicyclo[2.2.2]oct-2-ene. The molecular geometry is restricted by a crystallographic two-fold axis of symmetry passing between the nitrogen atoms and through the middle of the cyclohexane ring. The N–N double bond is 1.260 Å, the N–C bond 1.487 Å, and the C–C bonds are 1.542, 1.533 and 1.539 Å, respectively. The N=N–C angle is 114.7° which is very similar to the corresponding angle in aliphatic *trans*-azoalkanes (114.2–116.0°)[7] but slightly larger than that found for aromatic *trans* species (113°).[9] The conformation of the cyclohexane ring is that of a skew boatform. The skewing, shown by the asymmetry in bond angles around C6 (Fig. 1) brings about a small deviation (5.3°) from an eclipsed conformation about the C4–C5 and C7–C8 bonds. The dihedral angle C6–N1–N2–C3 is 1.5°; the arrangement is thus nearly planar.

Compound II, 7,8-diazatricyclo[4.2.2.$0^{2,5}$]-dec-7-ene. This molecule exhibits mirror symmetry, the mirror plane being situated between the nitrogen atoms and normal to the N=N bond. The N=N bond length is 1.259 Å, the N–C bond 1.497 Å, and the N=N–C angle 114.9°. The C6–N1–N2–C3 arrangement is bound by the symmetry to be strictly planar and the geometry is nearly equal to that of Compound I. Except for the C7–C8 bond the C–C bonds of the cyclohexane ring are of the same length as those of Compound I, 1.538, 1.540 and 1.537 Å, respectively; the conformation is that of a pure boat form with planar C3–C4–C5–C6 and C3–C8–C7–C6 arrangements. By symmetry the cyclobutane ring is strictly planar, and with the possible exception of the C9–C10 bond the bond lengths are significantly longer (by 0.010–0.015 Å) than the normal aliphatic C–C bond. The conformations about all C–C bonds not involving C3 and C6 are eclipsed.

Compound III, 7,8-diazapentacyclo-[4.2.2.$0^{2,5}$.$0^{3,9}$.$0^{4,10}$]dec-7-ene (diazabasketene). The experimental values of bond lengths and

angles show that any deviation from *mm* symmetry for this molecule is insignificant. Our discussion is based on mean values given in Fig. 1 assuming this symmetry actually to be present.

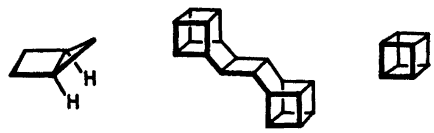
The N=N bond is 1.251 Å, the C–N bond 1.466 Å and the angle C–N=N 115.9°. The C6–N1–N2–C3 arrangement is planar. The molecular fragment may be different from the corresponding part of the other two molecules, the C–N bond being significantly shorter, the C–N=N angle larger, and possibly, the N=N bond shorter. Excepting C7–C8 all C–C bonds, being members in fused cyclobutane rings, are longer than normal aliphatic C–C bonds. Two of the cyclobutane rings (C4–C5–C10–C9 and C7–C8–C9–C10) are planar with C–C–C angles close to 90°. The two remaining cyclobutane rings are equal and non-planar. The angle between planes defined by C5–C6–C7 and by C5–C10–C7 (C4–C3–C8 and C4–C9–C8) is 153.7°. In the latter rings the bond angles are near 90° at C5 and C7 (C4 and C8) and 3–4° less at the other corners. The torsional angles are given in Table 2, the conformations about the bonds C4–C5, C7–C8 and C9–C10 are strictly eclipsed.

The geometry around the *cis*-azo moiety for I, II and III is very similar to that observed for two other *cis*-azoalkanes. An X-ray determination for a substituted pyrazoline [8] and a microwave study [19] of 2,3-diazabicyclo[2.2.1]-hept-2-ene yield C–N=1.50/1.52 and 1.506 Å, N=N=1.23 and 1.246 Å, ∠C–N=N=112/114 and 108.1°, respectively.

DISCUSSION OF THE STRUCTURES

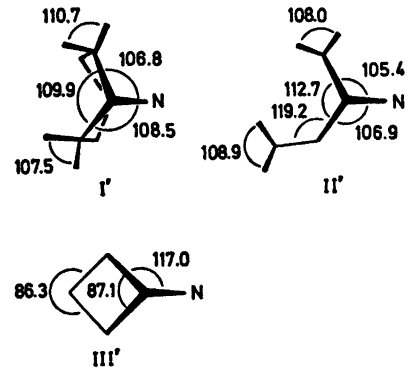
The influence of fused cyclobutane rings on the 2,3-diazabicyclo[2.2.2]oct-2-ene structure

Bond angles. As described above, I in the crystalline state has been found to possess a slightly twisted skeleton with C_2 symmetry. The most pronounced consequence of introducing a single four-membered ring (*i.e.* II) is to rigidify the [2.2.2]bicyclo system by reducing the bridge C–C dihedral angles to 0°. Concurrently the cyclobutane ring is required to be planar. Only three other pure organic substances bicyclo[2.1.0]pentane [20] (IV), the basketene photodimer [21] (V) and cubane [22] (VI), contain completely planar cyclobutane rings resulting from internal molecular forces alone. Except for a series of organometallic cyclobutane derivatives,[23] simple substituted cyclobutanes usually exhibit dihedral angles around 26°. Certain compounds with a crystallographic center of symmetry have planar four-membered rings presumably as a result of crystal packing forces.[23]



IV V VI

Angle distortion around the bridgehead carbons of II suggest the existence of H···H steric repulsion between the fused cyclobutane and the CH_2-CH_2 bridge. Thus the ∠CCC of 112.7° (II′) has expanded by 2.8° as compared with the same angle of I. Accordingly the complimentary angles 105.4° and 106.9° (II′) are compressed by 1.5–2.0°. The strain is likewise evidenced around the cyclobutane bridge with a ∠CCC of 119.2°. The corresponding angle for a cyclobutane fused to a non-bicyclic six-membered ring (*i.e.* a bicyclo[4.2.0]octane system) is 113±0.5°.[24] Finally the HCH angle of both the cyclobutane ring (108.9°) and the CH_2-CH_2 bridge (108.0°) is reduced relatively to cyclobutane [23] (110–114°) and I.



The formal act of bonding the cyclobutane ring of II to the CH_2-CH_2 bridge generates three new four-membered rings and leads to diazabasketene III. Two unique cyclobutane ribbons can be identified. The bicyclohexane

fragment bisected by the symmetry plane passing through the N=N bond contains two planar cyclobutane rings. The relative lengths of the three different C–C bonds involved in this unit are in the same relative order as those found for bicyclo[2.2.0]hexane [25] (VII). The bridge angle for III (86.3°), however, is considerably diminished relative to the 113.5° value for the latter. Each of the rings of VII exhibits a puckering angle (β) of 11.5°.

The second cyclobutane sequence in diazabasketene contains the rings bonded to nitrogen. The isolated three-ring fragment is depicted by structure VIII. The central cyclobutane is planar, whereas the flanking rings are both twisted outward with $\beta = 26.3°$. By contrast the cyclobutanes of *syn*-tricyclo[4.2.0.0^{2,5}]-octane [26] (IX) are all puckered ($\beta = 9.8°$) but in the same screw-sense. Consistent with the inclusion of IX in the condensed diazabasketene structure, the angle between adjacent ring carbons (108.5°) is considerably reduced relative to that in IX.

Bond lengths. Two noteworthy changes in bond distances for the I–III series concern the C–N and N=N bonds. The latter is constant in the transition from I to II, but drops by about 0.01 Å from II to III. By contrast the C–N bond lengthens by 0.01 Å from I to II, then shrinks by 0.03 Å from II to III.

Although these variations are small, their direction is significant and entirely consistent with the results of a recent photoelectron (PES) study for I, II and III.[4,6] The lone pair (n_- and n_+) and π(NN) molecular orbitals have been assigned as shown in Fig. 2 by assuming the validity of Koopmans theorem.[27] Whereas the antisymmetric n_- MO rises only slightly in energy along the I–III series, π(NN) drops slightly and n_+ falls markedly. The pattern of the changes has been rationalized by considering both conformational differences and the effect of through-space interaction [28] between the N=N unit and the cyclobutane and bicyclobutane fragments, respectively.[4,6] Fig. 3 illustrates the situation for n_+ of diazabasketene and the highest occupied a_1 MO of bicyclohexane.[29] Interaction of the a_1 symmetry species leads to the stabilization of n_+ and the destabilization of $8-a_1$. Of necessity lower energy a_1, b_1 and b_2 orbitals of bicyclohexane combine with the corresponding MO's of the azobicycle leading to the final MO distribution in III. The detailed interaction diagram will be presented in a forthcoming report.

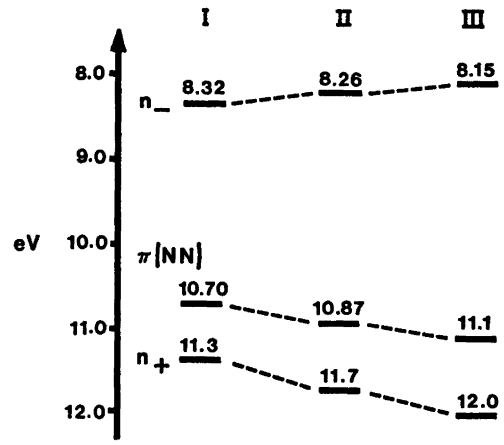


Fig. 2. Experimental ionization potentials (photoelectron spectroscopy) for the nitrogen lone pairs (n_- and n_+) and the π(NN) bonds of azoalkanes I, II and III. Refs. 4 and 6.

The implication of the PES results for III is obvious. The stepwise introduction of cyclobutane rings into the 2,3-diazabicyclo[2.2.2]oct-3-ene structure stabilizes the MO's associated with N–N bonding leading to a shorter N=N bond. Unfortunately the constancy of the same bond distance from I to II is not accomodated by this straightforward analysis. We have therefore performed CNDO calculations [30] for structures I–III in the hope of gaining additional insight. The MO energy trends implied by Fig. 2 are reproduced. For lower-lying MO's with the same symmetry as n_+ (b, c and d, Fig. 4), the calculations predict essentially no change or a moderate stabilization for analogous orbitals in I and II. In actuality, even if the low-lying MO energies were quantitatively evaluated by the calculation, the relative energies of nearly degenerate orbitals are only a very rough guide to the strength of the N–N and C–N bonding. All of the MO's in question are highly delocalized and contain non-trivial

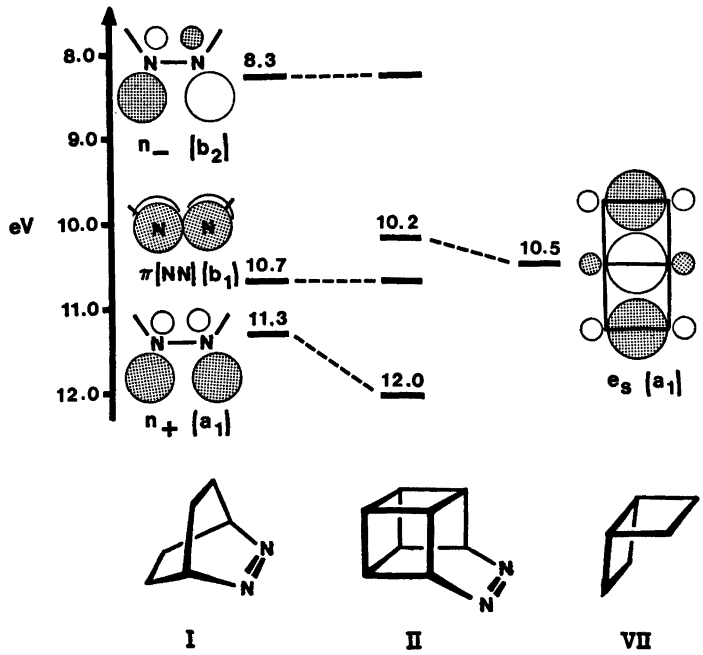


Fig. 3. Interaction of the a_1 molecular orbitals between azoalkane I and bicyclo[2.2.0]hexane leading to stabilization of n_+ in diazabasketene (III). Additional MO interactions not illustrated.

contributions from the carbon skeleton. By contrast the corresponding diazabasketene orbitals are calculated to be greatly stabilized relative to II (0.75 – 3.0 eV).

Of particular note is the prediction that low-lying N – N and C – N MO's for I and III are pure σ in composition, whereas certain of the corresponding energy levels for II contain considerable π-character (Fig. 4). This can be rationalized by noting that the cyclobutane plane makes a 120.8° angle with the plane of the CH – CH bridge to which it is fused. The orientation of the four-membered ring is such that it lies unsymmetrical with respect to the nodal plane of the hypothetically isolated π(NN) (*cf.* II′). In diazabasketene the two corresponding cyclobutane rings (*i.e.* the bicyclohexane moiety) are oriented symmetrically about this π-bond. Thus for II the appropriate cyclobutane MO (*i.e.* the e_s MO [31]) interacts through-space with the –CNNC– fragment leading to orbitals with mixed $\sigma - \pi$ character (*e.g.* –12.7, –13.0 and –13.8, b and c, Fig. 4). The latter is, no doubt, partly responsible for the similar energies of comparable low-lying MO's of I and II. More to the point, analogous orbitals with significant N – N bonding properties are expected to contribute less to the N – N bond energy the greater the degree of π-mixing. Thus we postulate that relative to I, the stabilization of π(NN) and n_+ is offset by the weaker π-bonding of the lower energy orbitals in II. The net effect is to leave the N = N distance unchanged in I and II.

Variations in the C – N bond lengths are described by a similar analysis. The characteristic MO's are shown in Fig. 4. The orbital containing the greatest C – N contribution is n_-, experimentally and theoretically higher in energy in II than in I. Lower-lying MO's for the former, as is the case for the N – N link, contain considerable π-type bonding. On this basis the C – N bond in II is expected to be weaker and therefore longer than in I as is observed.

The transition to diazabasketene reverses the trend. Although its n_- lies highest in the I – III series, III's low-lying C – N MO's are completely σ in character and significantly lower in energy than the corresponding energy levels

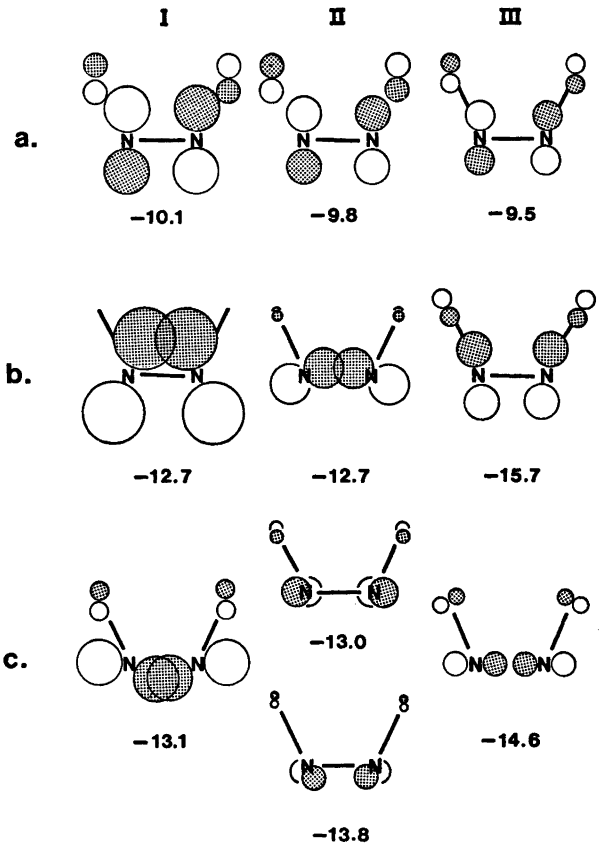


Fig. 4. Partial CNDO[30] eigenfunctions for certain N−N and N−C centered MO's of I, II and III. Only the nitrogen bridge and bridgehead carbon contributions are shown. Circle radii represent the squares of the relative atomic orbital compounds among the orbitals pictured. The numerical value associated with each MO is the CNDO predicted energy in eV.

for I and II. Accordingly the C−N bonds in diazabasketene are stronger and shorter by comparison.

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^{13}C NMR Studies of Some 2-Substituted Thieno[2,3-*b*]thiophenes and Thieno[3,2-*b*]thiophenes

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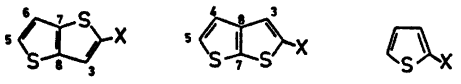
The ^{13}C NMR parameters of some 2-substituted thieno[2,3-*b*]thiophenes and thieno[3,2-*b*]thiophenes have been determined. The substituent-caused chemical shifts are discussed. Differences were observed for the transmittance of substituent effects to similar positions in the two systems. In comparison with the analogous shifts of the corresponding thiophenes great similarities were observed and good linear correlations were obtained. Linear correlations between some of the shifts and the reactivity parameters according to Swain and Lupton's two-parameter equation are also given.

In our previous papers, a detailed study of the ^{13}C NMR parameters of 2- and 3-substituted furans, thiophenes, selenophenes, and tellurophenes [1–4] was made. In order to study how substituent effects are transmitted over the heteroatoms, the thieno[3,2-*b*]thiophenes (Ia–c, e–i) and the thieno[2,3-*b*]thiophenes (IIa–i) were investigated and compared with the corresponding thiophenes (IIIa–i).



I II III

Fig. 1. X=

a, H	f, CHO
b, SCH_3	g, $COCH_3$
c, Cl	h, CN
d, Br	i, NO_2
e, CH_3	

Bugge [5] found linear correlations between the ^1H chemical shifts of I (a–e, g–i) and II (a–e, g–i) with the reactivity constants $\mathscr{F}$ and $\mathscr{R}$ of Swain and Lupton. A comparison was also made with similar calculations on thiophene by Rodmar *et al.*[6]

^{13}C NMR SPECTRA AND ASSIGNMENTS OF SIGNALS

In the ^{13}C NMR measurements, deuterioacetone was used as solvent. All shifts were determined from proton decoupled spectra using TMS as an internal standard.

In the ^{13}C spectra of 2-substituted thieno[3,2-*b*]thiophenes (Ib–i), C-8 and C-7 were identified by their lower intensity, as expected for quaternary carbons due to their relatively long relaxation times. For the unsubstituted Ia, the C-8 and C-7 absorptions are equivalent because Ia is symmetrical, while for the substituted derivatives (Ib–i) these positions are non-equivalent.

As seen from Fig. 3, C-7 in Ib–i should be compared with C-5 in 2-substituted thiophenes.[1] C-8 in Ib–i can in a similar way be compared with C-4 in 2-substituted thiophenes, showing small substituent-caused shifts.[1]

In Ib–i, C-2 is a quaternary carbon giving an absorption with lower intensity. The substituent influence on the C-2 shift is the same as on the C-2 shift in the corresponding 2-substituted thiophenes. For the unsubstituted compound (Ia) there is a shift difference of 8 ppm between C-α and C-β. In order to get further information for the shift assignments, the undecoupled spectrum of 2-chlorothieno[3,2-*b*]thiophene (Ic) was determined (Fig. 2). The direct coupling constants were found to

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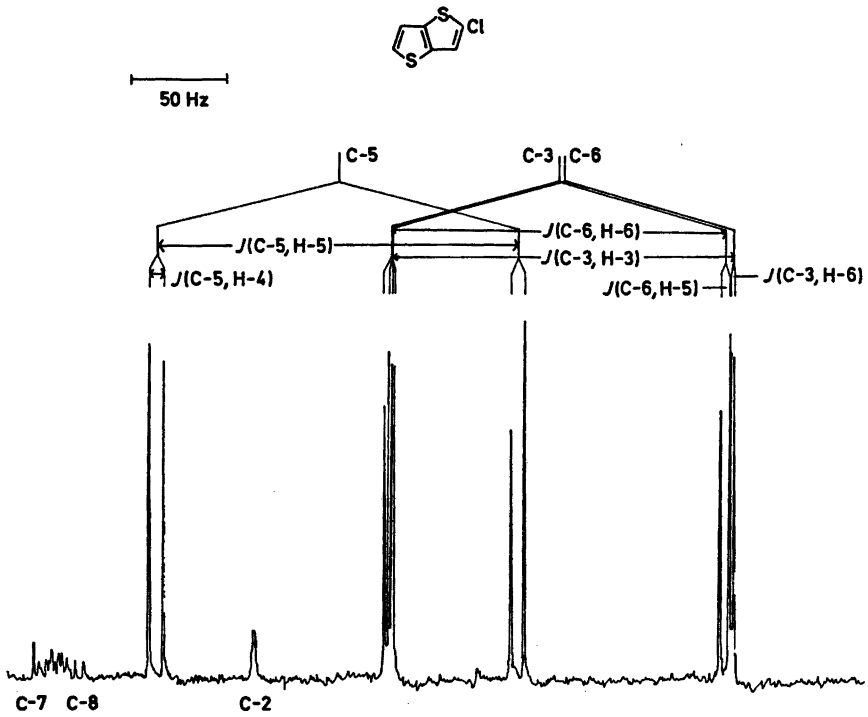

Fig. 2. ^{13}C NMR Spectra of 2-chlorothieno[3,2-*b*]thiophene in hexadeuterioacetone solution at 15.00 MHz.

be 177.7 Hz, 188.2 Hz and 174.8 Hz, showing that the C-5 absorption occurs at lower field than the C-3 and C-6 absorptions because $J_{C-\alpha,H-\alpha}$ is larger than $J_{C-\beta,H-\beta}$.[1] For C-3, adjacent to the substituent, the substituent-caused shifts are expected to vary more than those of the C-6-position. (*Cf.* Table 2: the intervals for C-3 are 11.3 – 1.7 ppm and for C-6 1.4 – 0.1 ppm.) The assignments of the C-3 and C-6 carbons in the 2-chloro derivative could also be obtained from the long-range couplings by comparison with the 2-substituted thiophenes,[1] where $J_{C-4,H-5}$ and $J_{C-5,H-4}$ are about 5 and 7 Hz, respectively. The long-range couplings obtained for Ic were $J_{C-3,H-6}$ 1.7 Hz, $J_{C-5,H-6}$ 7.1 Hz and $J_{C-6,H-5}$ 4.7 Hz (Fig. 2).

In the thieno[2,3-*b*]thiophene series (IIb – i), C-2, C-8 and C-7 are quaternary carbon atoms. These carbons can be identified in the same way as described above. C-2 exhibits the largest substituent effects. C-7, with the character of a *para* position (Fig. 3), should also be rather sensitive to the variation of substituent, while C-8, which can be compared with a *meta* posi-

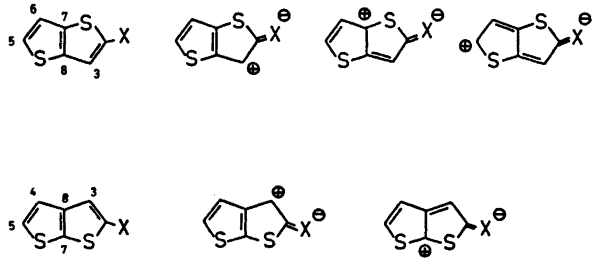

Fig 3.

Table 1. ^{13}C NMR chemical shifts for some 2-substituted thieno[3,2-*b*]thiophenes (I) in deuterioacetone solution at 15.0 MHz using TMS as an internal standard.

X	C-2	C-3	C-5	C-6	C-7	C-8	CH_3	C=O
H	127.9	119.9	127.9	119.9	139.9	139.9		
SCH_3	139.2	124.5	128.3	120.2	141.6	139.8	22.3	
Cl	130.7	120.0	127.8	120.0	137.4	137.6		
CH_3	142.8	118.2	126.3	120.1	138.3	139.6	16.2	
CHO	146.3	130.7	135.2	121.1	145.8	140.1		184.4
$COCH_3$	146.9	126.6	133.9	121.1	145.6	140.1	30.7	191.5
COOH	136.9	126.8	133.1	120.8	144.8	139.6		163.6
CN	114.9	131.2	134.9	121.0	144.1	139.3		110.4
NO_2	152.2	123.6	136.5	121.3				

Table 2. ^{13}C NMR chemical shifts for some 2-substituted thieno[2,3-*b*]thiophenes (II) in deuterioacetone solution at 15.00 MHz using TMS as an internal standard.

Substituent	C-2	C-3	C-4	C-5	C-7	C-8	CH_3	C=O
H	129.1	120.4	120.4	129.1	137.6	147.9		
SCH_3	140.3	126.0	120.5	129.0	139.0	147.3		
Cl	130.8	119.5	119.5	128.8	134.6	145.3		
Br	113.3	124.1	120.2	129.1	136.1	146.7		
CH_3	143.7	118.7	120.3	128.1	135.2	147.8	16.2	
CHO	148.0	130.6	121.7	131.4	145.9	147.5		184.1
$COCH_3$	148.7	126.6	121.7	131.0	145.0	147.7	26.3	191.2
COOH		126.9	121.6	130.8				
CN	114.8	131.5	121.1	132.5	143.3	146.5	111.8	
NO_2	153.4	122.9	122.4	131.0	144.0	146.4		
CHNOH		123.9	120.9	129.5	141.5	146.1		

Table 3. Chemical shifts (ppm)[a] of the carbons of 2-substituted thieno[3,2-*b*]thiophenes (I) and thieno[2,3-*b*]thiophenes (II) relative to corresponding carbons of unsubstituted I and II in deuterioacetone solution.

	I						II					
Substituent	ΔC-2	ΔC-3	ΔC-5	ΔC-6	ΔC-7	ΔC-8	ΔC-2	ΔC-3	ΔC-4	ΔC-5	ΔC-7	ΔC-8
H	0	0	0	0	0	0	0	0	0	0	0	0
SCH_3	11.3	4.6	0.4	0.3	1.7	−0.1	11.2	5.6	0.1	−0.1	1.4	−0.6
Cl	2.8	0.1	−0.1	0.1	−0.5	−0.3	1.7	−0.9	−0.9	−0.3	−3.0	−2.6
Br							−15.4	3.6	−0.1	0.0	−1.5	−1.2
CH_3	14.9	−1.7	−1.6	0.2	−1.6	−0.3	14.6	−1.7	−0.1	−1.0	−2.4	−0.1
CHO	18.4	10.8	7.3	1.2	5.9	0.2	18.9	10.2	1.3	2.3	8.6	−0.4
$COCH_3$	19.0	6.7	6.0	1.2	5.7	0.2	19.6	6.2	1.3	1.9	7.4	−0.2
COOH	9.0	6.9	5.2	0.9	4.9	−1.3	–	6.5	1.2	1.7	–	–
CN	−13.0	11.3	7.0	1.1	4.2	−0.6	−14.3	11.1	0.7	3.4	5.7	−1.4
NO_2	24.3	3.7	8.6	1.4	–	–	24.2	2.5	2.0	2.9	6.4	−1.5

[a] ΔC=C(substrate)−C(reference); positive values are downfield.

Table 4. Linear correlations obtained for ^{13}C chemical shifts of 2-substituted thieno[3,2-*b*]thiophenes (Ib–i) and thieno[2,3-*b*]thiophenes (IIb–k) *versus* those of corresponding 2-substituted thiophenes (III).

	r	*n*	
I			
C-7 = (0.62 ± 0.04)C-5 − (0.25 ± 0.27)	0.990	7	(1)
C-5 = (0.81 ± 0.09)C-5 − (0.46 ± 0.63)	0.966	7	(2)
C-3 = (1.00 ± 0.04)C-3 − (0.34 ± 0.27)	0.995	8	(3)
C-2 = (0.92 ± 0.03)C-2 + (0.67 ± 0.43)	0.998	8	(4)
II			
C-7 = (0.98 ± 0.09)C-5 − (2.04 ± 0.64)	0.972	8	(5)
C-5 = (0.33 ± 0.06)C-5 − (0.53 ± 0.31)	0.939	8	(6)
C-3 = (1.01 ± 0.05)C-3 − (0.43 ± 0.31)	0.992	9	(7)
C-2 = (0.93 ± 0.03)C-2 − (0.41 ± 0.50)	0.997	9	(8)

tion, should be rather insensitive to shift effects.

The other three carbons were assigned from the undecoupled spectrum of 2-formylthieno-[2,3-*b*]thiophene, IIf. The direct coupling constants were found to be 171.1, 173.1 and 190.0 Hz. The largest direct coupling was observed for the carbon atom that has its absorption at lowest field, demonstrating it to be C-5. C-3, adjacent to the substituent, also shows the largest substituent effects, while C-4 is almost uninfluenced. The assignments of C-3 and C-4 carbons in IIf could also be obtained with the same procedure as above from the long-range coupling constants. The long-range couplings obtained for IIf were $J_{C-3,H-5}$ 1.0 Hz, $J_{C-3,H(CHO)}$ 0.5 Hz, $J_{C-4,H-5}$ 4.2 Hz, $J_{C-4,H-3}$ 1.0 Hz and $J_{C-5,H-4}$ 7.6 Hz. The ^{13}C parameters are given in Tables 1 and 2 and the chemical shifts relative to the unsubstituted compounds Ia and IIa are presented in Table 3.

DISCUSSION

In both thieno[3,2-*b*]thiophene and thieno-[2,3-*b*]thiophene, the 3-carbon is *ortho* to the substituent and the substituent-caused shifts

Table 5. Regression equations relating ^{13}C chemical shifts [a] of 2-substituted thieno[3,2-*b*]thiophenes (I) and thieno[2,3-*b*]thiophenes (II) to substituent constants $\mathcal{F}$ and $\mathcal{R}$.

	G[b]	*R*[c]	*N*[d]
I			
ΔC-5 = (0.6 ± 0.6) + (3.9 ± 1.0)$\mathcal{F}$ + (18.0 ± 3.5)$\mathcal{F}$	0.7	0.99	6
ΔC-6 = (0.5 ± 0.2) + (0.2 ± 0.2)$\mathcal{F}$ + (2.8 ± 0.9)$\mathcal{R}$	0.2	0.95	6
ΔC-7 = (1.3 ± 0.9) + (0.9 ± 1.7)$\mathcal{F}$ + (17.2 ± 4.8)$\mathcal{R}$	1.0	0.95	5
II			
ΔC-5 = (−0.9 ± 0.4) + (1.7 ± 0.7)$\mathcal{F}$ + (7.4 ± 2.5)$\mathcal{R}$	0.5	0.96	6
ΔC-7 = (1.4 ± 0.7) + (0.1 ± 1.1)$\mathcal{F}$ + (27.5 ± 3.2)$\mathcal{R}$	0.8	0.99	5
ΔC-8 = (0.3 ± 0.4) − (2.7 ± 0.6)$\mathcal{F}$ + (5.0 ± 2.0)$\mathcal{R}$	0.4	0.91	5
III			
ΔC-5 = (2.1 ± 0.4) + (2.5 ± 0.7)$\mathcal{F}$ + (26.5 ± 2.5)$\mathcal{R}$	1.1	0.99	6

[a] The shifts are given in ppm relative to those of the parent compounds I and II (*cf.* Table 3). [b] Standard deviation 1 ppm. [c] Correlation coefficient. [d] Number of substituents in the regression analysis.

(ΔC-3) show good linear correlations with ΔC-3 in 2-substituted thiophenes (*cf.* eqns. 3 and 7 in Table 4). These equations also show that the sensitivity to the substituent effects is the same in the three systems (I–III). The substituted carbon atoms in the three series also show comparable sensitivity to the substituent effects (eqns. 4 and 8, Table 4). By correlating ΔC-5 of 2-substituted thiophenes with ΔC-7 of the two fused systems (eqns. 1 and 5, Table 4), it is found that the transmission over the heteroatom to C-7 is of the same magnitude in thiophene and system II, while it is much lower in system I. However, when ΔC-5 of I and ΔC-5 of II are correlated with ΔC-5 of thiophene, the sensitivity is much lower in the II-series than in the I-series. Simple resonance structures (Fig. 3) indicate that for an –I-M-substituent the positive charge can be placed in three positions, on C-3, C-5, and C-7 in the I-series, but only in two positions in the II-series (C-3 and C-7). This supports the assumption that the substituent effect can be efficiently transmitted to the 5-carbon in the I-series but not in the II-series.

In our previous investigations of ^{13}C spectra of furans, thiophenes and selenophenes,[1–3] we successfully correlated the substituent-caused shifts with the two-parameter equation of Swain and Lupton.[7] In this way, we obtained some information about the resonance and inductive contributions to the chemical shifts. Bugge [5] has obtained good correlations of the ^{1}H chemical shifts of I and II with the reactivity constants $\mathcal{F}$ and $\mathcal{R}$ of Swain and Lupton.

The correlations obtained with I and II are given in Table 5. The substituents Br and SCH_3 were excluded from the least-squares fit calculations as the values calculated from the regression equations deviate considerably more from the experimental ones for these substituents than for the others.

The only positions which gave good correlations in both I and II were C-5 and C-7. By comparing the equations for C-5 of I and II in Table 5 it is interesting to note that the resonance effect is much more efficiently transmitted over two sulfur atoms in system I than in system II; the r-values are 18.0 and 7.4, respectively. This is in agreement with reasoning based on simple resonance structures, as a positive charge in the 5-position can only be written for system I and not for system II without violating the octet rule for sulfur (Fig. 3).

On the other hand, for the 7-position, where the interaction with the substituent is only over one sulfur atom, system II shows great similarities with the monocyclic system (III). The equations for ΔC-7 of II and ΔC-5 of III have comparable f- and r-values, while the equation for C-7 of I shows a much lower r-value. In the latter system, the effects of the substituents are more equally divided between the 7- and 5-position ($r = 17.2$ and 18.0). The transmission of the resonance effects in system II is reflected in the r-values 7.4 and 27.5 for the 5- and 7-positions, respectively.

The low value of the slope of eqn. 1 compared to eqn. 5 in Table 4 is consequently due to the continued transmission of the resonance effects over both of the sulfur atoms of system I, while system II allows transmission mainly over one sulfur atom, and therefore shows similarities to the monocyclic system.

EXPERIMENTAL

All 2-substituted thieno[2,3-b]thiophenes (Ia-i) and thieno[3,2-*b*]thiophenes (IIa-i) used in this study are known.[5,6,9] The purity and structure of the compounds were checked by gas chromatography, IR and ^{1}H NMR analysis.

All ^{13}C NMR spectra were obtained at 15.0 MHz with a Jeol JNM-FX 60 spectrometer with a built-in Jeol 980A computer with 12 K memory. For all spectra the proton noise decoupling technique was used. The *Rf* pulse width was 6 μs, corresponding to a pulse angle of 45 degrees and the pulse repetition rate was 1 s. The shifts were determined with an accuracy of ± 0.1 ppm.

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On the Halogenation of Halothiophenes with *N*-Chloro- and *N*-Bromosuccinimide

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Isomer distribution and halogen exchange in the reaction of *N*-chlorosuccinimide and *N*-bromosuccinimide with some bromo- and iodothiophenes have been studied. It was found that from a preparative point of view these reagents are to be preferred to molecular chlorine or bromine.

The use of *N*-bromosuccinimide (NBS; Wohl-Ziegler reaction) for benzylic and allylic brominations is well established [1] and the mechanism of the reaction is known (for a review *cf.* Ref. 2). However, as early as 1944 Buu-Hoi [3] found that reactive aromatics, such as thiophene, gave aromatic substitution with NBS in carbon tetrachloride. Schmid found that in the presence of acids, nuclear bromination could also be obtained with benzene and toluene.[4] Recently, the nuclear halogenation of fluorene and acenaphthene with NBS has been studied.[5]

In the thiophene series NBS in inert solvents or in acetic acid or acetic acid-chloroform has been used in a few investigations for the bromination of for instance bromo-, methyl-, and phenylthiophenes,[6–8] as well as for deactivated thiophenes.[9] Also, *N*-chlorosuccinimide (NCS) has been used for nuclear chlorination of thiophenes.[10]

However, no systematic investigation of the use of NBS and NCS for the bromination and chlorination of especially halothiophenes, has been carried out.

We were especially interested to ascertain the tendency of these reagents to give halogen exchange, which often complicates the reaction of certain halothiophenes with molecular chlorine or bromine (for reviews, *cf.* Refs. 11, 12), and also the selectivity of these reagents in the substitution of 2- and 3-substituted halothiophenes. We needed mixed halothiophenes in connection with our study of the halogen-metal exchange reaction of such compounds with alkyllithia.[13,14] Some mixed halothiophenes have previously been prepared by introducing the lighter halogen first or by the use of organomercury or Grignard reagents as intermediates, especially for the introduction of iodine.[15–17]

CHLORINATION WITH *N*-CHLOROSUCCINIMIDE (NCS)

The chlorination of 2- and 3-bromothiophene, 2- and 3-iodothiophene, 2,3-, 2,4-, and 2,5-dibromothiophene with NCS was studied. Product analysis was carried out by GLC. Products of less than 1 % abundance in the reaction mixtures are not reported, if they are not of special interest.

The reaction of 2-bromothiophene with one equivalent of NCS in refluxing acetic acid gave a mixture consisting of 83 % of 2-bromo-5-chlorothiophene, 11 % of 2,5-dibromothiophene, and 6 % of 2,5-dichlorothiophene. In a preparative run, a 55 % yield of pure 2-bromo-5-chlorothiophene was obtained by fractional distillation. It has previously been obtained by bromination of 2-chlorothiophene with molecular bromine.[18] Somewhat surprisingly, 2-iodothiophene gave even a cleaner result: after refluxing for 4 h in acetic acid with one equiv.

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of NCS, a mixture consisting of 95 % of 2-chloro-5-iodothiophene, 5 % of 2,5-dichlorothiophene, and less than 1 % of diiodothiophene was obtained. Fractional distillation *in vacuo* gave a 55 % yield of pure 2-chloro-5-iodothiophene. This compound has previously been obtained by Steinkopf *et al.* *via* the mercury derivative.[19] We found that the most convenient method was to iodinate 2-chlorothiophene with iodine-iodic acid according to the general method of Wirth *et al.*,[20] which gave 2-chloro-5-iodothiophene in 53 % yield.

The chlorination of 3-bromothiophene with NCS (reflux 5 h in acetic acid) proceeded with very high selectivity in the 2-position, as the product contained much less than 1 % of 4-bromo-2-chlorothiophene and 96 % of 3-bromo-2-chlorothiophene. More important by-products were 2,3-dichlorothiophene (1 %) and 3-bromo-2,5-dichlorothiophene (3 %). The sensitivity factor of 2,3-dichlorothiophene was set equal to that of 2,5-dichlorothiophene. Fractional distillation gave a 65 % yield of pure 3-bromo-2-chlorothiophene. This is certainly the best method for the preparation of this compound, which has been used as starting material in connection with the synthesis of dithienocycloheptanones.[10] When two equivalents of NCS were used, 3-bromo-2,5-dichlorothiophene was, as expected, the main product (87 %). In addition, the product contained 5 % of 3-bromo-2-chlorothiophene, 2 % of tetrachlorothiophene, less than 0.1 % of 2,3-dichlorothiophene, and 6 % of a bromotrichlorothiophene, which was not identified. Its sensitivity factor was set equal to that of 3-bromo-2,5-dichlorothiophene. By fractional distillation *in vacuo* a 55 % yield of pure 3-bromo-2,5-dichlorothiophene was obtained. The behavior of 3-iodothiophene was similar to that of the 3-bromo derivative upon chlorination with one equivalent of NCS (reflux 3.5 h in acetic acid). The product consisted of 90 % of 3-iodo-2-chlorothiophene together with 8 % of 2,5-dichloro-3-iodothiophene, 2 % of 2,3-dichlorothiophene, and much less than 1 % of 2-chloro-4-iodothiophene. The sensitivity factor of 2,3-dichlorothiophene was again set equal to that of 2,5-dichlorothiophene. In spite of the bulkiness of the substituent in the 3-position, substitution thus occurs selectively in the 2-position. This is not unexpected, as it has been found that iodination of 3-iodothiophene with iodine and iodic acid gave a mixture consisting of 99.5 % of 2,3-diiodothiophene and 0.5 % of 2,4-diiodothiophene.[21] Fractional distillation of the chlorination products gave a 53 % yield of 2-chloro-3-iodothiophene. When two equivalents of NCS were used (reflux in acetic acid, 4 h), the main component was 2,5-dichloro-3-iodothiophene (72 %) together with 23 % of 2-chloro-3-iodothiophene, 4 % of 2,3,5-trichlorothiophene, less than 1 % of 2,3-dichlorothiophene, and 2 % of a mono-iodotrichlorothiophene. Its sensitivity factor was set equal to that of 2,5-dichloro-3-iodothiophene. By fractional distillation *in vacuo* pure 2,5-dichloro-3-iodothiophene was obtained in 39 % yield. For preparative purposes, it is more convenient to iodinate 2,5-dichlorothiophene with iodine and iodic acid, which gives a 70 % yield.[22]

Other examples of the preparative usefulness of this iodination method are the preparation of 2-bromo-3,5-diiodothiophene in 53 % yield from 2-bromo-3-iodothiophene, of 3-bromo-2-iodothiophene in 66 % yield from 3-bromothiophene, and of 2-chloro-5-iodothiophene from 2-chlorothiophene in 53 % yield.

The chlorination of the dibromothiophenes with NCS was not as selective. After refluxing in acetic acid for 24 h with one equivalent of NCS, 2,3-dibromothiophene gave a mixture consisting of 59 % of 5-chloro-2,3-dibromothiophene, 24 % of 2,3,5-tribromothiophene, 14 % of 3-bromo-2,5-dichlorothiophene, and 4 % of a dibromodichlorothiophene. Its sensitivity factor was set equal to that of 3-bromo-2,5-dichlorothiophene. Fractional distillation *in vacuo* gave 36 % of pure 5-chloro-2,3-dibromothiophene. After reflux for 6 h with one equivalent of NCS, 2,4-dibromothiophene gave 77 % of 2-chloro-3,5-dibromothiophene, 13 % of 3-bromo-2,5-dichlorothiophene, and 10 % of 2,3,5-tribromothiophene. Fractional distillation gave 43 % of pure 2-chloro-3,5-dibromothiophene.

The attempted chlorination of 2,5-dibromothiophene was quite unsuccessful. After 5 h reflux in acetic acid with one equivalent of NCS, 39 % of 2-bromo-5-chlorothiophene, 11 % of 2,3,5-tribromothiophene, 5 % of 2,5-dichlorothiophene, and 44 % of a mixture of at least two of the possible 2,3,5-substituted chlorodibromothiophenes (3-chloro-2,5-dibromo-, 2-chloro-3,5-dibromo-, and 5-chloro-2,3-dibromo-

thiophene) were formed. It was not possible to resolve this peak and the values given in this case are not calibrated. Nor was it possible to isolate any pure 3-chloro-2,5-dibromothiophene by fractional distillation. This compound should therefore most easily be prepared by dibromination of 3-chlorothiophene.

BROMINATIONS WITH *N*-BROMOSUCCINIMIDE (NBS)

We have also studied the bromination of 2- and 3-iodothiophene and of 2,3-diiodothiophene with NBS in acetic acid. When 2-iodothiophene was refluxed in acetic acid with one equivalent of NBS for 1.5 h, a mixture consisting of 88 % of 2-bromo-5-iodothiophene, 8 % of 2,5-dibromothiophene, and 4 % of 2,5-diiodothiophene was obtained, from which 38 % of pure 2-bromo-5-iodothiophene was isolated by fractional distillation. This compound has previously been prepared by Steinkopf *et al.*[17] from the Grignard reagent of 2,5-dibromothiophene and iodine.

If 2-iodothiophene was reacted with two equivalents of NBS, either at room temperature or at reflux, the main product was 2,5-dibromothiophene together with traces of 2-bromo-5-iodothiophene and other more high-boiling products, which were not characterised. This indicates that the second equivalent of NBS prefers to eliminate the iodine instead of entering the β-position.

The reaction of 3-iodothiophene with NBS was much more homogeneous. After 30 min reflux, a mixture consisting of 98 % of 2-bromo-3-iodothiophene, 1 % of 2,3-dibromothiophene, 1 % of 2,5-dibromo-3-iodothiophene, and much less than 1 % of 2-bromo-4-iodothiophene was obtained. Fractional distillation *in vacuo* gave an 83 % yield of pure 2-bromo-3-iodothiophene.

With two equivalents of NBS, 3-iodothiophene gave 2,5-dibromo-3-iodothiophene as the main product (90 %), together with 9 % of a mixture of 2,3,5-tribromothiophene and 3-iodo-2-bromothiophene, and 1 % of 2,3-dibromothiophene after 19 h at room temperature. By fractional distillation *in vacuo*, 2,5-dibromo-3-iodothiophene was isolated in 44 % yield. This method of synthesis is to be preferred, as we only managed to obtain an 11 % yield in the iodination of 2,5-dibromothiophene with iodine-iodic acid.

We also found in this bromination reaction that higher temperature and longer reaction time influenced the reaction (Table 1), and led to a decrease in the yield of 2,5-dibromo-3-iodothiophene.

The separation of all the products with GLC was not possible in this case and therefore no calibration was carried out.

Bromination of 2,3-diiodothiophene with NBS, either in acetic acid or in a 1:1 mixture of acetic acid-chloroform did not proceed cleanly. The mixture consisted of 59 % of 5-bromo-2,3-diiodothiophene, 22 % of 2,5-dibromo-3-iodothiophene, 13 % of 2,3,5-triiodothiophene, and 6 % of 2-bromo-3-iodothiophene. Only a low yield of pure 5-bromo-2,3-diiodothiophene could be obtained by fractional distillation *in vacuo*.

Table 1. Bromination of 3-iodothiophene with two equivalents of NBS in acetic acid.

Products	Yield (%) Room temp. 19 h	Reflux 2 h	Reflux 5 h
2,3-Dibromothiophene	1	2	2
2-Bromo-3-iodothiophene 2,3,5-Tribromothiophene	9	23	29
2,5-Dibromo-3-iodothiophene	90	75	69

DISCUSSION

The mechanism of halogenations with NCS and especially NBS has been much discussed [2] and it now seems accepted that these reagents can be considered as sources of molecular chlorine and bromine, which are continuously formed in very low steady-state concentrations.[23] The bromine thus formed may either give radicaloid allylic or benzylic bromination, addition to double bonds or electrophilic (or radicaloid) aromatic substitution, depending upon substrate, solvent and catalyst.[23] In the presence of acids and polar solvents, nuclear substitution is often favoured. The great preparative advantage of NCS and NBS over

molecular chlorine and bromine for the bromination of thiophenes lies most probably in the fact that no strong acids are formed which can cause decomposition of the heterocyclic derivatives. It also seems unlikely that addition products can be formed with these reagents. Such addition products complicate the chlorination [24] and possibly the bromination [25] with molecular chlorine and bromine, respectively. The formation of compounds such as 2,5-dibromothiophene in the reaction of 2-bromothiophene with NCS or of 2,3,5-triiodothiphene in the reaction of 2,3-diiodothiophene with NBS may be due to the formation of Br^+ and I^+ (or Br – Cl and I – Cl) from electrophilic halogen exchange of the substrates followed by electrophilic substitution in the 5-position. This is in agreement with the formation of 2,5-dichlorothiophene and 2-bromo-3-iodothiophene in the two above-mentioned reactions.

The advantage of the NCS and NBS halogenations also became apparent from experiments with other halogenating agents. Thus, sulfuryl chloride, which is the reagent of choice for the preparation of 2-chloro- and 2,5-dichlorothiophene,[27] cannot be used for the chlorination of 3-bromothiophene. A mixture of many compounds, with none predominating, was obtained and no effort was made to identify them. Nor did the reaction of 2,3-diiodothiophene with sulfuryl chloride give any 5-chloro derivative. Also the bromination of 2,3-diiodothiophene with one equivalent of bromine in carbon tetrachloride was quite unsuccessful. No main product was obtained, and 5-bromo-2,3-diiodothiophene could not be detected in the reaction mixture. By reacting 2,3-diiodothiophene with excess bromine, Steinkopf *et al.*[15] obtained 4-iodo-2,3,5-tribromothiophene. When 3-iodothiophene was reacted with two equivalents of bromine in carbon tetrachloride, the main part of the product consisted of one or several hexabromobithienyls together with 2,3,5-tribromothiophene. 2,5-Dibromo-3-iodothiophene could not be detected in the reaction mixture. In acetic acid, on the other hand, the main product was 2,5-dibromo-3-iodothiophene. The different behaviour of NBS and bromine towards 3-iodothiophene is quite striking and casts some doubt on the hypothesis that molecular bromine (or Br^+) is the active agent in both the Br_2 and NBS brominations.

EXPERIMENTAL

General. Gas chromatographic analyses were performed with a Perkin-Elmer 900 apparatus equipped with a flame ionisation detector and connected to a Varian 480 digital integrator. In some cases, however, a disc integrator was used for the evaluation of the gas chromatograms. The columns were made of stainless steel with 3 mm o.d. Nitrogen was used as carrier gas. The following columns were used: 5 % Neopentyl glycolsuccinate (NPBS) on Chrom. W (80/100 mesh), 2.0 m (A). 3 % OV 17 on Gas Chrom. Q (100/120 mesh), 2.5 m (B). 10 % Butane-1,4-diolsuccinate (BDS) on Chrom. W (80/100 mesh), 2.0 m (C) and 10 % Neopentylglycol sebacate on Chrom. W (80/100 mesh), 2.5 m (D).

Products of less than 1 % abundance in the reaction mixtures are not reported, if they are not of special interest. For all products obtained, calibration was, if not otherwise stated, made for the sensitivity factor of each compound to the flame.

NMR spectra were recorded on a Varian A-60 instrument. Tetramethylsilane (TMS) was used as internal standard. A Perkin-Elmer 257 IR spectrometer was used for IR spectra. Mass spectra were recorded on an LKB 9000 mass spectrometer at 70 eV. The mass spectra were mainly used for identification of the molecular ion.

Most of the elemental analyses were performed by the Department of Analytical Chemistry at the University of Lund, Sweden, and a few by Miss Ilse Beetz, Mikroanalytisches Laboratorium, Kronach, West Germany.

General procedure for NCS chlorination. To 0.050 mol of halothiophene in approximately 200 ml of acetic acid a small amount of a total of 6.7 g (0.050 mol) of NCS was added. The temperature was raised to reflux, whereupon the rest of the NCS was added in portions. After refluxing for 1 h, the reaction mixture was poured into water and extracted with ether. The combined ether phases were washed with water, dilute sodium hydroxide solution and more water. After drying over magnesium sulfate, the ether was evaporated, the product analyzed by GLC and the product distilled.

2-Bromo-5-chlorothiophene was obtained in 55 % yield from 2-bromothiophene,[26] b.p. 63 – 67 °C/11 mmHg (Column A). Lit. value [18] 69.5 – 70.0 °C/18 mmHg.

2-Chloro-5-iodothiophene was obtained in 55 % yield from 2-iodothiophene,[20] b.p. 89 – 90 °C/10 mmHg (Column A). Lit. value [19] 95 – 96 °C/14 mmHg.

3-Bromo-2-chlorothiophene was obtained in 62 % yield from 3-bromothiophene [28] (0.50 mol in 400 ml of acetic acid), b.p. 69 – 73 °C/11 mmHg (Column A). Lit. value [10] 68 – 72 °C/9 mmHg.

2-Chloro-3-iodothiophene was obtained in 53 % yield from 3-iodothiophene [29] (0.30 mol.

300 ml acetic acid), b.p. 96–98 °C/11 mmHg (Column A). NMR (CCl_4): δ 6.91 and 6.99. J_{45} 5.8 Hz. Calc. for C_4H_2ClIS (244.5): C 19.6; H 0.82; I 51.9. Found: C 19.8; H 0.92; I 51.9.

3-Bromo-2,5-dichlorothiophene was obtained in 55 % yield from 3-bromothiophene [28] using two equivalents of NCS, b.p. 89–92 °C/11 mmHg (Column A). Calc. for C_4HBrCl_2S (232.0): C 20.7; H 0.43; S 13.8. Found: C 20.7; H 0.39; S 13.8.

2,5-Dichloro-3-iodothiophene was obtained in 39 % yield from 3-iodothiophene [29] using two equivalents of NCS, b.p. 112–114 °C/11 mmHg (Column A). Calc. for C_4HCl_2IS (278.9): C 17.2; H 0.36; I 45.5 Found: C 17.6; H 0.48; I 45.3.

5-Chloro-2,3-dibromothiophene was obtained in 36 % yield from 2,3-dibromothiophene [31] (1.50 mol in 1500 ml acetic acid and 1.57 mol NCS), b.p. 106–109 °C/11 mmHg (distilled through a 35 cm long column filled with glass helices (Column B). Calc. for C_4HBr_2ClS (276.4): C 17.4; H 0.36; S 11.6. Found: C 17.5; H 0.33; S 11.6.

2-Chloro-3,5-dibromothiophene was obtained in 43 % yield from 2,4-dibromothiophene,[32] b.p. 104–108 °C/10 mmHg (Column B). Calc. for C_4HBr_2ClIS (276.4): C 17.4; H 0.36; S 11.6. Found: C 17.5; H 9.42; S 11.4.

From *2,5-dibromothiophene* [26] and *NCS* no 3-chloro-2,5-dibromothiophene could be identified with certainty, because of difficulties in the separation of the products in the reaction mixture (Column C).

General procedure for NBS bromination. To 0.100 mol of halothiophene in approximately 160 ml acetic acid 0.104 mol of NBS was added in portions. When the reaction did not start spontaneously, it was heated to 35–40 °C. After stirring for 2 h during which time the temperature did not exceed 40 °C, the reaction mixture was poured into water and worked up as described for the NCS chlorination.

2-Bromo-5-chlorothiophene was obtained in 47 % yield from 2-chlorothiophene,[27] b.p. 59–60 °C/9 mmHg (Column A): Lit. value [18] 69.5–70.0 °C/118 mmHg. NMR (CCl_4): δ 6.67 and 6.74, J_{34} 4.0 Hz.

2-Bromo-5-iodothiophene was obtained in 38 % yield from 2-iodothiophene,[20] b.p. 107–110 °C/11 mmHg (Column C). Lit. value [17] 116 °C/13 mmHg. NMR (CCl_4): δ 6.67 and 6.95, J_{34} 3.8 Hz.

2-Bromo-3-iodothiophene was obtained in 83 % yield from 3-iodothiophene,[29] b.p. 104–108 °C/8 mmHg (Column A). NMR (CCl_4): δ 6.91 and 7.17, J_{45} 5.6 Hz. Calc. for C_4H_2BrIS (288.9): C 16.6; H 0.70; S 11.1. Found: C 16.7; H 0.81; S 10.9.

2,5-Dibromo-3-iodothiophene was obtained in 44 % yield from 3-iodothiophene [29] using two equivalents of NBS, b.p. 145–150 °C/11 mmHg (Columns A, C). Higher temperatures and shorter reaction times did not improve the yields. Calc. for C_4HBr_2IS (367.8): C 13.1; H 0.27; I 34.5. Found: C 13.2; H 0.24; I 33.5.

5-Bromo-2,3-diiodothiophene was obtained in 5 % yield from 2,3-diiodothiophene.[30] The fractions boiling at 102–119 °C/0.2 mmHg were chromatographed on a column filled with neutral aluminium oxide. Petroleum ether (30–50 °C) was used as eluent. The fractions were checked by GLC. In this way pure title compound, m.p. 45–46 °C was obtained (Column C). Calc. for C_4HBrI_2S (414.8): C 11.6; H 0.24; I 61.2. Found: C 11.9; H 0.28; I 60.0.

2-Chloro-5-iodothiophene. To 11.8 g (0.100 mol) of 2-chlorothiophene,[27] 43 ml of acetic acid, 16 ml of water, 21 ml of carbon tetrachloride, 3.7 g (0.021 mol) of HIO_3, and 0.7 ml of concentrated sulfuric acid, at reflux, 10.2 g (0.040 mol) of iodine was added in portions. After reflux for 3 h, more water and carbon tetrachloride were added. The organic phase was separated, washed with water, sodium thiosulfate solution, and water, and dried over magnesium sulfate. Distillation gave 12.9 g (53 %) of 2-chloro-5-iodothiophene at 86–87 °C/11 mmHg. Lit. value [19] 95–96 °C/14 mmHg (Column A). NMR (CCl_4): δ 6.51 and 6.95, J_{34} 3.8 Hz.

3-Bromothiophene and sulfuryl chloride. To 24 g (0.15 mol) of 3-bromothiophene,[28] 25 ml (0.31 mol) of sulfuryl chloride was added dropwise. After the addition, the mixture was refluxed for 2 h. GLC analysis showed that several products had been formed. Reflux for a further 2 h did not change the result. No peak in the gas chromatogram was dominant. This method is thus not useful for the preparation of 3-bromo-2,5-dichlorothiophene. The products were not identified. (Column D).

2,3-Diiodothiophene and sulfuryl chloride. To 6.7 g (20 mmol) of 2,3-diiodothiophene,[30] 1.6 ml (20 mmol) of sulfuryl chloride was added dropwise. After reflux for 1.5 h, GLC analysis showed that most of the starting material was unreacted. An additional 1.5 ml (19 mmol) of sulfuryl chloride was added, whereupon reflux was continued for 6.5 h. Besides starting material, which was the largest peak, GLC analysis indicated the formation of 2-chloro-3-iodothiophene, 2,5-dichloro-3-iodothiophene and eventually 2,3,5-triiodothiophene. Thus, this method was not useful for the preparation of 5-chloro-2,3-diiodothiophene (Column A).

3-Iodothiophene and bromine. To 21 g (0.10 mol) of 3-iodothiophene [29] in 15 ml of carbon tetrachloride 11 ml (0.21 mol) of bromine in 15 ml of carbon tetrachloride was added during 2 h. After stirring at room temperature for 24 h, the reaction mixture was refluxed for 45 min. More carbon tetrachloride and 30 ml of 5 N sodium hydroxide were added. After reflux for 2 h, the reaction mixture was filtered. The separated solid tarlike product did not dissolve in boiling dioxane, but upon treatment with acetone, red-brown crystals were obtained after filtering. These crystals were only to a small degree soluble in carbon tetrachloride, which, however, was a better solvent than acetone, dimethyl sulfoxide, or chloroform. NMR spectrum showed

no proton absorptions. Combined GLC-mass spectrometry indicated that the crystals consisted of one or more hexabromobithienyls. The filtrate above contained mainly 2,3,5-tribromothiophene. 2,5-Dibromo-3-iodothiophene could not be detected in the reaction mixture.

If acetic acid was used as solvent, no hexabromobithienyl could be observed. In this case, 2,5-dibromo-3-iodothiophene was formed together with other products. GLC analysis indicated that if acetic acid is used as solvent, molecular bromine can be an alternative to the utilization of NBS for the synthesis of 2,5-dibromo-3-iodothiophene (Column B).

2,3-Diiodothiophene and bromine. To 9.0 g (0.027 mol) of 2,3-diiodothiophene [30] in 10 ml of carbon tetrachloride, 1.6 ml (0.031 mol) of bromine dissolved in 10 ml of carbon tetrachloride was added dropwise during a 35 min period. After stirring for 24 h, GLC analysis indicated that only products with retention times shorter than that of 2,3-diiodothiophene had been formed, with the exception of one product with a longer retention time. This product, which possibly was 2-bromo-4,5-diiodothiophene, was however only approximately 3 % of the total product distribution. Thus, this route is not useful for the synthesis of 5-bromo-2,3-diiodothiophene. The other products were not identified (Column B).

2-Bromo-3,5-diiodothiophene. 5.8 g (20 mmol) of 2-bromo-3-iodothiophene, 9 ml of acetic acid, 3.5 ml of water, 4.5 ml of carbon tetrachloride, 0.2 ml of concentrated sulfuric acid, and 0.80 g (4.6 mmol) of iodic acid were heated to reflux; 2.1 g (8.3 mmol) of iodine was then added in portions. After reflux for an additional 4 h, more water and carbon tetrachloride were added. The organic phase was separated and washed with water, sodium thiosulfate solution, and water, and dried over magnesium sulfate. The carbon tetrachloride was evaporated and the residue recrystallised from ethanol, whereupon 2-bromo-3,5-diiodothiophene precipitated. Weight after drying: 4.4 g (53 %). M.p.: 63–64 °C. Column: B. Calc. for C_4HBrI_2S (414.8): C 11.6; H 0.24; I 61.2. Found: C 11.6; H 0.21; I 60.9.

3-Bromo-2-iodothiophene. 41 g (0.25 mol) of 3-bromothiophene,[28] 80 ml of acetic acid, 30 ml of water, 20 ml of carbon tetrachloride, 1.5 ml of concentrated sulfuric acid, 20.4 g (0.080 mol) of iodine, and 8.2 g (0.047 mol) of HIO_3 were stirred at 40 °C until the iodine was consumed (2 h). Water, sodium hydrogen carbonate solution, and 20 ml of carbon tetrachloride were added. The organic phase was separated, washed with sodium hydrogen carbonate solution and dried over magnesium sulfate, whereupon it was chromatographed on a column filled with basic aluminium oxide. Low boiling petroleum ether was used as eluent. After evaporation of the petroleum ether, distillation gave 38 g (66 %) of 3-bromo-2-iodothiophene at 108–110 °C/9 mmHg (Column A). NMR (CCl_4): δ 7.30 (H-4), 7.80 (H-5). J_{45} 5.6 Hz. Calc. for C_4H_2BrIS (288.9): C 16.6; H 0.70; S 11.1. Found: C 16.8; H 0.74; S 11.2.

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Preparation of Porcine Pancreatic Lipase Free of Co-lipase Activity

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Porcine pancreatic lipase L_B was purified and released from co-lipase activity by reduction with β-mercaptoethanol in the presence of guanidine chloride. The amino acid and carbohydrate composition of the enzyme is presented. The following physical constants were measured or calculated: Molecular weight 52 000, sedimentation coefficient ($s^\circ_{20,w}$) 4.0 *S*, diffusion coefficient ($D^\circ_{20,w}$) 6.7×10^{-7} cm² s⁻¹. Stokes' radius (*r*) 30.3 Å, partial specific volume ($\bar{v}$) 0.72 cm³ g⁻¹, frictional ratio (f/f_0) 1.23 and isoelectric point (pI) 5.18.

Pancreatic lipase (EC 3.1.1.3) is inhibited by conjugated bile salts in concentrations above their CMC (critical micellar concentration).[1–3] Addition to such systems of co-lipase, a polypeptide co-factor for lipase,[4,5] restores the activity of lipase.[1,2] A method for preparing a pure lipase — as judged by disc electrophoresis — from porcine pancreas was described by Verger *et al.*[6] In our hands lipase prepared according to that method still exhibits co-lipase activity [3] to an extent of 20—30 % of the lipase activity. Similar values have also been reported by others.[7]

The mechanism of the interaction between lipase, co-lipase and conjugated bile salts is being investigated at present in this laboratory. It was thus of interest to see whether the residual co-lipase activity was an inherent property of lipase or a property due to contamination. A method is presented in this paper by which the co-lipase activity was lowered considerably. Besides, the resulting lipase was characterised further with respect to a few physico-chemical properties and for comparative purposes the carbohydrate and amino acid composition is also presented.

EXPERIMENTAL

Determination of lipase and co-lipase activity. Determination of lipase activity was done potentiometrically at pH 8.0 as described by Borgström *et al.*[2] using a Mettler titrator. Co-lipase activity is defined as the hydrolysis of tributyrin at pH 7.0 in the assay system for lipase modified such that the incubation mixture is 0.004 M with respect to sodium taurodeoxycholate and contains 50 lipase units of rat pancreatic lipase.[1] A lipase or a co-lipase unit of activity expresses the number of micromol of butyric acid released per min in the assay system used at 25 °C.

Determination of protein. Protein concentration was determined by measuring the absorbance at 280 nm. When the molecular weight of lipase was determined on a Sepharose 6B column a fluorescence method was used.[8]

Preparation of porcine pancreatic lipase free of co-lipase activity. Lipase was prepared from fresh porcine pancreas by the method of Verger *et al.*[6] and the homogeneity of the preparation was verified by disc electrophoresis. According to the method mentioned a delipidated water extract is chromatographed on DEAE-cellulose (pH 8.0) using a salt gradient, on Sephadex G-100 and finally on CM-cellulose (pH 5.0) whereby two lipases, lipase L_A and L_B, respectively, are separated when eluted from the CM-cellulose column with a pH-gradient.

A sample of lipase L_B was taken (specific activity 11 300, E (280 nm, 1 %) = 13.3) containing 240 000 lipase units and 54 000 co-lipase units (0.225 co-lipase units per unit of lipase) in 0.020 M Tris-HCl pH 7.0 and 0.02 % NaN_3 in a total volume of 20 ml. To the sample was added guanidine chloride, β-mercaptoethanol and EDTA to final concentrations of 0.7 M, 0.4 M, and 0.002 M, respectively. It was then

kept at 4 °C for 4 h under stirring. After centrifugation at 35 000 *g* for 20 min the sample was put on a Sephadex G-100 column (100 × 5 cm) equilibrated with 0.020 M Tris-HCl pH 7.0, 0.4 M β-mercaptoethanol, 0.002 M EDTA and 0.02 % NaN_3. After elution with the same buffer (flow rate 55 ml/h, fraction time 20 min) fractions containing more than 100 lipase units per ml were pooled. This pool of 200 ml was dialyzed against 5 l of 0.050 M Tris-HCl pH 7.0 and 0.02 % NaN_3 for 8 h twice.

Disc electrophoresis. To check the purity of the preparations disc electrophoresis was performed. A 7.5 % polyacrylamide gel was used with a pH 8.9 system [9] and the gels were stained with Coomasie Brilliant Blue G 250.[10]

Amino acid analysis. Amino acid analysis was done in a Jeol amino acid analyser* after 25 and 72 h hydrolysis in 6 M HCl at 110 °C *in vacuo*. Only samples that had been subjected to performic acid oxidation were taken after 18 h hydrolysis. 1.7 mg of protein were used for the analysis. After lyophilisation the samples were dried over phosphorus pentoxide in a drying gun with boiling toluene to constant weight as judged by measurements on a Cahn microbalance. The individual values obtained for each residue were averaged except in the cases of isoleucine, leucine, proline, and valine for which the highest concentration determined after 72 h was used, and of serine and threonine for which extrapolations to zero time were done. Norleucine was incorporated as internal standard. The 18 h values were used for cystine measured as cysteic acid and for methionine measured as methionine sulfone after performic acid oxidation. The cysteic acid value was corrected as described by Moore.[11] Tryptophan was determined by spectrophotometry.[12]

Carbohydrate analysis. The lyophilized samples were dried over phosphorus pentoxide as described above. Neutral sugars were determined with the aid of gas chromatography according to a method by Lindahl [13] which had been modified by Carlstedt.** 2.9 mg of lipase L_B was used for the analysis. Hexosamine was determined on a Biocal BC 200 amino acid analyzer.[14]*** 2.4 mg of lipase L_B was used for the analysis.

Molecular weight determinations. a. *Sedimentation equilibrium analysis.* Sedimentation equilibrium analysis for calculation of the molecular weight was performed according to the technique of Yphantis.[15] The protein was dissolved in 0.1 M sodium chloride to a final concentration of 0.5 mg/ml and centrifuged at 72 000 *g* for 23 h at 20 °C in a Spinco Model E ultracentrifuge.* An interferometric optical system was used. The partial specific volume of the protein was calculated from the amino acid and carbohydrate composition using the amino acid specific volume values given by Schachman [16] and carbohydrate specific volume values given by Gibbons.[17] The weight of each amino acid and carbohydrate species per unit weight of protein was multiplied by its specific volume value. The sum of the obtained volume values is the volume of the protein per unit weight.

b. *Sedimentation velocity analysis.* Sedimentation velocity analysis for determination of the sedimentation coefficient was done as described by Schachman.[16] The protein was dissolved at three different concentrations in 0.1 M NaCl and 0.003 M potassium phosphate pH 8.0 and centrifuged at 52 000 rpm at 20 °C in a Spinco Model E ultracentrifuge with a schlieren optical system. The sedimentation coefficient s was combined with the free diffusion coefficient D and the partial specific volume $\bar{v}$ in the Svedberg equation

$$M = (s/D)[RT/(1-\bar{v}\varrho)]$$

(where R is the gas constant, ϱ solvent density and T the absolute temperature) to yield the molecular weight. The free diffusion coefficient was obtained from the Stokes' radius by use of the Stokes-Einstein equation [18]

$$D = kT/6\pi\eta r$$

where k is the Boltzmann constant and η the viscosity of the medium. The Stokes' radius was calculated from gel filtration data according to the method of Laurent and Killander.[19] Sephadex G-100 was used and 7×10^{-8} cm was chosen as the value of r_T.[19] The concentration of the dextran chains, L, was calculated by determining the K_{av} for bovine serum albumin and soybean trypsin inhibitor on the gel when chromatographed at 22 °C in a phosphate buffer, 0.10 M pH 6.0 and 0.05 M pH 7.0, respectively. The Stokes' radius for the two proteins was calculated from the Stokes-Einstein equation quoted above.[18] For soybean trypsin inhibitor D was calculated from s and molecular weight data given by Sheppard *et al.*[20] using the Svedberg equation and D for human serum albumin has been measured by Ehrenberg.[21] Lipase was chromatographed at 22 °C in 0.10 M sodium chloride and 0.003 M potassium phosphate pH 8.0.

c. *Amino acid composition.* Calculations of the molecular weight were also done using the

* The analysis was kindly done by Miss Inger-Marie Karlsson and Dr. J. O. Jeppson at the Department of Clinical Chemistry, Malmö.

** This was kindly done by Dr. I. Carlstedt, Department of Medical Chemistry 2, Lund.

*** This was kindly done by Dr. D. Heinegård, Department of Medical Chemistry 2, Lund.

* The determination was kindly done by Doc. H. Pertoft at the Institute of Medical Chemistry, University of Uppsala.

amino acid composition as described by Delaage.[22*]

d. *Gel chromatography of reduced protein in guanidinium chloride.* The molecular weight of lipase was determined by means of gel chromatography in a 120 × 0.9 cm glass column with Sepharose 6B (Pharmacia Lot. No. 5686) as described by Mann and Fish.[23] The proteins were dissolved in 0.5 ml of the eluent, 6 M guanidinium chloride and 0.05 M potassium phosphate pH 7.0 for the reduction. The solution was made 0.01 M with respect to dithiothreitol and when carboxymethylation was to be started the solution was made 0.025 M with respect to iodoacetic acid. The following proteins were used for calibrating the column; bovine serum albumin (M 65 400) (Ref. 24), the heavy chain of human γ-globulin (M 53 000) (Ref. 25), ovalbumin (M 40 000) (Ref. 24).

Frictional ratio. The frictional ratio was calculated from the expression [18] $f/f_0 = r/(3\bar{v}M/4\pi N)^{1/3}$ where N is Avogadro's number.

Isoelectric focusing. The isoelectric focusing was performed as described by Vesterberg and Svensson [26] in a column of 110 ml volume purchased from LKB-Produkter. An Ampholine (LKB-Produkter) pH gradient between pH 4 and 6 was used and 0.1 mg of lipase was introduced into the column. The water circulating through the mantle was kept at +3 °C in a thermostated bath. The electrolysis was interrupted after 72 h and fractions of 1 ml volume were taken from the column and the lipase activity as well as the pH of the fractions were measured. The pH measurements were done at +3 °C in a Radiometer (Copenhagen) pHM 71 Mk 2.

RESULTS

Pancreatic lipase free of co-lipase activity. The dialyzed pool contained 125 500 lipase units and 618 co-lipase units, *i.e.* 0.005 co-lipase units per unit of lipase. The specific activity of lipase was unchanged by the procedure and the lipase was homogenous by disc electrophoresis. When co-lipase activity was looked for in the eluate from the G-100 column a peak was found with a K_D-value of 0.64. This result could not be reproduced if the sample of lipase was simply rechromatographed on G-100 in the presence of 0.4 M NaCl as in the original method [6] without preceding treatment with β-mercaptoethanol and guanidine chloride.

Amino acid composition. The amino acid composition of the lipase prepared in the above mentioned manner is given in Table 1.

* These calculations were kindly done by Dr. Jan Sternby.

Table 1. Amino acid and carbohydrate contents of lipase L_B free of co-lipase activity. Number of residues per mol of lipase L_B free of co-lipase activity using the mean value (52 000) of the molecular weight determinations given in this paper.

	Exp. values	Next integers	
Ala	22.7	23	
Arg	19.9	20	
Asx	58.5	59	
Cys	5.8	6	
Glx	40.2	40	
Gly	44.0	44	
His	9.6	10	
Ile	27.3	27	
Leu	30.9	31	
Lys	22.0	22	
Met	2.8	3	
Phe	25.2	25	
Pro	27.1	27	
Ser	33.1	33	
Thr	24.9	25	
Trp	9.2	9	
Tyr	15.0	15	
Val	35.2	35	
Fucose	0.7	1	1
Galactose	0.5	1	—[a]
Galactosamine	1.4	1	2[a]
Glucose	0.2	—	—[a]
Glucosamine	2.4	2	3[a]
Mannose	6.1	6	6

[a] In case galactose and glucose result from the deamination of galactosamine and glucosamine.

Carbohydrate composition. The carbohydrate composition is presented in Table 1 together with the amino acid composition using a value of 52 000 for the molecular weight of lipase L_B free of co-lipase activity.

Molecular weight. a. The molecular weight determined by sedimentation equilibrium ultracentrifugation was found to be 52 000 ± 5 %. The value of 0.72 for the partial specific volume was used as derived from the amino acid and carbohydrate composition. When log C is plotted against r^2 a straight line is obtained. This is an indication of homogenous molecular weight distribution in the sample.[15]

b. The sedimentation coefficient obtained by extrapolation to zero concentration ($s^\circ_{20,w}$) was found to be 4.0×10^{-13} s using $s_{20,w}$ for solutions with a lipase concentration of 1.25 mg/ml, 2.5 mg/ml and 5.0 mg/ml, respectively.

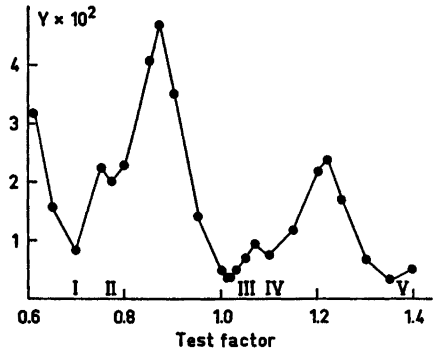

Fig. 1. The function $Y = \sum_i (n_i/N_i - 1)^2$ (see text) plotted against a computor program test factor. The minima indicated by numbers correspond to molecular weight as follows: I = 36 754, II = 40 258, III = 53 245, IV = 57 746, V = 71 070.

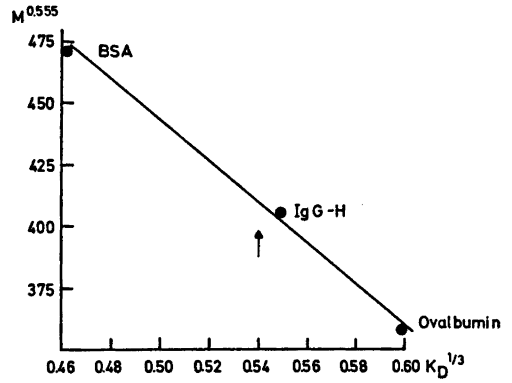

Fig. 2. Chromatography of reduced and carboxymethylated bovine serum albumin (BSA), lipase L_B free of co-lipase activity (indicated by the arrow), heavy chain of human γ-globulin and ovalbumin on Sepharose 6B in 6 M guanidine chloride.

The peaks were symmetrical. When gel filtration data were analysed according to the method of Laurent and Killander [19] the Stokes' radius was found to be 30.3 Å and the diffusion coefficient ($D_{20,w}$) 7.08×10^{-7} cm²/s. These values correspond to a molecular weight of 49 500 when combined in the Svedberg equation.

c. Amino acid composition. When molecular weight was calculated according to the method of Delaage [22] a value of 53 245 was obtained. The function $Y = \sum_i (n_i/N_i - 1)^2$ is represented in Fig. 1 as a function of a test factor in the computer program. n_i is the number of mol of the amino acid i per mol of protein (this value is varied by assuming different molecular weights of the protein) in the analysed material and N_i is the nearest integral. The result was the same whether methionine or cystine or both were omitted from the calculations. These amino acids occur least often (Table 1) in the protein and should be expected to influence the positions of the minima of the curve corresponding to different molecular weight values if the amino acid analysis was far from correct with respect to these amino acids. To the molecular weight value of 53 245 should be added the weight of the carbohydrate molecules in the lipase. That sum gives a molecular weight value of 55 200.

d. *Gel chromatography of reduced protein in guanidinium chloride.* The K_D values were analyzed as described by Porath.[27] According to that method $K_D^{1/3}$ is plotted against $M^{0.555}$. The interpolated value for lipase (see Fig. 2) corresponds to a molecular weight of 50 800.

Frictional ratio. Using the average value 52 000 of the molecular weight determinations the frictional ratio was found to be 1.23.

Isoelectric focusing. The pI value of lipase L_B free of co-lipase activity was found to be 5.18.

The physicochemical constants of lipase L_B free of co-lipase activity are summarized in Table 2 using the mean value of 52 000 for the molecular weight.

Table 2. Physicochemical properties of lipase L_B free of co-lipase activity.

Stoke's radius, r	30.3 Å
Sedimentation coefficient, $s^\circ_{20,w}$	4.0×10^{-13} s
Diffusion coefficient,[a] $D^\circ_{20,w}$	6.7×10^{-7} cm² s^{-1}
Partial specific volume,[b] $\bar{v}$	0.72 cm³ g^{-1}
Frictional ratio, f/f_0	1.23
Isoelectric point, pI	5.18

[a] Derived from the Svedberg equation using a value of 52 000 for the molecular weight. [b] Derived from carbohydrate and amino acid analyses.

DISCUSSION

Co-lipase activity is found in the eluate from the G-100 column in a peak with a K_D value of 0.64 which is separate from the lipase peak. This K_D value is the same as for pure co-lipase when reduced and chromatographed under the same conditions as described for lipase L_B in this paper. It is thus probable that the contaminating amount of co-lipase is bound to lipase by a disulfide bridge.

The residual co-lipase activity exhibited by lipase L_B after the treatment described in this article could be explained by incomplete inhibition by the sodium taurodeoxycholate. Co-lipase is rich in half-cystines [5] containing 10 mol of half-cystine per mol. It is noteworthy that the half-cystine value reported in this paper for lipase L_B free of co-lipase activity is less than half the value given by Verger *et al.*[6] for lipase L_B.

As can be seen from the results presented compared to those given by Verger *et al.*[6] no drastic changes in composition or in physicochemical constants have been introduced by the treatment to which the lipase has been subjected in this work. A method, which does not result in any major modification of protein structure, is thus available for the preparation of a lipase that can be used in experiments designed to elucidate the activating effect of co-lipase on lipase activity.

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Substituent Effects in ^{13}C NMR Spectra of Stabilised Arsenic Ylides

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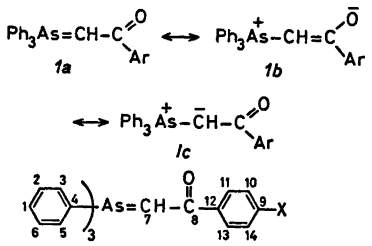
^{13}C chemical shift assignments are presented for a series of arsenic ylides with a *p*-substituted phenacyl group linked to the carbanion. Chemical shifts of the ylide carbon in this series show a linear dependence with the dual substituent parameters of Swain and Lupton, the resonance term being predominant. However, chemical shifts of the carbonyl carbon do not exhibit an analogous correlation. The chemical shifts of the carbon atoms in the phenyl groups linked to arsenic indicate limited interaction between aromatic ring and arsenic.

In ylides greater stability is conferred upon the molecule by attachment of electron-withdrawing groups to the site which bears a formal negative charge. In arsenic and phosphorus ylides there exists the further possibility of $d_\pi - p_\pi$ bonding between the heteroatom and the ylide carbon.[1] The bonding situation in these molecules is typically represented in terms of contributions from resonance hybrids of the type *1a-c*. Since ^{13}C chemical shifts represent sensitive indicators of physical environment we have undertaken an investigation of these shieldings in a number of ylide series containing substituted aroyl stabilising groups in order to assess the effect of ylide structure on the transmission of substituent effects against a background of work on other aromatic systems.[2-4] In this paper we present results obtained with a series of nine triphenylarsonium ylides *2a – i*. Previously it had been found that the bimolecular rate constants for reaction of a series of *p*-substituted triphenylarsonium ylides with *p*-nitrobenzaldehyde [5] correlated fairly successfully with the Hammett constant σ_p; accordingly a σ_p-like correlation might be expected for the chemical shifts of the ylide carbon C(7) in the series *2a – i*.



1a *1b* *1c*

2a X = H
2b X = Me
2c X = OMe
2d X = F
2e X = Cl
2f X = Br
2g X = CN
2h X = NO_2
2i X = Ph

RESULTS AND DISCUSSION

^{13}C chemical shifts for the series *2a – i*, determined at a common concentration in $CDCl_3$, are given in Tables 1 and 2. The ylide, C(7) and carbonyl, C(8), carbon absorptions were readily assigned. However, assignment of aromatic ring carbon absorptions presented more of a problem. Differentiation between carbons C(1) – C(6) and C(9) – C(14) could be made on the basis of signal intensities. Additionally the shieldings of C(1) – C(6) are related to those of the corresponding phosphorus ylides [6] and, in general, the C(9) – C(14) shieldings may be derived with reasonable accuracy from those for *2a* and the corresponding mono-substituted benzene.[7]

Table 1. ^{13}C chemical shifts of C(1)–C(8) in ylides (*2a–i*).

Compound	Substituent	C(1)	C(2), C(6)	C(3), C(5)	C(4)	C(7)	C(8)
2a	H	131.64	129.41	132.42	128.88	57.13	181.80
2b	Me	131.60	129.38	132.41	128.88	56.59	181.75
2c	MeO	131.70	129.44	132.40	128.73	56.54	179.71
2d	F	131.74	129.47	132.44	128.90	56.88	180.67
2e	Cl	131.75	129.47	132.38		57.43	180.31
2f	Br	131.76	129.48	132.41	128.48	57.48	180.40
2g	CN	131.92	129.54	132.30	127.94	59.23	179.01
2h	NO_2	132.01	129.60	132.37	127.93	59.90	178.76
2i	Ph	131.72	129.46	132.50	–	57.35	180.50

Table 2. ^{13}C chemical shifts of C(9)–C(14)/C(15) in ylides (*2a–i*).

Compound	Substituent	C(9)	C(10), C(14)	C(11), C(13)	C(12)	C(15)
2a	H	128.88	127.62	127.09	140.49	
2b	Me	138.86	128.35	126.96	137.62	21.29
2c	MeO	156.51	110.91	–	134.64	56.20
2d	F	163.60 [a]	114.34 [b]	128.98 [c]	136.87 [d]	
2e	Cl	134.74	128.68	128.08	138.77	
2f	Br	123.26	130.72	128.48	139.36	
2g	CN	111.97	131.56	127.47	144.68	119.10
2h	NO_2	148.01	123.02	127.76	146.63	
2i	Ph	–	–	–	–	

[a] $^1J_{C-F}=247.2$ Hz. [b] $^2J_{C-F}=21.1$ Hz. [c] $^3J_{C-F}=8.6$ Hz. [d] $^4J_{C-F}=2.8$ Hz.

Of particular interest is the chemical shift of the ylide carbon, C(7) which may be regarded as sp^2 hybridised. With respect to a 'normal' *i.e.* olefinic, sp^2 hybridised carbon C(7) in *2a–i* is shielded by *ca.* 100 ppm. Such behaviour is consistent with the presence of high electron density on the ylide carbon as is indicated, in the general case, by the resonance structure *1c*. A similar finding has been reported for a number of phosphorus ylides [8] and other examples of formally sp^2 hybridised carbons which absorb at higher field, than might be expected, include the terminal carbons of allene,[9] ketene [10] and diazomethane;[11] in the latter cases the enhanced shielding is attributed to high electron density.

In the series *2a–i* the ylide carbon, C(7), is deshielded by electron-withdrawing substituents, such that C(7) chemical shifts correlate with the dual substituent parameters [12] F and R according to eqn. 1

$$\delta_{C(7)} = 1.198F + 4.11R \quad (1)$$

where the correlation coefficient, $r = 0.949$. The weighting of R in eqn. (1) is 69.6 % and it is noted that a closely related weighting of R, 70.9 %, is obtained in the methyl proton chemical shift dependence on substituent character in *p*-substituted acetophenones [13] which is given in eqn. (2).

$$\delta_{H_3C} = 0.046F + 0.153R \quad (2)$$

The figures in eqn. (1), which represent a pronounced weighting in favour of R are thus in accord with comparable data for related systems, *e.g.* *p*-substituted phenylacetylenes [2] and styrenes [3] where for the ^{13}C chemical shifts of carbons β to the aromatic ring, ($\lambda = r/f$) is > 1; here r and f are the numerical coefficients in the dual substituent parameter (D.S.P) eqn. (3). We feel that, in general, the dual substituent parameter approach can provide for a

meaningful analysis of ^{13}C chemical shifts in substituted aromatic systems and a reaffirmation of the validity of this approach has recently been given.[2]

$$\delta C_\beta = fF + rR \quad (3)$$

By way of contrast the carbonyl chemical shifts in *2a–i* show no overall correlation with substituent parameters although it is observed that greater shielding of C(8) is associated with increased electron-withdrawing character of the substituent. This response of C(8) to substituent variation is opposite to that of C(7). However, the behaviour of C(8) in the *p*-methoxy derivative of *2c* is noteworthy in that here the carbonyl carbon absorbs at higher field than would be expected on any basis of substituent character. Preliminary results on the analogous phosphorus ylides [6] indicate similar 'anomalous' behaviour of the methoxy derivative. It is probable that the high field C(8) shift in *2c* is a reflection of enhanced conjugation between the methoxy substituent and the carbonyl group, mediated by the aromatic ring. This contention finds support in the fact that the methoxy induced substituent chemical shift for C(9), 27.6 ppm, is *ca.* 4 ppm less than found normally; in *p*-methoxyacetophenone the corresponding value is 30.4 ppm. Further, the C(12) substituent chemical shift in *2c*, 5.85 ppm, is again smaller than typically found for ring carbons *para* to a methoxy substituent, and the chemical shift of the methoxy carbon in *2c*, 56.2 ppm, shows deshielding by 1 ppm from its typical values; this deshielding is in accord with conjugative release of oxygen lone-pair electrons.

The carboxyl carbons in *p*-substituted benzoic acids experience enhanced shielding in the case of electron-donating substituents over that anticipated by σ values, and here calculations associate this effect with an increased bond order between the carboxyl carbon and the aromatic ring.[14] A parallel therefore appears to exist between the present results and those of Niwa and Yamazaki.[14]

In the series *2a–i* only methoxy, the most strongly electron releasing substituent, gives evidence for enhanced conjugation. In this connection the chemical shift behaviour brought about by the more strongly electron releasing *p*-dimethylamino substituent would be of considerable interest; however, we have been unable to extend the series *2a–i* to include this substituent.

Within the triphenylarsonium moiety minor but significant variation is found for the C(1) and C(4) shifts whereas those for C(2) and C(3) are essentially insensitive to C(9) substituent variation. It has been shown previously [15,16] in triphenylphosphonium ylides that resonance interaction of these phenyl rings across phosphorus is essentially absent and a similar situation probably obtains in the analogous triphenylarsonium ylides. At present it is not possible to be definitive about the C(1) and C(4) shift variation in the series *2a–i*. A number of proposals have been made in order to account for substituent chemical shifts of aromatic ring carbons. In the series *2a–i* an attractive possibility is that substituent induced variations of electron density on arsenic bring about π-polarisation of the three arsenic bonded aromatic rings thereby accounting for the observed chemical shift variation. It is extremely unlikely that the substituent inductive effect could act directly over such large distances.

EXPERIMENTAL

^{13}C NMR spectra were recorded with a Varian XL 100 spectrometer operating in the Fourier transform mode at 25.2 MHz. The spectra were determined at the University of Edinburgh by Dr. A. Boyd. Spectral data in Tables 1 and 2 pertain to solutions of ylide (0.1 M) in $CDCl_3$ and are quoted relative to internal tetramethylsilane as standard.

Triphenylarsonium 2-(4-cyanophenyl)-2-oxoethylide. To a stirred solution of 5.0 g (3.4×10^{-2} mol) *p*-cyanoacetophenone in 30 ml chloroform 5.5 g (3.4×10^{-2} mol) of bromine was slowly added. After the addition, the solution was evaporated to dryness in vacuum. The resulting α-bromo-*p*-cyanoacetophenone was dissolved in 40 ml chloroform together with 8.7 g (3.4×10^{-2} mol) triphenylarsine and refluxed for 5 h. The solution was cooled and 500 ml of ether was added. The precipitated arsonium salt was washed with ether, dissolved in methanol and a slight excess of sodium methoxide was added. The solvent was removed on a rotary evaporator and the crude ylide was purified by column chromatography using aluminium oxide as the substrate and chloroform as eluent, followed by recrystallisation from benzene-ether. For NMR data see Tables 1 and 2. M.p. 177 °C (dec.) (Found: C.72.21; H 4.43. Calc. for $C_{27}H_{20}AsNO$: C 72.16; H 4.49).

Triphenylarsonium 2-(4-fluorophenyl)-2-oxoethylide was prepared from *p*-fluoroacetophenone and triphenylarsine as described above. For NMR data see Tables 1 and 2. M.p. 152–153 °C. Found: C 70.42; H 4.59. Calc. for $C_{26}H_{20}AsFo$: C 70.59; H 4.56.

The other arsonium ylides were prepared as previously described.[5] For NMR data see Tables 1 and 2.

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Ring-opening Reactions of Heterocyclic Organometallics. IX.* The Opening of Some Chloro-, Methylthio- and Methoxy-substituted 3-Thienyl- and 3-Selenienyllithium Derivatives

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3-Thienyllithium derivatives with electron-withdrawing groups ($-I$-substituents) in the 2-position, *i.e.* 2-chloro-5-methyl-, 2,5-dichloro- and 2-methoxy-5-methyl-3-thienyllithium are stable at room temperature and do not undergo ring-opening to lithium enynethiolates. A strong tendency to undergo Wurtz-Fittig couplings was observed for the chlorinated 3-thienyllithium derivatives.

If the substituent in the 2-position has a weak $-I$-effect (*e.g.* methylthio), ring-opening occurs, as was observed for 5-methyl-2-methylthio-3-thienyllithium and 5-methyl-2-methylthio-3-selenienyllithium. From the latter compound, *1e* (Scheme 1) was obtained in 44 % yield.

Interestingly, 2,5-dichloro-3-selenienyllithium prepared by metalation of 2,5-dichloroselenophene with lithium diisopropylamide was stable, while the reaction of 2,5-dichloro-3-iodoselenophene with ethyllithium at −70 °C *via* ring-opening led to compound *8* (Scheme 2).

When the $-I$-substituent was in the 5-position, as in 5-methoxy-2-methyl-, 5-chloro-2-methyl- and 2-methyl-5-methylthio-3-thienyllithium, ring-opening occurred, although more slowly than with 2,5-dimethyl-3-thienyllithium.

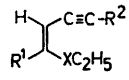
The synthetic applications of the ring-opening reaction are discussed.

It has previously been demonstrated [1,2] that 2,5-dialkyl-3-thienyl- and 2,5-dialkyl-3-selenienyllithium derivatives ring-open in a stereospecific manner to give lithium enynethiolates and lithium enyneselenolates which are alkylated by alkyl halides to give alkylthiovinyl acetylenes and alkylselenovinyl acetylenes. We were therefore interested in examining how other substituents than alkyl groups affected the stability of the 3-lithio heterocycles, and thus ascertaining the scope and limitations of the ring-opening reaction for the syntheses of substituted alkylthiovinyl acetylenes difficultly available by other synthetic approaches.

In the present paper the effect of changing the 2- and/or the 5-alkyl group for methoxy, chloro and methylthio groups will be discussed.

RESULTS

5-Chloro-, 5-methoxy- and 5-methylthio-3-thienyllithium derivatives. The ring-opening of 5-methoxy-2-methyl-3-thienyllithium was followed by GLC (hydrolysed samples), and it was shown that the reaction proceeded rather



1

2

3

Scheme 1. Compounds *1a–f*, *2a–c* and *3a–b*.

Compound	R^1 or R^5	R^2	X
1a	OCH_3	CH_3	S
1b	SCH_3	CH_3	S
1c	SCH_3	C_3H_7	S
1d, 3b	SCH_3	CH_3	Se
1e, 3a	CH_3	SCH_3	Se
1f	CH_3	H	Se
2a			OCH_3
2b			Cl
2c			SCH_3

* Part VIII, see Ref. 2.

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*** Taken in part from the Ph. D. Thesis of Torbjörn Frejd, University of Lund, 1975.

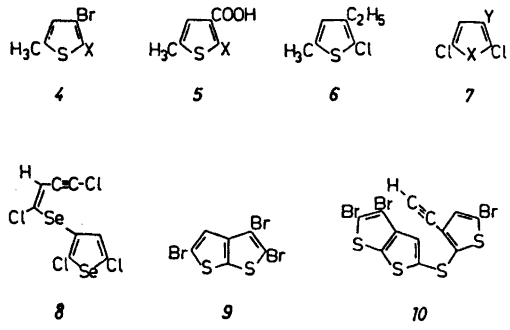
slowly. Even after 4 h at +21 °C, the peak originating from *1a* was still growing larger. Since a precipitate, probably 5-methoxy-2-methyl-3-thienyllithium, was formed immediately when ethyllithium and *2a* were mixed, the reaction became heterogeneous. This could be responsible for the apparently slow ring-opening. In order to make the reaction homogeneous, a small amount of hexamethylphosphoric triamide (HMPA) was added to the reaction mixture. In this way, a 33 % yield of *1a* was obtained and the ring-opening was complete in less than 2 h.

The ring-opening of 5-chloro-2-methyl-3-thienyllithium appeared to be more complex. As much as 21 % of the starting material (*2b*) and 42 % of 2-chloro-5-methylthiophene were present in the crude product, together with 37 % of 1-ethylthio-1,3-pentadiyne [23] (uncalibrated GLC values).

The ring-opening of *2c* and of *3b* has been described previously in a short communication.[24] On treatment of *2c* with ethyllithium followed by ethyl bromide, *1b* and *1c* were formed in 70 and 25 % yields, respectively. The ring-opening of the selenienyllithium derivative gave a more uniform crude product. Thus, *3b* gave an 80 % yield of 90 % pure *1d*. As mentioned previously,[24] these kinds of acetylenic mixed ketene monothioacetals (*1a*), ketene dithioacetals (*1b*) and ketene thioselenoacetals (*1d*) with pre-determined configuration do not seem to have been previously described in the literature, and are hardly available by any other synthetic route. We are pursuing our research on the synthetic possibilities of these types of compounds.

2-Chloro-, 2-methoxy- and 2-methylthio-3-thienyllithium derivatives. Upon treatment with ethyllithium/ethyl bromide at room temperature for 4 h *4a* and *4b* gave *5a* (70 %) and *5b* (36 %), respectively, after carbonation of the reaction mixture. In the latter case, the neutral phase contained about 20 % of *6*. When the reaction mixture was kept at room temperature for 15 h and then hydrolysed the yield of *6* was raised to 51 %.

In an experiment in which *7a* was treated with methyllithium and methyliodide at room temperature for 2 h followed by hydrolysis, only the Wurtz-Fittig product *7b* was formed in 60 % yield, indicating a higher stability of



Scheme 2. Compounds *4a–c*, *5a–b* and *6–10*.

Compound	X	Compound	X	Y
4a, *5a*	OCH_3	*7a*	S	I
4b, *5b*	Cl	*7b*	S	CH_3
4c	SCH_3	*7c*	Se	I
		7d	Se	$SeCH_3$
		7e	Se	H
		7f	Se	COOH

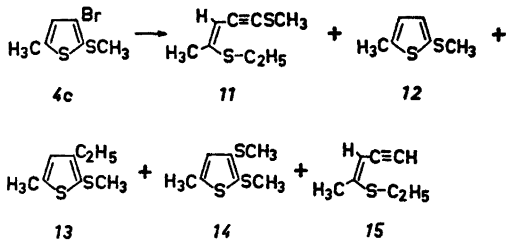
2,5-dichloro-3-thienyllithium than that of 2,5-dimethyl-3-thienyllithium.

Analogously, we therefore expected 2,5-dichloro-3-selenienyllithium to be more stable towards ring-opening than 3-selenienyllithium [7] and 2,5-dimethyl-3-selenienyllithium.[8] Surprisingly, this was not the case. Even at −70 °C and after 5 min, an acetylenic derivative (*8*) was formed in 50–70 % yield (NMR) when *7c* was treated with ethereal ethyllithium. The acetylenic derivative was too labile to survive isolation and purification attempts by TLC. For its structure determination, *cf.* below.

Unexpectedly, however, *7e* gave a 78 % yield of *7f* upon reaction with lithium diisopropylamide at −70 °C (2 h) followed by carbonation of the reaction mixture. The 2,5-dichloro-3-selenienyllithium thus prepared gave a 51 % yield of *7d* upon reaction with dimethyl diselenide.

In contrast to the behaviour of *4a* and *4b*, *4c* underwent ring-cleavage when reacted with ethyllithium and ethyl bromide and mainly *11* was obtained in 61 % yield. However, not only the type of by-products already observed in the ring-opening of 2,5-dialkyl-3-thienyllithium derivatives,[8] such as *12* [13] and the Wurtz-Fittig product *13*, were present in the crude product, but also *14* and what is believed to be *15* (*cf.* Table 1). No attempts to isolate *11* or *15* were made, but small enriched samples were

Table 1. Reaction product analysis of the reaction between *4c* and ethyllithium in the presence of ethyl bromide. Reaction time at +21 °C: 4 h, then hydrolysis. Uncalibrated GLC values.



4c *11* *12*

13 *14* *15*

Conditions		Products (%)				
C_2H_5Li (equiv)	Temp. (°C)	*11*	*12*	*13*	*14*	*15*
1	21	61	5	10	6	18
2	21	21	13	2	6	57
1	−70→21	33	9	19	27	12

obtained by TLC, which showed the expected spectroscopic properties. The structure of *14* was proven by comparison with an authentic sample prepared by halogen-metal exchange between *4c* and butyllithium at −70 °C followed by reaction with dimethyl disulfide.

In the selenophene series the reaction with one equivalent of ethyllithium at room temperature was cleaner. Thus, a 44 % yield of *1e* could be isolated (GLC yield 80 %) when *3a* was the substrate. Combined GLC-MS analysis showed that also in this case the methylthio group could be lost, giving about 10 % of *1f*. Four other unidentified compounds amounting to less than 10 % were also formed.

DISCUSSION

5-Methyl-2-methylthio-3-thienyllithium. Some indications of how the by-products in the ring-opening of 5-methyl-2-methylthio-3-thienyllithium may have been formed were obtained in the following way.

When two equivalents of ethyllithium were used together with *4c*, compound *15* was the main component, which made it likely that ethyllithium attacked the sulfur atom [14] of the acetylenic methylthio group of *11* or that of lithium 5-methylthio-2-penten-4-yne-2-thiolate, causing fission of the carbon-sulfur bond with formation of ethylmethyl sulfide and the salt of *15*. It also seems likely that 5-methyl-2-methylthio-3-thienyllithium acts as a nucleophile similar to ethyllithium, which explains the formation of the bis(methylthio) derivative *14*. Some further evidence for this was obtained from an experiment in which the halogen-metal exchange was carried out at −70 °C, whereupon the reaction mixture was allowed to reach room temperature. In this case, the amount of *14* formed increased to 27 %. This indicates that the thienyllithium derivative had time enough for the nucleophilic attack at sulfur to become rather extensive at temperatures where the ring-opening was slow. If *14* was formed only through a nucleophilic attack by the 3-thienyllithium derivative on the acetylenic methylthio derivative, the amount of *14* would be less than or equal to the amount of *15* (Table 1). A thermal decomposition of *15* in the injector of the gas chromatograph might be responsible for the actual values, since no calibration was made, and it is well known that terminal acetylenes are sensitive to heat.[15,16]

From a preparative point of view, this reaction was apparently too complex to be of value. Furthermore, *11* could probably be prepared by the addition of ethylthiolate to 1-methylthio-1,3-pentadiyne, since methylthiolate was reported to add so as to yield (*Z*)-2,5-bis(methylthio)-2-penten-4-yne.[17]

Gol'dfarb *et al.*[18] demonstrated that 5-methyl-2-methylthio-3-thienyllithium was stable enough at 0 °C to give a 42 % yield of 3,5-dimethyl-2-(methylthio)thiophene upon treatment with dimethyl sulfate after 1 h. The relatively low yield could be indicative of a ring-opening reaction, but no report of the formation of acetylenes was made. It was also possible to metalate 2-methyl-5-methylthiothiophene in the 4-position with butyllithium at room temperature in low yield.[19] These results thus indicate that 5-methyl-2-methylthio-3-thienyllithium is considerably more stable towards ring-opening than 2,5-dimethyl-3-thienyllithium.[1]

5-Chloro-2-methyl-3-thienyllithium. By assuming that 5-chloro-2-methyl-3-thienyllithium is rapidly formed, when ethyllithium is added to *2b*, a subsequent ring-opening should yield lithium (*Z*)-1-chloro-1-penten-3-yne-1-thiolate,

which should be able to eliminate hydrogen chloride in the presence of base (*cis* elimination) to give 1-ethylthio-1,3-pentadiyne. The base could of course be either ethyllithium or the thienyllithium derivative or both, but the results implied at least that ethyllithium was the base, since a substantial amount of the starting material was recovered.

2-Chloro-5-methyl-, 2-methoxy-5-methyl- and 2,5-dichloro-3-thienyllithium. In the case of 2-chloro-5-methyl-3-thienyllithium the relatively low yield of *5b* is attributed to the concurrent Wurtz-Fittig coupling with ethyl bromide to give *6*, which was the main product after longer reaction times.

The Wurz-Fittig reaction was even more pronounced with 2,5-dichloro-3-thienyllithium than with 2-chloro-5-methyl-3-thienyllithium, and it could be argued that the chlorine atoms might speed up this reaction, consuming all of the 2,5-dichloro-3-thienyllithium before any ring-opening could occur. By using phenyllithium instead of methyllithium and quenching the reaction mixture with dimethyl sulfate, it could be shown that no ring-opening had occurred after 2 h at room temperature, and that *7b* was formed in 99 % yield (GLC). It is thus obvious that 2,5-dichloro-3-thienyllithium is more stable towards ring-opening than 2,5-dimethyl-3-thienyllithium [1] and 3-thienyllithium.[7]

The high stability of 2-methoxy-5-methyl-3-thienyllithium could perhaps be expected from Sicé's metalation of 2-methoxy-5-methylthiophene.[4] After reaction of this compound with ethereal butyllithium at room temperature followed by reaction with carbon dioxide, he obtained *5a* in 50 % yield. However, it was quite likely that the ring-opened product, if it were formed in Sicé's experiment, would have escaped detection since no alkylating agent was present. In the experiments performed in this work no acetylenic compounds could be detected, despite the presence of an alkylating agent, which indicated that neither 2-methoxy-5-methyl-3-thienyllithium nor 2-chloro-5-methyl-3-thienyllithium underwent ring-cleavage.

Upon attempted metalation of 2-methoxy-5-methylselenophene it was not possible to isolate any carboxylic acid after reaction with carbon dioxide. Elemental selenium was deposited from the aqueous extract, which should have contained acidic products.

2,5-Dichloro-3-selenienyllithium. The unexpected formation of *8* could have occurred by the chlorinated lithium enyneselenolate, formed by ring-opening, attacking *7c* in a nucleophilic aromatic substitution. We found indeed that nucleophilic substitution of *7c* with lithium-methylselenolate gave *7d*. However, this reaction was incomplete, even after several hours at room temperature in ether or in liquid ammonia at -33 °C, which possibly could be due to the heterogenous reaction conditions. It is obvious that additional experiments are necessary before the mode of formation of *8* is understood. A similar kind of reaction is indicated in Bugge's [12] reaction of *9* with butyllithium which gave the acetylene derivative *10* or an isomer thereof. From a preparative point of view it should be emphasized that the use of lithium diisopropyl amide made it possible to obtain 2,5-dichloro-3-selenienyllithium as a potential reagent for preparing 3-substituted 2,5-dichloroselenophenes from the easily available 2,5-dichloroselenophene. This might indicate a stabilizing effect of diisopropylamine on 2,5-dichloro-3-selenienyllithium due to complex formation. It should be pointed out that since diisopropylamine is formed at the reaction centre, the proximity of the amine to the lithium atom could be very favourable for complexation, as it is not necessary to displace any solvent molecules.[38]

Spectroscopic data of 8. The following spectroscopic data for the crude product of the reaction between *7c* and ethyllithium indicated that the main component was *8*:

(1) By using the direct inlet system of the mass spectrometer the most heavy fragment appeared at *m/e* 398, the isotopic composition of which indicated a content of four chlorine atoms and two selenium atoms. The simulated mass distribution of $C_8H_2Se_2Cl_4$ was almost identical with the experimental one.

(2) Absorptions at 2180 cm^{-1} (strong) and 2260 cm^{-1} (weak) were observed in the IR spectrum. The absence of absorptions around 3200 cm^{-1} indicated the substance to be a non-terminal acetylene.

(3) An ^1H NMR spectrum showed the absorption of the starting material and two singlets at δ 6.96 (aromatic) and 6.10 (vinylic) with the intensities 1:1. No absorptions in the

region of terminal acetylenic protons were observed.

(4) The decoupled ^{77}Se NMR spectrum of the crude product showed the peak originating from the starting material (see below) together with one line at 129.6 ppm lower field and one line at 146.3 ppm higher field than the selenium resonance of selenophene, which was used as a reference. The low-field line was well inside the region where the aromatic selenium resonance was expected to appear.[9,10] The high-field line was on the borderline between the aromatic selenium and the selenide resonances,[11] which was also as expected for the side-chain selenium in compound *8* (see below). The non-decoupled spectrum showed two doublets; J(Se1,H4) 2.4 Hz and J(Se side-chain, H vinylic) 9.0 Hz.

Since the crude product contained *7c*, a decoupled spectrum of this compound was recorded. A single line appeared at 139.8 ppm lower field than the reference. The non-decoupled spectrum showed a doublet with a spacing of 2.0 Hz [*i.e.* J(Se, H4) 2.0 Hz]. In order to estimate where the absorption of the side-chain selenium would appear, a decoupled spectrum of *7d* was recorded. The aromatic selenium absorption was at 117.3 ppm lower field and the side-chain selenium absorbed at 434.5 ppm higher field than the reference. The non-decoupled spectrum showed that the low-field band was a doublet [J(Se1, H4) 2.40 Hz], and that the high-field band was a doublet with fine structure [J(Se3,CH_3) 12 Hz, J(Se3,H4) 0.4 Hz]. Now, since the side-chain selenium in compound *8* besides being attached to an aromatic ring was also in a vinylic position, it could be expected to give a resonance signal at lower field than that of the methylseleno group in *7d*.

CONCLUSIONS

Previous observations concerning the relative rate of the ring-opening reaction indicate that $+I$ substituents enhance the rate.[7] Disregarding steric effects one would then assume that $-I$ substituents should work in the opposite direction, especially if these substituents were situated in the *ortho* positions to lithium, *e.g.* 2-substituted 3-thienyllithium derivatives. On the other hand, if such substituents were placed in the *meta* position, *i.e.* in the 5-position, the $-I$ effect should have less influence. These ideas seem to be supported by the above presented results.

The introduction of strong inductively electron-withdrawing groups ($-I$ substituents), such as Cl and OCH_3 (F parameters[20] 0.690 and 0.413, respectively), in the 2-position of 3-thienyllithium derivatives, strongly enhances the stability of these derivatives, so that ring-opening does not occur. A weaker $-I$ substituent such as SCH_3 (F value[20] 0.332) cannot prevent ring-opening, but slows it down markedly compared to 2,5-dimethyl-3-thienyllithium. However, these substituents also have non-bonded electrons, and it has been generally accepted that the selective *ortho*-metalation of aromatics containing such substituents is due to complexation of the organolithium derivative to the free electron pair of the substituent.[21,22] This complexation both directs the metal-introducing reagent, such as butyllithium, to the *ortho*-position, and increases the rate of metalation, by making the *ortho*-hydrogen more acidic. It is possible that intermolecular association (dimerization) of such 3-thienyllithium derivatives also contribute to their increased stability towards ring-opening. Intramolecular chelation would involve four-membered rings and therefore seems less likely. However, the cleavage of the methylthio derivative leaves little doubt of the importance of the $-I$ effect for preventing ring-opening. Other factors may, however, complicate the picture, *cf.* 2,5-dichloro-3-selenienyllithium.

STARTING MATERIALS

A few comments are worth making about the synthesis of some of the intermediates and starting materials.

Selective bromination of 2-methoxy-5-methylthiophene in the 3-position with *N*-bromosuccinimide in acetic acid gave *4a* (34 %). Attempts to prepare 3-bromo-2-methoxy-5-methylselenophene in the same way were unsuccessful due to decomposition. By treating 2-iodo-5-methylselenophene with excess sodium methoxide in HMPA in the presence of copper(II) oxide at room temperature a 47 % yield of 2-methoxy-5-methylselenophene was obtained.

The use of HMPA as a solvent gave a better yield under milder conditions and shorter reaction time as compared to methanol (23 % after 6 days).

The direct chlorination of 2,4-dibromothiophene with one equivalent of *N*-chlorosuccinimide in acetic acid leads to a mixture consisting of 2-chloro-3,5-dibromothiophene (77 %), 3-bromo-2,5-dichlorothiophene (13 %) and 2,3,5-tribromothiophene (3 %).[3] We therefore used the following route to obtain *4b*. Bromination of 3-bromo-2-chlorothiophene [27] with *N*-bromosuccinimide in acetic acid yielded 2-chloro-3,5-dibromothiophene in 51 % yield. Subsequent halogen-metal exchange at −70 °C with butyllithium followed by reaction with dimethyl sulfate gave *4b* (85 %).

A 47 % yield of 2,5-dichloroselenophene was obtained by treating selenophene with sulfuryl chloride in benzene. This method is better than that previously published by Suginome and Umezawa.[28]

Since some methylselenophene derivatives showed a tendency to decompose under halogenation conditions,[2,8] somewhat greater care had to be taken with these compounds than with their sulfur analogs. Thus 4-bromo-2-methylselenophene was brominated with bromine in a mixture of carbon disulfide and acetic acid at −30 °C. The hydrogen bromide formed in the reaction was removed (at least to some extent) by passing a current of nitrogen rapidly through the reaction mixture while it was reaching room temperature. In this way a 63 % yield of 2,3-dibromo-5-methylselenophene was obtained. The α-bromine atoms of 2,3-dibromo-5-methylthiophene [27] and 2,3-dibromo-5-methylselenophene were substituted by lithium in halogen-metal exchange reactions with butyllithium. By adding the lithiated heterocycles to dimethyl disulfide in ether,[30] *4c* and *3a* were obtained in 66 % and 56 % yields, respectively. Gol'dfarb *et al.*[18] previously synthesized *4c* by brominating 2-methyl-5-methylthiothiophene with a bromide-bromate solution. The possible formation of the isomer *2c* [24] was not examined, and compound *4c* was not fully characterized so it was considered more suitable to prepare it free of the isomer by introducing the methylthio group in the last step.

EXPERIMENTAL

General remarks. See Ref. 2 (Part VI).

2-Methoxy-5-methylthiophene was prepared by refluxing 44.8 g (0.200 mol) of 2-iodo-5-methylthiophene,[25] 16 g (0.20 mol) of copper(II) oxide and 0.600 mol of sodium methoxide [from 13.8 g (0.600 mol) of sodium and 220 g of methanol]. After 6 days, 10 % of the starting material remained. After work-up and distillation, 13.9 g (54 %) of the title compound was obtained, b.p. 12 mmHg 58 – 60 °C (lit.[4] b.p. 10 mmHg 51 – 52 °C).

2-Iodo-5-methylselenophene. To a mixture of 43.5 g (0.300 mol) of 2-methylselenophene in 300 ml of CCl_4, 200 ml of acetic acid, 300 ml of water and 2.0 ml of conc. H_2SO_4 were added, followed by 30.0 g (0.118 mol) of iodine and 12.3 g (0.0700 mol) of iodic acid dissolved in water. The latter two reagents were added in portions. This mixture was stirred vigorously at room temperature for 4 h, whereupon it was poured into aqueous sodium thiosulfate and extracted with CCl_4. The organic phases were washed with 1 N NaOH, water and dried. Evaporation and distillation gave 65.7 g (81 %) of the title compound, b.p. 59 – 60 °C/0.6 mmHg. NMR (CCl_4): δ 7.15 (1 H, d, H3), 6.42 (1 H, d, q, H4), 2.50 (3 H, d, CH_3). J(H3, H4) 3.6 Hz, J(H4, CH_3) 1.2 Hz. Anal. C_5H_5ISe: C 22.21; H 1.88; Se 29.21.

2-Methoxy-5-methylselenophene. To a mixture of 25 ml of dry HMPA and 9 ml of methanol, 1.3 g (0.057 mol) of sodium was added. When all of the sodium was consumed, the methanol was evaporated at 10 mmHg, and 5.00 g (0.0185 mol) of 2-iodo-5-methylselenophene together with 2.0 g of copper(II) oxide, were added. After 48 h at room temperature, the starting material was consumed as shown by GLC (column SE 30, 3 %, 100 – 200 °C, 10 °C/min) on a hydrolysed sample. The reaction mixture was poured into water and extracted with ether. The ethereal phase was washed with water and dried. Evaporation and distillation gave 1.53 g (47 %) of the title compound, b.p. 73 – 76 °C/12 mmHg. The NMR spectrum (CCl_4) was identical with that of an authentic sample.[5] When the same method as for the preparation of 2-methoxy-5-methylthiophene was used, only a 23 % yield was obtained. Extensive decomposition took place during the reaction, which was evident from the formation of elemental selenium.

3-Bromo-2-methoxy-5-methylthiophene (4a). To 12.8 g (0.100 mol) of 2-methoxy-5-methylthiophene in 100 ml of acetic acid, 19.6 g (0.110 mol) of *N*-bromosuccinimide was added in portions at +17 °C (ice cooling). When the addition was complete, the mixture was stirred for 1/2 h, whereupon it was poured into water. The aqueous phase was neutralized with $NaHCO_3$ and extracted with ether. The ethereal phase was filtered through alumina (Brockm. I, neutral) in order to remove some black solid

material, and dried. Evaporation and distillation gave 7.0 g (34 %) of the title compound, b.p. 115–117 °C/20 mmHg (decomp.). The substance darkened rapidly at room temperature, and even at −25 °C in the dark. NMR (CCl_4): δ 6.31 (1 H, q, H4), 2.32 (3 H, d, CH_3), 3.83 (3 H, s, OCH_3). J(H4, CH_3) 1.2 Hz. Anal. C_6H_7BrOS: C 34.8; H 3.46; S 15.0.

Attempted preparation of 3-bromo-2-methoxy-5-methylselenophene. When 2-methoxy-5-methylselenophene was treated with *N*-bromosuccinimide as in the preceding experiment, only black, unidentified material was formed immediately upon the NBS addition.

2-Chloro-3,5-dibromothiophene. A mixture of 10.6 g (0.0537 mol) of 3-bromo-2-chlorothiophene [3,26] and 11.0 g (0.0618 mol) of *N*-bromosuccinimide in 100 ml of acetic acid was refluxed for 2.5 h and was then neutralized with $NaHCO_3$. The aqueous mixture was extracted with ether and the collected ethereal portions were washed with 1 N NaOH, dried and evaporated. Distillation gave 7.6 g (51 %) of the title compound, b.p. 108–110 °C/10 mmHg (lit.[3] b.p. 104–108 °C/10 mmHg).

3-Bromo-2-chloro-5-methylthiophene (4b). To 7.0 g (0.025 mol) of 2-chloro-3,5-dibromothiophene [3] in 50 ml of ether, 18 ml (0.026 mol) of 1.42 M butyllithium in hexane was added at −70 °C (yellow precipitate), followed by 3.27 g (0.026 mol) of dimethyl sulfate in 25 ml of ether. The mixture was stirred at −70 °C for 1.5 h and was then allowed to reach room temperature, whereupon conc. ammonium hydroxide was added. The usual work-up gave 4.5 g (85 %) of the title compound after distillation, b.p. 89–91 °C/10 mmHg. NMR (CCl_4): δ 6.50 (1 H, q, H4), 2.40 (3 H, d, CH_3). J(H4, CH_3) 1.1 Hz. Anal. C_5H_4BrClS: C 28.40; H 2.00; S 15.10.

2,5-Dichloroselenophene (7e). To a solution of 80.0 g (0.611 mol) of selenophene [33] in 100 ml of dry benzene, 168 g (1.24 mol) of sulfuryl chloride was added during 2 h, whereupon the mixture was refluxed for 6 h. After cooling, the benzene solution was neutralized with $NaHCO_3$, washed with water and dried. Evaporation of the solvent and distillation gave 56.8 g (46 %) of the title compound, b.p. 68–69 °C/14 mmHg (lit.[28] b.p. 67 °C/12 mmHg). NMR (CCl_4): δ 6.74 (s, H3) (lit.[34] 6.70).

2,5-Dichloro-3-iodothiophene (7a). A mixture of 25.0 g (0.163 mol) of 2,5-dichlorothiophene,[27] 14.0 g (0.0551 mol) of iodine, 9.6 g (0.055 mol) of iodic acid, 40 ml of acetic acid, 50 ml of water, 30 ml of CCl_4 and 5 ml of conc. H_2SO_4 was refluxed for 72 h, whereupon it was poured into aqueous sodium thiosulfate and extracted with CCl_4. The collected organic phases were washed with water, dried, and the solvent was evaporated to give a crude product, which was distilled. Thus 31.8 g (70 %) of the title compound was obtained, b.p. 123–126 °C/15 mmHg (lit.[3] b.p. 112–114 °C/11 mmHg). NMR (CCl_4): δ 6.69 (s, H4).

2,5-Dichloro-3-iodoselenophene (7c) was prepared as described above from 20.0 g (0.100 mol) of 2,5-dichloroselenophene, 8.9 g (0.035 mol) of iodine, 6.2 g (0.035 mol) of iodic acid, 40 ml of acetic acid, 50 ml of water, 30 ml of CCl_4 and 1 ml of conc. H_2SO_4; yield 21.4 g (66 %), b.p. 89–93 °C/1.0 mmHg. NMR (CCl_4): δ 6.85 (s, H4). Anal. C_4HCl_2ISe: C 14.85; H 0.40; Se 24.20.

2,5-Dichloro-3-(methylseleno)selenophene (7d) (a) A solution of lithium diisopropylamide was prepared from 10.1 g (0.100 mol) of diisopropylamine in 50 ml of ether and 68 ml (0.10 mol) of 1.50 M butyllithium in hexane. This solution was cooled to −70 °C and 10.0 g (0.0500 mol) of 2,5-dichloroselenophene in 100 ml of ether was added at such a rate that the temperature did not exceed −65 °C. After the addition, the reaction mixture was stirred at −70 °C for 4 h, whereupon 18.8 g (0.100 mol) of dimethyl diselenide [35] in 50 ml of ether was added (−65 °C). After stirring for 1 h, the reaction mixture was allowed to reach room temperature. Water was added and the organic layer was washed with 2 N HCl, water and dried. Evaporation and distillation yielded 7.5 g (51 %) of the title compound, b.p. 95–97 °C/0.7 mmHg. ^{1}H NMR (CCl_4): δ 6.83 (1 H, s, H4), 2.28 (3 H, t, $SeCH_3$). J(Se-CH_3) 12 Hz. ^{77}Se NMR (19.14 MHz, acetone-d_6): see discussion. Anal. $C_5H_4Cl_2Se_2$: C 20.60; H 1.40; Cl 24.28; Se 53.80.

(b) Lithium methylselenolate was prepared according to Ref. 36 in 100 ml of liquid ammonia from 0.28 g (0.040 mol) of lithium and 3.8 g (0.020 mol) of dimethyl diselenide. The solvent was evaporated and 100 ml of ether was added to the white residue. To this suspension, 3.26 g (0.0100 mol) of 2,5-dichloro-3-iodoselenophene in 25 ml of ether was added. Samples were withdrawn, hydrolysed and analysed by GLC (column OV 17, 3 %, 80–200 °C, 8 °C/min). It was evident upon mixing with authentic samples that the reaction was rather slow, and after 2.5 h about 30 % conversion to *7b* had occurred. When the reaction was performed entirely in liquid ammonia, about the same result was achieved, except that some 2,5-dichloroselenophene was also formed.

4-Bromo-2-methylselenophene. To 96.0 ml (0.125 mol) of 1.30 M butyllithium in hexane cooled to −70 °C 35.5 g (0.123 mol) of 2,4-dibromoselenophene [29] in 150 ml of ether was added in a slow stream. The temperature was thereafter allowed to rise to −40 °C and 44 g (0.35 mol) of dimethyl sulfate in 50 ml of ether was added at such a rate that the reaction temperature did not exceed −30 °C. After warming to room temperature the reaction mixture was stirred with ammonia to destroy the excess of dimethyl sulfate. The organic phase was separated, washed with water and dried with magnesium sulfate. After evaporation of the solvent, part of the residue

(26.3 g, 95 % crude yield) was distilled to give the pure title compound, b.p. 83–85 °C/15 mmHg. NMR (CCl_4): δ 2.50 (3 H, d, CH_3), 6.75 (1 H, pentet, H3), 7.45 (1 H, d, H5). J(H3, H5) 1.4 Hz, J(CH_3, H3) 1.2 Hz. Anal. C_5H_5BrSe: C 26.80; H 2.20; Se 35.22.

2,3-Dibromo-5-methylselenophene. To a solution of 18.8 g (0.0839 mol) of crude 4-bromo-2-methylselenophene in 200 ml of carbon disulfide, 13.5 g (0.084 mol) of bromine in 50 ml of acetic acid was added dropwise at −30 °C. When the addition was complete, nitrogen gas was led through the reaction mixture while it was allowed to reach room temperature. In this way some of the hydrogen bromide, formed in the reaction, was quickly removed. The mixture was poured into aq. sodium thiosulfate and extracted with carbon disulfide. The organic portions were washed with water and dried. After evaporation of the solvent, 15.9 g (63 %) of homogeneous crude product remained (GLC: column BDS, 10 %, 100–195 °C, 12 °C/min), part of which was distilled to give the pure title compound, b.p. 75–76 °C/1.0 mmHg. NMR (CCl_4): δ 2.43 (3 H, d, CH_3), 6.64 (1 H, q, H4). J(H4, CH_3) 1.2 Hz. Anal. $C_5H_4Br_2Se$: C 19.76; H 1.24; Br 52.89; Se 26.00.

3-Bromo-5-methyl-2-(methylthio)thiophene (4c). To 28.3 g (0.110 mol) of 2,3-dibromo-5-methylthiophene [27] in 100 ml of ether, 70 ml (0.11 mol) of 1.60 M butyllithium in hexane was added at −70 °C, whereupon this mixture was pressed with nitrogen into a stirred solution of 10.5 g (0.112 mol) of dimethyl disulfide in 100 ml of ether.[35] A white precipitate was formed immediately. The mixture was stirred at room temperature for 3 h and then hydrolysed with water. The ethereal phase was separated, washed with dilute sodium hydroxide solution and water, dried over magnesium sulfate and fractionated to yield 16.2 g (66 %) of the title compound, b.p. 75–80 °C/1.0 mmHg, (lit.[18] 86–87 °C/2 mmHg). NMR (CCl_4): δ 2.33 (s, SCH_3), 2.38 (d, CH_3), 6.59 (q, H4). J(H4, CH_3) 1.1 Hz.

3-Bromo-5-methyl-2-(methylthio)selenophene (3a) was prepared as above in 56 % yield (5.28 g) from 10.6 g (0.0350 mol) of 2,3-dibromo-5-methylselenophene in 100 ml of ether, 21 ml (0.036 mol) of 1.70 M butyllithium in hexane and 3.8 g (0.040 mol) of dimethyl disulfide, b.p. 100–110 °C/3 mmHg. NMR (CCl_4): δ 2.40 (3 H, s, SCH_3), 2.49 (3 H, d, CH_3), 6.72 (1 H, q, H4). J(H4, CH_3) 1.4 Hz. Anal. C_6H_7BrSSe: C 26.71; H 2.56; Br 29.68; S 11.81; Se 29.22.

5-Methyl-2,3-bis(methylthio)thiophene (14). From 2.23 g (0.0100 mol) of *4c* in 50 ml of ether, 7.0 ml (0.011 mol) of 1.60 M butyllithium in hexane and 1.13 g (0.012 mol) of dimethyl disulfide in 25 ml of ether, 1.60 g of crude title compound was obtained according to the preceding procedure. Distillation gave 0.72 g (38 %) of pure *14*, b.p. 94–96 °C/1.0 mmHg. NMR (CCl_4): δ 2.38 (d, CH_3), 6.51 (q, H4), 2.32 and 2.36 (s, SCH_3). J(H4, CH_3) 1.0 Hz. Anal. $C_7H_{10}S_3$: C 44.10; H 5.27; S 50.47.

3-Bromo-5-methoxy-2-methylthiophene (2a). To a solution of 28.6 g (0.112 mol) of 3,5-dibromo-2-methylthiophene [13] in 150 ml of ether, 68 ml (0.12 mol) of 1.69 M butyllithium in hexane was added at −70 °C, followed by 27 g (0.12 mol) of tributylborate after 15 min. The reaction temperature was allowed to rise to −10 °C during 1 h, whereupon 14 ml (0.12 mol) of 30 % hydrogen peroxide was added. The mixture was refluxed for 1 h and then poured into water. The alkaline aqueous layer was extracted with 50 ml of ether and acidified with 1 N HCl. The acidic aqueous solution was extracted with ether and the ethereal portions were dried. Evaporation of the solvent gave 18.7 g of the crude "hydroxythiophene"; IR (film): 1695 cm^{-1}. This crude product (~0.10 mol) and 25 g (0.20 mol) of dimethyl sulfate were dissolved in 100 ml of $CHCl_3$, and a mixture of 33.0 g (0.097 mol) of tetrabutylammonium hydrogensulfate, 8.0 g (0.20 mol) of NaOH and 97 ml of water was added with stirring (ice cooling). When the addition was complete, the mixture was stirred for 1 h, whereupon the organic layer was separated, washed with water and dried.[31,32] Evaporation of the solvent and distillation yielded 7.5 g (32 %) of the title compound, b.p. 57–61 °C/0.7 mmHg. NMR (CCl_4): δ 5.88 (1 H, s, H4), 2.23 (3 H, s, CH_3), 3.77 (3 H, s, OCH_3). Anal. C_6H_7BrOS: C 34.96; H 3.49; S 15.40.

3-Bromo-5-chloro-2-methylthiophene (2b) was prepared in analogy with its isomer *4b* from 14.8 g (0.0536 mol) of 2,3-dibromo-5-chlorothiophene [3] in 100 ml of ether, 40 ml (0.057 mol) of 1.42 M butyllithium in hexane and 7.56 g (0.0600 mol) of dimethyl sulfate in 50 ml of ether; yield 7.7 g (68 %), b.p. 84–85 °C/10 mmHg. NMR (CCl_4): δ 6.67 (1 H, s, H4), 2.32 (3 H, s, CH_3). Anal. C_5H_4BrClS: C 28.34; H 1.95; S 15.17.

General method for the ring-opening of 3-lithioheterocycles (G). See Ref. 2 (Part VI).

2-Methoxy-5-methyl-3-thiophenecarboxylic acid (5a). A solution of 2.07 g (0.0100 mol) of *4a* in 30 ml of ether was treated with 17 ml (0.010 mol) of 0.60 M ethereal ethyllithium and 5.45 g (0.0500 mol) of ethyl bromide according to the general method G, except that the reaction mixture was poured onto solid carbon dioxide in ether after 4 h. The usual work-up gave 1.2 g (70 %) of the title compound, m.p. ~145 °C, which after recrystallization from water had m.p. 146–148 °C (lit.[4] m.p. 147–148 °C, 50 %). NMR (acetone-d_6): δ 6.75 (1 H, q, H4), 2.32 (3 H, d, CH_3), 3.98 (3 H, s, OCH_3). J(H4, CH_3) 1.3 Hz. No signs of acetylenes were observed (IR) in the neutral phase, which amounted to 0.1 g of (mainly) 2-methoxy-5-methylthiophene (NMR).

2-Chloro-5-methyl-3-thiophenecarboxylic acid (5b). A solution of 1.0 g (4.7 mmol) of *4b* in 25 ml of ether was treated with 10.0 ml (4.7 mmol) of 0.47 M ethereal ethyllithium and

2.2 g (20 mmol) of ethyl bromide according to the general method G, except that the reaction mixture was poured onto solid carbon dioxide in ether after 4 h. Thus, 0.3 g (36 %) of the title compound was obtained after the usual work-up. The acid was recrystallized from ethanol:water, m.p. 165 – 168 °C. IR (KBr): 1680 cm^{-1}. NMR ($CDCl_3$): δ 7.06 (1 H, q, H4), 2.40 (3 H, d, CH_3), 11.25 (1 H, s, COOH). J(H4, CH_3) 1.1 Hz. Anal. $C_6H_5ClO_2S$: C 40.76; H 2.80; S 18.19.

The neutral ethereal phases contained 2-chloro-5-methylthiophene and *6* in the proportions 4:6 according to GLC analysis (OV 17, 3 %, 100 – 200 °C, 15 °C/min) and comparison of the retention times with those of authentic samples. Evaporation of the solvent gave 0.4 g of an oil, which showed no signs of acetylenes (IR).

2-Chloro-3-ethyl-5-methylthiophene (6). A solution of 10.0 g (0.0473 mol) of *4b* in 100 ml of ether was treated with 51 ml (0.048 mol) of 0.94 M ethereal ethyllithium and 16.4 g (0.150 mol) of ethyl bromide, according to the general method G, except that the reaction mixture was stirred at room temperature for 24 h before hydrolysis. Thus, 5.32 g of a crude product remained after work-up and evaporation of the solvent. The title compound was obtained through distillation, b.p. 83 – 85 °C/16 mmHg, 3.9 g (51 %). NMR (CCl_4): δ 6.37 (1 H, q, H4), 2.32 (d, CH_3); (C_2H_5), 2.50 (q) and 1.13 (3 H, t). J(H4, CH_3) 1.1 Hz, J(CH_2-CH_3) 7.0 Hz. Anal. C_7H_9ClS: C 51.7; H 5.51. No traces of acetylenes were found in the crude product (IR).

*2,5-Dichloro-3-methylthiophene (*7b*).* (a) The general method G was followed, except that the reaction mixture was hydrolyzed after 2 h. From 2.79 g (0.0100 mol) of *7a* in 10 ml of ether, 17 ml (0.010 mol) of 0.60 M ethereal methyllithium and 4.3 g (0.030 mol) of methyl iodide, 1.0 g (60 %) of crude *7b* was obtained. GLC (column NPGS, 5 %, 90 – 180 °C, 13 °C/min) and NMR showed the presence of only one component, b.p. 71 – 72 °C/14 mmHg, 0.9 g (55 %), $n_D^{20} = 1.5540$ (lit.[6] 65 °C/11 mmHg, $n_D^{20} = 1.5560$). IR showed no signs of acetylenes. NMR (CCl_4): δ 2.08 (3 H, s, CH_3), 6.45 (1 H, s, H4).

(b) To 11 ml (0.011 mol) of 1.0 M ethereal phenyllithium, 2.79 g (0.0100 mol) of *7a* was added. After 2 h at room temperature, a solution of 1.9 g (0.015 mol) of dimethyl sulfate in 25 ml of ether was added. One hour later the excess dimethyl sulfate was destroyed by adding 25 ml of conc. ammonium hydroxide solution to the reaction mixture. The ethereal layer was washed with water, 2 N HCl and water to neutral reaction. GLC (OV 1, 3 %, 70 – 220 °C, 10 °C/min) showed < 10 % of toluene, 0.5 % of 2,5-dichlorothiophene, 42 % of iodobenzene, and 40 % of *7b*, upon comparison of the retention times with those of authentic compounds. IR showed no signs of acetylenes in the evaporated crude product.

Attempted metalation of 2-methoxy-5-methylselenophene. To a solution of 0.46 g (2.6 mmol) of 2-methoxy-5-methylselenophene,[5] 6.0 ml (3.0 mmol) of 0.50 M ethereal ethyllithium was added at room temperature. The yellow solution darkened rapidly and was black after 1 min. The reaction mixture was poured onto solid carbon dioxide in ether after 15 min, but no selenophenecarboxylic acid could be isolated. Instead elemental selenium precipitated from the acidified alkaline extract. The neutral phase contained 0.2 g of the starting material.

2,5-Dichloro-3-selenienyl 1,4-dichloro-1-buten-3-yn-1-yl selenide (8). To 6.52 g (0.0200 mol) of *7c* 17 ml (0.010 mol) of 0.60 M ethereal ethyllithium was added dropwise at – 70 °C. After 5 min, the reaction mixture was poured onto solid carbon dioxide in ether. No carboxylic acid could be isolated from the alkaline aqueous extracts upon acidification with 2 N HCl. From the neutral, dried ethereal phase, 3.90 g of a brown oil remained after evaporation of the solvent. (Upon standing at room temperature the oil darkened and solidified.) MS of the oil (direct inlet): $m/e = 398$; calc. for $C_8H_2Cl_4{}^{80}Se_2 = 398$. ^1H NMR (CCl_4): singlets at δ 6.96 and 6.10, integrals 1:1. Absorptions from the starting material and 2,5-dichlorothiophene were also present, and the yield of *8* based on the NMR data and the weight of the crude product was ~ 60 %. IR (film): C≡C 2190 and 2265 cm^{-1}; C=C 1550 cm^{-1}. ^{77}Se NMR data are given in the theoretical part. Attempts to isolate *8* by preparative TLC, using a number of eluents, were unsuccessful, mainly due to the instability of the compound. The experiment was repeated several times, which gave mixtures, as mentioned above, containing 50 – 65 % of *8*.

*2,5-Dichloro-3-selenophenecarboxylic acid (*7f*).* Lithium diisopropylamide was prepared from 1.1 g (0.011 mol) of diisopropylamine in 20 ml of ether and 18 ml (0.011 mol) of 0.60 M ethereal ethyllithium. This solution was added dropwise to 2.00 g (0.0100 mol) of *7e* in 30 ml of ether at – 70 °C. After 2 h, the reaction mixture was poured onto solid carbon dioxide in ether. Upon acidification of the alkaline aqueous extracts with 2 N HCl, 1.9 g (78 %) of the title compound was isolated, m.p. ~ 145 °C. Recrystallisation from ethanol:water gave the pure acid, m.p. 151 – 153 °C. IR (KBr): C=O 1690 cm^{-1}. NMR (DMSO-d_6): δ 7.42 (s, H4). Anal. $C_5H_2Cl_2O_2Se$: C 24.70; H 0.91; Se 32.31.

*(Z)-1-Methoxy-1-methylthio-1-penten-3-yne (*1a*).* To 1.82 g (8.80 mmol) of *2a* in 50 ml of ether, 13 ml (9.1 mmol) of 0.70 M ethereal ethyllithium was added, followed by 4.9 g (45 mmol) of ethyl bromide. A white precipitate was formed after the addition of the ethyllithium. On the addition of 2.0 ml of dry HMPA, the precipitate dissolved with heat evolution. Samples of the reaction mixture were withdrawn with a pipette, hydrolysed

and analysed by GLC (OV 17, 3 %, 100–210 °C, 15 °C/min). It appeared that the ring-opening was complete in less than 2 h; 86 % of *1a* and 14 % of a compound with longer retention time were found. After 4 h, the reaction mixture was hydrolysed with water and the ethereal layer was separated, washed with 2 N HCl, water and dried. Preparative TLC (1 mm silica gel, hexane: ether 9:1) of the evaporated crude product (1.20 g) gave 0.45 g (33 %) of the title compound. IR (film): C≡C 2210 and 2040 cm^{-1}. NMR (CCl_4): δ 4.72 (1 H, q, H2), 1.95 (3 H, d, H5), 3.67 (3 H, s, OCH_3); (SC_2H_5), 2.75 (2 H, q) and 1.25 (3 H, t). J(H2, H5) 2.2 Hz, $J(SCH_2\text{-}CH_3)$ 7.0 Hz. Anal. $C_8H_{12}OS$: C 61.39; H 7.63; S 20.35.

When the experiment was performed as described above but without HMPA, the peak of the title compound increased in height even after 4 h, as was evident from GLC analyses (the same column as above) of hydrolysed samples from the reaction mixture. The peak originating from 2-methoxy-5-methylthiophene decreased simultaneously.

1-Ethylthio-1,3-pentadiyne. The general method G was followed. From 0.50 g (2.4 mmol) of *2b* in 7 ml of ether, 3.2 ml (2.4 mmol) of 0.75 M ethereal ethyllithium and 1.1 g (10 mmol) of ethyl bromide, 0.3 g of a crude product was obtained. (A yellow precipitate was formed in the reaction mixture.) IR: C≡C 2205 cm^{-1}. Combined GLC-MS analysis (column BDS, 10 %, 100–200 °C, 10 °C/min) of the washed and dried ethereal reaction mixture: 42 % of 2-chloro-5-methylthiophene, (m/e = 132; calc. for $C_5H_5{}^{35}ClS$ = 132); 21 % of the starting material (the same retention time as an authentic sample) and 37 % of the title compound, (m/e = 124; calc. for C_7H_8S = 124). NMR (CCl_4): besides absorptions from *2b* and 2-chloro-5-methylthiophene there were absorptions originating from the title compound: δ 1.95 (s, CH_3) (lit.[23] 1.97 ± 0.02); (SC_2H_5), 2.70 (2 H, q) and 1.40 (3 H, t). UV [cyclohexane] of a purified sample (TLC: 1 mm silica gel, hexane) λ_{max} (nm): 239.5, 255 (sh), 268, 286. Lit.[23] (no solvent given) 242, 253, 268, 284.

Reaction between ethyllithium and 3-bromo-5-methyl-2-methylthiothiophene (4c). (a) *(Z)-2-Ethylthio-5-methylthio-2-penten-4-yne (11).* The general method G was followed. From 2.23 g (0.0100 mol) of *4c*, in 50 ml of ether, 20 ml (0.012 mol) of 0.60 M ethereal ethyllithium and 3.3 g (0.030 mol) of ethyl bromide, 1.47 g of a crude product was obtained. Combined GLC-MS analysis (column BDS, 10 %, 130–190 °C, 16 °C/min) on the washed and dried ethereal reaction mixture showed 5 components (in the order of increasing retention times): 2-methyl-5-methylthiothiophene (*12*) (m/e = 144; calc. for $C_6H_8S_2$ = 144), (*Z*)-2-ethylthio-2-penten-4-yne (*15*) (m/e = 126; calc. for $C_7H_{10}S$ = 126), 3-ethyl-5-methyl-2-methylthiothiophene (*13*) (m/e = 172; calc. for $C_8H_{12}S_2$ = 172), (*Z*)-2-ethylthio-5-methylthio-2-penten-4-yne (*11*) (m/e = 172; calc. for $C_8H_{12}S_2$ = 172) and 5-methyl-2,3-*bis*(methylthio)thiophene (14) (m/e = 190; calc. for $C_7H_{10}S_3$ = 190) (see Table 1). Compound *14* was identified by comparison of its mass spectral data and its retention time with those of an authentic sample. IR (film) of the crude product: ≡CH 3280 cm^{-1}, C≡C 2138 and 2085 cm^{-1}. An enriched sample of *11* was obtained in the following way: (1) Column chromatography (Al_2O_3:hexane) was performed on the crude product. The acetylenes are eluted first. (2) The eluate from (1) was evacuated (1 mmHg) for 2 h. Most of the lower boiling acetylene was removed. (3) TLC (1 mm silica gel, hexane:ether 9:1) was carried out on the residue from (2). NMR (CCl_4): δ 2.01 (d, H1), 5.43 (1 H, q, H3), 2.40 (3 H, s, SCH_3); (SC_2H_5), 2.85 (2 H, q) and 1.28 (3 H, t). J(H1, H3) 1.4 Hz, $J(CH_2\text{-}CH_3)$ 7 Hz.

(b) *(Z)-2-Ethylthio-2-penten-4-yne (15).* The general method G was followed. From 2.23 g (0.0100 mol) of *4c*, 37 ml (0.022 mol) of 0.60 M ethereal ethyllithium and 3.3 g (0.030 mol) of ethyl bromide, 1.0 g of a crude product was obtained. The washed and dried ethereal reaction mixture had the composition presented in Table 1. Distillation of the crude product gave a few drops of almost pure *15*, b.p. 80–90 °C/12 mmHg. NMR (CCl_4): δ 2.06 (3 H, d, d, H1), 5.33 (m, H3), 3.08 (d, d, H5); (SC_2H_5), 2.83 (q) and 1.28 (3 H, t). J(H3, H5) 2.5 Hz J(H1, H3) 1.4 Hz, J(H1, H5) 0.6 Hz, $J(SCH_2\text{-}CH_3)$ 7.3 Hz.

(c) The reaction was repeated as in (a) but the reagents were mixed at −70 °C, after which the temperature was allowed to rise to +21 °C. After 4 h at this temperature, the reaction mixture was hydrolysed and worked-up as in the general method G, which gave 1.1 g of a crude product. The washed and dried ethereal reaction mixture had the composition presented in Table 1.

(Z)-2-Ethylseleno-5-methylthio-2-penten-4-yne (1e). The general method G was followed. From 3.53 g (0.0131 mol) of *3a* in 80 ml of ether, 20 ml (0.014 mol) of 0.70 M ethereal ethyllithium and 9.8 g (0.090 mol) of ethyl bromide, 2.50 g of a crude product was obtained. Combined GLC-MS analysis (column BDS, 10 %, 130–190 °C, 15 °C/min) of the washed and dried ethereal reaction mixture showed six components, of which the two most abundant ones were (*Z*)-2-ethylseleno-2-penten-4-yne (*1f*) (10 %, m/e = 174; calc. for $C_7H_{10}{}^{80}Se$ = 174) and *1e* (80 %, m/e = 220; calc. for $C_8H_{12}S^{80}Se$ = 220). IR(film): ≡CH 3280 cm^{-1}, C≡C 2100 and 2140 cm^{-1}. Distillation of the crude product gave 1.26 g (44 %) of the title compound, b.p. 74–77 °C/2 × 10^{-2} mmHg. IR (film): C≡C 2140 cm^{-1}, C=C 1570 cm^{-1}. NMR (CCl_4): δ 2.13 (3 H, d, H1), 5.70 (1 H, q, H3), 2.40 (3 H, s, SCH_3); (SeC_2H_5), 2.80 (2 H, q) and 1.40 (3 H, t). J(H1, 3H) 1.4 Hz, $J(SeCH_2\text{-}CH_3)$ 7.2 Hz. Anal. $C_8H_{12}SSe$: C 43.70; H 5.41; S 14.68; Se 36.20.

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Crystal and Molecular Structure of the Hydrogen-bonded Adduct of Triphenylarsine Oxide with Pentafluorophenol

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The 1:1 triphenylarsine oxide (TPAO) adduct of pentafluorophenol (PFP) crystallizes in the monoclinic space group $P2_1/c$, with a unit cell of dimensions $a = 8.790(5)$ Å, $b = 15.617(7)$ Å, $c = 16.576(4)$ Å and $\beta = 105.26(3)°$. There are 4 molecules in the unit cell. X-Ray data were collected on an off-line four-circle diffractometer. The structure was determined by three-dimensional Patterson and Fourier syntheses and refined by full-matrix least squares methodes to a final R factor of 0.043. The arsenic atom is tetrahedrally coordinated with angles ranging from 107.2 to 112.3°. The adduct consists of TPAO and PFP moieties hydrogen-bonded together. The overall O—H···O distance is 2.599(5) Å and the hydrogen atom H(1) is located at the phenol oxygen.

The present structure determination was undertaken in order to ascertain whether the 1:1 triphenyl arsine oxide—pentafluorophenol adduct has the hydrogen-bonded structure (*1*) or the ionic structure (*2*) corresponding to a proton transfer, or if the adduct has crystallized in the form suggested by Jensen [1] (*3*).

$Ph_3As^+ - O^- \cdots H - OC_6F_5$
1

$Ph_3As^+ - O - H \cdots {}^-OC_6F_5$
2

$Ph_3As^+ - O^{-\frac{1}{2}} \cdots H \cdots {}^{-\frac{1}{2}}OC_6F_5$
3

This complex has previously been studied by means of infrared spectroscopy and NMR (^1H and ^{19}F).[2] The results from these experiments indicate a strong hydrogen bond and no proton transfer.

EXPERIMENTAL

The crystals used were prepared by mixing 1:1 mol ratios of TPAO to PFP in methanol solution. After evaporation the isolated crystals were recrystallized from a mixture of light petroleum and benzene. The crystal used throughout the data collection had the dimensions 0.7 mm × 0.3 mm × 0.3 mm and was mounted along the a axis.

The space group was determined from Weissenberg and precession photographs. Unit cell dimensions were derived from diffractometer measurements of 2θ values for 16 reflections using Mo$K\alpha$ radiation.

3858 independent reflections with $2\theta \leq 50°$ were recorded on a four-circle diffractometer using $\theta - 2\theta$ scan. 623 of these reflections had net counts less than twice the estimated error in measurements and were given zero weight in the calculations. Lp corrections and absorption corrections were applied, the latter according to a procedure described by Coppens *et al.*[3]

Approximate coordinates for the arsenic atom were found from a three-dimensional Patterson synthesis, and the positions of the remaining non-hydrogen atoms were found from a subsequent Fourier map. The phenyl-hydrogen positions were calculated from geometrical considerations. The atomic parameters were refined by full-matrix least squares procedures, minimizing the function $\sum\omega(|F_o| - |F_c|)^2$ where $\omega = 1/\sigma_F^2$. The final hydrogen atom H(1) was localized from a difference Fourier map. The refinement converged at an R of 0.043, the weighted R factor is 0.050, with no shift at that stage greater than the corresponding standard deviation.

Final atomic coordinates and temperature parameters are listed in Table 1. Lists of observed and calculated structure factors may be obtained from the author.

Table 1a. Fractional coordinates and thermal parameters for non-hydrogen atoms with standard deviations. Thermal parameters are of the form $T = \exp[-2\pi^2(U_{11}h^2a^{*2} + U_{22}k^2b^{*2} + U_{33}l^2c^{*2} + 2U_{12}hka^*b^* + 2U_{23}klb^*c^* + 2U_{13}hla^*c^*)]$.

Atom	X/a	Y/b	Z/c	U_{11}	U_{22}	U_{33}	U_{12}	U_{23}	U_{13}
As	.14386(4)	.37597(2)	.35559(2)	.0420(2)	.0413(2)	.0516(2)	−.0004(2)	.0017(2)	.0105(1)
F2	.6140(4)	.1133(3)	.6416(3)	.091(2)	.164(3)	.181(3)	−.020(2)	−.033(3)	−.016(2)
F3	.5205(4)	−.0492(2)	.6554(2)	.148(3)	.154(3)	.113(2)	.048(3)	.046(2)	.006(2)
F4	.2508(5)	−.1078(2)	.5492(2)	.196(4)	.058(2)	.146(2)	−.026(2)	.014(2)	.037(2)
F5	.0770(4)	−.0031(2)	.4325(2)	.110(2)	.116(2)	.107(2)	−.037(2)	−.001(2)	−.005(2)
F6	.1654(4)	.1562(2)	.4170(2)	.098(2)	.106(2)	.107(3)	.015(2)	.062(2)	0.21(2)
O1	.2684(3)	.3365(2)	.4408(2)	.060(2)	.062(2)	.068(2)	.007(1)	.016(1)	.004(1)
O2	.4484(5)	.2170(2)	.5245(4)	.100(3)	.058(2)	.252(6)	−.031(2)	−.015(3)	.049(4)
C1	.3903(6)	.1389(3)	.5292(3)	.075(3)	.054(2)	.132(4)	−.007(2)	−.014(2)	.033(3)
C2	.4771(6)	.0853(4)	.5894(4)	.069(3)	.093(4)	.118(4)	−.012(3)	−.031(3)	.007(3)
C3	.4327(6)	.0016(3)	.5975(3)	.094(4)	.084(3)	.072(3)	.020(3)	.007(2)	.008(3)
C4	.2963(6)	−.0269(3)	.5446(3)	.113(4)	.049(2)	.080(3)	−.008(2)	.003(2)	.024(3)
C5	.2111(5)	.0267(3)	.4865(3)	.076(3)	.071(3)	.078(3)	−.011(2)	−.002(2)	.004(2)
C6	.2572(5)	.1081(3)	.4790(3)	.063(3)	.058(2)	.102(3)	.009(2)	.020(2)	.018(3)
C11	.1370(4)	.3084(2)	.2588(2)	.051(2)	.045(1)	.064(2)	−.002(2)	−.003(2)	.021(2)
C12	.2649(7)	.2562(4)	.2589(4)	.058(3)	.075(4)	.104(4)	.011(3)	−.016(3)	.020(3)
C13	.2598(9)	.2100(5)	.1877(5)	.091(5)	.098(5)	.128(6)	.018(4)	−.042(5)	.044(5)
C14	.1374(9)	.2121(4)	.1193(4)	.123(6)	.079(4)	.081(4)	−.009(4)	−.027(3)	.049(5)
C15	.0113(9)	.2643(4)	.1202(4)	.110(6)	.099(5)	.066(4)	.000(4)	−.013(3)	.008(4)
C16	.0079(7)	.3128(3)	.1900(3)	.079(4)	.073(3)	.055(3)	.002(3)	−.013(3)	.007(3)
C21	.2094(4)	.4891(2)	.3369(2)	.045(2)	.047(2)	.056(2)	−.003(2)	−.001(2)	.015(2)
C22	.3072(7)	.5318(3)	.4022(4)	.080(4)	.055(3)	.074(4)	−.011(3)	−.012(3)	.014(3)
C23	.3571(9)	.6135(4)	.3900(5)	.098(5)	.063(4)	.113(5)	−.022(3)	−.019(4)	.027(4)
C24	.3068(9)	.6524(4)	.3157(5)	.104(5)	.050(3)	.127(6)	−.016(3)	.001(4)	.050(5)
C25	.2140(9)	.6093(4)	.2496(5)	.159(8)	.070(4)	.102(5)	−.015(4)	.025(4)	.036(5)
C26	.1622(9)	.5271(4)	.2610(4)	.118(5)	.062(3)	.075(4)	−.024(4)	.014(3)	.003(4)
C31	−.0665(4)	.3833(2)	.3665(2)	.050(2)	.044(2)	.054(2)	.001(2)	−.001(2)	.017(2)
C32	−.1345(6)	.3111(3)	.3884(3)	.054(3)	.047(3)	.090(4)	−.000(2)	−.000(2)	.026(3)
C33	−.2875(7)	.3139(4)	.3967(4)	.055(3)	.065(3)	.111(5)	−.007(3)	.000(3)	.029(3)
C34	−.3686(7)	.3886(4)	.3840(4)	.046(3)	.087(4)	.105(4)	.000(3)	−.007(3)	.028(3)
C35	−.3038(8)	.4604(4)	.3615(5)	.069(4)	.068(4)	.160(4)	.023(3)	.020(4)	.052(4)
C36	−.1495(7)	.4585(4)	.3526(4)	.077(4)	.056(3)	.146(6)	.013(3)	.023(3)	.056(4)

Table 1b. Fractional coordinates and thermal parameters for hydrogen atoms with standard deviations. Thermal parameters are of the form $T = \exp[-8\pi^2 U(\sin^2\theta)/\lambda^2]$.

Atom	X/a	Y/b	Z/c	U
H12	.356(7)	.247(3)	.309(3)	.074(16)
H13	.333(7)	.177(4)	.185(3)	.103(18)
H14	.127(6)	.179(4)	.069(3)	.090(16)
H15	−.070(8)	.273(4)	.073(4)	.121(20)
H16	−.077(6)	.348(3)	.189(3)	.150(15)
H22	.342(6)	.503(3)	.462(3)	.071(14)
H23	.429(9)	.641(4)	.437(4)	.131(25)
H24	.340(6)	.708(4)	.309(5)	.086(16)
H25	.183(9)	.627(4)	.192(5)	.144(28)
H26	.097(7)	.496(3)	.212(3)	.076(16)
H32	−.075(7)	.253(3)	.397(3)	.083(17)
H33	−.347(8)	.261(4)	.409(4)	.125(21)
H34	−.467(6)	.392(3)	.392(3)	.070(13)
H35	−.364(6)	.513(4)	.353(3)	.097(18)
H36	−.113(7)	.513(4)	.335(3)	.103(17)
H1	.388(9)	.246(4)	.491(4)	.17(3)

CRYSTAL DATA

$C_{24}H_{16}AsF_5O_2$ M.W. = 506.31
Space group $P2_1/c$ (No. 14)
$a = 8.790(5)$ Å, $b = 15.617(7)$ Å, $c = 16.576(4)$ Å,
$\beta = 105.26(3)°$
$V = 2195(2)$ Å^3, $Z = 4$.
$D_c = 1.532$ g cm^{-3}, $D_m = 1.50$ g cm^{-3}.
$\mu(\mathrm{Mo}K\alpha) = 16.7$ cm^{-1}.

RESULTS AND DISCUSSION

The structure, shown in Fig. 1, consists of TPAO and PFP moieties hydrogen-bonded together giving monomeric adducts. Some important bond distances are shown in the same figure in which the atomic numbering used in this paper is also given. The essential bond distances and bond angles are listed in Table 2.

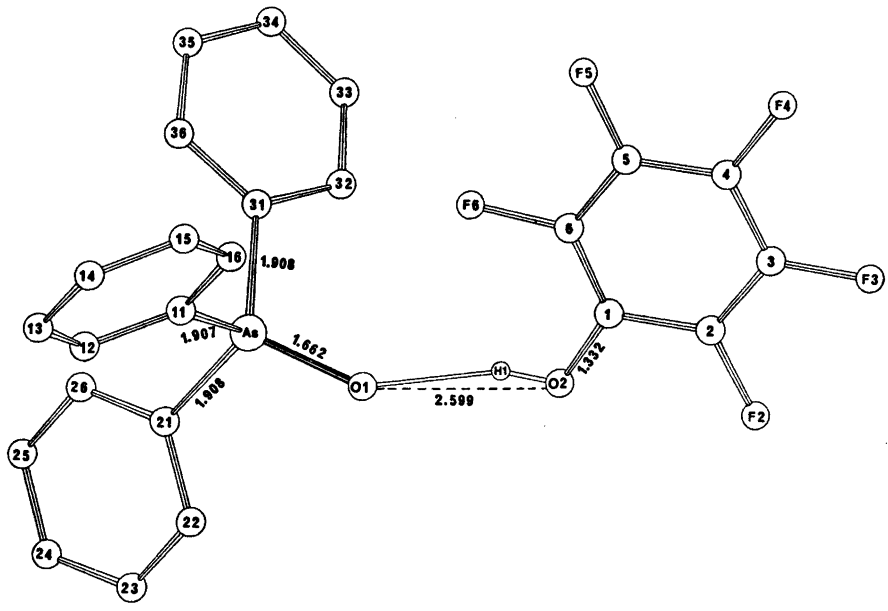

Fig. 1. TPAO-PFP with numbering of atoms and a few parameters.

Table 2. Selected bond distances and bond angles with corresponding standard deviation in parentheses.

	(Å)	Angle	(°)
As–O(1)	1.662(2)	O(1)–As–C(11)	112.2(1)
As–C(11)	1.907(4)	O(1)–As–C(21)	108.8(1)
As–C(21)	1.908(3)	O(1)–As–C(31)	112.3(1)
As–C(31)	1.908(4)	C(11)–As–C(21)	108.7(1)
C(1)–O(2)	1.332(6)	C(11)–As–C(31)	107.2(1)
O(1)–O(2)	2.599(5)	C(21)–As–C(31)	107.5(1)
O(1)–H(1)	0.80(6)	As–O(1)–O(2)	153.2(2)
O(2)–H(1)	1.83(7)	O(1)–O(2)–C(1)	120.0(3)
		H(1)–O(2)–C(1)	111(5)
		O(1)–H(1)–O(2)	162(5)

Table 3. Some average bond angles and bond distances in triphenylarsine oxide complexes.

Compound	Average C–As–C (°)	Average C–As–O (°)	As–O (Å)	Average * O···O (Å)
$Ph_3AsO.H_2O$ [4]	108.0	110.9	1.644	2.800 *
$Ph_3AsO.C_6H_2Cl_4O_2$ [5]	108.4	110.6	1.694	2.616 *
$(Ph_3AsO.HgCl_2)_2$ [6]	108.9	110.1	1.66	
$(Ph_3AsO)_2.HgCl_2$ [7]	107.7	111.2	1.69	
$Ph_3AsO.C_6F_5OH$	107.8	111.1	1.662	2.599

The mean C–C bond distance in the phenyl groups are 1.369(4) Å, while the C–H distances range from 0.84 to 1.04 Å. Standard deviations of the individual C–H bond distances are approximately 0.06 Å. In the PFP moiety the mean C–C bond distance is 1.373(3) Å and the mean C–F bond distance 1.346(3) Å. The bond distances have not been corrected for rigid-body libration.

The geometry around the arsenic atom is essentially tetrahedral with angles ranging from 107.2 to 112.3°. The average C–As–C and C–As–O angles, 107.80(6) and 111.10(6)° respectively, are comparable with corresponding values in analogous complexes [4–7] (see Table 3). The TPAO moiety as a whole possesses no symmetry because of unequal rotations of the phenyl groups about the As–C bonds. The twist angles between the planes through the benzene rings and the planes containing As, O(1) and C(11), C(21) and C(31), respectively, are 22, 20, and 54°. The three phenyl rings are planar within experimental error.

It is interesting to note that the conformation of the TPAO moiety is practically the same in the present study as in triphenylphosphine oxide.[8] The average C–P–C and C–P–O angles are 107.1(5) and 111.7(4)°, respectively, and the twist angles of the phenyl groups are 24.7, 21.1, and 59.3°. In the analogous arsenic compounds [4–7] one also finds one large twist angle and two smaller ones; however, the values of the angles differ from those already mentioned.

The spectroscopic data [2] on this compound indicate a strong hydrogen bond. The overall O···O distance of 2.599(5) Å found in the present X-ray diffraction study supports the spectroscopic evidence for a strong bond.

In X-ray work on analogous triphenylarsine oxide complexes [4–7] no evidence for proton transfer is found, indicating that these complexes belong to the hydrogen-bonded structure (*1*).

In the present case the structure (*1*) is supported by the location of the hydrogen atom

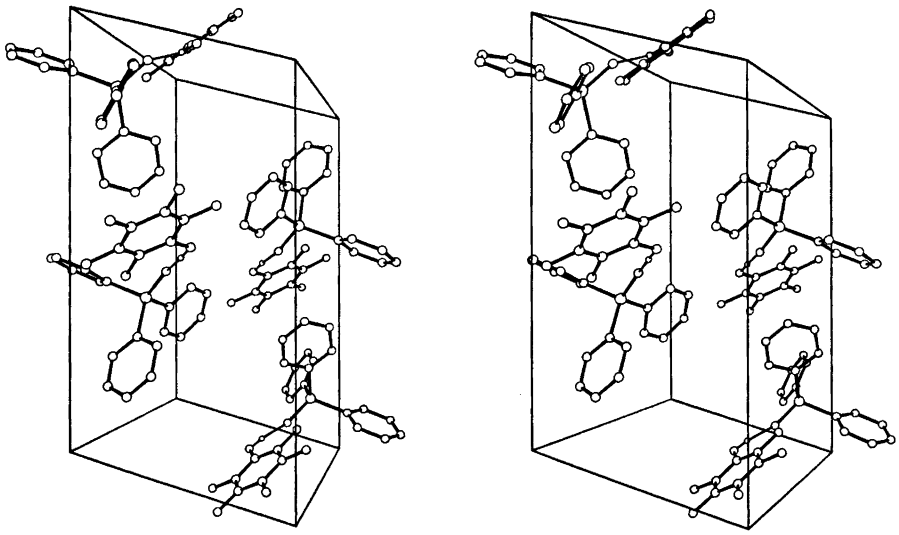

Fig. 2. Stereo drawing showing the packing of the molecules in the unit cell. The *b*-axis is pointing away from the viewer, the *c*-axis is running vertically, bottom to top, and the *a*-axis diagonally left to right. Figs. 1 and 2 were drawn by use of the ORTEP program.[12]

H(1) in the difference Fourier map and also by consideration of the bond distances As – O(1) and C(1) – O(2) found. The As – O(1) bond distance of 1.662(2) Å is appreciable shorter than the As – O bond distance of 1.87 Å calculated from covalent bond radii, and also significantly shorter than the calculated As – O double distances of 1.73 Å.[9] Similar shortening effects are found in analogous compounds of phosphorus and sulfur. Cruickshank [10] has explained this shortening by overlap between the filled p_π-orbitals of the oxygen atom and the unfilled $3d_\pi$-orbitals of the phosphorus and sulfur atoms. In the present case the unfilled $4d_\pi$-orbitals of the arsenic atom are presumably involved.

The C(1) – O(2) bond distance of 1.332(6) Å is comparable to the C – O bond distance of 1.33 Å in pentachlorophenol.[11]

A stereo drawing of the molecular packing in the unit cell is shown in Fig. 2. There are no intermolecular contacts shorter than the corresponding van der Waals distances.

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Preparation, Physical Properties, and Kinetics of the Hydrolysis of Substituted α-Chlorobenzyl Benzoates

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There have been prepared 34 α-chlorobenzyl benzoates with substituents in the *para*- or *meta*-position of the acyl or/and benzyl group. Their physical properties have been determined and the kinetics of their hydrolysis in aqueous acetone, acetonitrile, and dioxane solutions has been investigated. The hydrolysis of the esters with substituents in the acyl group obey the Okamoto-Brown equation with $\varrho = -0.8$. For the esters with substituents in the benzyl group the value of ϱ is -2.8 in 60 "%" acetone-water. The points for the *p*-methoxy-substituted compounds do not, however, lie on the line determined by the other substituents. These facts together with the values found for solvent and deuterium solvent isotope effects and for activation parameters show that the reaction is an S_N1 solvolysis of the α-chlorine in all the cases studied.

One of the present authors [1,2] has shown that the neutral hydrolysis of α-haloalkyl esters may proceed either by general-base catalysed ester hydrolysis ($B_{AC}3$) with water as the base or by a nucleophilic displacement (S_N) of the α-halogen atom. It is known that α-chloroethyl acetate [1] and benzoate [3] are solvolysed by the S_N1 mechanism and that α-phenyl substituents facilitate the unimolecular solvolysis by resonance much more than alkyl groups do.[4] Therefore it is to be expected that at least α-halobenzyl esters of acids without strongly electron-withdrawing substituents are solvolysed by the S_N1 mechanism. This was shown to be true in the case of α-chlorobenzyl acetate by Cleve and Euranto.[5] It was also found that α-chlorobenzyl formate follows the same mechanism, but that α-chlorobenzyl trifluoro- and trichloroacetates are hydrolysed by the $B_{AC}3$ mechanism.[5] It is therefore probable that α-chlorobenzyl benzoate follows the S_N1 mechanism, but strongly electron-withdrawing substituents could possibly change the mechanism, which then could be seen as a deviation from linearity in the Hammett plot. A study of the hydrolysis of substituted α-chlorobenzyl benzoates was performed to test the validity of these hypotheses. Also information about the transmission of substituent effects through the carboxylic group was thought to be desirable because the influence of structural changes in the acyl group of α-haloethyl esters was earlier found to be relatively strong.[6]

Several α-halobenzyl benzoates have already been prepared by the reaction of benzoyl halides with benzaldehydes.[7–9] The method was improved later.[10,11] Of the 34 α-chlorobenzyl benzoates now studied, only the unsubstituted [7,10,11] and three other esters [8,9,12,13] seem to have been prepared earlier. Of their physical properties, except melting points, only few data on their IR-spectra can be found in the literature.[10,12] To our knowledge, no quantitative kinetic investigations of their reactions have been performed earlier.

EXPERIMENTAL

Solvents. The solvent mixtures used in the kinetic experiments were prepared as described earlier.[1,14] Weight percentages were employed in the case of dioxane-water mixtures. In the case of aqueous acetone (E. Merck AG, *pro analysi*) and acetonitrile (Fluka, AG, *purissimum*) solutions the symbol *p* "%" indicates that 100 cm³ of the solvent mixture contained $(100-p)$ g of water. In the deuterium oxide (Norsk hydro-elektrisk kvaelstofaktieselskab, 99.8 g D_2O/100 g) solutions containing acetonitrile, the molarity of D_2O was the same

as that of water in the corresponding ordinary water solutions.

Esters. The α-chlorobenzyl benzoates were prepared from benzoyl chlorides and benzaldehydes under nitrogen with zinc chloride as catalyst.[10] After the reaction mixture was allowed to stand for 15 min to several days at room temperature, the ester was precipitated by adding hexane. Occasionally cooling was necessary for precipitation. In some cases the yield of the crystalline ester was low. Sometimes the formed ester could not be separated from the reaction mixture at all. The esters were analysed by hydrolysing weighed amounts in acetone-water and titrating potentiometrically with standard sodium hydroxide and silver nitrate solutions. The IR-spectra were recorded in benzene solution on a Perkin-Elmer Model 337 Grating Infrared Spectrophotometer. The NMR-spectra were recorded on a 60 MHz Perkin-Elmer Model R 10 NMR Spectrometer in tetrahydrofuran solution using TMS as internal standard. The analytical results are given in Table 1.

Table 1. Analytical results, melting points, and spectral data for α-chlorobenzyl benzoates Y-$C_6H_4CO_2CHClC_6H_4$-Z.

Y	Z	Acid % [a]	Chlorine % [a]	Melting point °C	IR (in benzene) $\nu_{C=O}$ cm^{-1}		ν_{C-O-C} cm^{-1}		NMR (in THF) δ (benzylic H)
H	H	99.9	99.8	45–52	1746.2	1249	1077	1059	
					1749.9 [b]	1251 [b]	1078 [b]	1059 [b]	
				51–52 [11]	1730 [b,10]	1255 [b,10]	1080 [b,10]	1060 [b,10]	
H	*m*-Br	102.5	99.0	43–45	1742.6	1247	1073	1055	7.87
H	*p*-Br	84.5	75.3	80–85	1743.6	1247	1073	1057	
				109–110 [9]					
H	*m*-Cl	104.4	98.1	45–65	1744.4	1247	1071	1054	
H	*p*-Cl	99.2	99.5	75–78	1745.8	1249	1077	1060	7.82
H	*m*-NO_2	103.9	102.6		1746.4	1247	1077	1060	
H	*p*-NO_2	103.2	98.1	90	1744.8	1247	1075	1058	
H	*m*-CH_3				1741.3	1247	1077	1059	
H	*p*-CH_3				1743.4	1250	1077	1058	
H	*m*-OCH_3				1741.6	1253	1077	1060	
H	*p*-OCH_3				1739.7	1251	1079	1060	
m-Br	H			120–130	1744.4	1245	1066	1057	7.86
p-Br	H			85	1744.0	1243	1066		7.81
m-Cl	H			82–84	1746.0	1247	1070	1055	
p-Cl	H	99.3	92.3	70–75	1743.6	1249	1072		7.77
p-Cl	*m*-NO_2	96.5	93.9	*ca.* 100	1744	1240	1066		
m-NO_2	H	103.5	79.1	60	1748.7	1240	1075	1058	
				87–88 [8]					
m-NO_2	*m*-NO_2	97.4	101.4	106	1748	1232	1070	1053	
m-NO_2	*m*-CH_3				1747.9	1253	1071	1055	
p-NO_2	H	101.7	102.4	130	1746.5	1249	1077		7.84
				118–118.5 [8]					
				119–120 [12]	1745 [c,12]				
				115–116 [13]					
p-NO_2	*p*-OCH_3	96.1	79.3		1742	1247	1077		
m-CH_3	H	84.3	78.7	58–68	1740.0	1260	1084	1062	7.87
m-CH_3	*m*-NO_2			91–93	1745.4	1259	1081	1060	
p-CH_3	H	94.9	92.4	49–51	1740.1	1247	1066		7.78
m-CF_3	H				1747.9	1245	1064		
m-CF_3	*m*-NO_2	97.8	99.4	95	1748	1221	1064		
p-CF_3	H				1748.9	1250	1077		
m-CH_3O	H	99.8	94.9		1738.7	1257	1080	1071	7.78
p-CH_3O	H	99.3	95.2	48	1737.3	1251	1069		
p-CH_3O	*m*-Cl	98.9	97.9	60–65	1739.1	1247	1068		7.83
p-CH_3O	*p*-Cl			73–80	1731.2	1251	1069		7.87
p-CH_3O	*m*-NO_2	99.8	99.6	90–94	1739.1	1249	1066		

[a] Percentages of the theoretical amounts of hydrolysable acids and chlorine according to the scheme $RCO_2CHClR' + H_2O \rightarrow RCO_2H + HCl + R'CHO$. [b] In 2,3-dimethylbutane. [c] In chloroform.

Kinetic experiments. The reaction rates were measured by a conductometric method employing a Philips PR 9501 or a Radiometer CDM 3 conductometer. The ester concentration was usually *ca.* 5×10^{-3} mol dm^{-3}, but in the case of the least soluble esters a saturated solution was used. The reactions followed first-order kinetics indicating that the hydrogen ion produced by the reaction did not catalyse it in these concentrations. In the case of the hydrolysis of α-chlorobenzyl *p*-nitrobenzoate in H_2O-D_2O mixtures the ester concentration was *ca.* 10^{-3} mol dm^{-3} and the rate coefficients were calculated by Guggenheim's method. The hydrolysis of α-chlorobenzyl benzoate in dioxane-water mixtures was studied also in solutions containing added electrolytes. The ester concentration was then 0.02 to 0.04 mol dm^{-3} and the time-average $\bar{c}_t$ of the hydrogen-ion concentration and the rate coefficient k_0 for zero acid concentration were calculated as described earlier.[1] The kinetic data are given in Tables 2 – 5.

Errors. Some of the esters contained appreciable amounts of impurities, which could not be removed. Benzoyl chlorides, if present, disturbed the kinetic determinations, because their hydrolysis rates sometimes were quite similar to those of the esters. In these cases, marked in Table 3, their hydrolysis rates were determined separately and appropriate corrections, not exceeding 18 %, were made in the conductivity values. Other impurities did not interfere with the spectral and kinetic determinations.

When the rate coefficients were calculated conventionally from the integrated first-order rate equation, their accuracy was estimated

Table 2. Kinetic data for the hydrolysis of α-chlorobenzyl benzoate in *p* "%" acetone-water (A-W) or *p* wt. % dioxane-water (D-W).

p	Solvent	*t* / °C	Added electrol. / mol dm^{-3}	$\bar{c}_t$ / mol dm^{-3}	*k* / 10^{-3} s^{-1}	$\Delta H^{\ddagger}$ / kJ mol^{-1}	$\Delta S^{\ddagger}$ / J K^{-1} mol^{-1}
50	A-W	25	–		43.4		
60	A-W	5	–		1.27		
		15	–		3.91		
		25	–		9.63	66.8 ± 1.5	−60 ± 5
		35	–		23.9		
67.5	A-W	25	–		3.65		
75	A-W	25	–		1.09		
60	D-W	35		0	21.1 [a]		
			–	0.0124	21.8		
			0.0527 $HClO_4$	0.0644	24.6		
		45		0	44.7 [a]	61	−87
			–	0.0081	41.4		
			–	0.0090	51.7		
			0.0527 $HClO_4$	0.0707	58.7		
			0.0617 $HClO_4$	0.0801	62.1		
70	D-W	45		0	10.0 [a]		
			–	0.0057	10.2		
			–	0.0068	10.2		
			0.0505 $HClO_4$	0.0595	12.2		
			0.050 $NaClO_4$	0.0044	12.0		
			0.050 $NaClO_4$	0.0164	12.1		
75	D-W	25		0	0.594		
			–	0.0051	0.628		
			–	0.0063	0.634		
			–	0.0069	0.595		
			0.0526 $HClO_4$	0.0639	0.857	74.4 ± 1.1	−57 ± 3
		35		0	1.66		
			–	0.0031	1.77		
			–	0.0066	1.65		
			0.0526 $HClO_4$	0.0691	2.38		
		45		0	4.16		
			–	0.0059	4.57		
			–	0.0070	4.10		
			0.0529 $HClO_4$	0.0670	5.99		

[a] The value of the rate coefficient extrapolated to zero acid concentration.

as the standard deviation of the mean. The so estimated relative errors were found to vary only little, the average value being 0.75 %. When parallel runs were performed, the mean of their average deviations was found to be 0.95 %. The method of least squares was used when the rate coefficients were calculated by Guggenheim's method, when the extrapolated values at zero acid concentration were computed, and when the Arrhenius equation was applied. In the first case the average value of the relative standard deviations was found to be 0.58 %. The errors given for the parameters in the Arrhenius equation are standard deviations.

DISCUSSION

Physical properties. α-Chlorobenzyl benzoates in general, but especially those with electron-donating substituents in the benzyl group, are unstable and decompose partly during purification or when standing for a few hours or days even at a low temperature under nitrogen. However, esters with strong electron-withdrawing substituents are more stable.[5]

The IR-frequencies for the carbonyl group (1730 – 1750 cm^{-1} in benzene solution, Table 1) and the -CO_2C- group (1221 – 1260 and one or two peaks at 1053 – 1084 cm^{-1}) vary only slightly with structure. The NMR signal for the benzylic proton (δ 7.77 – 7.87 in tetrahydrofuran) lies among those for the aromatic protons and could be identified sometimes only by the aid of the NMR spectra of the corresponding substituted benzyl benzoates. No clear correlation between the spectral data and various substituent constants could be found.

Kinetics and mechanism of the hydrolysis of α-chlorobenzyl benzoate. The data (Table 2) from the kinetic runs carried out in dioxane-water solutions containing perchloric acid or sodium perchlorate show that both of these electrolytes increase the rate slightly and equally. The acceleration is thus a salt effect rather than acid catalysis and similar to that found in the case of α-chloroethyl benzoate[3] and acetate.[6] The following facts show that the reaction mechanism is S_N1 for the unsubstituted ester. The thermodynamic parameters of activation (Table 2) resemble closely those found for α-chloroethyl benzoate in 75 wt. % dioxane-water ($\Delta H^{\ddagger} = 82.1 \pm 0.8$ kJ/mol, $\Delta S^{\ddagger} = -71 \pm 3$ J K^{-1} mol^{-1})[3] and for α-chloroethyl acetate in 50 wt. % acetone-water ($\Delta H^{\ddagger} = 74.8 \pm 0.2$ kJ/mol, $\Delta S^{\ddagger} = -56 \pm 1$ J K^{-1} mol^{-1}), although the influence of different solvent composition has to be taken into account in the latter case.[1] Also the solvent effect is typical of S_N1 reactions. Plots of log k against log c_{H_2O} for α-chlorobenzyl benzoate (not shown) are slightly curved. Their slopes, 4.9 for 70 wt. % dioxane-water at 45 °C and 4.6 for 70 "%" acetone-water at 25 °C, compare with the values 4.4 for α-chloroethyl benzoate and 4.3 for α-chloroethyl acetate under corresponding conditions.[3]

Table 3. Rate coefficients $k/10^{-3}$ s^{-1} for the hydrolysis of α-chlorobenzyl benzoates Y-$C_6H_4CO_2CHClC_6H_4$-Z in p "%" acetone-water mixtures at 25 °C.

Y	Z	$p = 60$	$p = 75$
H	H	9.63	1.09
H	*m*-Br	0.432	
H	*p*-Br	2.76	
H	*m*-Cl	0.465	
H	*p*-Cl	3.48	
H	*m*-NO_2	0.110	
H	*p*-NO_2	0.067	
H	*m*-CH_3	12.8	1.52
H	*p*-CH_3	75.6[a]	12.8[a]
H	*m*-CH_3O	4.52	0.596
H	*p*-CH_3O	74.5[a]	6.12[a]
m-Br	H	2.89	
p-Br	H	3.78	
m-Cl	H	2.71	
p-Cl	H	6.43	
m-NO_2	H	1.89	
p-NO_2	H	2.40	
m-CH_3	H	9.98	
p-CH_3	H	13.7	
m-CF_3	H	2.28	
p-CF_3	H	2.73	
m-CH_3O	H	6.91	
p-CH_3O	H	30.3	
m-NO_2	*m*-NO_2	0.0238	
m-NO_2	*m*-CH_3	2.76	
m-NO_2	*p*-CH_3	18.3	
p-NO_2	*p*-CH_3	28.7	
p-NO_2	*p*-OCH_3	25.9	
p-CH_3O	*m*-Cl	1.52	
p-CH_3O	*p*-Cl	9.44	
p-CH_3O	*m*-NO_2	0.326	
p-Cl	*m*-NO_2	0.0848	
m-CH_3	*m*-NO_2	0.119	
m-CF_3	*m*-NO_2	0.0278	

[a] The reaction mixture contained benzoyl chloride; its hydrolysis was taken into account when calculating the rate coefficient.

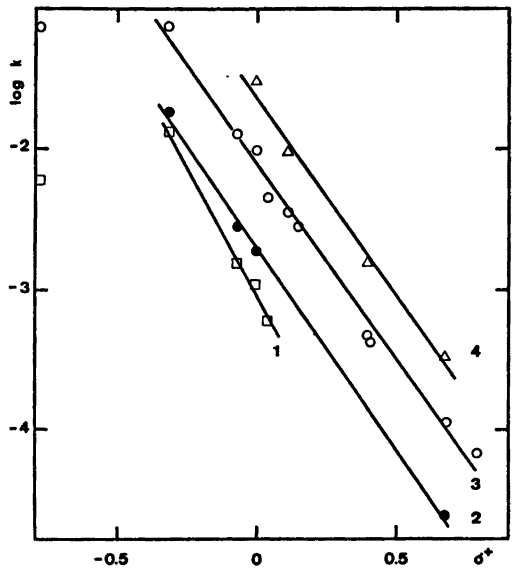


Fig. 1. The σ^+ correlation for the hydrolysis rates of α-chlorobenzyl benzoates Y-$C_6H_4COOCHClC_6H_4$-Z with variable substituents Z in the benzyl group. Solvent *p* "%" acetone-water; temperature 25 °C. The solid lines are the least-square fits for all other points than that for the *p*-methoxy compound. 1. Y=H, *p*=75; 2. Y=*m*-NO_2, *p*=60; 3. Y=H, *p*=60; 4. Y= *p*-CH_3O, *p*=60.

The rate coefficients for α-chlorobenzyl benzoate are on an average 120 times as great as those for α-chloroethyl benzoate.[3] This is in accordance with the supposed S_N1 mechanism for both esters and with the influence of this structural change. The rate difference is due to the lower activation enthalpy and the less negative activation entropy of the α-chlorobenzyl ester.

Structural effects. When substituents in the benzyl group were varied, the rate coefficients (Table 3) obeyed the Hammett equation $\log k = \log k^\circ + \varrho\sigma$ (Fig. 1). Best correlation was obtained with σ^+ substituent constants [15] (the Okamoto–Brown equation). The reaction constants ϱ (Table 4) are negative as they ought to be for an S_N1 reaction. They are, however, less negative than found for the solvolysis of tertiary halides in corresponding solvents; *e.g.*, Okamoto and Brown [16] calculated for ϱ the value −4.11 for the hydrolysis of substituted benzhydryl chlorides in 70 % acetone-water and the value −4.62 for that of phenyldimethylmethyl chlorides in 90 % acetone-water. The less negative values for α-chlorobenzyl benzoates may be due to the fact that the positive charge of the supposed intermediate is not only on the benzylic carbon atom but partly on the ethereal oxygen, *cf.* Ref. 17. In fact, similar values are found also for other reactions leading to oxonium-carbenium ions; as examples the following values can be presented: −1.9 for the hydrolysis of α-acetoxystyrenes in aqueous sulfuric acid,[17] −2.25 for α-methoxystyrenes [18] and −2.29 for acetophenone dimethyl acetals [19] in 5 "%" dioxane-water.

α-Chloro-*p*-methoxybenzyl benzoates are omitted from the above correlations because they essentially lower the correlation coefficients (see Fig. 1). The reason could be the inaccuracy of the rate measurements for these very fast reacting, thermally unstable esters, which could not be prepared free from impurities, especially from benzoyl chlorides. It is,

Table 4. Values of the reaction constants ϱ for the hydrolysis of α-chlorobenzyl benzoates in *p* "%" acetone-water at 25 °C. *n*=number of substituents, r^+=correlation coefficient for the presented σ^+ correlation, *r*=correlation coefficient for the corresponding σ correlation.

Substrate	*p*	*n*	ϱ	r^+	*r*
$C_6H_5CO_2CHClC_6H_4$-Z	75	4	−3.67	0.997	0.923
	60	10	−2.80	0.995	0.981
p-$CH_3OC_6H_4CO_2CHClC_6H_4$-Z	60	4	−2.86	0.995	0.982
m-$NO_2C_6H_4CO_2CHClC_6H_4$-Z	60	4	−2.89	0.999	0.980
Y-$C_6H_4CO_2CHClC_6H_5$	60	13	−0.81	0.967	0.938
Y-$C_6H_4CO_2CHClC_6H_4$-*m*-NO_2	60	6	−0.82	0.983	0.962

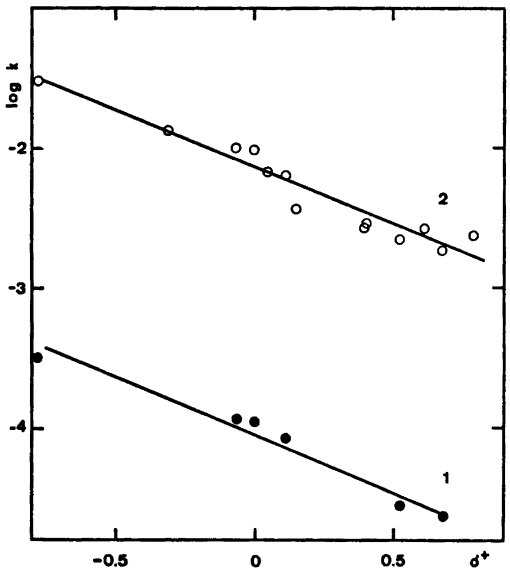
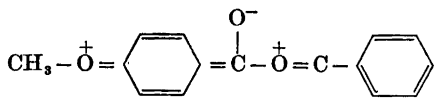


Fig. 2. The σ^+ correlation for the hydrolysis rates of α-chlorobenzyl benzoates Y-$C_6H_4COOCHClC_6H_4$-Z with variable substituents Y in the acyl group. Solvent 60 "%" acetone-water; temperature 25 °C. The solid lines are the least-square fits for all experimental points.
1. Z = *m*-NO_2; 2. Z = H.

however, improbable that the exceptional rate coefficients are seriously wrong, because similar results were obtained under various conditions of the hydrolysis for two esters of several batches. In fact, *p*-methoxy-substituted compounds have been found to deviate downward in σ^+ correlations also in several other cases.[18–20] Loudon and Berke [19] have examined this kind of substituent effect with the Yukawa-Tsuno relation and extended Hückel molecular orbital calculations. Their explanation, however, does not explain quantitatively the present case, because the points for the *p*-methyl-substituted esters lie on the lines fixed by the other substituents, thus requiring a value close to unity for Yukawa's and Tsuno's resonance parameter *r*. On the other hand, the *p*-methoxy-substituted compounds reacted in all cases studied at lower rates than the *p*-methyl compounds and require that $r < 0.1$. The deviating behaviour of the methoxyl group as substituent may reflect the partial oxonium-ion character of the intermediate,[20] which makes its resonance effect less important.

When the variable substituent is in the acyl group, the correlation is worse (Fig. 2), but even then σ^+-values give better correlation than σ (Table 4). This is in contrast with the first expectation, because the substituent cannot be in direct resonance with the developing positive charge on the benzylic carbon atom. Resonance forms like



are, however, possible in the present case. If their contribution is sufficient, they may explain the observed correlation.

The values of the reaction constants ϱ are lower in the latter case (Table 4), which evidently is due to the larger distance between the substituent and the reaction centre. The ratio of the reaction constants is about 0.3, which is larger than the corresponding ratio (about 0.2) [21] for two intervening methylene groups. This is a further indication that the transmission of electronic effects through the carboxylic group takes place easier than through a saturated hydrocarbon chain.[6]

Hydrolysis mechanism of substituted α-chlorobenzyl benzoates. The structural effects considered above show that also substituted α-chlorobenzyl benzoates obey the same S_N1 mechanism as the unsubstituted ester. They thus confirm quantitatively the conclusion drawn by Filler and Miller on the basis of qualitative hydrolysis experiments which α-chlorobenzyl benzoate and *p*-nitrobenzoate that "attack of water on the carbonyl carbon is not involved in the rate-determining step".[12] A slight curvature, which possibly could be found in some of the Okamoto-Brown plots (Figs. 1 and 2), could be an indication of a shift from S_N1 to $B_{AC}3$ mechanism in the case of the most electronegative substituents. A rough estimation based on the known rate of the $B_{AC}3$ hydrolysis of α-chlorobenzyl trichloroacetate [5] and on reasonable structural and solvent effects gives for the rate coefficient of the hydrolysis of α-chloro-*m*-nitrobenzyl *m*-nitrobenzoate in 60 "%" acetone-water at 25 °C a value of the order of 10^{-6} s^{-1}, whereas

Table 5. Kinetic data for the hydrolysis of α-chlorobenzyl benzoates Y-$C_6H_4CO_2CHClC_6H_5$ in 22.20 M solutions of water (60 "%" acetonitrile-water) or its mixtures with deuterium oxide in acetonitrile at 25 °C. n = deuterium atom fraction.

Y	n	$k/10^{-3}$ s^{-1}	k_n/k_0
H	0.00	31.4	1.000
	1.00	21.3	0.678
p-NO_2	0.00	4.90	1.000
	0.30	4.26	0.869
	0.60	3.88	0.792
	0.80	3.58	0.731
	1.00	3.37	0.688

the experimental value is 2.38×10^{-5} s^{-1}. This indicates that a change in the mechanism is possible. The change is, however, ruled out by the fact that no deviation can be observed in the case of the above-mentioned ester. This conclusion was confirmed by determining the temperature dependence of its rate. The obtained rate coefficients [2.38 (Table 3), 6.28, 16.6, and 41.9×10^{-5} s^{-1} at 24.98, 34.95, 45.01, and 55.07 °C, respectively] yield the values 76.9 ± 0.3 kJ/mol for $\Delta H^{\ddagger}$ and -76 ± 1 J K^{-1} mol^{-1} for $\Delta S^{\ddagger}$. They differ only slightly from those found for the unsubstituted ester (Table 2) and show that the observed lower rate is due to both the higher activation enthalpy and the lower activation entropy. A significant contribution of ester hydrolysis should have produced a lower activation enthalpy. No change in mechanism could thus be found in the hydrolysis of the esters studied.

Solvent isotope effects. The deuterium solvent isotope effect $k_{D_2O}/k_{H_2O} = 0.68$ (Table 5) found for the hydrolyses of both α-chlorobenzyl benzoate and *p*-nitrobenzoate in acetonitrile-water mixtures is typical for an S_N1 reaction in water.[22] Relatively small amounts of organic solvents have only a small influence on the isotope effect.[22] In the case of α-chlorobenzyl *p*-nitrobenzoate also the rates in $H_2O - D_2O$ mixtures were measured. The plot (not shown) of k_n/k_H against n (the deuterium atom fraction) is slightly bent downward, but does not fit any simple equation based on the equilibrium theory of solvent isotope effects.[23]

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Short Communications

1-Substituted 3,5-Diaryl-s-triazolo-[3,4-*c*]-s-triazoles

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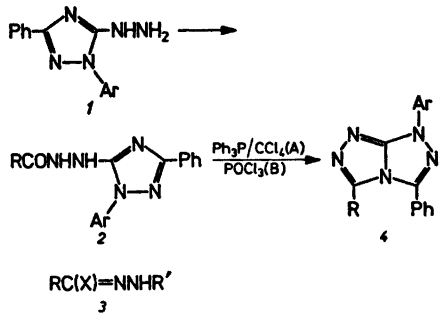
Hydrazonyl halides (*3*, X = halogen) have been used extensively in the preparation of five-membered heterocyclic compounds *via* 1,3-dipolar cycloaddition reactions,[1] and by displacement reactions at the hydrazonyl carbon atom.[2] Internal dipolar cyclization reactions are also known,[2,3] and recently internal 1,3-dipolar cycloaddition reactions have been reported.[4]

5-Hydrazino-1,2,4-triazoles (*1*) have now become readily available from the reaction of hydrazonyl halides with thiosemicarbazide in the presence of base,[5] and these compounds were thought to be suitable as precursors, *via* the acylhydrazide (*2*) and the hydrazonyl halide (*3*), of the hitherto unknown 1,3,5-trisubstituted-s-triazolo[3,4-*c*]-s-triazoles (*4*). This ring system (3,5-disubstituted) has previously been reported in the literature.[6,7]

The acylhydrazides (*2*) were easily prepared by acylation [aroyl chloride (*2a*—*c*) or acetic anhydride (*2d*)] of the free hydrazine. Treatment of the benzohydrazide (*2a*) with triphenylphosphine and carbon tetrachloride (Ph_3P/CCl_4) (Method A), did not give the expected [8] hydrazonyl chloride, instead internal cyclization took place with formation of 1,3,5-triphenyl-s-triazolo[3,4-*c*]-s-triazole (*4a*) (*cf.* Ref. 9). Phosphoryl chloride ($POCl_3$) was also found to be a convenient reagent (Method B) for the conversion of *2* into *4*.



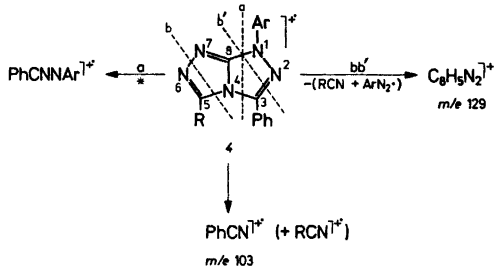
Scheme 1. *a*, Ar = R = Ph; *b*, Ar = 2,4-$Br_2C_6H_3$ and R = Ph; *c*, Ar = Ph and R = 4-$CH_3OC_6H_4$; *d*, Ar = Ph and R = CH_3.

The mass spectra of *4* (see Table 1) are compatible with the structural assignment. The mass spectra are characterized by molecular ions, base peaks of the spectra, abundant $[PhCN]^+$ (also $[RCN]^+$ in case of *4c*), $[PhCNNAr]^+$ and $[M-(ArN_2\cdot+RCN)]^+$ ions. The latter two types of ions are of particular significance. The $[PhCNNAr]^+$ ions (route a) confirm the integrity of the original 1,2,4-triazole ring of *1*. The *m/e* 129 ion $[C_6H_5N_2]^+$ is present in all mass spectra and corresponds to the loss of $(ArN_2\cdot+RCN)$ from the molecular ion (route bb') (confirmed by metastable defocusing [10]). This ion may be formed also by a different route involving atoms C- (5 and 8) and N- (6 and 7) of the ring as seen by the

Table 1. The principal peaks [a] in the mass spectra [b] (70 eV) of compounds *4*.

Compound	M^+	$[PhCNNAr]^+$	M^{2+}	$[C_6H_5N_2]^+$ *m/e* 129	$[PhCN]^+$ *m/e* 103	$[ArN]^+$
4a (140 °C)[c]	339 (100)	194 (6)	(5)	(8)	(60)	91 (17)
4b (160 °C)	497 (100)	350 (1)	(5)	(13)	(33)	249 (8)[d]
4c (120 °C)	369 (100)	194 (9)	(7)	(5)	(11)	91 (28)[e]
4d (100 °C)	275 (100)	194 (1)	(4)	(18)	(67)	91 (5)[f]

[a] *m/e* (rel. int.). [b] Recorded on an A.E.I. MS 902 mass spectrometer. [c] Ion source temperature. [d] $[C_7H_3Br_2N]^+$ 261 (46). [e] $[C_9H_7N_2O]^+$ 159 (1.5), 133 (35), 118 (4). [f] 117 (8).



Scheme 2. MS fragmentation of *4*.

formation of *m/e* 159 in the spectrum of *4c*.

Several attempts to convert (with nitrous acid) the hydrazino compounds (*1*) to azides, invariably resulted in decomposition to the corresponding amines (*cf.* Ref. 11).

Experimental. 5-(N'-Acylhydrazino-1-aryl-3-phenyl-1,2,4-triazoles (2). Crude hydrochloride of *1a* [5] (10.0 mmol) was stirred for 30 min in conc. ammonia (20 ml), filtered and dried to give the free hydrazine *1a* (7.5 mmol). This was dissolved in pyridine (10 ml) and treated with benzoyl chloride (3.75 mmol). After 30 min the reaction mixture was poured into water and stirred. The precipitate was filtered off, dried and crystallized from ethanol to give *2a* (40 %), m.p. 164–165 °C. Anal. $C_{21}H_{17}N_5O$: C, H, N.

Compounds *2b* and *2c* were prepared in a similar manner. Compound *1b* and benzoyl chloride gave *2b* (40 %), m.p. 241–243 °C (from ethanol). Anal. $C_{21}H_{15}Br_2N_5O$: C, H, N.

Compound *1a* and anisoyl chloride gave *2c* (60 %), m.p. 198–200 °C (from ethanol). Anal. $C_{22}H_{19}N_5O_2$: C, H, N.

Compound *1a* (2.5 mmol) and acetic anhydride (2.5 mmol) were refluxed together in benzene (10 ml) for 30 min. Removal of the solvent by evaporation and crystallization of the residue from ethanol gave *2d* (60 %), m.p. 191–194 °C. Anal. $C_{16}H_{15}H_5O$: C, H, N.

1-Substituted 3,5-diaryl-s-triazolo[3,4-c]-s-triazoles (4). Method A. Carbon tetrachloride (1.50 mmol) was added to a stirred suspension of *2a* (1.50 mmol) and Ph_3P (1.87 mmol) in dry acetonitrile (5 ml) as described previously.[8] After 7 days at room temperature the mixture was filtered, and the solid was crystallized from acetonitrile (80 ml) to give *4a* (23 %), m.p. 244–246 °C. Anal. $C_{21}H_{15}N_5$: C, H, N.

Method B. The benzohydrazide *2a* (0.31 mmol) and $POCl_3$ (1 ml) were refluxed together for 15 min, allowed to cool and followed by removal of the solvent. The residue was treated with ice-water, and the solid was crystallized from ethanol to give *4a* (34 %), m.p. 240–245 °C.

Compounds *4b*, *4c* and *4d* were prepared in a similar manner. Compound *2b* and $POCl_3$ gave *4b* (70 %), m.p. 198–200 °C. Anal. $C_{21}H_{13}Br_2N_5$: C, H, N. Compound *2c* and $POCl_3$ gave *4c* (50 %), m.p. 219–221 °C. Anal. $C_{22}H_{17}N_5O$: C, H, N. Compound *2d* and $POCl_3$ gave *4d* (60 %), m.p. 158–160 °C. Anal. $C_{16}H_{13}N_5$: C, H, N.

Nitrosation of the hydrazines (1). Sodium nitrite (2.5 mmol) in water (0.5 ml), previously cooled, was added dropwise to a stirred suspension of the hydrochloride of *1a* [5] (1.0 mmol) in conc. hydrogen chloride (2.5 ml) cooled to 5–10 °C. After 30 min the solution was filtered, and the solid was crystallized from acetonitrile, containing a few drops of conc. HCl, to give the hydrochloride of the corresponding amine (45 %), solvated as a hydrate. Anal. $C_{14}H_{12}N_4.HCl.H_2O$: C, H, N.

Treatment of this with conc. ammonia gave the free amine, m.p. 148–150 °C (from hexane/benzene, 1:1) (reported [5] 148–150 °C).

Similarly, after work-up the hydrobromide of *1b* [5] gave the free amine, m.p. 226–229 °C (reported [5] 219–221 °C); an IR spectrum was identical to that of an authentic specimen.[5]

Nitrosation of the free hydrazine *1b* at 0 °C did not afford any reaction, the hydrochloride of *1b* was recovered, m.p. 257–261 °C. Anal. $C_{14}H_{11}Br_2N_5.HCl$: C, H, N.

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The Metabolism of Quinidine in Man: Structure of a Main Metabolite

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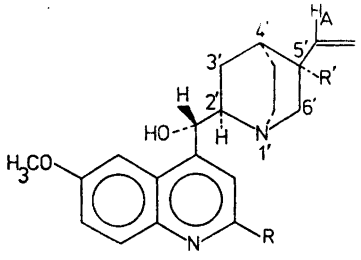
The chinchona alkaloid quinidine (*1*) is used as a drug for the treatment of cardiac arrythmia. Several metabolites of quinidine (*1*) in man have previously been isolated and characterised but the structure of only one of them (*2*) has been established.[1,2] One of the main metabolites, previously considered to be 6′-hydroxyquinidine (earlier denoted 2′-hydroxyquinidine),[1] is now demonstrated to have structure *3*.



1, R = R′ = H
2, R = OH; R′ = H
3, R = H; R′ = OH

Mass spectrometry shows that the metabolite *3* has a molecular weight of 340, which corresponds to quinidine (*1*) with an additional oxygen atom. The ^{1}H NMR spectrum of quinidine (*1*) exhibits a complex multiplet (1 H) at δ 5.9 – 6.4 attributed to H_A in formula *1*. The corresponding hydrogen atom in the metabolite appears as two doublets at δ 6.41, which establishes that the additional oxygen atom is situated at C-5′, and hence that the metabolite is 5′-hydroxyquinidine (*3*). The dissociation constants for *3* (in ethanol-water, 1:2) were found to be 7.3 and 4.0. The former value is in good agreement with that calculated for 5′-hydroxyquinidine.[3]

The absolute configuration at C-5′ is not at present known but, since enzymatic hydroxylation generally takes place with retention of the configuration,[4] the absolute configuration depicted in formula *3* is proposed for the metabolite.

Brodie *et al.*[1] have also studied the metabolism of quinine in man, and they reported that 6′-hydroxyquinine is one of the main metabolites. The assignment of structure was based on UV and pK_a measurements. The dissociation constants were found to be 7.24 and 4.12 (in 27 % ethanol), which are of the same magnitude as those here found for 5′-hydroxyquinidine (*3*). It thus seems probable that the metabolite formulated as 6′-hydroxyquinine in fact is 5′-hydroxyquinine.

Experimental. Melting points are corrected. Optical rotations were measured on a Perkin-Elmer 141 polarimeter, NMR spectra on a Varian XL-100 spectrometer, UV spectra on a Bausch and Lomb spectronic UVd instrument and the mass spectra on an LKB 2091 mass spectrometer. Solvents were evaporated under reduced pressure at bath temperatures not exceeding 25 °C. Plates pre-coated with silica gel F_{254} (2 mm, Merck) were used for preparative TLC.

Isolation of 3. Urine (4 l) from human subjects chronically treated with quinidine (0.8 – 1.2 g/day) was made alkaline (pH 9) with aqueous ammonia and extracted with ethyl acetate (3 × 1.6 l). The organic phase was washed twice with a saturated solution of sodium chloride, dried (Na_2SO_4) and evaporated to dryness. The residue was chromatographed on preparative silica gel plates (methanol) giving *3* (30 mg). Metabolite *3* had R_F 0.6 (R_F for quinidine 0.5). Needles (water-ethanol), m.p. 209 – 212 °C. (Lit. 1 m.p. 210 – 212 °C). UV, indistinguishable from that previously reported.[1] $[\alpha]_D^{22}$ + 16° (*c* 1.0, methanol). pK_a (ethanol-water, 1:2) 7.3 and 4.0.

^{1}H NMR (CD_3OD): δ 1.0 – 1.3 (m, 2 H), 1.7 – 2.3 (m, 3 H), 2.64 (d, 1 H, *J* 14 Hz), 2.8 – 3.2 (m, 3 H), 3.94 (d, 1 H, *J* 14 Hz), 4.01 (s, 3 H), 5.21 (dd, 1 H, J_1 10.8 Hz, J_2 1.6 Hz), 5.45 (dd, 1 H, J_1 17.6 Hz, J_2 1.6 Hz), 5.66 (d, 1 H, *J* 2.6 Hz), 6.41 (dd, 1 H, J_1 17.6 Hz, J_2 10.8 Hz), 7.32 – 7.50 (m, 2 H), 7.70 (dd, 1 H, J_1 4.5 Hz, J_2 1 Hz), 7.99 (ddd, 1 H, J_1 10.0 Hz, J_2 1.6 Hz, J_3 1 Hz), 8.68 (d, 1 H, *J* 4.5 Hz). MS, *m/e* (rel. intensity): M^+ 340 (11), 189 (15), 152 (100).

Acknowledgement. We are indebted to Dr. Carl-Gunnar Swahn for recording the NMR spectra.

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Synthesis of 1,9-Diazacycl[3.3.3]azine

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2-Methylpyridines substituted in the 6-position with a function containing an activated methylene group adjacent to the ring, *1*, (X = CN or COOEt) react with ethoxymethylenemalononitrile, *2* (Y = CN),[1] or 3-ethoxy-2-cyanoacrylate, *2* (Y = COOEt),[2] to form bicyclic compounds. When these are treated with an acid anhydride (or a second mol of *2*), 7,9-disubstituted 1-azacycl[3.3.3]azines, *3*, result.*

The present communication describes a variation of this approach. The use of *ca.* 3 mol of ethyl *N*-cyanoformimidate, *4*,[3] instead of *2* and the anhydride, leads to the monosubstituted diazacycl[3.3.3]azines *6a* and *6b*.[4,5] Reaction of 2,6-dimethylpyridine, *5*, with *4* analogously yields the unsubstituted diazacycl-[3.3.3]azine, *6* (*cf.* Chart 1).

6-Methyl-2-pyridineacetonitrile, *1a*,[6,7] and ethyl 6-methyl-2-pyridineacetate, *1b*,[8] react with ethyl *N*-cyanoformimidate, *4*, to form 3-cyano- and 3-carbethoxy-1,9-diazacycl[3.3.3]-azine, *6a* and *6b*, respectively. The proposed structures of these green crystalline compounds are confirmed by high resolution mass spectrometrical molecular weights, infrared spectra (C=O and C≡N absorption) and ^{1}H NMR spectra (H-2 singlet, H-4, H-5, and H-6 AMX-type absorption and H-7, H-8 of AX-type in both compounds; *cf.* Experimental). The chemical-shift value for H-4 (δ 7.08) is unusually high due to the effect of the 3-carbethoxy group.

Attempts to decyanate *6a* with PPA [9] to the parent system were unsuccessful due to decomposition of the ring system. Decarbethoxylation of *6b* in diphenyl ether containing traces of *p*-toluenesulfonic acid, on the other hand, gave the unsubstituted cyclazine *6*. 1,9-Diazacycl[3.3.3]azine is a blue-green crystalline compound, rather insoluble in nonpolar solvents and fairly unstable both in air and in solution. The assignment of its structure is supported by an exact mass spectrometrical molecular weight determination and by the presence of one A_2X (H-4, H-5, and H-6) and two AX (H-2, H-3 and H-7, H-8) type absorptions in the NMR spectrum.

1,9-Diazacycl[3.3.3]azine is the first unsubstituted isomer of the seven possible ones in the diaza group.[10] The chemical-shift values for the protons (δ 4.3 – 6.2) are between those for the "corresponding" protons in 2-methyl-1-azacycl[3.3.3]-azine (δ 3.7 – 5.6) [11] and 1,3,4-triazacycl[3.3.3]azine (δ 5.3 – 6.9).[12] This order indicates that the degree of aromaticity increases with the number of peripheral *N*-atoms in the cyclazine.

Treatment of *6* with *N*-bromosuccinimide (NBS) under conditions employed earlier for similar systems [13,14] caused decomposition of the cyclazine, and no brominated products could be isolated.

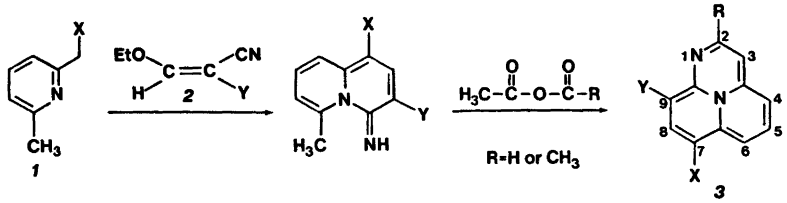
Since the 6-methyl groups in *1a* and *1b*, which are not activated by conjugation, reacted smoothly, we hoped that both methyl groups in *5* would be reactive enough to condense with 2 mol of *4*. This turned out to be the case, and in a one-flask reaction *6* was formed, although in very low yield. This modification should prove to be synthetically useful since methylsubstituted heterocycles are often more stable than their amino analogues and since they are not likely to tautomerize.

Experimental. General. NMR spectra were recorded with a Varian Model A-60 and a Bruker WH 270 spectrometer, using tetramethylsilane (TMS) as internal reference.

Ultraviolet and visible spectra were measured in ethanol with a Beckman DK-2A spectrophotometer. IR spectra were determined in KBr with a Perkin-Elmer 337 spectrophotometer. Mass spectra were obtained with a GEC-AEI 902 mass spectrometer at the Department of Medical Biochemistry, University of Göteborg. TLC was performed on Silica Gel 60F 254 (Merck). For column chromatography, Silica Gel 60 (0.063 – 0.2 mm; Merck) was used.

The cyclazines were obtained in low yields. Attempts to increase them by variation of mol ratios, reaction temperatures and reaction times were not successful as judged from TLC. A detailed study of the reaction conditions could lead to better yields. This has, however, not been pursued since our main object was to synthesize these compounds in amounts sufficient for spectrometric investigations.

* For definitions and nomenclature of cyclazines, *cf.* Refs. 15 and 16.



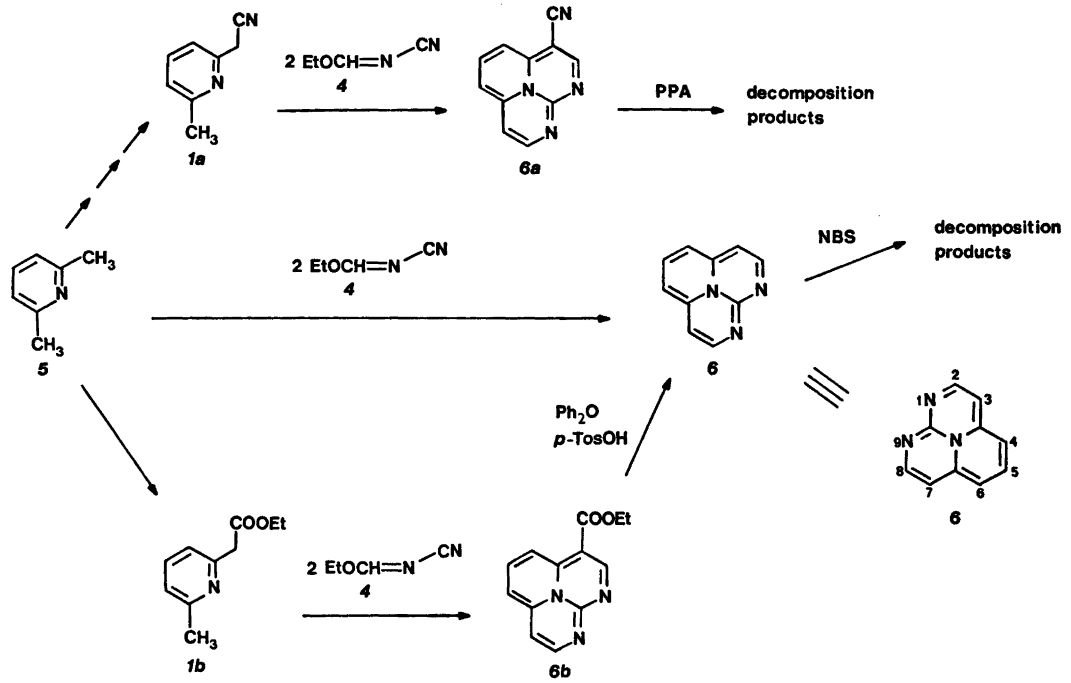


Chart 1.

The ring-closure reactions have been performed at elevated temperatures without solvents which results in large amounts of dark unidentifiable products. Since the cyclazines were easily discovered and isolated by TLC on account of their characteristic colours, no attempts were made to isolate other products from the reaction mixtures.

3-Cyano-1,9-diazacycl[3.3.3]azine, 6a. A mixture of 0.23 g (1.74 mmol) of *1a* [6,7] and 0.60 g (6.12 mmol) of *4* was kept at 110 °C for 15 min. Higher reaction temperature and/or lower *4*/*1a* ratio decreased the yield of *6a*. The reaction product, a dark, oily liquid, was then dissolved in ethyl acetate and chromatographed on 10 g of silica gel. With EtOAc/MeOH (3:1), a dark-green band was eluted. It was further purified by preparative TLC (EtOAc/MeOH; 3:1). Yield: 15 mg (4 %) of *6a* as dark-green needles, m.p. 230 °C. MS: $M^+ = 194.0585 \pm 0.003$. Calc. for $C_{11}H_6N_4$: 194.0592. *m/e* (Rel. int.) 194(100, M^+), 168 (8.4, M−CN), 167 (7.3, M−HCN), 140 (4.6, M−2HCN); *m** (calc.): 145.4 (145.48) 194→168, 143.8 (143.76) 194→167, 117.3 (117.37) 167→140. IR: 2208 cm^{-1} (C≡N). UV: $\lambda_{max}(\varepsilon)$ 254 (28 000), 268 sh. (19 400), 361 (21 600), 391 (19 660), 412 (18 370), 452 (860), 570 sh. (230), 623 (360), 686 nm (420). NMR ($CDCl_3$): δ 5.09 (1 H, d, H-7), 5.43 and 5.58 (2 H, 2d, H-4 and H-6), 6.54 (1 H, t, H-5), 6.67 (1 H, d, H-8), 6.80 (1 H, s, H-2), $J_{4,5} = J_{5,6} = 8.1$, $J_{4,6} = 1.5$, $J_{7,8} = 5.9$ Hz.

3-Carbethoxy-1,9-diazacycl[3.3.3]azine, 6b. A mixture of 0.35 g (1.96 mmol) of *1b* [8] and 0.61 g (6.22 mmol) of *4* was heated at 140 °C for 8 min. Further heating decreased the yield of *6b*. The reaction was followed by TLC (EtOAc/MeOH; 3:1). The product, a very dark, oily liquid, was dissolved in EtOAc and chromatographed on 10 g of silica gel. With EtOAc/MeOH (3:1), a dark-green band was eluted. It was further purified by preparative TLC. The clear, green solution gave on evaporation dark-green crystalline needles. Yield of *6b*: 12 mg (3 %), m.p. 178−179 °C. MS: $M^+ = 241.084 \pm 0.003$. Calc. for $C_{13}H_{11}N_3O_2$: 241.0868. *m/e* (Rel. int.) 241 (97.0, M^+), 213 (100, $M-C_2H_4$), 196 (33.3, M−OEt), 169 (22.7, 196−HCN), 168 (37.9, 196−CO), 141 (17.3, 168−HCN), 114 (16.4, 168−2HCN). *m** (calc.): 188.1 (188.25) 241→213, 180.5 (180.36) 213→196, 144.0 (144.00) 196→168, 118.2 (118.34) 168→141, 92.1 (92.17) 141→114. IR: 1675 cm^{-1} (C=O). UV: $\lambda_{max}(\varepsilon)$ 256 (23 500), 282 sh. (7550), 354 (9400), 384 sh. (7350), 399 (10 700), 420 (11 100), 564 sh. (130), 612 (210), 672 nm (220). NMR ($CDCl_3$): δ 1.28 (3 H) and 4.12 (2 H) [ester group], 5.14 (1 H, d, H-7), 5.52 (1 H, d, H-6), 6.60 (1 H, t, H-5), 6.69 (1 H, d, H-8), 7.08 (1 H, d, H-4), 7.48 (1 H, s, H-2); $J_{4,5} = J_{5,6} = 8.1$, $J_{7,8} = 6.0$ Hz.

Decarbethoxylation of 6b. To a solution of 30 mg (0.12 mmol) of *6b* in 3 ml of diphenyl ether kept at 200 °C, 3 mg of *p*-toluenesulfonic acid

was added. After 2.5 h the reaction mixture was cooled and the diphenyl ether dissolved in petroleum ether. The residue, containing *6*, was then taken up in 2 ml of methanol and purified by preparative TLC (MeOH; $R_F = 0.21$). Yield: 15 mg (71 %) of *6* as blue-green needles, m.p. 216–218 °C (decomp.). MS: $M^+ =$ 169.0637. Calc. for $C_{10}H_7N_3$: 169.0640. *m/e* (Rel. int.) 169 (100, M^+), 143 (17.9, M–CN), 142 (12.5, M–HCN), 116 (6.8, 143–HCN), 115 (10.7, M–2HCN). UV: $\lambda_{max}(\varepsilon)$ 246 (9500), 264 (12 750), 268 sh. (12 000), 365 sh. (10 500), 378 (13 600), 385 sh. (11 300), 399 (5800), 452 sh. (650), 483 (680), 503 sh. (370), 602 sh. (150), 650 nm (240). NMR ($CDCl_3$): δ 4.34 (2 H, d, H-3, H-7), 4.70 (2 H, d, H-4 and H-6), 5.92 (1 H, t, H-5), 6.17 (2 H, d, H-2 and H-8); $J_{2,3} = J_{7,8} = 5.8$, $J_{4,5} = J_{5,6} = 8.0$ Hz.

Preparation of 1,9-diazacycl[3.3.3]azine, 6, from 2,6-dimethylpyridine 5. A mixture of 1 g (9.34 mmol) of *5* and 1.8 g (18.36 mmol) of ethyl *N*-cyanoformimidate, *4*, was kept at 100 °C for 45 min. It was cooled to room temperature and poured onto a column of silica gel. A blue product was eluted with EtOAc/MeOH (4:1) and further purified by preparative TLC (MeOH). Yield: < 5 % of *6*. Mass and NMR spectral data showed it to be identical with the compound obtained by decarbethoxylation of *6b*.

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Synthesis of Methyl-substituted 5-Thia- and 5-Selena-1,3,6-triazacycl-[3.2.3]azines

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For the study of properties of a nonbenzenoid aromatic system the parent compound is usually the most desired member. In many cases, however, available synthetic methods allow only the preparation of derivatives. Of these the phenyl analogues can be of limited value for an investigation of aromaticity.[1,2] The methyl-substituted systems, on the other hand, in general show properties which are very similar to those of the parent compound. Often a methyl group has a stabilizing effect on the system.[3]

We have recently described the synthesis of phenyl-substituted thia- and selena-1,3,6-triazacycl[3.2.3[azines *,[4,5] from 5-phenyl-2,4-diaminothia- and selenazole and ethyl 2-cyano-3-ethoxyacrylate, *2*. We did not succeed then in preparing the unsubstituted thiasystem since 2,4-diaminothiazole reacts with *2* at C-5 in preference to the amino groups,[6] or the methyl-substituted system since 5-methyl-2,4-diaminothiazole is unstable as the free base. Attempts to liberate it from the hydrochloride gave 4-amino-2-hydroxy-5-methylthiazole instead.[7]

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We have recently described the synthesis of phenyl-substituted thia- and selena-1,3,6-triazacycl[3.2.3[azines *,[4,5] from 5-phenyl-2,4-diaminothia- and selenazole and ethyl 2-cyano-3-ethoxyacrylate, *2*. We did not succeed then in preparing the unsubstituted thiasystem since 2,4-diaminothiazole reacts with *2* at C-5 in preference to the amino groups,[6] or the methyl-substituted system since 5-methyl-2,4-diaminothiazole is unstable as the free base. Attempts to liberate it from the hydrochloride gave 4-amino-2-hydroxy-5-methylthiazole instead.[7]

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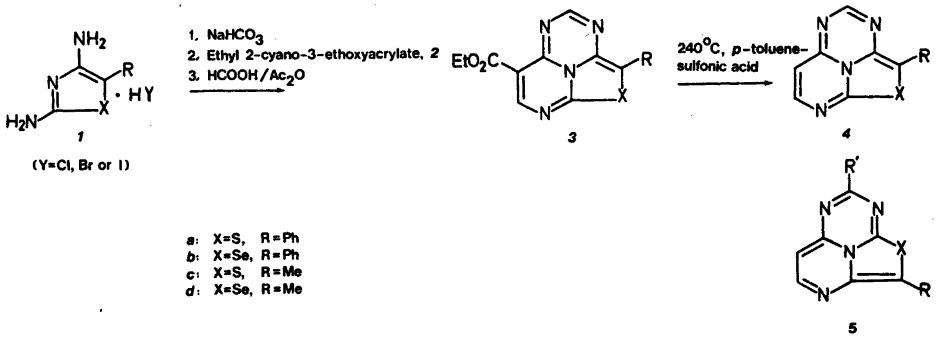

ated and reacted *in situ*. The same procedure was now successfully employed for the synthesis of *4c* and *4d*.

2,4-Diamino-5-methylthiazole was liberated from its hydrochloride with sodium bicarbonate and *2* was immediately added to the solution. The condensation product was then extracted and directly formylated without isolation. Ring closure and dehydration to *3c* occurred spontaneously under the formylating conditions.

2,4-Diamino-5-methylselenazole hydrohalide, *1d*, was prepared from α-chloropropionitrile and selenourea in analogy with the method used for the thiazole analogue.[7] Attempts to purify *1d* by recrystallization led to decomposition. Therefore the crude product was used directly and *3d* was obtained in a procedure analogous to the one described above.

Decarbethoxylation of *3c* and *3d* was subsequently achieved by heating in diphenyl ether containing *p*-toluenesulfonic acid.

The synthetic route we have followed could also yield the isomeric systems *5c* and *5d* (R′=H). The NMR chemical shifts for H-8 in *4a* and *5a* (R′=CH_3), whose structures were proved independently, were δ 5.49 and 4.64, respectively. In the spectra of the isolated methyl analogues the same proton signals appear at δ 5.40 and 5.51. We believe this to be sufficient evidence [8] to assign them the structures *4c* and *4d*.

The chemical shifts for the corresponding protons in *4a*, *4b*, *4c*, and *4d* are very close and $J_{7,8}$ is 6 Hz in all compounds. This indicates that the phenyl group has only a small effect on the electron distribution and consequently on the aromaticity of the two cyclazine systems.

Experimental. General. NMR spectra were recorded with a Varian Model A-60 or a Bruker Model WH-270 spectrometer using TMS as internal reference. UV and visible spectra were measured in ethanol with a Cary Model 15 spectrophotometer. The mass spectra, obtained from the Department of Medical Biochemistry, University of Göteborg, were recorded with a GEC-AEI 902 instrument, at an ionizing potential of 70 eV. TLC was performed on silica gel GF_{254} (Merck) plates with ethyl acetate:methanol, 5:1, as the developing solvent, and the spots were visualized with short-wave UV light and with iodine vapour. The cyclazines were obtained in low yields. Attempts to increase the yields by variation of molar ratios, reaction temperatures and reaction times were not successful as judged from thin-layer chromatography. A detailed study of the reaction conditions could possibly lead to better yields. This has, however, not been pursued since our main object was to synthesize these compounds in amounts sufficient for spectrometric investigations.

8-Carbethoxy-4-methyl-5-thia-1,3,6-triazacycl-[3.2.3]azine, 3c. A vigorously stirred solution of 2,4-diamino-5-methylthiazole hydriodide (2.06 g; 8.00 mmol) in 100 ml of water was neutralized with sodium bicarbonate (0.67 g; 8.00 mmol), *2* (1.35 g; 8.00 mmol) was immediately added followed by 100 ml of ethanol. The solution was stirred for 1 h at room temperature. The ethanol was then evaporated under reduced pressure and the residue extracted with 3 × 100 ml of ether. The combined extracts were dried (magnesium sulfate) and the ether was evaporated. The brown residue was dissolved in 40 ml of formic acid and 15 ml of acetic anhydride was added. The solution was kept at 90 °C for 3 h, cooled, and poured into 100 ml of ice water, neutralized with aqueous saturated sodium bicarbonate solution and extracted with 3 × 75 ml of methylene chloride. The combined extracts were washed with water, dried (magnesium sulfate), and evaporated to dryness. The brown residue was chromatographed on silica gel using methylene chloride – ethyl acetate as the eluent. The fractions containing *3c* (R_F=0.40) were recrystallized from anhydrous ethanol to give 110 mg (5 %) of *3c* as brown needles, m.p. 191 – 192 °C. UV $\lambda_{max}(\varepsilon)$: 646 (55), 588 (300), 536 (620), 501 (780), 469 (750), 391 (9100), 292 sh (3200), 279 sh (4700), and 251 nm (12 700). NMR ($CDCl_3$): δ 1.31 (3 H) and 4.27 (2 H) [ester group], 2.03 (3 H, Me-4), 7.10 (1 H, H-2), 8.03 (1 H, H-7). MS: M^+ found 262.052 ± 0.002; $C_{11}H_{10}N_4O_2{}^{32}S$ requires 262.0524. $(M+2)^+$ found 264.049 ± 0.002; $C_{11}H_{10}N_4O_2{}^{34}S$ requires 264.0482, *m/e*

(relative intensity): 264 (5), 263 (10), 262 (73), 217 (18), 191 (12), 190 (100), 189 (21), 162 (8), 150 (7), 149 (6), 111 (5), 104 (6), 85 (13), 77 (12), 59 (23). *m/2e* (relative intensity): 108.5 (5.5), 94.5 (2).

8-Carbethoxy-4-methyl-5-selena-1,3,6-triazacycl[3.2.3]azine, 3d. To a stirred suspension of selenourea (2.46 g; 20.0 mmol) in 10 ml of anhydrous ethanol under nitrogen was added a solution of α-chloropropionitrile (1.79 g; 20.0 mmol) in 20 ml of anhydrous ethanol and then sodium iodide (3.00 g; 20.0 mmol). The mixture was kept at 70 °C for 4 h and then at room temperature for 10 h. The ethanol was evaporated and the residue was dissolved in 100 ml of water, sodium bicarbonate (1.68 g; 20.0 mmol) was added, and then immediately *2* (3.38 g; 20.0 mmol) dissolved in 50 ml of ethanol. The reaction mixture was treated as described for *3c* and 60 mg (1 %) of *3d* (R_F = 0.40) was obtained as brown needles, m.p. 163 – 164 °C. UV $\lambda_{max}(\varepsilon)$: 650 (70), 590 (370), 542 (750), 504 (920), 467 (970), 397 (7900), 290 sh (3100), 279 sh (5000), 248 sh (13200) and 236 nm (14 300). NMR ($CDCl_3$): δ 1.28 (3 H) and 4.23 (2 H) [ester group], 2.10 (3 H, Me-4), 7.05 (1 H, H-2), 7.92 (1 H, H-7). MS: M^+ found 307.996 ± 0.003 and 309.996 ± 0.003; $C_{11}H_{10}N_4O_2$ ^{78}Se requires 307.9977 and $C_{11}H_{10}N_4O_2$ ^{80}Se requires 309.9969. *m/e* (relative intensity): 312 (7), 311 (5), 310 (40), 309 (3), 308 (19), 307 (8), 306 (8), 240 (16), 239 (12), 238 (84), 237 (20), 236 (43), 235 (21), 234 (19), 182 (33), 158 (43), 149 (21), 137 (52), 110 (29), 95 (41), 86 (63), 84 (100), 77 (55). *m/2e* (relative intensity): 132.5 (2), 131.5 (1), 113.5 (1).

Decarbethoxylation of 3c to 4c. A suspension of *3c* (100 mg) in 5 ml of diphenyl ether was heated to 240 °C. At that temperature *p*-toluenesulfonic acid (200 mg) was added, the solution left for 25 min and then allowed to cool to room temperature. The dark solution was passed over a column of silica gel (50 g), and the diphenyl ether was washed out with petroleum (b.p. 60 – 85 °C). The coloured material was eluted with ethyl acetate – methanol. From these fractions, 15 mg (21 %) of *4c* (R_F = 0.27), brown crystals with m.p. 145 – 146 °C, was isolated by preparative TLC, UV $\lambda_{max}(\varepsilon)$: 656 (90), 590 (390), 544 (690), 505 (730), 470 sh (590), 357 (6000), 293 (3500), 282 (4900), 257 (13 000), and 250 nm (13 500). NMR ($CDCl_3$): δ 1.94 (3 H, Me-4), at 6.80 (1 H, H-2), 7.30 (1 H, H-7), 5.40 (1 H, H-8); $J_{7,8}$ = 6 Hz. MS: M^+ found 190.032 ± 0.002; $C_6H_6N_4$ ^{32}S requires 190.0313. $(M+2)^+$ found 192.027 ± 0.002; $C_6H_6N_4$ ^{34}S requires 192.0271. *m/e* (relative intensity): 192 (5), 191 (18), 190 (100), 189 (70), 162 (20), 157 (28), 111 (17), 110 (18), 85 (40), 70 (20), 59 (20), 52 (17). *m/2e* (relative intensity): 95.5 (1), 94.5 (0.5), 81.5 (1).

Decarbethoxylation of 3d to 4d. Compound *3d* (20 mg) was decarbethoxylated in 1 ml of diphenyl ether in the presence of *p*-toluenesulfonic acid (40 mg) at 240 °C and purified as described for *3c* to give 3 mg (19 %) of *4d* (R_F = 0.27) as brown crystals m.p. 172 – 174 °C. UV λ_{max} (ε): 663 (80), 603 (400), 551 (730), 510 (780), 475 (630), 378 (7400), 366 (7200), 292 (3600), 282 (4900), 244 (15 800), and 236 nm (16 300). NMR ($CDCl_3$): δ 2.02 (3 H, Me-4), 6.79 (1 H, H-2), 7.25 (1 H, H-7), 5.51 (1 H, H-8) $J_{7,8}$ = 6 Hz. MS: M^+ found 235.976 ± 0.003 and 237.975 ± 0.003; $C_6H_6N_4$ ^{78}Se requires 235.9765 and $C_6H_6N_4$ ^{80}Se requires 237.9757. *m/e* (relative intensity): 240 (19), 239 (17), 238 (100), 237 (40), 236 (50), 235 (35), 234 (25), 210 (11), 185 (17), 158 (30), 157 (14), 156 (12), 133 (26), 106 (14), 79 (14), 52 (28). *m/2e* (relative intensity): 119.5 (0.5), 117.5 (0.5), 105.5 (0.5).

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Mass Spectrometric Studies of 1,6,6aλ^4-Trithiapentalenes Bearing Functional Groups or Alkyl Substituents

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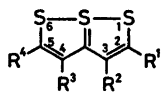
The fragmentation taking place upon electron impact of benzoyl substituted 1,6,6aλ^4-trithiapentalenes is strongly dependent on the substitution pattern. The loss of a 2-benzoyl group gives rise to a highly stable ion, whereas the stability of the ion formed by loss of a 3-benzoyl group is lower as seen from the more pronounced fragmentation. This difference is not observed in alkyl or methoxycarbonyl substituted trithiapentalenes.

The geometry of the 1,6,6aλ^4-trithiapentalene nucleus is strongly dependent on substitution as indicated by CNDO calculations [1] and observed experimentally in X-ray studies.[2,3] Furthermore it is known from chemical evidence, *e.g.* 3-amino-1,6,6aλ^4-trithiapentalenes show basic properties in contrast to the 2-amino-compound, which is acidic rather than basic,[4] that the 3 and 4 positions are more electron deficient than the 2 and 5 positions although this difference is not reflected in *ab initio* calculations.[5] Accordingly the fragmentation upon electron impact of a series of previously studied aryl substituted trithiapentalenes [6] was found to be dependent on the substitution pattern. However, significant differences which primarily may be ascribed to the influence of substitution and electron distribution on the fragmentation patterns of 2- and 3-monophenyl substituted isomers, are smaller than might be anticipated. The predominant difference, the elimination of the phenyl group from the molecular ions, takes place preferentially in the case of 2-substituted compounds, giving rise to an $[M-C_6H_5]^+$ ion of 20 % (in the case of the 3-substituted compound only 3 %).



X-Ray studies [7] have shown that the introduction of functional groups in the trithiapentalene nucleus gives rise to a higher degree of asymmetry than observed with simple substituents. Therefore it was found of interest to study whether this was reflected in the electron impact induced fragmentation of trithiapentalenes with various groups in the 2 or 3 position.

RESULTS

Three groups of compounds were studied. 1. Benzoyl substituted trithiapentalenes. 2. Alkoxycarbonyl substituted trithiapentalenes. 3. Methyl substituted trithiapentalenes.

1. Benzoyl substituted trithiapentalenes

	R^1	R^2	R^3	R^4
I	COC_6H_5	H	H	H
II	H	COC_6H_5	H	H
III	COC_6H_5	H	H	C_6H_5
IV	H	COC_6H_5	H	C_6H_5
V	COC_6H_5	C_6H_5	H	C_6H_5
VI	C_6H_5	COC_6H_5	H	C_6H_5

* Author to whom correspondece should be addressed.

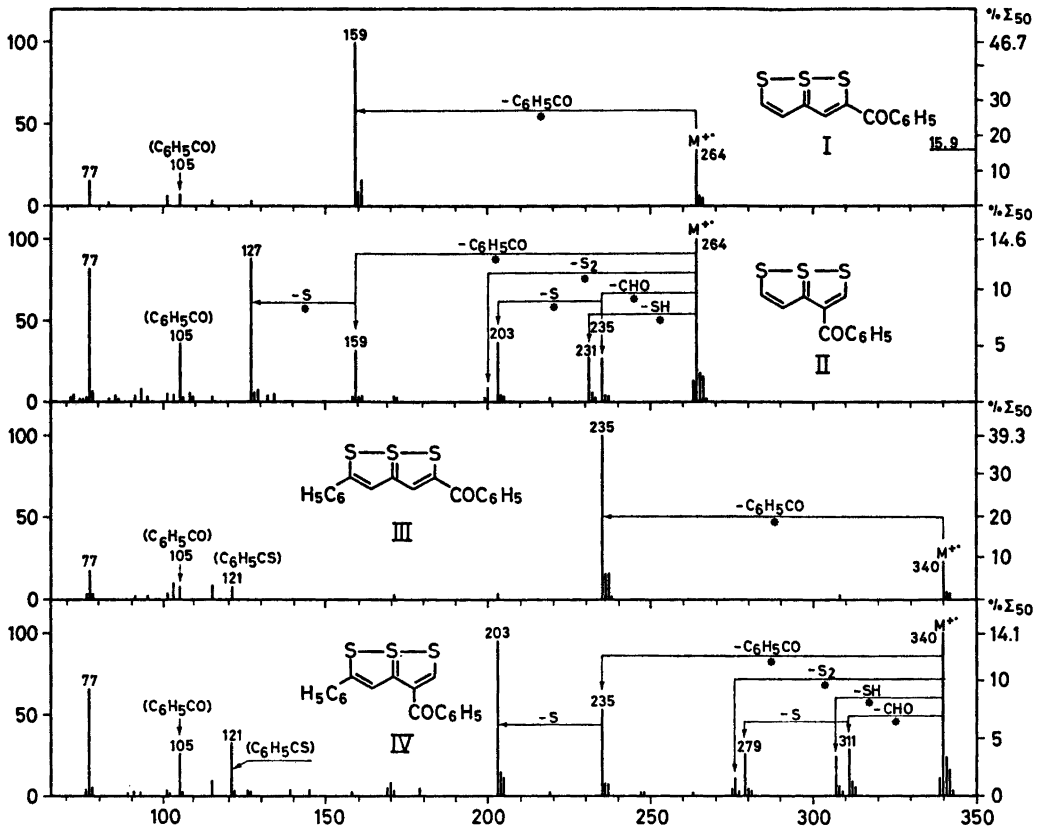


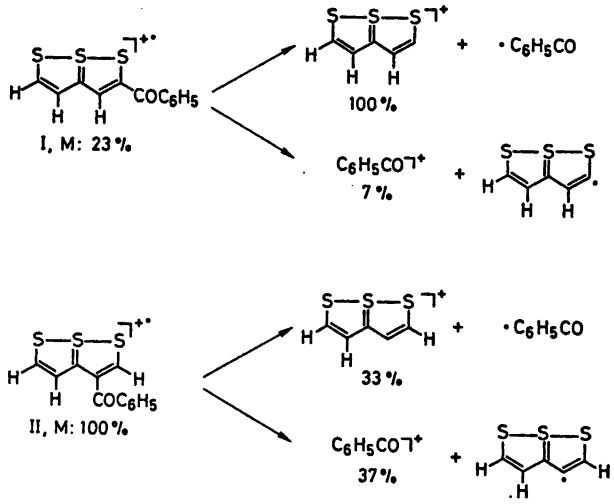
Fig. 1. MS of compound I–IV.

If the phenyl group is replaced by a benzoyl group the position dependent differences in the fragmentation pattern are increased drastically, *cf.* Fig. 1. The only fragmentation mode of importance for I is loss of the 2-benzoyl group with charge retention preferentially on the trithiapentalene moiety yielding the base at *m/e* 159. The corresponding $[M-C_6H_5CO]^+$ ion possesses a considerable stability carrying 47 % of the total ion current (Σ_{50}).

The corresponding loss of the 3-benzoyl group also takes place in the fragmentation of II. However, in this case charge localization on the benzoyl moiety competes effectively giving rise to a $[C_6H_5CO]^+$ ion (*m/e* 105) of relatively high abundance (Scheme 1). The $[M-C_6H_5CO]^+$ ion is readily decomposed by elimination of a sulfur atom and is only responsible for 5 % of Σ_{50}.

In addition, other marked differences between I and II exist. Loss of ·CHO, requiring a skeletal rearrangement, followed by elimination of a sulfur atom takes place only from the molecular ion of II. The same difference was observed for the losses of ·SH and S_2 (Fig. 1).

By additional phenyl substitution in the trithiapentalene nucleus it is found that these differences (except for the ·SH and S_2 losses, not observed in the spectrum of VI) are repeated and thus primarily may be due to the position of the benzoyl group. In no case is direct elimination of a phenyl group from the molecular ion observed.



Scheme 1.

2. Alkoxycarbonyl substituted trithiapentalenes

	R^1	R^2	R^3	R′
VII	CO_2CH_3	H	H	H
VIII	H	CO_2CH_3	H	H
IX	CO_2CH_3	H	H	C_6H_5
X	H	CO_2CH_3	H	C_6H_5
XI	CO_2CH_3	C_6H_5	H	H
XII	C_6H_5	CO_2CH_3	H	H
XIII	$CO_2C_2H_5$	H	H	C_6H_5
XIV	H	$CO_2C_2H_5$	H	C_6H_5
XV	CO_2CH_3	C_6H_5	H	C_6H_5
XVI	C_6H_5	CO_2CH_3	H	C_6H_5

The influence of the alkoxycarbonyl group on the fragmentation mode is completely different from that of the benzoyl group and none of the differences found between 2- and 3-benzoyl substituted compounds are reproduced with correspondingly substituted alkoxycarbonyl trithiapentalenes as seen from Fig. 2 where the spectra of compounds VII and VIII are given.

The predominant fragmentation process is also in this group of compounds elimination of the functional group. The resulting $[M-COOCH_3]^+$ ions in VII and VIII formally correspond to the $[M-C_6H_5CO]^+$ ions in the spectra of I and II, respectively. The $[M-COOCH_3]^+$ ions, however, in the case of VII loses a sulfur atom whereas the corresponding loss from $[M-C_6H_5CO]^+$ in I is not observed. The sulfur loss is considerably reduced in VIII as compared with II.

The position of the methoxycarbonyl group affects the fragmentation pattern in another way, however. Thus, instead of losing the OCH_3 group as observed for VIII, a hydrogen rearrangement with loss of CH_2O takes place in VII. CH_3CH_2O and CH_3CHO, respectively, are eliminated from the molecular ions of XIV and XIII. Apart from this difference, the fragmentation modes of the methyl and ethyl esters are analogous. Furthermore the spectrum of VIII exhibits a peak at *m/e* 158 not present in that of VII. It corresponds to the loss of a hydrogen atom from the $[M-CO_2CH_3]^+$ ion and since a corresponding ion is completely absent in the spectra of XII and XVI but present in that of X and XIV it is likely that the hydrogen lost in this process originates from the 2 position. Apart from this loss, additional phenyl substitution on the trithiapentalene nucleus in the spectrum of XI only, gives rise to different behaviour (Fig. 3). In this case, the loss of CH_2O from the molecular ion is completely absent. Instead, the loss of a hydrogen atom from the molecular ion gives rise to an abundant peak. This process is probably due to the proximity of the phenyl group and the carbonyl oxygen which enables the ejection of a phenyl hydrogen under formation of the following ion.

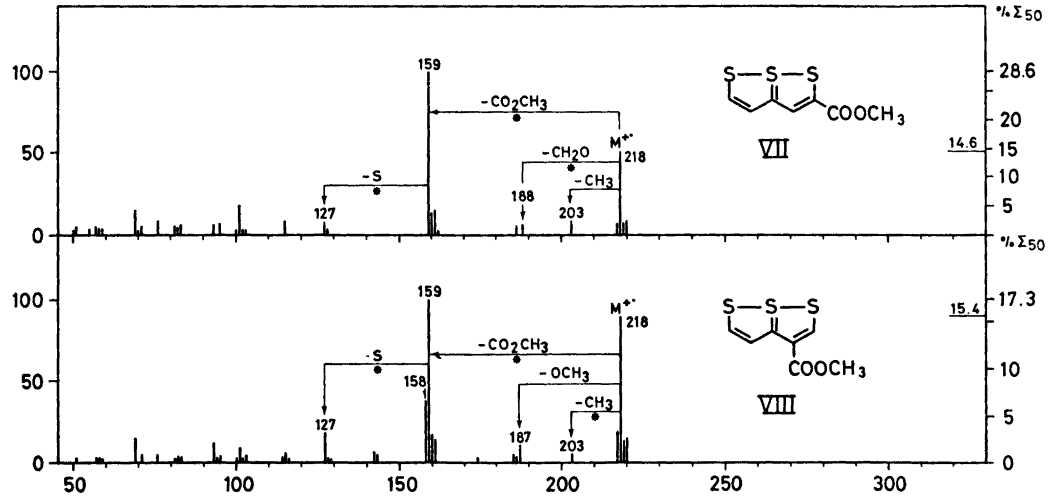


Fig. 2. MS of compound VII and VIII.

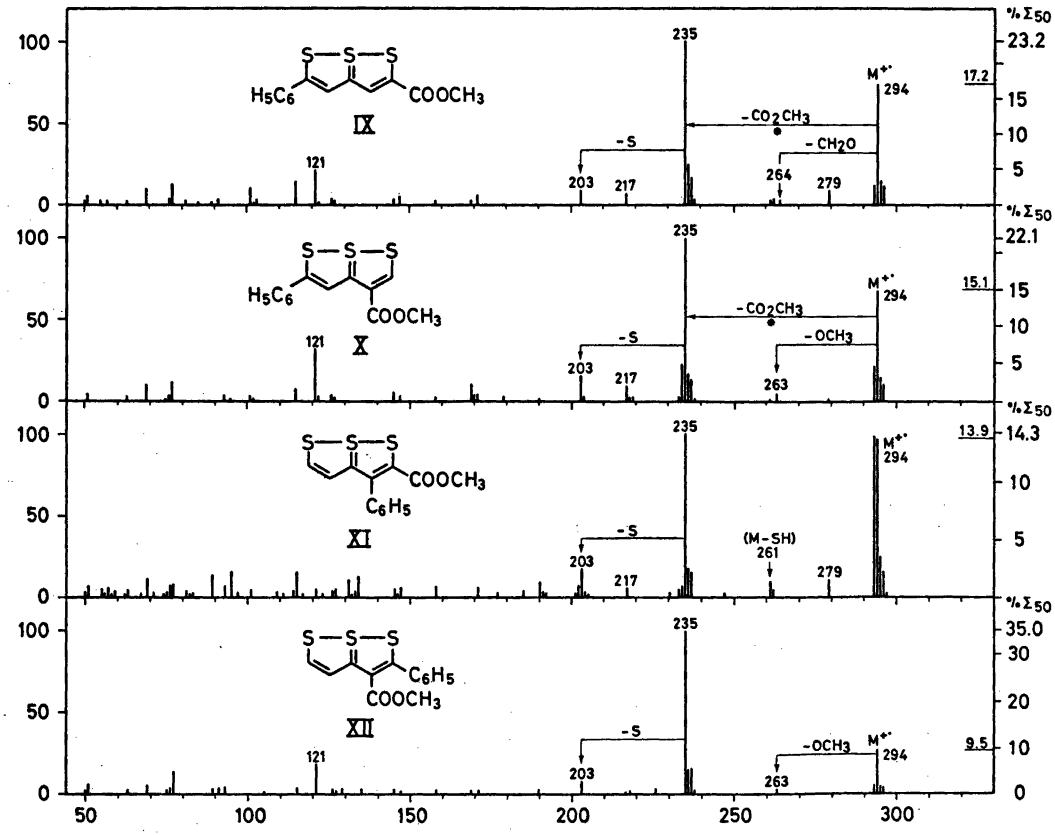
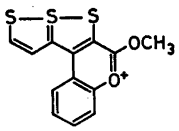


Fig. 3. MS of compound IX–XII.



An analogous ion is not formed from XII in accordance with the previous observation [6] that a phenyl group in position 2 or 5 does not participate in the expulsion of a hydrogen atom.

3. Methyl substituted trithiapentalenes

	R^1	R^2	R^3	R^4
XVII	CH_3	H	H	H
XVIII	H	CH_3	H	H
XIX	CH_3	H	H	CH_3
XX	H	CH_3	CH_3	H
XXI	CH_3	H	H	C_6H_5

The fragmentation patterns of these compounds (Fig. 4) are similar to those of the corresponding phenyl substituted compounds.[6] Ions corresponding to losses of a hydrogen atom and of ·SH from the molecular ions are characteristic features for both groups of compounds.

However, the difference in ability for elimination of the substituent from the 2 and 3 position is much less pronounced. While *m/e* 159 corresponding to $[M-C_6H_5]^+$ in the mono phenyl substituted compounds was 20 and 3 % for the 2 and 3 substituted isomer, respectively, the corresponding $[M-CH_3]^+$ ions appear with abundance of 52 and 33 % in the spectra of XVII and XVIII, respectively.

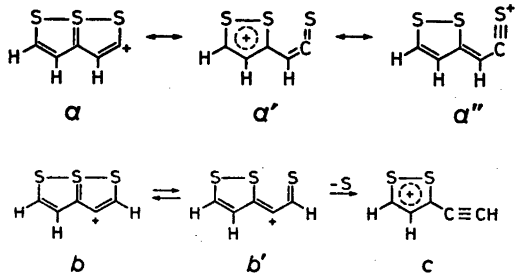
The resemblance of the influence of a methyl and a phenyl group in the 2-position on the fragmentation modes of the molecular ions is clearly demonstrated in the spectrum of XXI. The $[M-C_6H_5]^+$ ion (*m/e* 173) and the $[M-CH_3]^+$ ion give rise to peaks with approximately the same abundances. (*Ca.* 10 % of *m/e* 173 corresponds to the loss of CS from the $[M-SH]^+$ ion in accordance with the behaviour of XVII to XX). Furthermore the peaks corresponding to $[C_6H_5CS]^+$ and $[CH_3CS]^+$ appear with similar abundances.

DISCUSSION

Not only was the behaviour of the molecular ions found to vary with the substituents and their positions but also the further fragmentation of formally identical fragment ions such as the trithiapentalenyl ions *a* and *b* formed by elimination of the substituents were found to be strongly dependent on the original substituent pattern of the molecular ions.

These differences in behaviour might be explained in terms of different amounts of excess energies for *a* and *b* in the various spectra. Thus *b* might for some reason be generated with a large amount of excess energy in case of II as compared with VIII and XVIII, and *a* with a minimum of excess energy in I relative to VII and XVII. However, this explanation did not find any support in the low voltage spectra, since the characteristic differences observed at 70 eV were maintained even at the lowest obtainable ionizing energy.

An alternative explanation of the differences in fragmentation can be expressed in terms of resonance stabilization of *a* and *b* (Scheme 2). Thus, *a* is stabilized by the resonance ($a \leftrightarrow a' \leftrightarrow a''$) without further fragmentation whereas this mode of stabilization is not open in the case of *b*. Additional loss of a sulfur atom therefore appears to be more likely from *b* (to yield *c*) than from *a*. The very high selectivity between the 2 and 3 position in the case of benzoyl substituents can be explained by the assumption that this substituent favours the thiocarbonyl form *b'* and thus facilitates the loss of sulfur, when other modes of stabilization are not open.



Scheme 2.

EXPERIMENTAL

The mass spectra were obtained on an MS 902 mass spectrometer using the direct sample insertion system with the lowest feasible ion source temperature. 70 eV electrons were used. Peaks corresponding to doubly charged ions

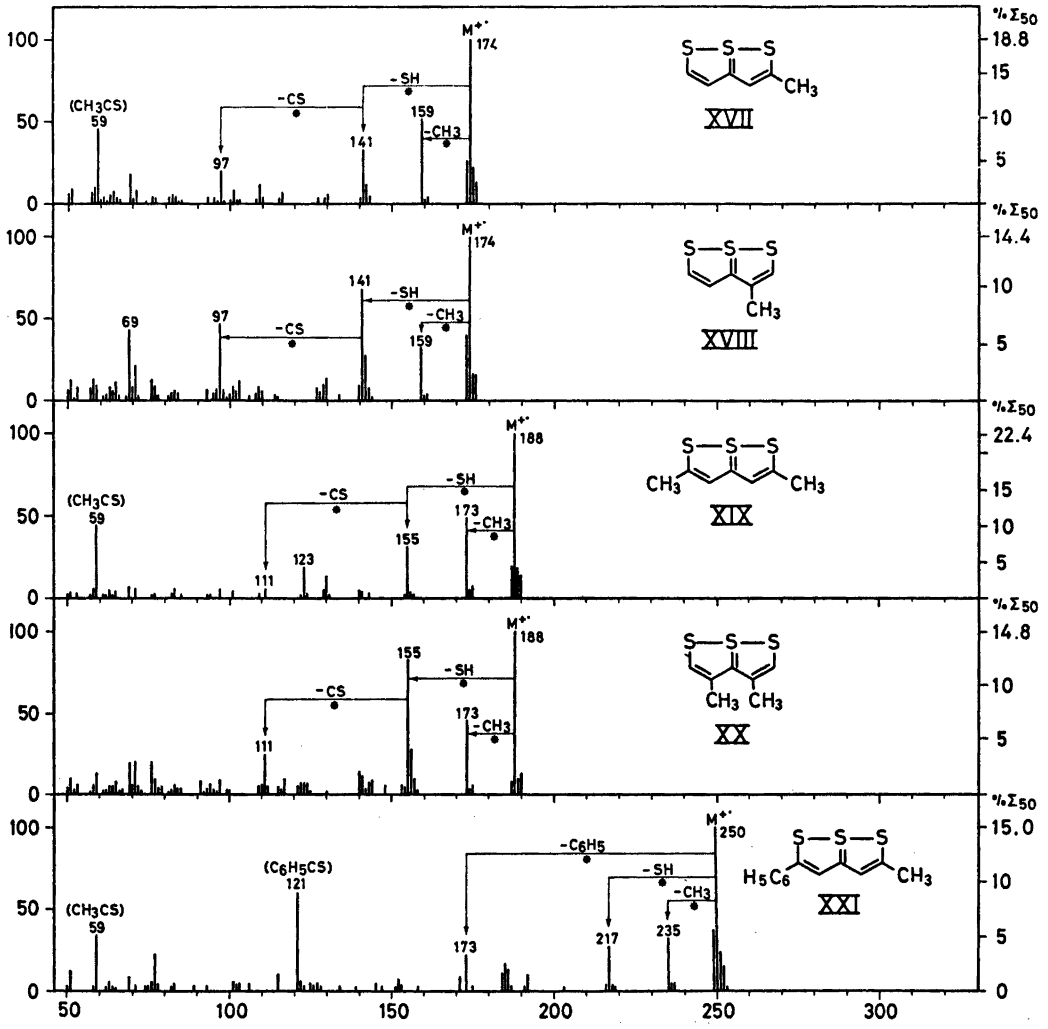


Fig. 4. MS of compound XVII–XXI.

appearing at half mass numbers as well as peaks of abundance lower than 2 % were omitted from the spectra shown.

Benzoyl, alkoxycarbonyl and monomethyl substituted 1,6,6aλ⁴-trithiapentalenes were prepared as described by Davy [8] from 1,2-dithiole-3-thiones and acetylenes with subsequent isomerization.

2,5-Dimethyl-1,6,6aλ⁴-trithiapentalene was prepared according to Arndt *et al.*[9]

3,4-Dimethyl-1,6,6aλ⁴-trithiapentalene was prepared according to Dingwall *et al.*[10]

2-Methyl-5-phenyl-1,6,6aλ⁴-trithiapentalene was prepared according to Pedersen.[11]

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The Electrochemistry of Organic Sulfur Compounds Part VI.[1] The Anodic Dimerization of α-(1′, 2′-Dithiol-3′-ylidene)-acetophenones

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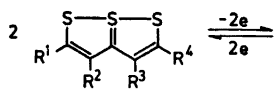
A series of aryl substituted α-(1′,2′-dithiol-3′-ylidene)acetophenones, *1*, has been examined by voltammetric and exhaustive electrochemical techniques. One electron oxidation of compounds *1* was accompanied by the formation of the corresponding dimeric dications, *2*, which were not capable of undergoing further electrochemical oxidation. Reaction of *2* with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) resulted in hydrogen abstraction and formation of a new dication, *4*, which upon electrochemical reduction gave the uncharged dimer of *1*, the bi[α-(1′,2′-dithiol-3′-ylidene)-phenacyl], *3*. The effect of the nature and degree of substitution on the reaction is discussed. By reaction with P_2S_5, the dimers, *3*, could be converted to the corresponding dimeric 1,6,6aλ⁴-trithiapentalenes, *5*.

In part III of this series we reported the reversible electrochemical interconversion of several 1,2-dithiol-3-thiones.[2] Anodic oxidation resulted in formation of dimeric dications, linked through a disulfide bond, from which the starting materials could be regenerated by reduction. A similar reversible process was observed for a series of 1,6,6aλ⁴-trithiapentalenes, *6*, during the oxidation of which *7* were formed.[3] We have now extended the study to include the structurally related α-(1′,2′-dithiol-3′-ylidene)-acetophenones, *1*, and this paper deals with the results obtained by anodic oxidation of compounds having an unsubstituted α-position. Compounds *1* can be prepared



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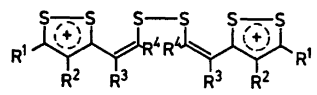

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by oxidative desulfuration of *6*,[4] but further oxidation of the dithiolylidene ketones has not been observed. We now report the non-destructive anodic oxidation of a series of aryl substituted compounds *1*.

RESULTS

For convenience of reference, the structures of the compounds studied are tabulated in Table 1.

Voltammetry of α-(1′,2′-dithiol-3′-ylidene)-acetophenones, 1. All of the compounds showed essentially the same voltammetric behavior in acetonitrile or the mixed solvent acetonitrile/dichloromethane (1/1) containing sodium perchlorate as supporting electrolyte (0.1 M). The voltammogram of *1f* in the mixed solvent is illustrated in Fig. 1. On the anodic sweep an irreversible oxidation peak, O_1, was observed

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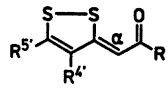
Table 1. Structures of α-(1′,2′-dithiol-3′-ylidene)acetophenones, *1*.



Structure[a]	R5′	R4′	R
1a	Phenyl	H	Phenyl
1b	*p-tert*-Butylphenyl	H	Phenyl
1c	*p*-Methoxyphenyl	H	Phenyl
1d	H	Phenyl	Phenyl
1e	H	*p*-Methylphenyl	Phenyl
1f	Phenyl	Phenyl	Phenyl
1g	Phenyl	Phenyl	*p*-Bromophenyl

[a] The same letters, *a*–*g*, are used for the corresponding substitution in compounds *2*–*5*.

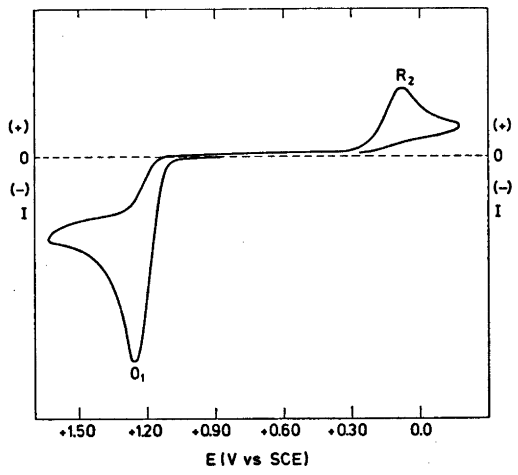


Fig. 1. Cyclic voltammetry of *1f* in acetonitrile/dichloromethane (1/1) containing sodium perchlorate (0.1 M). Sweep-rate = 86 mV/s.

at +1.26 V.* On the reverse sweep a reduction peak, R_2, appeared at +0.18 V. An increase of the sweep-rate from 86 mV/s to 20 V/s did not bring about any significant change in this picture. The voltammetric data for all the compounds are tabulated in Table 2.

When the voltammetry of the compounds *1a*–*1c* was carried out in dichloromethane containing Bu_4NBF_4 (0.25 M) as supporting electrolyte, slightly different behavior was observed. This is illustrated by the voltammogram of *1a* in Fig. 2. The reduction peak R_2 was barely visible (not shown in Fig. 2) and instead a redox couple, R_3–O_3, appeared at +0.72 V (R_3) and +0.96 V (O_3).

Exhaustive electrolysis of 2 mM solutions (50 ml) of *1d*–*1g* in the acetonitrile/dichloro-

* All peak potentials refer to the aqueous saturated calomel electrode (SCE).

Table 2. Voltammetric data for α-(1′,2′-dithiol-3′-ylidene)acetophenones, *1*, bi[α-hydro-α-(1′,2′-dithiol-3′-ylio)phenacyl]s, *2*, and bi[α-(1′,2′-dithiol-3′-ylidene)phenacyl]s, *3*.

Structure	E_{O_1}[a]	Structure	E_{R_2}[a]	Structure	E_{O_3}[b]	E_{R_3}[b]
1a	+1.24	*2a*	−0.14	*3a*	+0.96	+0.72
1b	+1.22	*2b*	−0.19	*3b*	+0.97	+0.61
1c	+1.20	*2c*	−0.30	*3c*	+0.90	+0.63
1d	+1.24	*2d*	+0.31	*3d*	+0.88	+0.60
1e	+1.25	*2e*	+0.27	*3e*	+0.87	+0.52
1f	+1.26	*2f*	+0.18	*3f*	+0.89	+0.45
1g	+1.27	*2g*	+0.15	*3g*	+0.94	+0.49

[a,b] Peak potentials in V *vs.* SCE measured at a platinum button electrode at the sweep-rate 86 mV/sec. [a] Solvent = acetonitrile/dichloromethane (1/1) containing sodium perchlorate (0.1 M). [b] Solvent = dichloromethane containing Bu_4NBF_4 (0.25 M). Substrate conc. = 1.0×10^{-3} M.

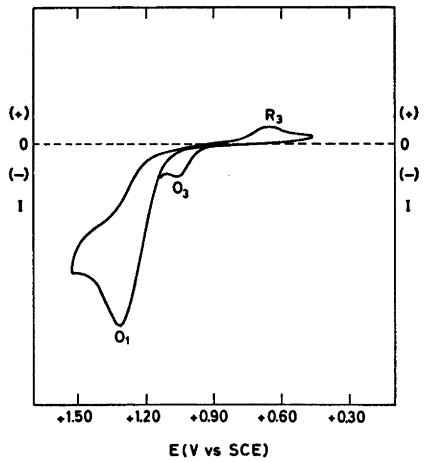


Fig. 2. Cyclic voltammetry of *1a* in dichloromethane containing Bu_4NBF_4 (0.25 M). Sweep-rate = 86 mV/s.

methane solvent mixture, using constant current coulometric techniques,[5] demonstrated that one F/mol was transferred resulting in species which reduce at R_2. When $R^{4'}=H$ (*1a–1c*) coulometric *n*-values ranging from 1.0 to 1.8 were found. Voltammetry of the resulting solutions showed the presence of a mixture of the compounds responsible for R_2 and R_3. *1d–1g* could be regenerated by coulometric reduction (~1 F/mol) in nearly quantitative yields as judged from the corresponding peak currents. However, when $R^{4'}=H$ the products connected with O_3 were present as well.

Preparative anodic oxidation of α-(1',2'-dithiol-3'-ylidene)acetophenones. Constant current oxidation of *1* on a millimolar scale in acetonitrile/dichloromethane (1/1) containing sodium perchlorate as supporting electrolyte (0.1 M) gave products dependent on the substitution in the 4'-position of the starting material as expected from the voltammetric experiments. For *1a–1c* mixtures of the corresponding *2* and *3* were obtained, the product ratio varying with both the nature of $R^{5'}$ and the solvent composition. When $R^{4'}$ = aryl quantitative formation of *2* as the perchlorate salt was observed.

Attempts to deprotonate *2d–2g* in order to obtain *3d–3g* were unsuccessful, as were attempts to electrochemically oxidize *2d–2g* to *4d–4g*. However, the latter oxidation was found to take place when 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was added to the voltammetric solutions and *4* were obtained in good yields. Compounds *3* could now be prepared by electrochemical reduction with consumption of one F/mol (corresponding to starting material). By following this procedure, increased yields of *3a–3c* were also obtained. Isolated yields of the dimers are given in Table 3 as well as melting points and visible spectral data.

The dimers, *3*, are coupled through the α-positions. This was shown by an experiment with the C_6D_5 analogue of *1d*, *1d*(d_{10}). The ^1H NMR spectrum of this compound consisted of only two peaks, H(5'), δ 7.78 and H(α), δ 7.30. Preparative oxidation according to the procedure described above resulted in the isolation of a product, the ^1H NMR spectrum of which showed only the presence of a single peak at δ 7.87 consistent with the structure *3d*(d_{10}).

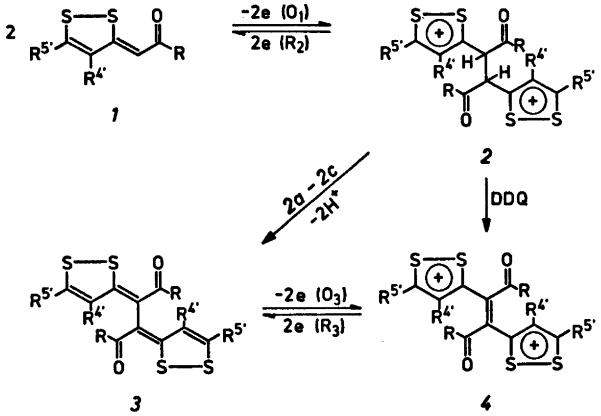


Table 3. Isolated yields, melting points and visible spectral data for bi-[α-(1′,2′-dithiol-3′-ylidene)phenacyl]s, *3*.

Structure	Yield[a]/%	M.p./°C	λ_{max}/nm[b]	ε[b]
3a	39	266–267	473	31 900
3b	37	205–206	472	34 000
3c	28	248–249	472	36 000
3d	68	299–300	467	26 100
3e	49	283–284	473	29 000
3f	54	282–283	475	27 100
3g	49	303–304	483	32 000

[a] After purification by chromatography (see experimental section). [b] In dichloromethane.

Voltammetry of bi[α-(1′,2′-dithiol-3′-ylidene)-phenacyl]s. The voltammetric measurements on all of the dimers, *3*, gave very similar results, and will only be described for one model compound, *3f*. The cyclic voltammogram in dichloromethane containing Bu_4NBF_4 (0.25 M) as supporting electrolyte is shown in Fig.3. During the anodic scan a single irreversible peak, O_3, was observed at +0.89 V. A cathodic counterpart, R_3, at +0.45 V appeared when the direction of the scan was changed. Voltammetric data for all the dimers are listed in Table 2. Constant current coulometry demonstrated that two electrons per molecule were transferred.

The resulting solution showed no signs of instability even after standing for several hours. Reduction back to starting material required two F/mol, and comparison of the height of peak O_3 before and after the experiment showed that *3f* was regenerated in 97 % yield. It was not possible to detect the presence of any intermediate cation radicals during this procedure.

The identity of the products from coulometric oxidation was supported by oxidation of *3e* by $SbCl_5$. The resulting compound showed satisfactory analysis for a dication salt, *4e*, $Sb_2Cl_{12}^{2-}$, and the voltammetric behavior was indistinguishable from that of the solution from coulometric oxidation.

Reaction of bi[α-(1′,2′-dithiol-3′-ylidene)-phenacyl]s with P_2S_5. The compounds *1* can be transformed into 1,6,6aλ^4-trithiapentalenes, *6*, upon reaction with phosphorus pentasulfide.[4,6] The reaction of *3a* with P_2S_5 resulted in formation of the corresponding dimeric trithiapentalene, *5a*. Such compounds have hitherto been unknown. The structure of *5a* was supported by mass spectrometry and visible spectroscopy.

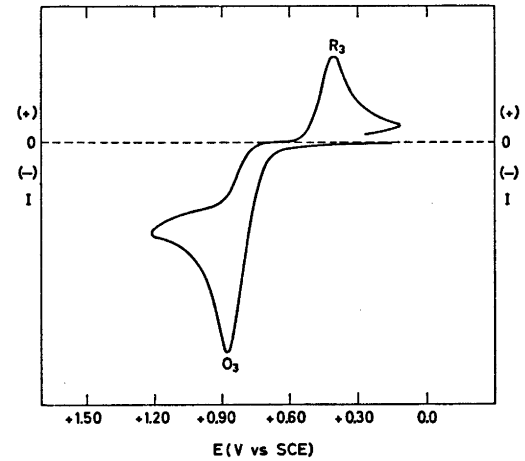
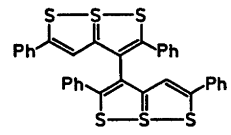


Fig. 3. Cyclic voltammetry of *3f* in dichloromethane containing Bu_4NBF_4 (0.25 M). Sweep-rate = 86 mV/s.



5a

DISCUSSION

Scheme 2 explains the experimental results.

The voltammetric and coulometric oxidation of *1* is consistent with a one-electron charge transfer to form the cation radicals $1^{+\cdot}$, which dimerize to the dications *2*. Under conditions

where *2* are not consumed in further reactions, reduction regenerates *1*. This type of voltammetric behavior has earlier been reported for other reactions including the anodic oxidation of 1,2-dithiole-3-thiones [2] and 1,6,6aλ^4-trithiapentalenes [3] and the cathodic reduction of 1,2-dithiolylium ions.[1,7]

The peak potentials of *1* vary surprisingly little with the degree of substitution and the nature of the substituents (Table 2). Substitution of phenyl by the electron donating *p*-methoxyphenyl at C(5′) facilitates oxidation by 40 mV (*1a* and *1c*) and introduction of an extra phenyl group at C(4′) results only in a 20 mV increase of the peak potential (*1a* and *1f*). For comparison the corresponding changes observed for the trithiapentalene series were 140 mV and 290 mV, respectively.[3] Since the molecular geometry of *1* is very similar to that of *6*, the reason for the observed differences has to be sought in the electronic structure of the two types of molecules. The presence of one oxygen and two sulfur atoms in *1* instead of three sulfur atoms as in *6* makes the distribution of charge more uneven. Dipole moments [8] and ESCA spectra [9] suggest a strong contribution of mesoionic forms like *8a*, and from studies of the carbonyl vibration in ^{18}O enriched compounds it was found that the contribution of ketonic forms in some cases is as low as 27 %.[10] It is also believed that the observed oxidation potentials reflect this dipolar structure, the nearly constant values being associated with

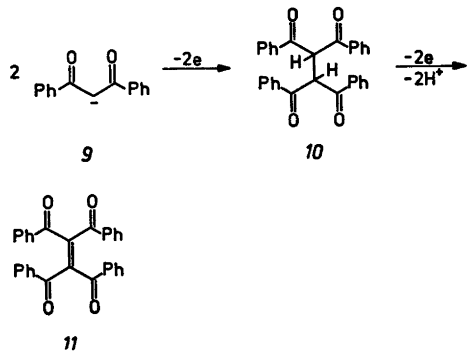
8a *8b*

the high electron density in the oxygen containing part of the molecule (*8a* – *8b*). Substitution in the dithiole ring does not affect the polarity of the molecule significantly as measured from dipole moments.[8,11]

Considering now the reduction potentials of *2* the figures in Table 2 indicate that ions *2a* – *2c* are more difficultly reduced than ions carrying an aryl group at C(4′), *2d* – *2g*. The peak potentials found for *2a* – *2c* (approximately −0.20 V) are close to those observed (approximately −0.30 V) for the analogous 3,5-diaryl-1,2-dithiolylium salts.[1] Molecular models of *2* show that the presence of large substituents in the 4′-position causes considerable steric interactions, which could be released by reduction to *1*. Thus, the anodic shift in peak potentials observed for *2d* – *2g* can be rationalized from the effect of steric acceleration of the bond cleavage resulting in a kinetic shift of the reduction potential.

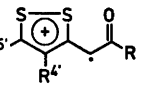
The reactivity of *2* is likewise significantly dependent on the nature of R$^{4'}$. Where R$^{4'}$ = aryl, dications, stable on the time scale of coulometry, were formed which only very slowly reverted to starting materials in an unknown redox process. When R$^{4'}$ = H, *2* deprotonated to *3* in a solvent dependent chemical step. The lack of reactivity of *2* substituted at C(4′) can be explained on steric grounds. The preferred conformation of *3* seems to be one in which the two planar halves of the molecule are twisted around the C(α) – C(α) bond. This is supported by the observation that λ_{max} of *3* (Table 3) are only approximately 17 nm higher than λ_{max} for *1*,[12] indicating little or no increase in conjugation. However, substitution at C(4′) effectively prevents a transition state involving planarity of the α-(1′,2′-dithiol-3′-ylidene)acetophenone ring system resulting in increased stability of *2*. The formation of *3* directly from *2* was only sufficiently rapid in dichloromethane to be observed by voltammetry. In the mixed solvent and in acetonitrile the only observable peak after changing the direction of the sweep was R_2. It is believed that the lower reactivity of *2* in the polar solvents reflects the better solvation of positive ions in these systems. In non-polar dichloromethane the uncharged *3* are favored.

The possibility of transforming the products from preparative electrolysis to dimeric 1,6,6aλ^4-trithiapentalenes, *5*, is only consistent with coupling between carbon atoms, and the actual position was shown by anodic oxidation of *1d*(d_{10}), the product of which, *3d*(d_{10}), was demonstrated to be a C(α) – C(α) dimer by ^1H NMR spectroscopy. Coupling through this position parallels the observation by VandenBorn and Evans,[13] who recently reported the formation of C(α) – C(α) dimers, *10* – *11*, from exhaustive oxidation of the enolate of dibenzoylmethane, *9*, which is analogous to the resonance structure *8b* for *1*.



9 10 11

The coupling took place *via* the free radical corresponding to *9*, and similarly a resonance structure like *12* for 1^+ plays an important role in determining the nature of the resulting products.



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EXPERIMENTAL

General procedures, purification of solvents and supporting electrolytes and apparatus for voltammetry and coulometry have been described earlier.[5,14]

^{1}H NMR spectra were recorded on a Varian model A 60 spectrometer, mass spectra on a MS 902 spectrometer and visible spectra on a Bechman Acta III spectrometer.

α-(1',2'-Dithiol-3'-ylidene)acetophenones, 1, were prepared by conventional methods.[15–17]

Anodic oxidation of α-(1'2'-dithiol-3'-ylidene)-acetophenones, 1. The compound (1 mmol) to be oxidized was dissolved in acetonitrile/dichloromethane, 1/1, (60 ml), containing sodium perchlorate (0.1 M) as supporting electrolyte. This solution was subjected to constant current oxidation (50 mA) in a two-compartment cell kept at room temperature. The anode was a platinum gauze electrode and the cathode a platinum wire. When the calculated amount of current had been passed through the cell (32.2 min required for one F/mol at 50 mA) the electrolysis was stopped, and DDQ (0.6 mmol) was added together with sodium carbonate (250 mg). The solution was allowed to stand overnight after which it was dark red colored. Constant current reduction (50 mA) was now performed for another 32.2 min. The resulting solution was evaporated to near dryness in *vacuo* and then diluted with dichloromethane (200 ml) in which the supporting electrolyte is insoluble. This solution was washed several times with water, aqueous sodium carbonate and again water followed by drying over sodium sulfate. After evaporation of the solvent the residue was chromatographed on neutral alumina (100 g) (Woelm W 200, 10 % water). Elution was started with ligroin/dichloromethane (10/1) and continued with increasing amounts of dichloromethane in the mixture ending with a 1/1-mixture (total approximately 900 ml). Small amounts of purple and yellow biproducts were eluted with the less polar solvent mixtures and finally the bi[α-(1',2'-dithiol-3'-ylidene)phenacyl]s, *3.* Some polymeric material and $DDQH_2$ were left on the column. Recrystallization was in general not necessary, but if very pure samples were wanted, *3* were recrystallized from 2-ethoxyethanol. Yields are given in Table 3. All compounds showed satisfactory elemental analysis. However, these data are not included because the monomers and the dimers have the same calculated analysis within the experimental error. Mass spectra showed in all cases the presence of M^+.

Anodic oxidation of α-(4'-pentadeuteriophenyl-1',2'-dithiol-3'-ylidene)pentadeuterioacetophenone, 1d(d_{10}).[17] The procedure was essentially that described above. *1d*(d_{10}) (82 mg, 0.268 mmol) was dissolved in acetonitrile/dichloromethane (2/3) (25 ml) containing sodium perchlorate (0.1 M) as supporting electrolyte. The time of oxidation (50 mA) required for 0.268 mmol was 8.63 min, and the amount of DDQ was reduced to 0.3 mmol. After standing over night and cathodic reduction, work-up was as described above. Chromatography on alumina (25 g) gave 42 mg of *3d*(d_{10}), (52 %) * ^{1}H NMR ($CDCl_3$): δ 7.87 (s).

Oxidation of bi[α-(4'-(p-methylphenyl)-1',2'-dithiol-3'-ylidene)phenacyl], 3e, by $SbCl_5$. Compound *3e* (13 mg) was dissolved in dichloromethane (1 ml). To this solution was added dropwise a solution of $SbCl_5$ in dichloromethane in excess. The resulting suspension was added to ether and filtered. The yellow precipitate was washed several times with ether and dried at +80 °C. (Found C 33.2; H 2.37; S. 9.95; Cl 31.9 Calc. for $C_{36}H_{26}O_2S_4Sb_2Cl_{12}$: C 33.5; H 2.03; S 9.89; Cl 33.4).

Reaction of bi[α-(5'-phenyl-1',2'-dithiol-3'-ylidene)-phenacyl], 3a, with P_2S_5. Compound *3a* (50 mg) was refluxed for 2h with P_2S_5 (200 mg) in toluene (50 ml). The toluene solution was dried over sodium sulfate and evaporated *in vacuo*. The resulting dark residue was chromatographed on alumina (MERCK neutral, 4 % water) (cyclohexane). Recrystallization from cyclohexane gave 25 mg of *5a*. Yield 47 %, m.p. 214–216 °C, $M^+ = 622$ (52 %), λ_{max} (dichloromethane) = 480 nm, $\varepsilon = 26\,000$.

* The yield is lower than that obtained for *3d* (68 %). This is probably due to the smaller amount of material taken into work.

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Ring-opening Reactions of Heterocyclic Organometallics. X.* Phenyl- and *N*,*N*-Dimethylaminomethyl-substituted 3-Thienyllithium Derivatives

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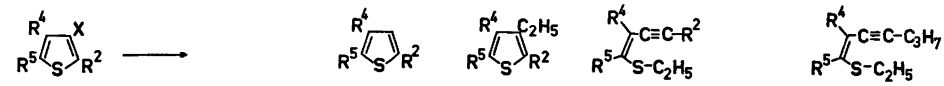
The usefulness of the ring-opening of phenyl substituted 3-thienyllithium derivatives for the synthesis of substituted alkylthiovinyl acetylenes is demonstrated. The stability of 2-*N*,*N*-dimethylaminomethyl-5-methyl-3-thienyllithium (*20*) towards ring-opening is ascribed to intramolecular chelation. On the other hand, 5-*N*,*N*-dimethylaminomethyl-2-methyl-3-thienyllithium smoothly ring-opened to give (*Z*)-1-(*N*,*N*-dimethylamino)-2-ethylthio-2-hexen-4-yne (*23*) in 50 % yield, upon ethylation. This gives additional evidence for the usefulness of this reaction for the preparation of otherwise difficultly accessible alkylthiovinyl acetylenes.

In recent papers, we have demonstrated that 2,5-dialkyl-3-thienyllithium derivatives ring-open at room temperature to lithium enynethiolates, while 2,5-dialkyl-3-selenienyllithium derivatives even at −70 °C undergo the corresponding reactions to lithium enyneselenolates.[1,2] We have also found that strong −I-substituents such as Cl or OCH_3 in the 2-position of 3-thienyllithium derivatives have a stabilizing influence so that ring-opening does not occur.[3] As mentioned previously,[3] intermolecular complexation of the organolithium derivative to the free electron pair of the −I-substituent could possibly also contribute to the stabilization of the above-mentioned 3-thienyllithium derivatives. We were therefore interested in studying the effect on ring-opening of a substituent which makes intramolecular chelation possible, and have chosen the dimethylaminomethyl group for this purpose.

With a weaker −I-substituent such as methylthio in the 2-position ring-opening took place.[3] It seemed therefore reasonable that 2-phenyl-substituted 3-thienyllithium derivatives should also ring-open, as C_6H_5 has a weaker inductive effect than SCH_3, judging from Swain's and Lupton's *F*-values[4] (*F* = 0.139 and 0.332, respectively). Therefore, in order to elucidate the scope and limitation of the ring-opening for

* Part IX, see Ref. 14.
** To whom correspondence should be addressed.
*** Taken in part from the Ph.D. thesis of Torbjörn Frejd, University of Lund, 1975.

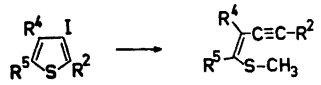


1	X = Br, $R^3 = C_6H_5$, $R^4 = H$, $R^5 = CH_3$	*4*	9 %	*5*	13 %	*6*	75 %	(47 %)			
2	X = I, $R^3 = R^5 = CH_3$, $R^4 = C_6H_5$	*7*	35 %	*8*	18 %	*9*	47 %	(12 %)			
3	X = Br, $R^3 = CH_3$, $R^4 = H$, $R^5 = C_6H_5$	*4*	20 %	*10*	5 %	*11*	65 %	(55 %)	*12*	10 %	

Scheme 1. Product analysis of the reaction between different phenyl substituted 3-halothiophenes and ethyllithium in the presence of a fivefold excess of ethyl bromide. Reaction conditions: 4 h at +21 °C, then hydrolysis. Uncalibrated GLC values. Figures within parentheses represent isolated yields.

the stereospecific synthesis of substituted alkylthiovinyl acetylenes, we have studied some phenyl substituted 3-thienyllithium derivatives.

Phenyl substituted 3-thienyllithium derivatives. As expected, when *1*, *2*, and *3* were treated in the standard way [2,3] with ethereal ethyllithium followed by ethyl bromide at room temperature and hydrolysis after 4 h, ring-opening occurred (Scheme 1). However, the relatively large amounts of dehalogenated heterocycles formed, such as 35 % of *7* from the reaction of *2* and 20 % of *4* from *3*, indicated that the corresponding thienyllithium derivatives were rather stable or that the enynethiolates were slowly alkylated. If the ring-cleavage is slow, such lithium derivatives would also have greater opportunity to undergo Wurtz-Fittig couplings. The formation of *5* from *1*, *8* from *2*, and *10* from *3* was indicated by combined GLC-MS, but since the fragmentation patterns (MS) of these compounds were not conclusive their structures are uncertain. For preparative purposes, the ring-opening of *1* and *3* with ethyllithium appears useful, since 47 % of *6* and 55 % of *11* could be isolated. In the reaction of *3*, also a small amount of *12* was formed through ethylation at the propargylic methyl group. The assignment of structure *12* was based on its mass spectrum, which showed similar features to that of (*Z*)-3-ethylthio-3-nonen-5-yne,[2] *e.g.* the prominent peak at M − 57 and the relatively high abundance of *m/e* = 91 ($C_7H_7^+$, tropylium). However, it was more difficult to isolate *9* formed from the reaction of *2* with ethyllithium and ethyl bromide, since its properties were too similar to those of some by-products. In order to suppress the formation of these by-products, halogen-metal exchange was carried out with phenyllithium. In such a case it is most convenient to use iodothiophenes in order to achieve a rapid reaction. The enynethiolates formed were then methylated with dimethyl sulfate. In this way, a 69 % yield of *15*, was obtained from *13*, a 50 % yield of *16* from *14* and a 63 % yield of *17* from *2*.

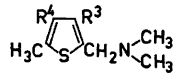


13	$R^2 = C_6H_5, R^4 = H, R^5 = CH_3$	*15*	69 %
14	$R^2 = CH_3, R^4 = H, R^5 = C_6H_5$	*16*	50 %
2	$R^2 = R^5 = CH_3, R^4 = C_6H_5$	*17*	63 %

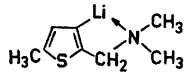
Scheme 2. Isolated yields of enyne-methylthioethers when some phenyl substituted 3-iodothiophenes were treated with phenyllithium at +21 °C for 4 h, followed by dimethyl sulfate.

These results give additional evidence for the preparative usefulness of the ring-opening of 3-thienyllithium derivatives for the preparation of substituted alkylthiovinyl acetylenes, corresponding to a stereo- and regiospecific addition of alkylthiolates to unsymmetrically disubstituted diacetylenes. In the cleavage of the lithium derivatives of *2*, a fully substituted alkylthiovinyl acetylene is obtained, which should be more difficult to prepare by other routes.

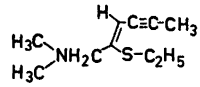
N,N-*Dimethylaminomethyl-substituted 3-thienyllithium derivatives.* The effect of a 2-*N*,*N*-dimethylaminomethyl group in stabilizing 3-thienyllithium is indicated in Slocum and Gierer's [9] work (which appeared when our work was in progress) in which it was found that *18* was metalated in the 3-position by butyllithium at room temperature. The lithium compound was trapped with benzophenone, which gave a 65 % yield of *19*. The possibility for the lithium compound to ring-open was not mentioned. We could, however, show by ^1H NMR spectroscopy that no vinylic hydrogen absorption was present even after 12 h at room temperature in an experiment in which *20* was sealed in an NMR tube and the vinyl region was scanned at different times. The thienyllithium derivative was prepared through halogen-metal



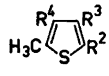
18 $R^3 = R^4 = H$
19 $R^3 = -C(OH)(C_6H_5)_2$, $R^4 = H$
21 $R^3 = Br$, $R^4 = H$
22 $R^3 = H$, $R^4 = Br$



20



23



24 $R^2 = R^4 = H$, $R^3 = Br$
25 $R^2 = COOH$, $R^3 = Br$, $R^4 = H$
26 $R^2 = CON(CH_3)_2$, $R^3 = Br$, $R^4 = H$
27 $R^2 = COOH$, $R^3 = H$, $R^4 = Br$
28 $R^2 = CON(CH_3)_2$, $R^3 = H$, $R^4 = Br$

exchange between *21* and ethyllithium in ether. In a parallel experiment it was shown that only *18* was formed upon hydrolysis after 4 h. Thus in this case the Wurtz-Fittig coupling did not interfere.

That intramolecular chelation was of importance in the stabilization of *20* was evident from the facile opening taking place when the *N,N*-dimethylaminomethyl function was situated in the 5-position. Thus *22* gave a 50 % yield of *23* when treated with ethyllithium and ethyl bromide at room temperature for 4 h. It thus seems clear that both strongly electron-attracting groups, and substituents giving intramolecular chelates of suitable geometry, can stabilize 3-thienyllithium derivatives

Synthesis of starting material. Starting from the known 3,5-dibromo-2-phenylthiophene,[6] prepared through bromination of 2-phenylthiophene, *1* was obtained by halogen-metal exchange with butyllithium followed by reaction with dimethyl sulfate. Halogen-metal exchange between *1* and butyllithium at −70 °C followed by reaction with iodine gave *13* in good yield.

Compound *2* was prepared from the known 2,5-dimethyl-3-phenylthiophene [6] by iodination with the iodine/iodic acid method.[7] In order to prepare *3*, we started from 3,5-dibromo-2-methylthiophene, which upon reaction with butyllithium and cyclohexanone gave 3-bromo-5-(1-cyclohexenyl)-2-methylthiophene. This compound was then aromatized in the usual way [8] with chloranil to give *3*. Substance *14* was obtained through halogen-metal exchange between *3* and butyllithium at −70 °C followed by reaction with iodine.

The compound *21* was prepared in the following manner: *24* [10] was metalated in the 5-position with lithium diisopropylamide in ether (*cf.* Ref. 11). Upon reaction with solid carbon dioxide, *25* was obtained in 65 % yield. *Via* the acid chloride, *25* was transformed to *26*, which upon $LiAlH_4$ reduction gave *21*. In a similar way, starting from *27*, *28* was prepared *via* the acid chloride. Reduction gave *22* in 72 % yield. The acid *27* was obtained by treating 3,5-dibromo-2-methylthiophene with butyllithium at −70 °C followed by solid carbon dioxide.

The structures of starting materials and products isolated in the reactions described in this paper were evident from their spectroscopical properties (IR, NMR and mass spectra) and elemental analyses (*cf.* Experimental part).

EXPERIMENTAL

General remarks. (See Ref. 2).

3-Bromo-5-methyl-2-phenylthiophene (1). To 50.0 g (0.157 mol) of 3,5-dibromo-2-phenylthiophene [6] in 250 ml of ether, 115 ml (0.161 mol) of 1.40 M butyllithium in hexane was added at −70 °C followed by 20.5 g (0.163 mol) of dimethyl sulfate in 100 ml of ether. The temperature was kept below −65 °C. When the addition was complete, the reaction mixture was stirred at −70 °C for 4 h, whereupon it was allowed to reach room temperature. Conc. ammonium hydroxide was added and the ethereal layer was washed with 2 N HCl, water and dried. Evaporation and distillation gave 22.3 g (56 %) of the title compound, b.p.$_{1.0\ mmHg}$ 116–120 °C. NMR (CCl_4): δ 7.1–7.7 (C_6H_5, m, 5 H); 6.60 (4-H, q, 1 H); 2.33 (5-CH_3, d, 3 H). $J_{5CH_3,4H}$ = 1.1 Hz. [Found: C 52.12; H 3.60; Br 31.60; S 12.61. Calc. for $C_{11}H_9BrS$ (253.16): C 52.19; H 3.58; Br 31.56; S 12.67.]

3-Iodo-5-methyl-2-phenylthiophene (13). To a solution of 8.38 g (0.0331 mol) of *1* in 100 ml of ether, 25 ml (0.035 mol) of 1.40 M butyllithium in hexane was added at −70 °C, followed by 8.9 g (0.035 mol) of iodine in 100 ml of ether. The reaction mixture was allowed to reach room temperature, whereupon it was poured onto aq. sodium thiosulfate. The organic layer was washed with water to neutral reaction and dried. Evaporation of the solvent yielded 8.7 g of crude *13*, which was distilled to give 5.4 g (54 %) of the title compound, b.p.$_{10^{-3}mmHg}$ 118–119 °C. NMR (CCl_4): δ 7.15–7.65 (C_6H_5, m, 5 H); 6.66 (4-H, q, 1 H); 2.38 (5-CH_3, d, 3 H). $J_{4H,5CH_3}$ = 1.1 Hz. [Found: C 43.98; H 3.04; S 10.78. Calc. for $C_{11}H_9IS$ (300.16): C 44.02; H 3.02; S 10.68.]

3-Iodo-2,5-dimethyl-4-phenylthiophene (2). A mixture of 5.00 g (26.6 mmol) of 2,5-dimethyl-3-phenylthiophene,[12] 3.6 g (14 mmol) of iodine, 1.2 g (6.8 mmol) of iodic acid, 15 ml of acetic acid, 20 ml of water, 15 ml of CCl_4 and 0.1 ml of conc. H_2SO_4 was stirred vigorously at 60 °C for 4.5 h. After cooling, the reaction mixture was poured into aqueous sodium thiosulfate and extracted with CCl_4. The organic portions were washed with water, dried and the solvent was evaporated. The crystalline residue, 7.5 g (90 %), was recrystallized from hexane in the cold (−25 °C) giving 6.5 g (79 %) of the pure title compound, m.p. 53–56 °C. NMR (CCl_4): δ 7.1–7.5 (C_6H_5, m, 5 H); 2.40 (CH_3, s, 3 H); and 2.27 (CH_3, s, 3 H). [Found: C 45.90; H 3.60; S 10.16. Calc. for $C_{12}H_{11}IS$ (314.19): C 45.87; H 3.53; S 10.21.]

3-Bromo-2-methyl-5-phenylthiophene (3). To 25.6 g (0.100 mol) of 3,5-dibromo-2-methylthiophene in 250 ml of ether, 100 ml (0.110 mol) of 1.10 M butyllithium in hexane was added at −70 °C. After 0.5 h 10.0 g (0.102 mol) of cyclohexanone in 100 ml of ether was added and the mixture was allowed to reach room temperature, whereupon 100 ml of 5 N HCl was added. After stirring for 0.5 h the ethereal layer was washed with water and dried. Evaporation yielded 25.0 g of crude 3-bromo-5-(1-cyclohexenyl)-2-methylthiophene, which was aromatized with 40 g (0.18 mol) of DDQ in 400 ml of refluxing benzene for 2.5 h. The mixture was cooled and filtered and the filter was washed with benzene several times. The filtrate was washed repeatedly with 5 N NaOH solution and finally with water. Evaporation of the solvent and recrystallization from ethanol:water yielded 12.9 g (51 %) of the title compound, m.p. 72–73 °C. NMR (CCl_4): δ 7.00 (4-H, s, 1 H); 7.1–7.6 (C_6H_5, m, 5 H); 2.37 (2-CH_3, s, 3 H). [Found: C 52.16; H 3.51; Br 31.57; S 12.71. Calc. for $C_{11}H_9BrS$ (253.16): C 52.19; H 3.58; Br 31.56; S 12.67.]

3-Iodo-2-methyl-5-phenylthiophene (14) was prepared in a way analogous to that used for the isomeric 4-halo-2-methyl derivative *13*, from 3.00 g (0.0119 mol) of *3* in 75 ml of ether, 10 ml (0.012 mol) of 1.20 M butyllithium in hexane and 3.2 g (0.013 mol) of iodine dissolved in 75 ml of ether. Thus 3.56 g of a crystalline crude product was obtained, which was recrystallized from ethanol to give the title compound, m.p. 65–66 °C, 2.16 g (60 %). NMR (CCl_4): δ 7.1–7.6 (C_6H_5, m, 5 H); 7.08 (4-H, s, 1 H); 2.45 (2-CH_3, s, 3 H). [Found: C 44.1; H 2.99; I 41.8. Calc. for $C_{11}H_9IS$ (300.16): C 44.02; H 3.02; I 42.48.]

3-Bromo-5-methyl-2-thiophenecarboxylic acid (15). To 24.0 g (0.237 mol) of diisopropyl amine in 100 ml of ether, 160 ml (0.240 mol) of 1.50 M butyllithium in hexane was added. After 10 min, 35.4 g (0.200 mol) of 4-bromo-2-methylthiophene [10] in 100 ml of ether was added rapidly at room temperature. The reaction mixture was poured onto solid carbon dioxide in ether after 45 min and extracted with aq. 2 N NaOH. Upon acidification with 5 N HCl, 29.1 g (56 %) of the title compound was isolated, which was sufficiently pure to use in synthetic work according to NMR. Recrystallization from ethanol:water gave the pure acid, m.p. 205–206 °C. NMR (acetone-d_6): δ 6.93 (4-H, q, 1 H); 2.53 (5-CH_3, d, 3 H); 9.10 (COOH). $J_{5CH_3,4H}$ = 1.0 Hz. [Found: C 32.63; H 2.30; S 14.55. Calc. for $C_6H_5BrO_2S$ (221.07): C 32.60; H 2.30; S 14.50.]

3-Bromo-2-(N,N-dimethylcarboxamido)-5-methylthiophene (26). To a solution of 17.9 g (0.150 mol) of thionyl chloride in 50 ml of ether, 22.1 g (0.100 mol) of *25* was added in portions, followed by 2 ml of pyridine. The mixture was refluxed with stirring for 2.5 h, whereupon the solvent, together with the excess of thionyl chloride, was evaporated. The residue (white needles) was dissolved in 100 ml of anhydrous benzene and dimethylamine was led through the reaction mixture (ice cooling). When 20 g of dimethylamine had been introduced, the reaction mixture was filtered and the filter was washed several times with ether. The filtrate was evaporated and the residue dissolved in ether. The organic layer was washed with 2 N HCl, 2 N NaOH, water and dried. After evaporation and distillation, 11.8 g (48 %) of the pure amide was obtained, b.p.$_{0.5\ mmHg}$ 128–130 °C: IR: C=O 1630 cm^{-1}. NMR (CCl_4): δ 6.55 (4-H, q, 1 H); 2.45 (5-CH_3, d, 3 H); 2.98 (NCH_3, s, 6 H). $J_{5CH_3,4H}$ = 1.1 Hz. [Found: C 38.75; H 4.05. Calc. for $C_8H_{10}BrNOS$ (248.14): C 38.72; H 4.06.]

3-Bromo-2-(N,N-dimethylaminomethyl)-5-methylthiophene (21). To 1.05 g (0.0277 mol) of $LiAlH_4$ in 25 ml of ether, 10.0 g (0.0403 mol) of *26* in 25 ml of ether was added. When the addition was complete, the reaction mixture was refluxed for 3 h, cooled and 1.0 ml of water was added. The reaction mixture was filtered and the filter was washed with ether. The filtrate was dried over KOH pellets, evaporated and distilled (some decomposition), which gave 5.0 g (53 %) of the title compound, b.p.$_{0.6\ mmHg}$ 69–73 °C. NMR (CCl_4): δ 6.49 (4-H, q, 1H); 2.43 (5-CH_3, bs, 3 H); 3.47 (2-CH_2-, s, 2 H); 2.20 (NCH_3, s, 6 H). $J_{5CH_3,4H}$ = 1.0 Hz. [Found: C 41.12; H 5.14; S 13.65. Calc. for $C_8H_{12}BrNS$ (234.16): C 41.04; H 5.17; S 13.69.]

3-Bromo-2-methyl-5-thiophenecarboxylic acid (27). To 46.3 g (0.181 mol) of 3,5-dibromo-2-methylthiophene [6] in 200 ml of ether, 140 ml (0.186 mol) of 1.33 M butyllithium in ether was added at −70 °C. The mixture was stirred for 20 min, whereupon it was poured onto solid carbon dioxide in ether. The ethereal phase was extracted with 2 N NaOH and the alkaline portions were acidified with 5 N HCl, which gave 35.2 g of the crude acid. Recrystallization from acetic acid:water gave the pure title compound, m.p. 197–200 °C, 30.2 g (75 %). NMR (acetone-d_6): δ 7.60 (4-H, s, 1 H); 2.47 (2-CH_3, s, 3 H). [Found: C 32.70; H 2.30; Br 36.09; S 14.52. Calc. for $C_6H_5BrO_2S$ (221.07): C 32.60; H 2.28; Br 36.14; S 14.50.]

3-Bromo-5-(N,N-dimethylcarboxamido)-2-methylthiophene (28) was prepared in analogy with its isomer *(26)* from 35.2 g (0.159 mol) of *27* to give after work-up and distillation, 29.0 g (74 %) of the title compound; b.p.$_{1\ mmHg}$ 144–145 °C. IR: C=O 1625 cm^{-1}. NMR (CCl_4): δ 7.08 (4-H, s, 1 H); 2.38 (2-CH_3, s, 3 H); 3.10 (NCH_3, s, 6 H). [Found: C 38.80; H 4.11; S 12.85. Calc. for $C_8H_{10}BrNOS$ (248.14): C 38.72; H 4.06; S 12.92.]

3-Bromo-5-(N,N-dimethylaminomethyl)-2-methylthiophene (22) was prepared in analogy with its isomer *21* from 27.2 g (0.110 mol) of *28* to yield 18.7 g (73 %), b.p.$_{1\ mmHg}$ 85–87 °C. NMR (CCl_4): δ 6.65 (4-H, s, 1 H); 3.47 (5-CH_2, s, 2 H); 2.37 (2-CH_3, s, 3 H); 2.20 (NCH_3, s,

6 H). [Found: C 41.00; H 5.20; S 13.75. Calc. for $C_8H_{12}BrNS$ (234.16): C 41.04; H 5.17; S 13.69.]

General method for the ring-opening of 3-lithioheterocycles (G). See Ref. 2.

(Z)-2-Ethylthio-5-phenyl-2-penten-4-yne (6). The general method G was followed. From 5.06 g (0.0200 mol) of *1* in 50 ml of ether, 28 ml (0.021 mol) of 0.76 M ethereal ethyllihium and 10.9 g (0.100 mol) of ethyl bromide, 3.23 g of crude product was obtained. Combined GLC-MS analysis (column OV 17, 3 %, 100–230 °C, 12 °C/min) of the washed and dried ethereal reaction mixture showed three components, namely 2-methyl-5-phenylthiophene (*4*) (*m/e* = 174; calc. for $C_{11}H_{10}S = 174$), 3-ethyl-5-methyl-2-phenylthiophene (*5*) (*m/e* = 202; calc. for $C_{13}H_{14}S = 202$), and *6* (*m/e* = 202; calc. for $C_{13}H_{14}S = 202$) (see Scheme 1). After distillation, 1.9 g (47 %) of the title compound was obtained, b.p.$_{.5\times10^{-4}\ mmHg}$ 105–110 °C.
IR: C≡C 2190 cm^{-1}. NMR (CCl_4): δ 2.00 (1-H, d, 3 H); 5.57 (3-H, q, 1 H); 7.1–7.6 (C_6H_5, m, 5 H); (SC_2H_5) 2.80 (q, 2 H) and 1.23 (t, 3 H). $J_{1H,3H} = 1.4$ Hz; $J_{SCH_2-CH_3} = 7.0$ Hz. [Found: C 76.9; H 7.01; S 15.7. Calc. for $C_{13}H_{14}S$ (202.32): C 77.18; H 6.98; S 15.85.]

(Z)-1-Ethylthio-1-phenyl-1-penten-3-yne (11). The general method G was followed. From 5.06 g (0.0200 mol) of *3* in 50 ml of ether, 30 ml (0.021 mol) of 0.70 M ethereal ethyllithium and 7.80 g (0.0500 mol) of ethyl iodide, 3.52 g of a crude product was obtained. Combined GLC-MS analysis (column OV 17, 3 %, 200–290 °C, 12 °C/min) showed the presence of four compounds, namely *4* (*m/e* = 174; calc. for $C_{11}H_{10}S = 174$), 3-ethyl-2-methyl-5-phenylthiophene (*10*) (*m/e* = 202; calc. for $C_{13}H_{14}S = 202$), *11* (*m/e* = 202; calc. for $C_{13}H_{14}S = 202$) and *(Z)-1-ethylthio-1-phenyl-1-hepten-3-yne (12)* (*m/e* = 230; calc. for $C_{15}H_{18}S = 230$) (see Scheme 1). TLC (1 mm silica gel, hexane) of 1.50 g of the crude product gave 0.94 g (55 %) of the title compound (R_F 0.2–0.4). IR: C≡C 2215 and 2040 cm^{-1}. NMR (CCl_4): δ 5.73 (2-H, q, 1 H); 2.05 (5-H, d, 3 H); 7.17–7.60 (C_6H_5, m, 5 H); (SC_2H_5) 2.50 (q, 2 H) and 1.08 (t, 3 H). $J_{3H,5H} = 2.40$ Hz; $J_{SCH_2-CH_3} = 7.0$ Hz. [Found: C 77.10; H 7.01; S 15.85. Calc. for $C_{13}H_{14}S$ (202.32): C 77.18; H 6.98; S 15.85.]

(Z)-2-Ethylthio-3-phenyl-2-hexen-4-yne (9). The general method G was followed. From 2.00 g (6.37 mmol) of *2* in 30 ml of ether, 14 ml (6.40 mmol) of 0.46 M ethereal ethyllithium and 3.27 g (30.0 mmol) of ethyl bromide, 0.90 g of a crude product was obtained. Combined GLC-MS analysis (column OV 1, 3 %, 100–290 °C, 10 °C/min) of the washed and dried reaction mixture showed the presence of three compounds, namely 2,5-dimethyl-3-phenylthiophene (*7*) (*m/e* = 188; calc. for $C_{12}H_{12}S = 188$), 3-ethyl-2,5-dimethyl-4-phenylthiophene (*8*) (*m/e* = 216; calc. for $C_{14}H_{16}S = 216$), and *9* (*m/e* = 216; calc. for $C_{14}H_{16}S = 216$) (see Scheme 1). TLC (1 mm silica gel, hexane) gave two fractions; Z 1 (0.23 g, R_F 0.30–0.54), which according to NMR and GLC analysis consisted of *7* and *8*; Z 2 (0.16 g, 12 %, R_F 0.12–0.05), which was the crystalline title compound. Recrystallization from hexane at −25 °C gave *9*, m.p. 58–60 °C. IR: C≡C 2110 and 2040 cm^{-1}. NMR (CCl_4): δ 2.02 (1-H, 6-H, bs, 6 H); 7.20 (C_6H_5, m, 5 H); (SC_2H_5) 2.83 (q, 2 H) and 1.32 (t, 3 H). $J_{SCH_2-CH_3} = 7$ Hz. [Found: C 77.79; H 7.46; S 14.77. Calc. for $C_{14}H_{16}S$ (216.35): C 77.72; H 7.45; S 14.82.]

(Z)-2-Methylthio-5-phenyl-2-penten-4-yne (15). To 1.50 g (5.00 mmol) of *13* in 25 ml of ether, 5.0 ml (5.0 mmol) of 1.0 M phenyllithium was added (with a syringe) at room temperature and in a glove-box supplied with dry oxygen-free nitrogen. After 4 h, 0.63 g, (5.0 mmol) of dimethyl sulfate in 10 ml of ether was added and the reaction mixture was hydrolyzed with 10 ml of conc. ammonium hydroxide solution after another hour. The mixture was washed with 2 N HCl and water to neutral reaction and dried. After evaporation of the ether and lower-boiling substances (*e.g.* iodobenzene), 0.90 g of crude product remained, which was chromatographed (TLC, 1 mm silica gel, hexane) to give 0.65 g (69 %) of the title compound. IR: C≡C 2185 cm^{-1}. NMR (CCl_4): δ 2.10 (1-H, d, 3 H); 5.54 (3-H, q, 1 H); 7.1–7.5 (C_6H_5, m, 5 H); 2.37 (SCH_3, s, 3 H). $J_{1H,3H} = 1.4$ Hz. [Found: C 76.44; H 6.39; S 17.18. Calc. for $C_{12}H_{12}S$ (188.29): C 76.55; H 6.42; S 17.03.]

(Z)-1-Methylthio-1-phenyl-1-penten-3-yne (16). This compound was prepared by the same method and on the same scale as the preceding experiment from *14*. The crude product (0.60 g) was purified by TLC (1 mm silica gel, hexane), giving 0.47 g (50 %) of the title compound. IR: C≡C 2220 cm^{-1}. NMR (CCl_4): δ 5.65 (2-H, q, 1 H); 2.05 (5-H, d, 3 H); 7.1–7.6 (C_6H_5, m, 5 H); 2.03 (SCH_3, s, 3 H). $J_{2H,5H} = 2.4$ Hz. [Found: C 76.50; H 6.40; S 17.13. Calc. for $C_{12}H_{12}S$ (188.29): C 76.55; H 6.42; S 17.03.]

(Z)-2-Methylthio-3-phenyl-2-hexen-4-yne (17). As in the preceding experiment, compound *17* was prepared from *2*. The crude product (0.85 g) was purified as above, which gave the title compound, 0.64 g (63 %), m.p. 84–86 °C. IR: C≡C 2200 cm^{-1}. NMR (CCl_4): δ 2.00 (1-H, 6-H, bs, 6 H); 7.17 (C_6H_5, s, 5 H); 2.32 (SCH_3, s, 3 H). [Found: C 77.24; H 7.01; S 15.80. Calc. for $C_{13}H_{14}S$ (202.32): C 77.18; H 6.98; S 15.85.]

The stability of 2-(N,N-dimethylaminomethyl)-5-methyl-3-thienyllithium. 21 (0.117 g, 0.500 mol) was introduced into an NMR tube. The tube was sealed with a plastic cap and cooled to −70 °C, whereupon 0.50 ml of 1.0 M ethereal ethyllithium was injected through the cap by a syringe and the tube was sealed off with a glassblower's torch. The vinyl region was scanned every two hours during 12 h, but no traces of vinylic protons were observed.

2-(N,N-Dimethylaminomethyl)-5-methylthiophene. The general method G was followed. From 2.34 g (0.0100 mol) of *21* in 30 ml of ether, 13 ml (0.010 mol) of 0.80 M ethereal ethyllithium and 5.45 g (0.0500 mol) of ethyl bromide, 1.40 g (90 %) of the crude title compound was obtained after evaporation of the solvent. GLC (column OV 17, 3 %, 100–250 °C, 20°/min) showed only one component. IR: no acetylenic absorptions. Distillation gave the pure title compound, b.p.$_{12\ mmHg}$ 80–81 °C, $n_D^{20}=1.5170$. (Lit.[13] b.p.$_{15\ mmH}$ 83–84 °C, $n_D^{20}=1.5150$.) NMR (CCl_4): δ 6.53 (3-H, bd); 6.42 (4-H, d, q); 2.43 (5-CH_3, bs, 3 H); 3.48 (-CH_2-, s, 2 H); 2.20 (NCH_3, s, 6 H). $J_{3H,4H}=3.2$ Hz; $J_{4H,5CH_3}=1.0$ Hz.

(Z)-1-(N,N-dimethylamino)-2-ethylthio-2-hexen-4-yne (23). The general method G was followed. From 10.0 g (0.0427 mol) of *22* in 75 ml of ether, 75 ml (0.045 mol) of 0.60 M ethereal ethyllithium and 16.4 g (0.150 mol) of ethyl bromide, 7.6 g of a crude product was obtained. The title compound was obtained pure by distillation, b.p.$_{0.6\ mmHg}$ 76–80 °C, 3.8 g (49 %). (Extensive decomposition took place during the distillation.) IR: C≡C 2220 cm^{-1}. NMR (CCl_4): δ 2.97 (1-H, bs, 2 H); 5.53 (3-H, m, 1 H); 1.98 (6-H, d, 3 H); 2.17 (NCH_3, s, 6 H); (SC_2H_5) 2.97 (q, 2 H) and 1.25 (t, 3 H). $J_{3H,6H}=2.4$ Hz; $J_{SCH_2-CH_3}=7.2$ Hz. [Found: C 65.44; H 9.29; S 17.39. Calc. for $C_{10}H_{17}NS$ (183.32): C 65.52; H 9.35; S 17.49.]

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Heterogeneity of Hepatic Tyrosine Aminotransferase. Separation of the Multiple Forms* from Rat and Frog Liver by Isoelectric Focussing and Hydroxylapatite Column Chromatography and Their Partial Characterization

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L-Tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5.; TAT) and other enzymes that transaminate tyrosine in rat liver cytosol have been separated into four fractions by isoelectric focussing. One of the forms is probably identical to mitochondrial L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1.; mASAT). The other three forms have pI's of 4.72, 4.98 and 5.30 and K_m values of 1.3 and 0.3 mM for tyrosine and α-ketoglutarate. These heat stable forms have little or no ASAT activity. Rat liver TAT is also separated into three peaks by hydroxylapatite. Each fraction gives only one peak of activity when electrofocussed separately.

In the frog, three groups of peaks of TAT activity have been separated by hydroxylapatite column chromatography. The first group is connected with ASAT activity. These peaks (pI's 6.35, 6.50 and 6.90) are heat stable and have a K_m value for tyrosine of 4 mM. These fractions probably represent cytoplasmic ASAT (sASAT). The second group of peaks has at least two subforms (pI's 9.0 and 9.4, K_m for tyrosine 15 mM). These forms probably represent mASAT. The third group consists of three forms that resemble the major forms of rat liver TAT.

These results indicate that heterogeneity is common to many aminotransferases and independent of regulation by glucocorticoids.

Tyrosine aminotransferase (TAT) is the first enzyme in the catabolic pathway of the amino acid tyrosine. It has been very widely studied because of its complicated regulation. Many agents such as corticosteroids,[1] glucagon, insulin,[2] dibutyryl-3′,5′-cyclic AMP and adrenalin [3] are able to induce the enzyme in the liver of the rat and in rat hepatoma cell culture. This is why the enzyme of the rat has been largely used as a model in studies of enzyme regulation.[4] TAT from frog liver is known to have different hormonal regulation. It is unaffected by corticosteroids. Tyrosine and dibutyryl-3′,5′-cyclic AMP enhance the activity, while glucose decreases it to less than half.[5]

It has been reported that TAT from rat liver can be separated into three or four multiple forms by hydroxylapatite column chromatography, [6,7] electrophoresis in polyacrylamide gel [8] and by CM-Sephadex.[9]

At first it was thought that the multiple forms are regulated separately by different effectors.[6] However, it has been shown that the forms are interconverted in an orderly sequence in the homogenate.[9] A recent report suggests that TAT is a phosphoprotein;[10] it has been proposed that the heterogeneity results from different phosphate content. Johnson and

* It is essential that the reader be familiar with the following definitions:

"Multiple forms of TAT" refer to all proteins that can transaminate tyrosine; it will be shown that other enzymes than EC 2.6.1.5. can be detected as "multiple forms of TAT" due to their broad substrate spectra.

"Isoenzymes": ASAT is known to have two isoenzymes (sASAT and mASAT) that have different primary structures.

"Subforms": It will be shown in this paper that TAT, sASAT and mASAT can be divided into several subforms. The only known difference between the subforms is in their net charges.

Grossman have reported that the elution pattern of TAT activity is strongly affected by the concentration of phosphate in the original homogenization medium.[11] It has also been suggested that some of these multiple forms are in fact other aminotransferases such as ASAT.[12] Summing up, the physiological role of the multiple forms has remained obscure; we do not know whether they are degradation products or some other post-translational modifications or mere artifacts.

The aim of the present work was to obtain more information about the matters described above by separating the multiple forms by isoelectric focussing and correlating the fractions obtained by different techniques to each other. The enzyme from frog liver was studied and compared to that of the rat, because the former is known to have different and far less complicated hormonal regulation.[5] We also regarded it necessary to investigate the interrelations of TAT, sASAT, mASAT and L-alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2.; ALAT) to exclude any errors that might arise due to broad substrate spectra.[11] Further, we wanted to find out whether or not aminotransferses other than TAT consisted of subforms.

MATERIAL AND METHODS

Animals and tissue preparations. Male Wistar rats (average weight 200 g) of a strain bred in our laboratory were used. The animals were fed *ad libitum* with a standard diet (Hankkija, Helsinki). The frogs were provided by Porla Fisheries, Lohja, Finland. They were kept at +4 °C and "equilibrated" at +18 °C for 5 days before killing. Only male frogs were used; their average weight was about 30 g.

All the animals were killed between 1 and 2 p.m. to avoid the effects of the diurnal variations.[13] The animals were decapitated and the livers were removed immediately, rinsed in ice-cold 100 mM potassium phosphate buffer, pH 6.8 (in hydroxylapatite column chromatography experiments) or 320 mM glycerol (in electrofocussing experiments) and homogenized in 2 volumes of the same buffer with a Potter-Elvehjem glass homogenizer using a tightly fitting Teflon pestle (10 strokes). The homogenates were centrifuged at 100 000 *g* for 60 min in a Spinco Model L-50 ultracentrifuge. The resulting supernatant fractions were immediately used in the experiments. These procedures were performed at 0–4 °C.

Hydroxylapatite column chromatography. Hydroxylapatite was prepared in our laboratory as described previously [14] and equilibrated with excess 100 mM potassium phosphate buffer, pH 6.8. After packing the columns (1.5 cm × 12 cm) were washed with excess buffer. The sample was applied on top and the column was washed with the buffer until no more protein was eluted. The bound proteins were then eluted with a continuous potassium phosphate gradient 100–500 mM (pH 6.8). The total volume of the gradient was 1000 ml in all experiments. The flow rate was kept at about 0.5 ml/min. Fractions of about 5 ml were collected by an Isco fraction collector. Protein was estimated by following the absorbancy at 280 nm.

Isoelectric focussing. The isoelectric focussing apparatus (Model 8101) was purchased from LKB, Bromma, Sweden. A glycerol gradient 60–0 % v/v was used. The Ampholines were used at 1 % concentration and they were divided 1:3 between the light and dense gradient solutions. The gradient was mixed by a device manufactured by LKB, Bromma, for this purpose. The sample was divided equally between both mixing vials. The focussing was started (anode at top) with a voltage of 400 V. After 20 h the voltage was elevated to 500 V and after another 20 h the current was stopped and fractions (2 ml) were collected. The pH of every fraction was immediately measured by a Radiometer Titrator (Radiometer, Copenhagen). The enzyme activities were determined in the fractions directly, since preliminary experiments showed that the Ampholines do not appreciably disturb the assays.

When hydroxylapatite fractions were electrofocussed, the samples were dialyzed against water to remove excess ions so as to keep down the current.

Concentration of the fractions. This was performed with an Amicon ultrafiltrator under 3.5 atm pressure in nitrogen atmosphere using PM-10 membranes. The fractions from electrofocussing were diluted at least 1:10 with 100 mM potassium phosphate buffer, pH 6.8, concentrated, diluted again at least 1:10 with the same buffer and reconcentrated.

Estimation of heat stability. The heat stability of separated fractions was studied by heating them (in a volume of 300 µl) to +60 °C for 5 min in the presence of 1 mg/ml of bovine serum albumin and 0.5 mM pyridoxal-5′-phosphate. The control tubes (containing equal amounts of the two stabilizers) were kept at +0 °C. After the incubation a normal tyrosine aminotransferase assay was performed adding the usual amount of pyridoxal-5′-phosphate.

Determination of kinetic constants. The K_m values for tyrosine and α-ketoglutarate were determined by the method of Lineweaver and Burk.[15] The linear regression coefficients were calculated by computer using the least squares

method. The calculations are based on six to eight rate determinations.

Assays. Tyrosine aminotransferase was assayed by the following method. A mixture containing 80 volume units of 7 mM tyrosine in 125 mM potassium phosphate buffer, pH 7.6, 2 volume units of 0.5 – 0.05 M α-ketoglutarate and 1 volume unit 5 mM pyridoxal-5′-phosphate was prepared. The sample to be assayed (150 μl) was pipetted in test tubes, and 1.245 ml of the mixture described above was added. After shaking, a portion of 465 μl was taken out of the assay mixture and pipetted in a test tube containing 35 μl of 10 M KOH; the tubes were kept on ice. The assay mixture was incubated at 37 °C for 30 – 45 min, after which another portion of 465 μl was taken and handled similarly. After this the KOH-tubes were incubated at + 37 °C for another 30 min. The absorbancy of each sample was read against its own zero time blank in 0.5 ml quartz cuvettes at 331 nm in a Gilford spectrophotometer. This method is a technical modification of that of Diamondstone.[16]

ASAT and ALAT activities were estimated with a previously described method [17] using the assay sets of Boehringer Mannheim GmbH. In hydroxylapatite column chromatography, the protein elution profile was studied by following the absorbancy at 280 nm.

Reagents. The following reagents were used: L-tyrosine, pyridoxal-5′-phosphate and bovine serum albumin (Sigma), α-ketoglutaric acid, ethylenediamine, and glycerol (Merck). Ampholines were purchased from LKB, Bromma, Sweden.

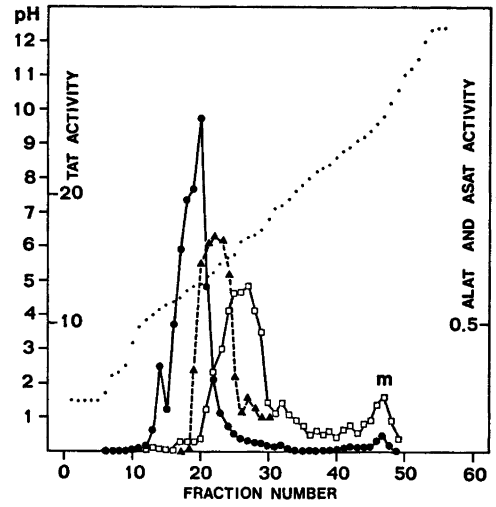

Fig. 1. Isoelectric focussing of rat cytosol in the pH range 3.5 – 10. Rat supernatant fraction (2 ml in 320 mM glycerol) was electrofocussed as described in the text. TAT was assayed with 0.5 mM α-ketoglutarate. ●, TAT; □, ASAT; ▲, ALAT; ···, pH. TAT activity is expressed as milliunits per fraction and ALAT and ASAT activities as units per fraction (the ASAT and ALAT peaks are somewhat smaller than in reality because the assay was performed so that the highest activities were in the unlinear region).

RESULTS

Isoelectric focussing of rat liver cytosol. Fig. 1 shows the tyrosine aminotransferase activity profile in isoelectric focussing in the pH range 3.5 – 10. A broad peak was seen around pH 5. This peak is distinctly separate from those of aspartate and alanine aminotransferases. It was often divided into three fractions. Another peak was seen above pH 9. This peak also had aspartate aminotransferase activity. It is a minor form but was consistently found. A trace of activity was sometimes seen around pH 8, but due to its minute amount no characterization of this form was possible.

To obtain better separation, rat liver cytosol was then electrofocussed in a narrower pH range (3 – 6). The broad peak at about pH 5 was distinctly divided into three forms having p*I*'s of 4.72, 4.98 and 5.30 (Fig. 2a).

In hydroxylapatite column chromatography, three distinctly separate peaks (designed 1, 2, and 3) were resolved (Fig. 3). Most of the aspartate and alanine aminotransferase activities were eluted in the buffer front, but some aspartate aminotransferase activity was connected with the middle peak. The elution pattern varied so that the first peak sometimes dominated and only traces of the other two were seen.

The hydroxylapatite fractions were electrofocussed separately to correlate the fractions obtained by the two different techniques to each other. Each hydroxylapatite fraction gave only one peak in isoelectric focussing (Fig. 2 b – d). The fractions had p*I*'s 5.2, 5.0 and 4.8 (fractions 1,2 and 3, respectively). These p*I*'s are in good agreement with those seen in Fig. 2a.

The multiple forms of hepatic TAT of Rana temporaria. Three groups of multiple forms of TAT were obtained from frog liver by hydroxylapatite column chromatography (Fig. 4), The

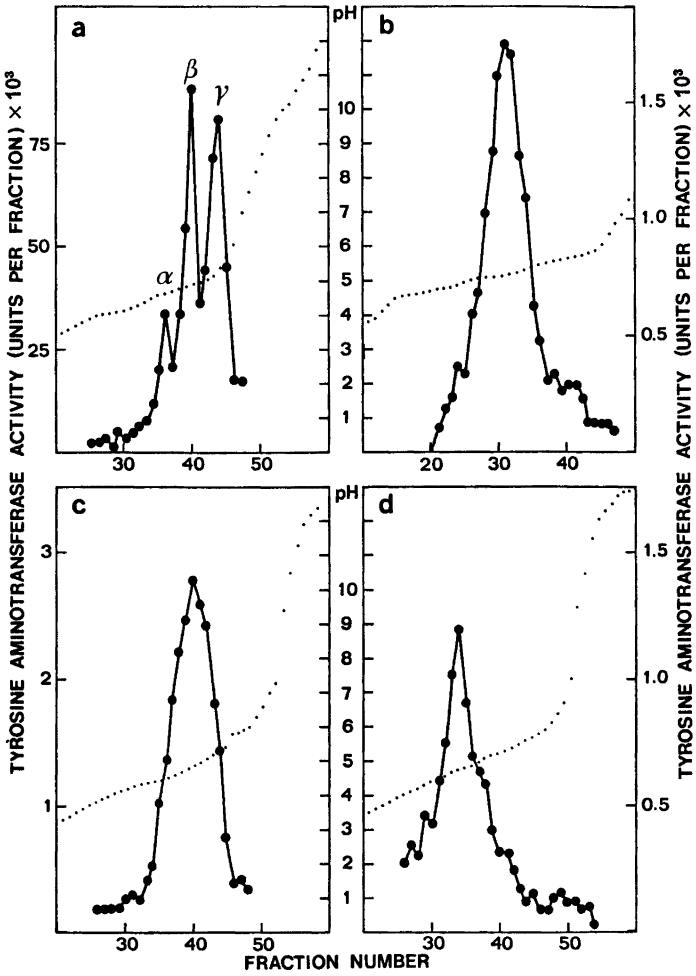

Fig. 2. a: Isoelectric focussing of rat cytosol in pH range 3 – 6. b-d: Isoelectric focussing of the three subforms of TAT obtained by hydroxylapatite column chromatography (b = 1, c = 2, and d = 3 in Fig. 3). The unit of TAT activity is μmol of *p*-hydroxyphenylpyruvate formed/min. TAT was assayed by 10 mM α-ketoglutarate. ●, TAT; ···, pH.

first group (designated group A) travelled in the front as did aspartate and alanine aminotransferases. This peak was hardly discernible when the assay was performed using 10 mM α-ketoglutarate but it was observed that this peak was strongly inhibited at the unphysiologically high concentration of the substrate used in the standard assay. Another band of activity was eluted by 150 mM phosphate. This group (designated group B) was often divided into two peaks, though in the experiment shown in Fig. 4 this was not so clearly seen. These fractions were consistently associated with ASAT activity that was often divided into two peaks. These two closely travelling peaks were only minor forms comprising not more than 5 % of total TAT activity.

In the middle region of the phosphate gradient a group of three peaks of TAT activity (group C) was eluted. Of these multiple forms, the two first ones (C1 and C2) were constantly obtained; the third, however, was generally smaller than the other two and was often ab-

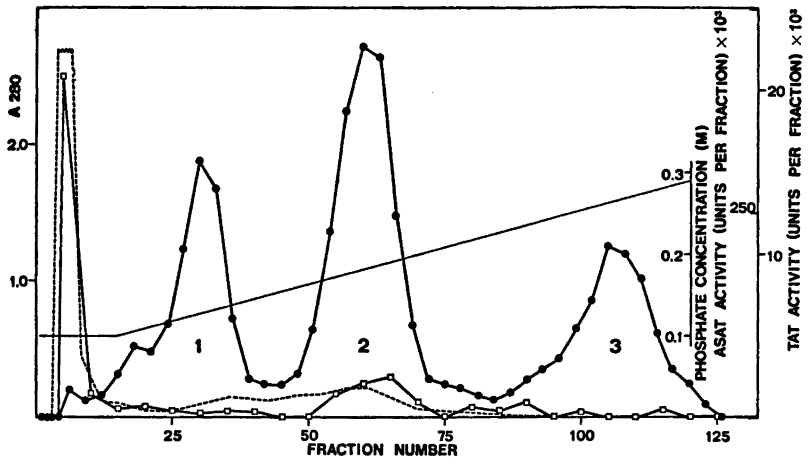

Fig. 3. Hydroxylapatite column chromatography of rat cytosol. A 2.5 ml portion of rat liver cytosol (in 100 mM potassium phosphate buffer, pH 6.8) was applied on top of a hydroxylapatite column and eluted as described in Material and Methods. TAT activity was assayed using 0.5 mM α-ketoglutarate to avoid any substrate inhibition. The activities are expressed as μmol of product formed/(fraction min). ●, TAT; □, ASAT; - - -, absorbancy at $\lambda = 280$ nm; —, phosphate concentration. ALAT activity (not shown) was eluted in the front.

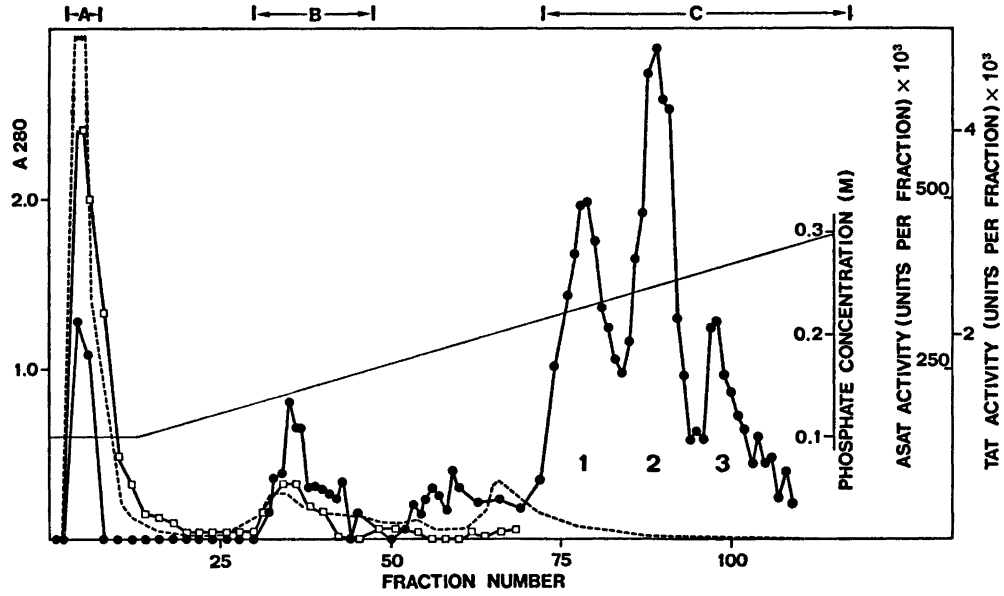

Fig. 4. Hydroxylapatite column chromatography of frog cytosol. The procedure as well as the symbols and units are as in Fig. 3. TAT was assayed with 0.5 mM (fractions 1 – 20) or 10 mM (fractions 20 – 110) α-ketoglutarate.

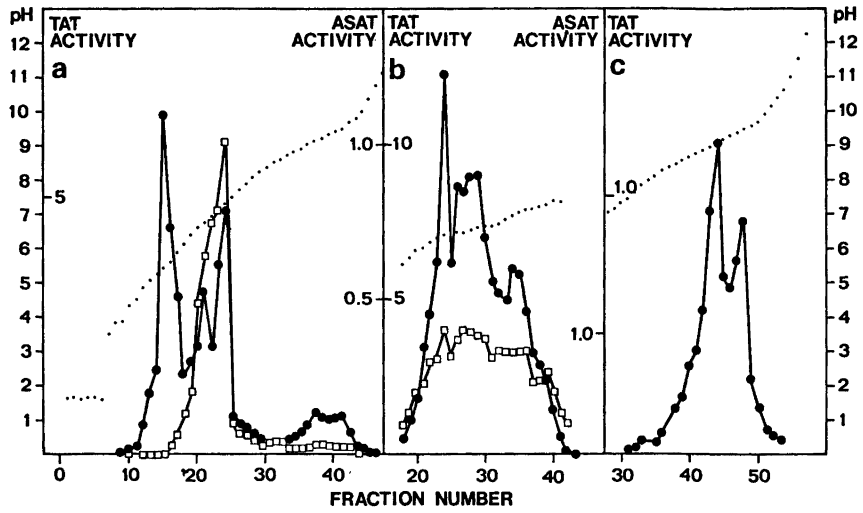

Fig. 5. a: Isoelectric focussing of frog liver cytosol (2 ml of supernatant focussed). b: Isoelectric focussing of fraction A obtained from frog liver by hydroxylapatite column chromatography. c: Electrofocussing of the combined fraction B from hydroxylapatite column chromatography. α-Ketoglutarate was used at 0.5 mM concentration in TAT assays. ASAT activity is expressed as μmol of product formed/(fraction min) and that of TAT as nmol/(fraction min). The symbols are as in Fig. 1.

sent. None of these three multiple forms had any ASAT or ALAT activity.

The isoelectric focussing profile showed that most activity was found around pH 5. When the analyses were performed at low α-ketoglutarate concentration this fraction seemed smaller compared to the others (Fig. 5a). Most often the peak was divided into two; three fractions were seldom seen. Another group of multiple forms was seen below pH 7. It also had aspartate aminotransferase activity. Both enzyme activities were divided into two or three fractions. Two peaks were found above pH 9; these multiple forms, again, had ASAT activity.

When group A from hydroxylapatite column chromatography was electrofocussed separately, three peaks of TAT activity were obtained (Fig. 5b). All three were accompanied by similar peaks of strong ASAT activity. The p*I*'s of these multiple forms were 6.35, 6.50 and 6.90. Sometimes, some activity was found at pH 7.2. We suggest that here the TAT and ASAT activities are due to the same enzyme, the cytoplasmic isoenzyme of ASAT, which, as can be seen in Fig. 5, has subforms as does TAT.

When group B from hydroxylapatite column chromatography was electrofocussed separately, two peaks with p*I*'s 9.0 and 9.4 were seen (Fig. 5c). This suggests that these fractions represent the mitochondrial isoenzyme of ASAT, which would thus also have subforms. A study of the subcellular localization of TAT activity (using 10 mM α-ketoglutarate in the assay) revealed that about one sixth of the activity is associated with mitochondria. This may be sufficient to give a contamination to the cytosol that could be responsible for the two peaks of activity; the possibility that this enzyme is partly cytoplasmic in nature must not, however, be overlooked.

These experiments show that even other aminotransferases have subforms and this is not a special property of TAT.

Characterization of the multiple forms from the two sources. The kinetic constants of the different multiple forms for tyrosine and α-ketoglutarate were determined (Table 1). The K_m-values for tyrosine of the three main fractions from rat liver were about 1 mM, as well as those of the three hydroxylapatite fractions of the frog proposed to be subforms of TAT (*i.e.*

Table 1. Summary of the properties of the multiple forms of TAT. The inhibition by α-ketoglutarate was calculated as percent of the highest activity measured, heat denaturation as percent of activity lost by heating to 60 °C for 5 min. The K_m-values for tyrosine were determined using 10 mM (rat HA 1–3, rat IF fractions and frog HA C 1, C 2 and C 3) or 0.5 mM (frog HA A and B) α-ketoglutarate.

Species	K_m(Tyr)/ mM [d]	K_m(α-KG)/ mM [d]	Isoelectric point	ASAT activity	Heat denaturation/%	Inhibition by 10 mM α-KG at pH 7.6/%	Suggested proper name
Rat, HA I [e]	1.4	0.4	5.2	–	0	None	TAT
Rat, HA 2 [e]	1.1	0.3	5.0	±	0	None	TAT
Rat, HA 3 [e]	1.3	0.3	4.8	–	0	None	TAT
Rat, IF α [f]	1.1	0.3	4.72	–	0	None	TAT
Rat, IF β [f]	1.4	0.3	4.98	–	0	None	TAT
Rat, IF γ [f]	1.3	0.4	5.30	–	0	None	TAT
Rat, IF m [f]	8.0	0.04	9.8	+	15	25	mASAT
Frog, HA A [b,g]	4.2	0.05	6.35, 6.50 and 6.90	+	0	75	sASAT
Frog, HA B [b,g]	15	0.05	9.0 and 9.4	+	80	30	mASAT
Frog, HA C 1	1.1	[a]	N.D.[c]	–	0	[a]	TAT
Frog, HA C 2	1.0	[a]	N.D.	–	0	[a]	TAT
Frog, HA C 3	1.1	[a]	N.D.	–	0	[a]	TAT

[a] = Substrate activation (see text). [b] = Mixtures of at least two forms (see Fig. 5). [c] = Not determined. [d] K_m(Tyr) = K_m for tyrosine; K_m(α-KG) = K_m for α-ketogluterate. [e] See Fig. 3. [f] See Figs. 1 and 2. [g] See Fig. 4.

C 1, C 2 and C 3). The fraction of TAT activity from rat liver that had a p*I* above 9 had a K_m for tyrosine of 8 mM, which further suggests that this form is, in fact, identical to mASAT.[18] Group A of multiple forms of TAT activity from hydroxylapatite column chromatography fractionation of frog liver cytosol (studied as a combination of all three subforms in roughly equal amounts) had a K_m for tyrosine of 4 mM. It is possible that this form can transaminate tyrosine under physiological conditions. The multiple forms of group B, when combined, had somewhat higher K_m-values for tyrosine. The K_m's for α-ketoglutarate will be discussed under a separate heading.

All the multiple forms except those of group B of the frog were found to be heat stable. The labile multiple forms were denaturated to 20 % by the heating (Table 1).

Mitochondrial ASAT separated from frog liver (unpublished) was found to share the following properties with group B: the ratio of ASAT and TAT activities, isoelectric point(s), K_m for tyrosine, heat lability, K_m for α-ketoglutarate, inhibition by α-ketoglutarate and ability to use pyruvate as the keto acid. Thus, group B is probably identical to mASAT.

Effects of α-ketoglutarate on the activity of the multiple forms of TAT. The K_m-values for α-ketoglutarate for the multiple forms of TAT of the rat are shown in Table 1. It can be seen that there are no gross differences between the three major forms. The form that has a high p*I* has a significantly lower K_m for α-ketoglutarate than the three main fractions. This further lends support to the suggestion that this form is identical to mitochondrial ASAT.[18] The K_m-values of the major forms are quite near to those reported by Iwasaki *et al.*[6]

The combined fractions of group A from frog liver (suggested to be subforms of sASAT) were strongly inhibited by α-ketoglutarate under the normal assay conditions (α-ketoglutarate, 10 mM). The inhibition was stronger at lower pH values. The K_m-value of the combination was very low, about 0.05 mM, which indicates that it is always saturated with this substrate.[19]

The combined fractions of group B (that showed similarities to mASAT) had also a low K_m for α-ketoglutarate but were inhibited to a much lesser extent by this substrate at pH 7.6 (25 % *vs.* 75 % inhibition at 10 mM α-ketoglutarate). This property is shared with mASAT from frog liver. The peaks of group B were combined in these studies because so little enzyme protein was obtained.

The three forms proposed to be subforms of TAT (C 1, C 2 and C 3) seemingly did not follow Michaelis-Menten kinetics. Instead, they exhibited substrate activation by α-ketoglutarate. All the forms showed this same tendency. Studies are in progress to further characterize this phenomenon.

DISCUSSION

Three major fractions that have tyrosine aminotransferase activity have been resolved from rat liver by isoelectric focussing, hydroxylapatite column chromatography, electrophoresis in agar gel [8] and ion exchange chromatography by CM-Sephadex.[9] Sepharose fails to separate these forms [9] as does Sephadex G-200 gel filtration.[6] This suggests that these subforms differ in their isoelectric points and net charges but probably not in molecular weights. These multiple forms are interconverted in homogenates [9] which, together with the above-mentioned facts, suggests that the different forms are posttranslational modifications that arise from a single molecular species. The physiological significance of the heterogeneity is not known at present. The present results show, however, that this phenomenon is independent of the complex hormonal regulation of the enzyme seen in rat liver, because hepatic TAT of the frog also has subforms but is not induced by corticosteroids.[5]

It was interesting to note that, in addition to tyrosine aminotransferase, both the mitochondrial and soluble isoenzymes of aspartate aminotransferase obtained from frog liver have subforms that differ in their isoelectric points. This indicates that the heterogeneity is a property common to at least three aminotransferases (TAT, sASAT and mASAT). Earlier reports on ASAT reveal that the two isoenzymes of ASAT obtained from different mammalian tissues also have subforms. The only difference so far known between them is in their isoelectric points.[20–22] The heterogeneity of the cytoplasmic isoenzyme of aspartate aminotransferase has been noted in preparations from

sheep liver,[20] beef heart [21] and rat brain.[22] Aspartate aminotransferase is far better characterized enzymologically than TAT.[20–28] The amino acid sequence for sASAT is completely known and that for mASAT almost completely known.[27,28] The subforms of sASAT from pig heart have identical amino acid compositions.[22] The subforms from sheep liver are immunologically identical.[20]

Summing up, we conclude that the heterogeneity is common to many aminotransferases. It is possible that in each case it is based on similar, minor posttranslational modification. The heterogeneity may be important in the regulation of gluconeogenesis from amino acids in higher animals.

While this manuscript was in preparation, Smith *et al.*[29] reported on a particle bound factor that interconverts the subforms of TAT. It will be very interesting to see if this factor can interconvert the subforms of sASAT and mASAT as well.

The cytoplasmic isoenzyme of ASAT from frog liver could also transaminate tyrosine while that of the rat is known to have little TAT activity.[30] It is possible that the aminotransferases have evolved from a common ancestrial gene during evolution. This would explain their common properties such as their heterogeneity and overlapping substrate spectra.

Another fraction of tyrosine aminotransferase activity that has a high K_m for tyrosine and is eluted from hydroxylapatite columns before the three "major" subforms has been reported and designated "form 1" by Iwasaki *et al.*[6] This form was denaturated by heating to 60 °C for 5 min and had a high K_m for α-ketoglutarate, all properties that clearly separate it from the aspartate aminotransferases. Other authors have failed to demonstrate this form. In our studies we have sometimes seen a small notch in the beginning of the phosphate gradient in hydroxylapatite column chromatography. One such notch is demonstrated in Fig. 3. We also sometimes noticed a minor form of activity at about pH 8 (see Results) in isoelectric focussing experiments of rat cytosol. It is not known if these forms are identical; because of the small amount of the enzyme characterization was not feasible. It is possible that the cathodal "isoenzymes" reported by Mertvetsov *et al.*[31,32] are related to these forms; most probably, however, they represent mitochondrial ASAT activity (also known as mitochondrial TAT).

From the results presented in this paper and from a study of the literature we conclude that several aminotransferases have subforms. The subforms probably arise from single molecular species through minor chemical modifications; this can be seen *in vitro* but the modification may be catalyzed by specific factors *in vivo*. The peculiar kinetic behaviour of the subforms of TAT from frog liver suggests a possible allosteric mechanism of regulation; this is presently being studied in our laboratory.

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The Crystal and Molecular Structure of DL-α-Methyltyrosine

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The crystal structure of DL-α-methyltyrosine has been determined by X-ray diffraction methods using 2095 observed reflections. The crystals are monoclinic, space group $P2_1/c$, with four molecules in the unit cell of dimensions $a = 12.763(2)$ Å; $b = 5.867(1)$ Å; $c = 17.653(4)$ Å; $\beta = 132.12(1)°$. The structure was solved by Patterson methods and refined to a conventional R-factor of 0.048. Estimated standard deviation in bond lengths not involving hydrogen atoms is 0.002 Å and in angles 0.1–0.2°. The bond lengths and angles are consistent with those found in L-tyrosine. The conformational angles describing the positions of the amino and carboxyl groups relative to the aromatic ring are nearly identical to those of L-tyrosine and thus different from those reported for α-methyl-*m*-tyrosine.

DL-α-Methyltyrosine has been examined as a part of the study of structural and conformational characteristics in phenylalanine derivatives in progress in this laboratory. The compound appears interesting both by being biologically active as a specific inhibitor of the enzyme tyrosine 3-hydroxylase [1] thus interfering with the biosynthesis of catecholamines, as well as by offering data in the study of the conformational effect of α substituents in phenylalanine derivatives.

EXPERIMENTAL

Crystals of DL-α-methyltyrosine were formed by very slow evaporation of a saturated solution of the compound in formic acid at 60 °C. The specimen used for the X-ray experiments had approximate dimensions $0.15 \times 0.30 \times 0.35$ mm³. Oscillation and Weissenberg photographs indicated monoclinic symmetry; systematically absent reflections proved the space group to be $P2_1/c$. Unit cell dimensions were determined from diffractometer measurements of 23 general reflections using Cu$K\alpha$-radiation ($\lambda = 1.3922$ Å).

The intensity data were recorded using a Picker automatic four-circle diffractometer with graphite crystal monochromated Mo$K\alpha$ radiation ($\lambda = 0.71069$ Å). 2710 independent reflections with $\sin\theta/\lambda < 0.7$ were measured using the $\omega - 2\theta$ scanning mode. The 2θ scan speed was 1° min^{-1} and the range from 0.8° below $2\theta(\alpha_1)$ to 0.8° above $2\theta(\alpha_2)$, background counts were taken for 30 s at each of the scan range limits. Three standard reflections were measured after every 100 reflections; they showed no systematic variation during the experiment. 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. 2095 reflections had net intensity larger than $2\sigma(I)$ and were regarded as observed, whereas the remaining reflections were excluded from the calculations. The intensity data were corrected for Lorentz and polarization effects.

Atomic form factors used were those of Doyle and Turner [2] for oxygen, nitrogen, and carbon atoms, and of Stewart, Davidson and Simpson [3] for hydrogen. A description of the computer programs employed during the structure determination is given in Ref. 6. In the full-matrix least-squares program the quantity minimized was $\sum w\Delta F^2$ where w is the inverse of the variance of the observed structure factors.

CRYSTAL DATA

DL-α-Methyl(4-hydroxyphenyl)alanine (α-methyltyrosine), $C_{10}H_{13}NO_3$, monoclinic, $a = 12.763(2)$ Å; $b = 5.867(1)$ Å; $c = 17.653(4)$ Å; $\beta = 132.12(1)°$, ($t = 18 \pm 1$ °C). $V = 980.47$ Å³; $F(000) = 416$; $Z = 4$; $D_{obs} = 1.30$ g cm^{-3}; $D_{calc} = 1.322$ g cm^{-3}. Absent reflections: $(0k0)$ for k odd, $(h0l)$ for l odd. Space group $P2_1/c$ (No. 14).

STRUCTURE DETERMINATION

The structure was solved by Patterson methods. From a sharpened three-dimensional Pat-

Table 1. Fractional atomic coordinates and thermal parameters with estimated standard deviations ($\times 10^4$). The temperature factor is given by exp $-(B11h^2+B22k^2+B33l^2+B12hk+B13hl+B23kl)$.

	x	y	z	$B11$	$B22$	$B33$	$B12$	$B13$	$B23$
O1	−2643(1)	−616(2)	−3281(1)	98(2)	221(4)	43(1)	−20(4)	55(2)	0(3)
O2	3668(1)	−2098(2)	−430(1)	98(2)	199(3)	44(1)	−40(3)	93(2)	−49(3)
O3	3729(1)	−3711(2)	743(1)	145(2)	136(3)	66(1)	22(4)	144(2)	22(3)
N1	3887(1)	2091(2)	157(1)	85(2)	130(3)	41(1)	9(4)	80(2)	6(3)
C1	1238(1)	468(3)	−338(1)	69(2)	176(4)	40(1)	12(5)	74(2)	−5(3)
C2	623(2)	2175(3)	−1071(1)	84(2)	155(4)	54(1)	1(5)	81(2)	9(4)
C3	−665(2)	1856(3)	−2061(1)	86(2)	179(5)	48(1)	25(5)	82(2)	34(4)
C4	−1377(2)	−191(3)	−2321(1)	75(2)	197(5)	40(1)	11(5)	69(2)	−7(3)
C5	−800(2)	−1894(3)	−1593(1)	88(2)	173(5)	51(1)	−32(5)	84(2)	−3(4)
C6	493(2)	−1560(3)	−618(1)	89(2)	191(5)	44(1)	6(5)	86(2)	23(3)
C7	2676(2)	763(3)	721(1)	80(2)	182(5)	38(1)	2(5)	75(2)	−19(3)
C8	3917(1)	312(2)	777(1)	71(2)	130(4)	31(1)	5(4)	59(2)	5(3)
C9	3757(1)	−2043(3)	327(1)	64(2)	142(4)	39(1)	7(4)	62(2)	−10(3)
C10	5342(2)	487(3)	1865(1)	80(2)	258(5)	37(1)	1(5)	55(2)	−12(4)

Table 2. Fractional atomic coordinates ($\times 10^3$) and B-values with estimated standard deviations for hydrogen atoms.

	x	y	z	B
HC2	112(2)	366(4)	−89(1)	3.8(4)
HC3	−107(2)	306(4)	−261(2)	4.3(4)
HC5	−127(2)	−335(4)	−175(1)	4.1(4)
HC6	87(2)	−276(3)	−12(1)	3.4(4)
H1C7	182(2)	−34(3)	121(1)	3.2(4)
H2C7	276(2)	228(3)	97(1)	3.1(4)
H1C10	540(3)	−61(5)	230(2)	6.5(6)
H2C10	550(3)	201(5)	213(2)	6.7(7)
H3C10	612(3)	18(4)	190(2)	5.1(5)
H1N	312(2)	190(3)	−55(2)	3.6(4)
H2N	466(2)	196(4)	20(1)	4.6(5)
H3N	383(2)	361(4)	37(2)	5.0(5)
HO1	−294(3)	68(5)	−370(2)	7.3(7)

terson map the position and orientation of the molecular fragment comprising the benzene ring and the two non-hydrogen atoms attached to it could be determined. This fragment was sufficient to bring out the positions of the molecular framework in the subsequent Fourier syntheses. The refinement proceeded by full-matrix least-squares calculations. Anisotropic thermal parameters were introduced for non-hydrogen atoms; hydrogen atomic positional parameters (calculated from stereochemical considerations) and isotropic thermal parameters were refined. The refinement converged to a final conventional R-factor of 0.048 for the 2095 observed reflections ($R_w = 0.051$). The corresponding atomic parameters are listed in Tables 1 and 2. The structure factor list may be obtained from the authors upon request.

The anisotropic thermal parameters were analysed in terms of rigid-body motion both for the whole molecule and for the hydroxybenzyl and alanine parts separately. The latter description was adopted when correcting bond lengths for libration effects. Standard deviations in interatomic distances are calculated from the correlation matrix ignoring uncertainties in cell dimensions.

Table 3. Bond lengths (Å) and bond angles (°). Estimated standard deviations in parentheses.

Bond		(corr)	Angle	
C1 – C2	1.390(2)	1.397	C6 – C1 – C7	120.7(1)
C2 – C3	1.392(2)	1.395	C7 – C1 – C2	121.8(1)
C3 – C4	1.385(2)	1.393	C2 – C1 – C6	117.5(1)
C4 – C5	1.387(2)	1.394	C1 – C2 – C3	121.7(1)
C5 – C6	1.384(2)	1.387	C2 – C3 – C4	119.5(1)
C1 – C6	1.391(2)	1.398	C3 – C4 – C5	119.6(1)
C1 – C7	1.514(2)	1.516	C3 – C4 – O1	122.4(1)
C7 – C8	1.542(2)		O1 – C4 – C5	117.9(1)
C8 – C9	1.538(2)	1.544	C4 – C5 – C6	120.0(1)
C8 – C10	1.524(2)	1.530	C5 – C6 – C1	121.6(1)
C8 – N1	1.494(2)	1.500	C1 – C7 – C8	113.7(1)
C9 – O2	1.263(2)	1.267	C7 – C8 – C9	110.3(1)
C9 – O3	1.239(2)	1.244	C7 – C8 – C10	112.1(1)
C4 – O1	1.368(2)	1.370	C7 – C8 – N1	108.2(1)
			C9 – C8 – C10	110.1(1)
			C9 – C8 – N1	108.6(1)
			C10 – C8 – N1	107.4(1)
			C8 – C9 – O2	117.1(1)
			C8 – C9 – O3	116.7(1)
			O2 – C9 – O3	126.2(1)
Hydrogen bonds (Å)			Dihedral angles (°)	
O1 – O2($-x,\frac{1}{2}+y,\frac{1}{2}-z$)		2.675(2)	C2 – C1 – C7 – C8	278.4
N – O1($-x,\frac{1}{2}+y,\frac{1}{2}-z$)		2.871(2)	C1 – C7 – C8 – C9	306.5
N – O2($1-x,-y,-z$)		2.821(2)	C1 – C7 – C8 – N	65.2
N – O3($x,1+y,z$)		2.731(2)	C1 – C7 – C8 – C10	183.4
			O2 – C9 – C8 – N	1.6

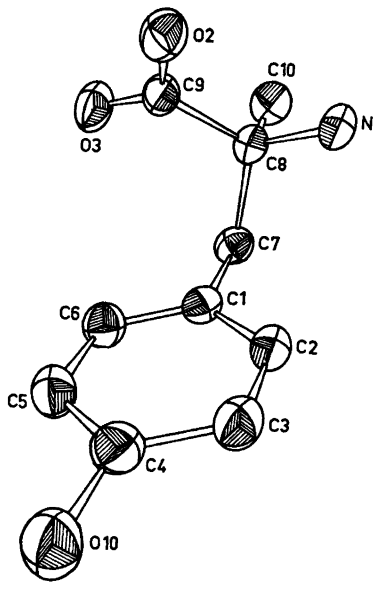

Fig. 1. Structure of α-methyltyrosine.

DISCUSSION

The bond lengths and angles are given in Table 3; Fig. 1 shows an illustration of the molecular structure with the numbering of the atoms. Conformational angles in the molecule and hydrogen bond data are also listed in Table 3. Dihedral angles are given as positive for the clockwise rotation.

The bond lengths and angles found in α-methyltyrosine are close to those reported for L-tyrosine.[5] Significant differences are found only in the α-amino acid moiety; in L-tyrosine the C – O2 bond (*cis* relative to the C – N bond) is 1.246 Å and the C – O3 bond 1.260 Å whereas the corresponding bond lengths in α-methyltyrosine are 1.267 and 1.244 Å, respectively. The difference may be explained by the hydrogen bonding system in the two crystals. In both cases the oxygen atom of the longer C – O bond is acceptor in *two* hydrogen bonds and the other in only one such bond. The C7 – C8 – N angle in α-methyltyrosine (108.2°) is smaller

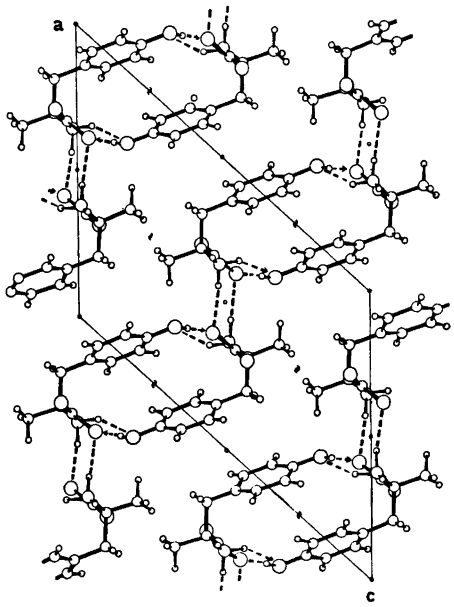

Fig. 2. The crystal structure of α-methyltyrosine as viewed along the *b*-axis.

than the corresponding angle in L-tyrosine (111.3°); this may be a result of the α-substitution of the more bulky methyl group. The six atoms of the benzene ring are coplanar.

The conformation about the C7–C8 bond of the α-methyltyrosine molecule corresponds to that found for L-tyrosine, *i.e.* with the amino and carboxyl groups both in *gauche* positions relative to the phenyl group. The fact that the isomers of these compounds, *m*-tyrosine and α-methyl-*m*-tyrosine, both appear in the conformation where the carboxyl group is in *trans* position with respect to the aromatic ring in the solid phase [6,7] may indicate that the different conformations are energetically close and mainly determined by crystal forces.

The nitrogen atom in α-methyltyrosine is situated close to the plane of the carboxyl group, the dihedral angle O2–C9–C8–N is as small as 1.6°.

The crystal structure is characterized by bimolecular layers parallel to (1,0,−2) connected only through van der Waals' forces as illustrated in Fig. 2. Within the layers the molecules are linked together by hydrogen bonds. The nitrogen and phenol oxygen atoms act as hydrogen donors in *four* hydrogen bonds to other molecules; the phenol oxygen atom (O1) is acceptor in one and the carboxylic oxygen atoms in three such bonds. The molecule is thus linked to other molecules within the double molecular layer by *eight* hydrogen bonds of lengths given in Table 3.

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On the Halogen—Metal Exchange Reaction between Mixed Dihalothiophenes and Alkyllithium

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The halogen-metal exchange of 2-bromo-4-iodo-, 2-chloro-4-bromo-, 2-chloro-3-iodo-, and 2-chloro-3-bromothiophene and butyllithium has been studied. Exchange occurred in all cases first with the β-halogen. 2-Bromo-4-thienyllithium rearranged even at −100 °C, while the other β-thienyllithium compounds were stable enough at −70 °C to be of preparative use. Upon warming 2-chloro-3-thienyllithium underwent rearrangements, while 2-chloro-4-thienyllithium ring-opened.

Halogen-metal exchange between bromo- or iodothiophenes and alkyllithium derivatives is one of the most important preparative reactions in thiophene chemistry.[1,2] The reaction is very rapid at −70 °C and it is also known that an α-bromine [3] or α-iodine [4] exchanges much faster than a corresponding β-halogen. This has for instance been demonstrated in the halogen-metal exchange reaction between 2,3-dibromothiophene or 2,4-dibromothiophene with butyllithium at −70 °C, in which cases only the α-bromines are exchanged.[3] It is also well known from the classical investigations by Wittig *et al.* and Gilman *et. al.* that iodides react much faster than bromides. (For a review *cf.* Ref. 5.)

Chlorothiophenes give halogen-metal exchange only when there is no free α-position. Such exchange has been observed with 2,5-dichlorothiophene [6] and with tetrachlorothiophene [6,7] and also with β-chlorine derivatives such as 2,5-dimethyl-3,4-dichlorothiophene.[8] 2-Chlorothiophene on the other hand is metalated to 5-chloro-2-thienyllithium.[6] No halogen-metal interconversion has been observed with fluorothiophenes.[9]

During our work certain complications have been observed in these halogen-metal exchanges. Thus it was found that when the lithium atom does not replace the most acidic hydrogen, rearrangement by a series of rapid halogen-metal exchanges and metalations leads to the most stable lithium derivative, as for instance observed for 4-bromo-3-thienyl- and 4-bromo-2-thienyllithium.[3] It has also been found that at higher temperatures certain 3-thienyllithium derivatives ring-open to lithium thioenynes.[10–13] On the other hand, the high stability of *ortho*-bromothienyllithium derivatives such as 3-bromo-2-thienyllithium [14] is markedly different from that of *ortho*-bromophenyllithium. [15] These compounds showed no tendency to eliminate lithium bromide to give dehydrothiophene. Many different attempts to trap dehydrothiophenes have failed,[16–19] and it has been shown that the *cine*-substitution occurring during the amination of halothiophenes does not involve aryne pathways.[18] Recently, however, Reinecke and Newson [20] claimed the intermediacy of five-membered hetarynes in the thermolysis of heterocyclic anhydrides. We were therefore interested to study the halogen-metal exchange of mixed dihalothiophenes with the more reactive halogen in the β-position. If 2-halo-3-thienyllithium derivatives could be obtained, these should be suitable intermediates for the formation of 2,3-dehydrothiophene.

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** Taken in part from the Ph.D. thesis of Boris Holm, University of Lund 1974.

In a short communication some years ago we described the reaction between 2-bromo-3-iodothiophene and ethyllithium.[21] At −100 °C halogen-metal exchange occurred in the β-position, giving 2-bromo-3-thienyllithium in at least 84 % yield, characterized as 2-bromo-3-thiophenecarboxylic acid. Upon hydrolysis, 95 % of 2-bromothiophene was obtained.

However, attempts to detect 2,3-dehydrothiophene by carrying out the halogen-metal exchange in the presence of furan were unsuccessful. 2-Bromo-3-thienyllithium, however, was very prone to undergo rearrangement to a thermodynamically more stable lithium derivative. Thus if the halogen-metal exchange was carried out at −70 °C, 3-bromo-2-thienyllithium was the main product.[21] A reaction scheme for the transformation of 2-bromo-3-thienyllithium to 3-bromo-2-thienyllithium was suggested.

STARTING MATERIALS

2-Bromo-4-iodothiophene was prepared in 36 % yield from 2,4-diiodothiophene,[4] through halogen-metal exchange followed by reaction with bromine. A possible route to 2-bromo-4-iodothiophene consisting of halogen-metal exchange of 5-bromo-2,3-diiodothiophene followed by hydrolysis was inferior due to the low yield obtained in the bromination of 2,3-diiodothiophene.[28]

4-Bromo-2-chlorothiophene has been prepared from 4-bromo-2-thienyllithium through the reaction with chlorine [9] or with hexachloroethane.[30] Since we had 5-chloro-2,3-dibromothiophene [28] available, we reacted it with butyllithium followed by hydrolysis which gave 4-bromo-2-chlorothiophene in 53 % yield.

For identification purposes the unknown 3-bromo-2-chloro-5-thiophenecarboxylic acid was prepared from 2-chloro-3,5-dibromothiophene through halogen-metal exchange with butyllithium followed by reaction with carbon dioxide. The isomeric 3-bromo-5-chloro-2-thiophenecarboxylic acid was analogously obtained from 5-chloro-2,3-dibromothiophene.[28]

RESULTS

As could be expected from the previous results with 2-bromo-3-iodothiophene, halogen-metal exhange of 2,5-dibromo-3-iodothiophene led to extensive rearrangement and several compounds were formed upon hydrolysis. This reaction therefore cannot be used for the preparation of 2-bromo-4-iodothiophene. Thus as 2-bromo-3-iodothiophene, 2,5-dibromo-3-iodothiophene also gives exchange of the β-iodine faster than of α-bromine.

When 2-bromo-4-iodothiophene was allowed to react with butyllithium it was found that the intermediate 2-bromo-4-thienyllithium was more unstable than 2-bromo-3-thienyllithium and underwent rapid rearrangement even at −100 °C. The results were here somewhat more difficult to reproduce, and in Table 1 the analytical results obtained are given. GLC analyses were carried out both after ethanolysis and after reaction with carbon dioxide followed by esterification with diazomethane.

From Table 1 it can be seen that a large excess of butyllithium suppresses the rearrange-

Table 1. Product distributions in the reactions of 2-bromo-4-iodothiophene with butyllithium in ether after protonation or carbonation followed by acidification and esterification

Temp./°C	X	2-Bromo-4-X-thiophene/%	3-Bromo-5-X-thiophene/%	3-Iodo-5-X-thiophene/%	2,4-Dibromo-5-X-thiophene/%	Excess of BuLi/%
<−60	H	8	48	39	5	110
<−60	H	66	22	11	trace	150
<−100	H	9	24	35	33	10
<−100	H	60	trace	19	21	10
<−100	H	24	12	32	32	10
<−60	CO_2CH_3	trace	21	62	16	10
<−100	CO_2CH_3	64	2	26	8	10
<−100	CO_2CH_3	92	1	7	—	10
<−100	CO_2CH_3	78	6	17	—	10

ments of initially formed 2-bromo-4-thienyllithium. The observation of methyl 3,5-dibromo-2-thiophenecarboxylate is somewhat unexpected, as it implies the formation of 3,5-dibromo-2-thienyllithium through metalation at −100 °C. Metalation is usually slow at this temperature, but this observation is in accordance with the faster metalation of 2,4-dibromothiophene compared to 2,3-dibromothiophene.[3] Thus 2-bromo-4-iodothiophene is obviously not a suitable starting material for the preparation of 2-bromo-4-thienyllithium and products derived from it.

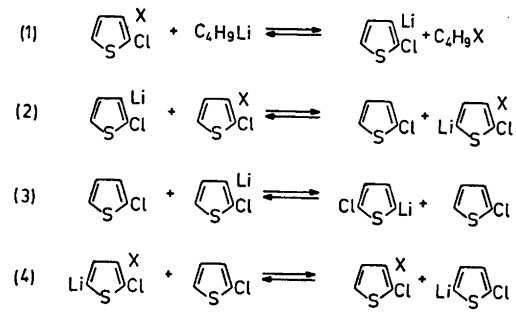
3-Bromo-2-chloro-[28] and 2-chloro-3-iodothiophene,[28] on the other hand, showed no tendency to rearrange when treated with alkyllithium at −70 °C. Protonation of the intermediate 2-chloro-3-thienyllithium gave only 2-chlorothiophene, and reaction with carbon dioxide 2-chloro-3-thiophenecarboxylic acid in good yield.

However, as the lithium of 2-chloro-3-thienyllithium does not substitute the most acidic hydrogen, rearrangement to the thermodynamically more stable lithium derivatives can be expected.[3] If 2-chloro-3-thienyllithium, prepared at −70 °C, was allowed to reach room temperature slowly and stirred overnight, interesting differences were observed

Table 2. Product distributions in the reactions of 3-bromo-2-chloro- and 2-chloro-3-iodothiophene [a] with butyllithium in ether at −70 °C followed by temperature rise, carbonation, acidification, and esterification. A, methyl 2-chloro-5-thiophenecarboxylate; B, methyl 2-chloro-3-thiophenecarboxylate; C, methyl 2-chloro-3-bromo-5-thiophenecarboxylate.

A/%	B/%	C/%
18	71	10
53	5	42
31	45	23
32	68	—
25	75	—
13	87	—

[a] The first three results were obtained with 3-bromo-2-chlorothiophene and the following three with 2-chloro-3-iodothiophene. [b] The sensitivity factor of methyl 2-chloro-5-thiophenecarboxylic acid was set equal to that of methyl 2-chloro-3-thiophenecarboxylic acid.



Scheme 1. X = Br or I.

depending upon whether the 2-chloro-3-thienyllithium was derived from 3-bromo-2-chlorothiophene or 2-chloro-3-iodothiophene. In the former case, rearrangement appeared to be more extensive and large relative amounts of 5-chloro-2-thienyllithium and especially of 3-bromo-2-chloro-5-thienyllithium were formed (Table 2). The lithium derivatives were as previously characterized by reaction with carbon dioxide followed by methylation. In the latter case no formation of 2-chloro-3-iodo-5-thienyllithium was observed.

This is somewhat unexpected, since according to the general mechanism previously suggested [3] for such rearrangements, 2-chloro-3-halo-5-thienyllithium should be an intermediate in the formation of 5-chloro-2-thienyllithium (*cf.* reaction formulae (1)−(3) in Scheme 1). It is possible that reaction (3) is much faster than reaction (2) when X = I, and therefore only a very low concentration of 2-chloro-3-iodo-5-thienyllithium will be formed, or that this lithium derivative is removed by a reaction such as (4) to a much greater extent than when X = Br. As is also evident from Table 2, it was quite difficult to obtain reproducible proportions of the compounds formed. As stressed previously,[3] this depends upon the fact that small and difficultly controllable differences in the experimental conditions cause great differences in the final product proportions. For instance small amounts of 2-chlorothiophene formed by unintentional hydrolysis will catalyze the rearrangement to 5-chloro-2-thienyllithium (eqn. 3). Also differences in the reaction rate of the various lithium derivatives with the "trapping" reagents might contribute to the difficulties in obtaining

reproducible results. No ring-opening to acetylenic derivatives was observed with 2-chloro-3-thienyllithium. The same is also true for 2-chloro-5-methyl- and 2,5-dichloro-3-thienyllithium.[29]

When 4-bromo-2-chlorothiophene was reacted with butyllithium at −70 °C, 2-chloro-4-thienyllithium was obtained, which at this temperature gave no rearrangement yielding 2-chloro-4-(1-cyclohexenyl)thiophene with cyclohexanone,[30] and 2-chloro-4-thiophenecarboxylic acid and 2-chloro-4-iodothiophene with carbon dioxide and iodine, respectively. However, when 2-chloro-4-thienyllithium prepared at −70 °C was stirred overnight at room temperature, no chlorothiophenecarboxylic acids could be detected after reaction with carbon dioxide. IR analysis indicated that compounds containing triple bonds had been formed. One peak in the gas chromatogram, with longer retention time than that of the starting material, showed in the GLC/MS analysis a molecular ion at $m/e = 138$. The intensities of this peak and those at $m/e = 139$ and $m/e = 140$ indicated this compound to have the composition $C_8H_{10}S$, and the intense fragments at $m/e = 81$ ($M^+ - C_4H_9$) and $m/e = 82$ ($M^+ - C_4H_8$) showed the compound to be butylthiodiacetylene. This compound is formed by ring-opening of 2-chloro-4-thienyllithium to lithium 1-chloro-1-buten-3-yne-1-thiolate, which is alkylated by the butyl bromide to give 1-butylthio-1-chloro-1-buten-3-yne. This yields the observed 1-butylthiodiacetylene upon elimination of hydrogen chloride.

A similar observation was made when 5-chloro-2-methyl-3-thienyllithium, prepared by halogen-metal exchange between 3-bromo-5-chloro-2-methylthiophene and ethyllithium, ring-opened to yield 1-ethylthio-1,3-pentadiyne.[29]

CONCLUSIONS

Our investigations thus indicate that α-chloro-β-thienyllithium compounds prepared through halogen-metal exchange between α-chloro-β-iodo- or α-chloro-β-bromothiophenes and alkyllithia at −70 °C are stable enough to allow the preparation of 2-chloro-3-substituted and 2-chloro-4-substituted thiophenes. If the temperature is allowed to rise, 2-chloro-3-thienyllithium rearranges to thermodynamically more stable lithium derivatives, predominantly 5-chloro-2-thienyllithium, while 2-chloro-4-thienyllithium ring-opens. α-Bromo-β-thienyllithium compounds prepared from α-bromo-β-iodothiophenes rearrange much faster. However, 2-bromo-3-substituted thiophenes can be obtained by carrying out the reaction below −100 °C. No evidence for the formation of 2,3-dehydrothiophene from 2-bromo-3-thienyllithium was found.

EXPERIMENTAL

The equipment for obtaining NMR, IR, and mass spectra has been described in Ref. 28, as well as the GLC equipment used. The following columns were used: 5 % Neopentyl glycol-succinate (NPGS) on Chrom. W (80/100 mesh), 2.0 m (A), 3 % OV 17 on Gas Chrom. Q (100/120 mesh), 2.5 m (B), 10 % butane-1,4-diol succinate (BDS) on Chrom. W (80/100 mesh), 2.0 m (C), and 5 % Apiezon L on Chrom. W (80/100 mesh), 2.0 m (D).

Products of less than 1 % abundance in the reaction mixtures are not reported, if they are not of special interest. For all products obtained calibration was, if not otherwise stated, made for the sensitivity factor of each compound to the flame.

Most of the elemental analyses were performed by the Department of Analytical Chemistry at the University of Lund, Sweden, and a few by Miss Ilse Beetz, Mikroanalytisches Laboratorium, Kronach, West Germany.

Experiments with lithium compounds and titration of alkyllithium solution were carried out as described in Ref. 13.

The purities of the compounds were checked by gas chromatography and NMR. Acids were esterified with diazomethane before being injected into the gas chromatograph.

Authentic samples of 2-bromo-,[22] 3-bromo-,[23] 3-iodo-,[24] 2,4-dibromothiophene [25] as well as of 2-bromo-4-thiophenecarboxylic acid,[26] 4-bromo-2-thiophenecarboxylic acid,[25] 4-iodo-2-thiophenecarboxylic acid,[4] and 3,5-dibromo-2-thiophenecarboxylic acid [27] were used for identification of the products.

Halogen-metal interconversion with 2,5-dibromo-3-iodothiophene. To an ethereal solution of 5.5 g (0.015 mol) of 2,5-dibromo-3-iodothiophene [28] cooled to −70 °C, 28 ml of 0.55 M (0.015 mol) butyllithium in ether was added at such a rate that the temperature was kept below −60 °C. After stirring for 10 min at −70 °C, the reaction mixture was poured into water. After work-up in the usual manner, GLC analysis showed that several products had been produced, indicating rapid rearrangement reactions. The method was therefore not

suitable for the preparation of 2-bromo-4-iodothiophene. (Column: C.)

2-Bromo-4-iodothiophene. To 120 ml of 0.90 M (1.08 mol) butyllithium in ether cooled to −70 °C, 33.6 g (0.100 mol) of 2.4-diiodothiophene [4] dissolved in 100 ml of ether was added in a slow stream. After stirring for an additional 20 min, bromine, dried with concentrated sulfuric acid, was added, while the temperature was kept at −70 °C. The bromine was added in portions, and at regular intervals aliquots were analyzed by GLC after hydrolysis. When the ratio between 3-iodothiophene and 2-bromo-4-iodothiophene no longer declined according to their relative concentrations in the reaction mixture, the addition of bromine was interrupted. After pouring the reaction mixture into water, the ether phase was separated and the water phase extracted with ether. The combined ether phases were washed with sodium thiosulfate solution, dilute sodium hydroxide solution, and water, and dried over magnesium sulfate. Distillation gave 10.5 g (36 %) of 2-bromo-4-iodothiophene, b.p. 106–108 °C/10 mmHg. The product was further purified by preparative gas chromatography (BDS 20 % on Chrom. W, 9 mm × 2 m). (Column: C.) NMR (CCl_4): δ 6.95 (H3 or H5, d, 1 H) and 7.20 (H5 or H3, d, 1 H). J (H3, H5) 1.5 Hz. [Calc. for C_4H_2BrIS (288.9): C 16.6; H 0.70; I 43.9. Found: C 17.0; H 0.84; I 43.6.]

Halogen-metal interconversion with 2-bromo-4-iodothiophene. 5.0 ml of 1.12 M (5.6 mmol) butyllithium in ether was cooled to −70 °C, whereupon 1.1 g (3.8 mmol) of 2-bromo-4-iodothiophene dissolved in 5 ml of ether was added at such a rate that the temperature was kept below −60 °C. After stirring for an additional 20 min, carbon dioxide gas, dried by bubbling through concentrated sulfuric acid, was led into the reaction mixture during 45 min at −70 °C. If protonation was desired instead, ethanol was added after stirring for 20 min. Work-up and analyses were performed as in the halogen-metal interconversion reactions with 2-bromo-3-iodothiophene (*cf*, above). When the reaction was performed at about −110 °C, the same method was used while keeping the temperature below −100 °C. The results of the analyses are given in Table 1 (Columns: A, C).

2-Chloro-3-thiophenecarboxylic acid. A. *From 2-chloro-3-iodothiophene.* To 50 ml of 0.90 M (0.045 mol) butyllithium in ether at −70 °C, 10 g (0.041 mol) of 2-chloro-3-iodothiophene dissolved in ether was added at such a rate that the temperature was kept below −55 °C. After stirring for 15 min, the reaction mixture was poured onto dry ice in ether. When the bubbling of the carbon dioxide had finished, the mixture was hydrolyzed with water and acidified with hydrochloric acid. After extracting the water phase with ether, the combined ether phases were extracted several times with dilute sodium hydroxide. The combined water phases were acidified with hydrochloric acid, whereupon the acid precipitated. After recrystallization from water-ethanol (1:1) and drying, 3.4 g (51 %) of 2-chloro-3-thiophenecarboxylic acid was obtained with m.p. 162–163 °C; lit. value:[31] 163 °C (Column: C). NMR (DMSO): δ 7.38 (H4 or H5, d, 1 H), 7.50 (H5 or H4, d, 1 H), and 9.08 (COOH, s, 1 H). J (H4, H5) 5.8 Hz. B. *From 3-bromo-2-chlorothiophene.* The same method as above utilizing 3-bromo-2-chlorothiophene gave a 58 % yield of 2-chloro-3-thiophenecarboxylic acid (Column: C).

Stability of 2-chloro-3-thienyllithium. 57 ml of 0.51 M (0.029 mol) butyllithium in ether was cooled to −70 °C, whereupon 5.0 g (0.025 mol) of 3-bromo-2-chlorothiophene [28] dissolved in ether was added, keeping the temperature below −55 °C. After stirring for 10 min the cooling bath was removed and the reaction mixture was stirred at room temperature overnight. The reaction mixture was carbonated and worked up in the usual manner. The resulting acid mixture was esterified with diazomethane. GLC/MS showed the formation of methyl 2-chloro-5-thiophenecarboxylate, methyl 2-chloro-3-thiophenecarboxylate, and methyl 3-bromo-2-chloro-5-thiophenecarboxylate. The NMR spectrum of the acid mixture also indicated the formation of 2-chloro-5-thiophenecarboxylic acid (J = 4.1 Hz) and 2-chloro-3-thiophenecarboxylic acid (J = 5.8 Hz). IR analysis of the ether phase did not reveal the formation of any compounds containing carbon-carbon triple bonds, indicating that no ring-opening reaction had occurred.

When 2-chloro-3-iodothiophene was used in a corresponding reaction, methyl 2-chloro-5-thiophenecarboxylate and methyl 2-chloro-3-thiophenecarboxylate were formed. However, in this case, no methyl 2-chloro-3-iodo-5-thiophenecarboxylate was obtained. IR analysis gave the same result as above. The results are given in Table 2 (Column: C).

3-Bromo-2-chloro-5-thiophenecarboxylic acid. To 40 ml of 0.85 M (0.034 mol) butyllithium in ether 8.2 g (0.030 mol) of 2-chloro-3,5-dibromothiophene [28] in 30 ml of ether was added in a slow stream, keeping the temperature below −47 °C. After stirring for an additional 20 min at −70 °C, the reaction mixture was poured onto dry ice in ether. After work-up in the usual manner and recrystallization from water-ethanol (1:1) 4.5 g (62 %) of 3-bromo-2-chloro-5-thiophenecarboxylic acid (m.p. 206–207 °C) was obtained (Column: A). [Calc. for $C_5H_2BrClO_2S$ (241.5): C 24.9; H 0.83; S 13.3. Found: C 24.8; H 0.85; S 13.2.]

4-Bromo-2-chloro-5-thiophenecarboxylic acid. To 40 ml of 0.85 M (0.034 mol) butyllithium in ether cooled to −70 °C 8.6 g (0.031 mol) of 5-chloro-2,3-dibromothiophene [28] in 40 ml of ether was added in a slow stream, keeping the temperature below −42 °C. After stirring

for an additional 20 min at −70 °C, the reaction mixture was carbonated and worked up in the usual way. The crude acid was recrystallized from water-ethanol (1:1), whereupon 5.0 g (67 %) of 4-bromo-2-chloro-5-thiophenecarboxylic acid (m.p. 198−199 °C) was obtained (Column: A). [Calc. for $C_5H_2BrClO_2S$ (241.5): C 24.9; H 0.83; S 13.3. Found: C 24.9; H 0.80; S 13.4.]

4-Bromo-2-chlorothiophene. 450 ml of 0.87 M (0.40 mol) butyllithium in ether was cooled to −70 °C, whereupon 96.5 g (0.350 mol) of 5-chloro-2,3-dibromothiophene [28] dissolved in 200 ml of ether was added in a slow stream. After stirring for 20 min the reaction mixture was poured into water and worked up in the usual manner. Distillation gave 36.3 g (53 %) of 4-bromo-2-chlorothiophene at 68.0−71.5 °C/10 mmHg. Lit. value:[9] 70−72 °C/12 mmHg (Column: A).

2-Chloro-4-thiophenecarboxylic acid. 40 ml of 0.82 M (0.033 mol) butyllithium in ether was cooled to −70 °C, whereupon 6.0 g (0.030 mol) of 4-bromo-2-chlorothiophene dissolved in 30 ml of ether was added in a slow stream keeping the temperature below −48 °C. After stirring for an additional 20 min, the reaction mixture was poured onto dry ice in ether. Water was added and the ether phase separated and extracted with dilute sodium hydroxide solution. The combined water phases were acidified with hydrochloric acid, whereupon the acid precipitated. Recrystallization from water-ethanol (1:1) gave 2.3 g (47 %) of 2-chloro-4-thiophenecarboxylic acid with m.p. 163−164 °C. Lit. value:[26] 156−157 °C (Column: B). NMR (acetone): δ 7.39 (H3, d, 1 H), 8.14 (H5, d, 1 H), and J (H3, H5) 1.6 Hz.

2-Chloro-4-iodothiophene. 70 ml of 0.89 M (0.062 mol) butyllithium in ether was cooled to −70 °C, whereupon 11 g (0.056 mol) of 4-bromo-2-chlorothiophene dissolved in 60 ml of ether was added in a slow stream, keeping the temperature below −60 °C. After stirring for an additional 25 min, the reaction mixture was poured through a rubber tube into a solution of 14.2 g (0.056 mol) of iodine in 70 ml of ether, also cooled to −70 °C. Stirring was then continued for 1 h at −70 °C. The cooling bath was removed, and after the temperature had reached 0 °C, the reaction mixture was poured into water. After separation of the ether phase, the water phase was extracted with ether. The combined ether phases were washed with water, sodium thiosulfate solution, and water, and dried over magnesium sulfate. Distillation gave 6.0 g (44 %) of 2-chloro-4-iodothiophene at 89.5−91.5 °C/10 mm (Column: A). NMR (CCl_4): δ 7.14 (H3 or H5, d, 1 H) and 7.36 (H5 or H3, d, 1 H). J (H3, H5) 1.5 Hz. [Calc. for C_4H_2ClIS (244.5): C 19.6; H 0.82; I 51.9. Found: C 19.6; H 0.86; I 50.2.]

3-Iodo-2-thiophenecarboxylic acid. To 15 ml of 1.00 M (0.015 mol) ethyllithium in ether cooled to −70 °C, 4.8 g (0.014 mol) of 2,3-diiodothiophene [4] dissolved in ether was added. The temperature was kept below −60 °C. After stirring for an additional 10 min, the reaction mixture was carbonated and worked up as described for 2-chloro-4-thiophenecarboxylic acid. Recrystallization from water-ethanol (1:1) gave 1.6 g (45 %) of 3-iodo-2-thiophenecarboxylic acid with m.p. 199−201 °C. Lit. value:[32] 193−195 °C (Column: A). NMR (acetone): δ 7.33 (H4, d, 1 H), 7.77 (H5, d, 1 H), and 7.95 (COOH, s, 1 H). J (H4, H5) 5.1 Hz.

Stability of 2-chloro-4-thienyllithium. To 40 ml of 0.85 M (0.034 mol) butyllithium in ether, 6.2 g (0.031 mol) of 4-bromo-2-chlorothiophene in 70 ml of ether was added, keeping the temperature below −51 °C. After stirring for an additional 15 min at −70 °C, the cooling bath was removed and the reaction mixture stirred for 19 h at room temperature. One sample was taken up and hydrolyzed with water, a second sample was carbonated, and a third part was stirred at room temperature for an additional 24 h. By absorption at approximately 2180 cm^{-1}, IR analysis of the ether phase indicated the formation of acetylenic products from ring-opening reactions. Combined GLC-mass spectrometry also indicated the prevalence of acetylenic compounds. The carbonated product was worked up and esterified. However, no methyl thiophenecarboxylates could be observed upon analysis, which also indicates that ring-opening had occurred. Analysis of the part of the reaction mixture which was stirred for a longer period gave the same result (Columns: C, D).

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Indolizine Derivatives. VI.* Indolizines from the Acylative Cyclization of 3-(2-Pyridyl)-2-propen-1-ones and Related Compounds

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3-(2-Pyridyl)-2-propen-1-ones with acetic anhydride/potassium acetate give rise to 1-substituted 2-acetyl-3-methylindolizines. In the presence of ethyl acetoacetate, analogous 2-ethoxycarbonylindolizines are obtained. The reaction using propionic anhydride/potassium propionate gives 4-(2-pyridyl)-2-pyrones, or products requiring a reduction step. 1-(2-Pyridyl)-2-propen-1-ones in neat acetic anhydride cyclize to 1-acetoxyindolizines. The reactions of some related compounds were also studied. The mechanisms are discussed.

It has been established that the Perkin reaction of 2-pyridinecarbaldehyde, as well as 3-(2-pyridyl)acrylic acid and 2-(2-pyridyl)methylene-1,3-dicarbonyl compounds under the conditions of the Perkin reaction, give rise to indolizine derivatives.[1,2] A logical extension of this is to treat (2-pyridyl)propenones in the same way with boiling acetic anhydride/potassium acetate. Thus, the present paper deals with the acylative cyclization of 3-(2-pyridyl)-2-propen-1-ones and related compounds as a novel synthetic route to indolizines.[3]

RESULTS

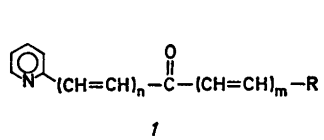
When the pyridylpropenone *1a* (Table 1) was heated with an excess of acetic anhydride/potassium acetate for 2 h, the indolizine *2a* was obtained in *ca.* 80 % yield. Its elemental formula $C_{21}H_{19}NO_3$, supported by the parent peak in the mass spectrum, $m/e = 333$ corresponds to a condensation of *1a* with 2,4-pentanedione and acetylation. Its NMR spectrum shows that there is no strongly deshielding substituent, such as an acetyl group,[4] at C-1 or C-3 of the heterocyclic ring. Thus, the strong IR absorption at 1665 cm^{-1} is due to the acetyl group at C-2. The other strong peak in the IR spectrum (1750 cm^{-1}) originates from the vinyl acetate group on the C-1 side chain. The mass spectrum fragmentation pathway: M − 42 ($CH_2=C=O$) − 105 (PhCO) to give the base peak ($m/e = 186$) corresponding to the quinolizinium ion [5] *3a* is very typical. Similarly, the pyridylpropenones *1b* and *1c* gave with acetic anhydride/potassium acetate the indolizines *2b* and *2c*, respectively. The mass spectra of *2b* and *2c* both exhibit the base peak $m/e = 186$.

The reaction of the pyridylpropenone *1a* with acetic anhydride/potassium acetate in the presence of ethyl acetoacetate afforded the analogous 2-indolizinecarboxylic esters *2d* and *4b*, both showing in their mass spectra the base peak at $m/e = 216$ corresponding to the ion *3b*. The structure of *2d* is manifested by the similarity of its spectral properties to those of *2a*. The absence of the vinyl acetate peak in the IR spectrum, but instead the peak 1695 − 1670 cm^{-1}, and a two proton singlet at δ 4.64, prove that *4b* carries a free phenacyl group at C-1. The treatment of *4b* with boiling acetic anhydride/potassium acetate gave *2d*. Hydrolysis of *4b* to the acid *4c* and subsequent cyclization of the latter by acetic anhydride afforded the lactone *5* (IR: 1715 cm^{-1}), the structure of which is obvious on the basis of its spectral resemblance to *2d*.

The similar reactions of *1a* and *1b* with propionic anhydride/potassium propionate gave the 2-pyrones *6a* and *6b*, respectively. Their

* Part V; Ref. 2.

Table 1. Compounds *1 – 8*.



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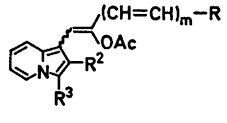

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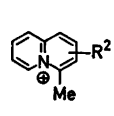

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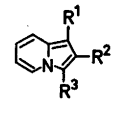

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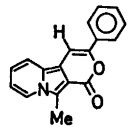

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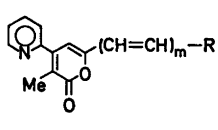

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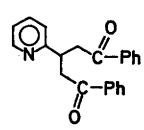

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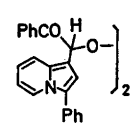

8

Compound No.	n	m	R	R^2	R^3	Compound No.	R^1	R^2	R^3
1a	1	0	Ph			*4a*	CH_2COPh	Ac	Me
1b	1	1	Ph			*4b*	CH_2COPh	CO_2Et	Me
1c	1	0	Me			*4c*	CH_2COPh	CO_2H	Me
1d	0	1	Ph			*4d*	OAc	H	Ph
1e	1	0	OEt			*4e*	CH_2COPh	H	Ph
2a		0	Ph	Ac	Me	*4f*	H	H	Me
2b		1	Ph	Ac	Me	*4g*	COEt	H	Me
2c		0	Me	Ac	Me	*4h*	OAc	Me	Me
2d		0	Ph	CO_2Et	Me	*4i*	CH_2CO_2Et	Ac	Me
2e		0	Ph	H	Ph				
3a				Ac					
3b				CO_2Et					
6a		0	Ph						
6b		1	Ph						

structures are evident on the basis of the spectra, that is, they are 2-pyrones (IR, 1710 – 1680 cm^{-1}) carrying methyl, 2-pyridyl and phenyl (styryl) groups (NMR) at the denoted positions (MS, for example, *6a*: M – PhCO – CO). These pyrones are oxidized products. No reduced species were detected, whereas from the reaction of *1c* with propionic anhydride/potassium propionate the reduction products *4f* and *4g* (NMR, H-8 at δ 8.14), but no oxidized species, were isolated.

One example of the compounds *1*, where $n = 0$, was examined. When *1d* was refluxed with acetic anhydride alone, the indolizine *4d* was obtained in good yield. Its structure is easily obtained from the spectra; that is, it is an indolizine with an acetoxy group (IR, 1760 cm^{-1}) at C-1 (MS, for example, $m/e = 106$[2]) and a phenyl group at C-3 (NMR, H-5 at δ 8.00).

Interestingly, the compound *7* is cyclized with acetic anhydride/potassium acetate to a mixture of the indolizines *2e* and *4e* if worked under nitrogen atmosphere. When exposed to air the indolizine *4e* changes into a compound $C_{44}H_{32}N_2O_4$ for which the structure *8* (without stereochemistry) was proposed (MS, 20 eV, *m/e*, for example, 652, 634, 620, 618 which are very weak, and 310, base peak; NMR, a singlet at δ 6.14 instead of δ 4.16 in *4e* with half the intensity; IR, 1670 cm^{-1}). Use of the aldol derivatives of *1a* (*9a*) and *1c* (*9b*) did not cause any change among the products, while 4-hydroxy-3-methyl-4-(2-pyridyl)-2-butanone (*9c*) is known to yield the indolizine *4h* on cyclization.[4] The methyl substituent of *9c* has therefore a profound effect on the course of reaction.

Ethyl 3-(2-pyridyl)acrylate (*1e*) gave with acetic anhydride/potassium acetate the indolizine *4i*, the structure of which is obvious on the basis of the spectral data.

DISCUSSION

Stereochemistry of the indolizines 2

According to NMR studies, the indolizines *2a*, *2b* and *2d* each consisted of a single isomer, whereas *2c* was a mixture of both geometric isomers. Experiments with molecular models indicated that the *Z*-isomers (the aromatic rings in *trans*-relation) of *2a* and *2d* are sterically less hindered than the corresponding *E*-isomers. According to this and the spectral (particularly NMR) resemblance to compound *5*, the configuration of *2a* and *2d* is assumed to be *Z*. Similarly, the double bond adjacent to the indolizine ring of *2b* possesses *Z*-configuration, whereas the geometry of the other double bond (next to the phenyl group) is *E* (NMR: $J = 16$ Hz). A comparison of the NMR spectra of the two isomers of *2c* reveals great differences. For example, one of the isomers (*2c'*) exhibits the allylic methyl signal higher (δ 1.87) and the acetoxy methyl signal lower (δ 2.17) than the other (*2c''*), at δ 2.13 and δ 1.82, respectively. Assuming that the C-2 acetyl group lies almost in the plane (IR: 1660 cm^{-1}) and the C-1 double bond is twisted out of the ring plane, the acetoxy methyl group of the *Z*-isomer and the allylic methyl group of the *E*-isomer are strongly shielded by the aromatic ring. This leads to assignment of *2c'* as the *E*-isomer and of *2c''* as the *Z*-isomer. The δ value of the vinyl hydrogen of *2c'* is estimated by means of additive shielding increments [6] to be *ca.* 0.25 ppm greater than that of *2c''*. The observed difference of *ca.* 0.15 pmm (δ $H_{vinyl}(2c') = 6.27$, δ $H_{vinyl}(2c'') = 6.14$) is in accordance with the assessed configurations.

The stereochemistry of *2e* is believed to be *Z*, analogously to the other compounds *2*. The absence of a C-2 substituent allows the acetoxy group of *2e* to be situated in the plane of the molecule, and the acetoxy methyl hydrogens appear *ca.* 0.4 ppm downfield relative to those in the other compounds *2*, owing to a larger deshielding by the aromatic rings.

Formation of the indolizines 2

The formation of the indolizines *2* from the pyridylpropenones *1* may be explained as a Michael-addition of 2,4-pentanedione or an equivalent self-condensation product of acetic anhydride [2] to give the addition products, which then cyclize to the corresponding indolizines *4*. Acetylation of the C-1 side chain ketone of *4* furnishes the indolizines *2*.

Owing to the methyl substituent the readiness of the addition compound of *1* and a possible self-condensation product of propionic anhydride to cyclize to an indolizine is reduced. Alternatively, the preferred formation of 2-pyrones arises from the notably higher refluxing temperature needed for propionic anhydride than acetic anhydride, enabling propionic anhydride to attack the pyridylpropenone. Both routes involve a dehydrogenation step. The formation of the reduced indolizine products in the reaction of *1c* with propionic anhydride/potassium propionate probably follows the disproportionation routes suggested before [2] for some analogous reactions.

EXPERIMENTAL

Instruments and chromatographic procedures have been described earlier.[2] Elemental analyses (C, H, N) were carried out by Mrs. A. M. Horko. Mass spectra were obtained through the cooperation of Mr. P. Karvonen.

Preparation of pyridylketones

1-Phenyl-3-(2-pyridyl)-2-propen-1-one (*1a*), *3-phenyl-1-(2-pyridyl)-2-propen-1-one* (*1d*), *1,5-diphenyl-3-(2-pyridyl)-1,5-pentanedione*(*7*) and *3-hydroxy-1-phenyl-3-(2-pyridyl)-1-propanone* (*9a*),[7] *4-hydroxy-4-(2-pyridyl)-2-butanone* (*9b*),[8] and *ethyl 3-(2-pyridyl)acrylate* (*1e*) [9] were prepared as described in the literature.

1-(2-Pyridyl)-5-phenyl-1,4-pentadien-3-one (*1b*). 2-Pyridinecarbaldehyde (10.7 g, 0.10 mol), benzylideneacetone (14.6 g, 0.10 mol), MeOH (100 ml), H_2O (100 ml) and $Ca(OH)_2$ (0.5 g) were heated at 60 °C for 1.5 h. After cooling, addition of water, extraction with ether and drying over Na_2SO_4 gave a dark sticky product mixture, from which *1b* was separated by CC (silica, benzene as eluent), yield 9.6 g (41 %), m.p. (light petroleum, b.p. 40 – 60 °C) 83 °C. Anal. $C_{16}H_{13}NO$.

4-(2-Pyridyl)-3-buten-2-one (*1c*). 2-Pyridinecarbaldehyde (10.7 g, 0.10 mol), AcMe (50 ml), H_2O (100 ml) and $Ca(OH)_2$ were refluxed for 1 h to produce after extraction with $CHCl_3$ and drying (Na_2SO_4) rather pure *1c*, as an oil,[8] accompanied by self-condensation products of acetone (< 5 %, NMR, TLC). Yield 10.1 g. This pyridylpropenone was used without further purifications.

Cyclizations of pyridylketones

General procedure. The pyridylketone with an excess of Ac_2O/KOAc or $(EtCO)_2O$/KOCOEt was refluxed for 1 to 2 h. After cooling, the black product mass was decomposed with water. The product was extracted with ether, neutralized ($NaHCO_3$) and dried (Na_2SO_4). After evaporation, the dark residue was fractionated, when necessary, by CC and purified by recrystallization. LP refers to light petroleum, b.p. 40 – 60 °C.

1a with Ac_2O/KOAc. *1a* (2.09 g, 0.010 mol), Ac_2O (20 ml) and KOAc (10 g) gave *(Z)-2-(2-acetyl-3-methyl-1-indolizinyl)-1-phenyl-vinyl acetate* (*2a*), yield 2.64 g (79 %), m.p. (MeOH) 128 °C. Anal. $C_{21}H_{19}NO_3$. UV (log ε): 394 (3.64), 331 (3.73), 267 (sh, 399), 250.5 (4.23), 231 (sh, 4.04). ^{1}H NMR: δ 7.70 – 7.15 (7 H, m), 7.03 (1 H, s), 6.90 – 6.35 (2 H, m), 2.58 (3 H, s), 2.54 (3 H, s), 1.95 (3 H, s). MS, *m/e* (%): 333 (M^+, 30), 291 (21), 290 (24), 274 (30), 262 (17), 249 (24), 187 (15), 186 (100).

1b with Ac_2O/KOAc. *1b* (2.35 g, 0.010 mol), Ac_2O (20 ml), and KOAc (10 g) gave *(1Z,3E)-1-(2-acetyl-3-methyl-1-indolizinyl)-4-phenyl-2-buta-1,3-dienyl acetate* (*2b*), yield 2.91 g (81 %), m.p. (EtOH) 201 °C. Anal. $C_{23}H_{21}NO_3$.

1c with Ac_2O/KOAc. *1c* (14.7 g, 0.010 mol), Ac_2O (20 ml) and KOAc (10 g) gave *1-(2-acetyl-3-methyl-1-indolizinyl)-2-propenyl acetate* (*2c*) as an oil, yield 1.68 g (62 %). Fractionation by CC afforded the oily *E-2c* (*2c'*), ^{1}H NMR: δ 6.27 (1 H, q 0.9 Hz), 2.64 (3 H, s), 2.46 (3 H, s), 2.17 (3 H, s), 1.87 (3 H, d 0.9 Hz) and *Z-2c* (*2c''*), m.p. (LP) 64 °C. Anal. $C_{16}H_{17}NO_3$. IR: 1750 (s), 1740 (s), 1660 (s). ^{1}H NMR: δ 6.14 (1 H, q 1.2 Hz), 2.53 (3 H, s), 2.43 (3 H, s), 2.13 (3 H, d 1.2 Hz), 1.82 (3 H, s).

1a with Ac_2O/KOAc in the presence of ethyl acetoacetate. *1a* (2.09 g, 0.010 mol), Ac_2O (30 ml), KOAc (15 g) and $AcCH_2CO_2Et$ (1.95 g, 0.015 mol) gave similarly after chromatographic purification *(Z)-2-(2-ethoxycarbonyl-3-methyl-1-indolizinyl)-1-phenyl-vinyl acetate* (*2d*), yield 0.76 g (21 %), m.p. (LP) 113 °C. Anal. $C_{22}H_{21}NO_4$. IR: 1755 (s), 1685 (s), 1680 (s); *ethyl 1-phenacyl-3-methyl-2-indolizinecarboxylate* (*4b*), yield 1.57 g (49 %), m.p. (benzene/LP) 146 °C. Anal. $C_{20}H_{19}NO_3$. IR: 1690 – 1670 (s); and *2a*, 0.23 g (7 %).

Treatment of *4b* with an excess of Ac_2O/KOAc gave *2d* in a good yield. Hydrolysis of *4b* (3.2 g, 0.010 mol) with H_2O/MeOH/NaOH (100 ml/50 ml/2 g) afforded after acidification *1-phenacyl-3-methyl-2-indolizinecarboxylic acid* (*4c*), yield 1.75 g (60 %), m.p. (AcOEt/LP) 190 °C. Anal. $C_{18}H_{15}NO_3$. Heating this acid (1.46 g, 0.005 mol) with Ac_2O (10 ml) afforded *10-methyl-3-phenyl-1H-pyrano[4,3-a]indolizine-1-one* (*5*), yield 1.07 g (85 %), m.p. 140 °C. Anal. $C_{18}H_{13}NO_2$. MS, *m/e* (%): 275 (M^+).

1a with $(EtCO)_2O$/KOCOEt. *1a* (2.09, 0.010 mol), $(EtCO)_2O$ (25 ml) and KOCOEt (15 g) gave *3-methyl-6-phenyl-4-(2-pyridyl)-2H-pyran-2-one* (*6a*), yield 1.10 g (42 %), m.p. (LP) 136 °C Anal. $C_{17}H_{13}NO_2$. UV, (log ε): 336 (3.96), 265 (3.98), 253 (sh, 4.04), 248 (4.12), 240 (sh, 4.01), 231 (sh, 3.90). ^{1}H NMR: δ 8.64 (1 H, broad d 5), 6.78 (1 H, s), 2.10 (3 H, s). MS, *m/e* (%): 263 (M^+, 79), 236 (17), 235 (100), 234 (23), 206 (32), 158 (28), 130 (64), 105 (17).

1b with $(EtCO)_2O$/KOCOEt. *1b* (2.35 g, 0.010 mol), $(EtCO)_2O$ (25 ml) and KOCOEt (15 g) gave *3-methyl-6-styryl-4-(2-pyridyl)-2H-pyran-2-one* (*6b*), yield 1.16 g, (40 %), m.p. (LP) 167 °C. Anal. $C_{19}H_{15}NO_2$.

1c with $(EtCO)_2O$/KOCOEt. *1c* (1.47 g, 0.010 mol), $(EtCO)_2O$ (25 ml), KOCOEt (15 g) gave after chromatographic purification *3-methylindolizine* (*4f*)[5] as liquid and *1-(3-methyl-1-indolizinyl)ethanone* (*4g*),[5] m.p. (LP) 83 °C. Treatment of *4f* with $(EtCO)_2O$/KOCOEt afforded *4g*. The total yield of *4f* and *4g* was *ca.* 20 %.

1d with Ac_2O. *1d* (2.09 g, 0.010 mol) in 25 ml of Ac_2O was refluxed for 0.5 h. After evaporation of the excess of Ac_2O *in vacuo* and recrystallization *3-phenyl-1-indolizinyl acetate* (*4d*) was obtained, yield 1.96 g (78 %), m.p. (LP) 51 °C. Anal. $C_{16}H_{13}NO_2$. MS, *m/e* (%): 251 (M^+, 24), 210 (12), 209 (100), 208 (21), 180 (11), 106 (15).

7 with Ac_2O/KOAc. *7* (3.29 g, 0.010 mol), Ac_2O (25 ml) and KOAc (10 g) were refluxed for 3 h to produce *(Z)-2-(3-phenyl-1-indolizinyl)-1-phenyl-1-vinyl acetate* (*e*), yield 1.63 g (46 %), m.p. (LP/benzene) 200 °C. Anal. $C_{24}H_{19}NO_2$. UV, (log ε): 359 (sh, 4.36), 346 (4.41). IR: 1745 (s), 1640 (m). ^{1}H NMR: δ 8.10 (1 H, broad d 7), 7.08 (1 H, s), 7.02 (1 H, s), 2.38 (3 H, s). MS, *m/e* (%): 353 (M^+, 67), 312 (20), 311 (93), 310 (98), 282 (47), 207 (26), 206 (100), 205 (25), 204 (44) ,120 (52), 119 (53), 105 (21), 103 (25); and *1,1'-diphenyl-2,2'-di(3-phenyl-1-indolizinyl)-2,2'-dioxyethanone* (*8*), yield 0.68 g (21 %), m.p. ($CHCl_3$) 242 °C. Anal. $C_{44}H_{32}N_2O_4$. A similar reaction of *7* under nitrogen and with added AcOH (10 ml) afforded *1-phenyl-2-(3-phenyl-1-indolizinyl)ethanone* (*4e*) as liquid, yield *ca.* 2.2 g (70%). IR: 1680 (s). MS, *m/e* (%): 311 (M^+, 11), 207 (13), 206 (100). During attempted crystallizations *4e* was oxidized to *8*.

9a with Ac_2O/KOAc. *9a* (2.27 g, 0.010 mol), Ac_2O (30 ml) and KOAc (15 g) gave *2a* in 75 % yield.

9b with Ac_2O/KOAc. *9b* (1.65 g, 0.010 mol), Ac_2O/KOAc (30 ml/15 g) gave *2c* in 55 % yield.

1e with Ac_2OKOAc. *1e* (1.77 g, 0.010 mol), Ac_2O/KOAc (20 ml/10 g) gave *ethyl 2-acetyl-3-methyl-1-indolizinylacetate* (*4i*), yield 1.55 g (60 %), m.p. (LP) 107 °C. Anal. $C_{15}H_{17}NO_3$. IR: 1720 (s), 1640 (s). ^{1}H NMR: δ 3.88 (2 H, s).

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Some Optically Active *N*-(4-*tert*-Amino-1-methyl-2-butynyl)-substituted Succinimides and 2-Pyrrolidones and their Absolute Configurations*

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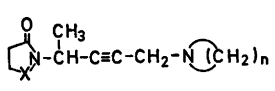
The enantiomers of *N*-(1-methyl-4-pyrrolidino-2-butynyl)-2-pyrrolidone, *N*-(1-methyl-4-pyrrolidino-2-butynyl)succinimide and *N*-(1-methyl-4-perhydroazepino-2-butynyl)succinimide have been prepared from the enantiomers of 1-methyl-2-propynylamine in order to study their stereoselectivity as oxotremorine antagonists. The absolute configurations of the optically active compounds have been established in two independent ways by correlation to (*S*)-1-methylpropylamine and to (*R*)-alanine.

Previous work in our laboratories has shown that a number of *N*-(*tert*-aminoalkynyl)-substituted succinimides and 2-pyrrolidones are rather potent in blocking the motor effects of the muscarinic agent oxotremorine, *N*-(4-pyrrolidino-2-butynyl)-2-pyrrolidone.[1-6]

Common to the most active compounds is the intermediate chain connecting the two nitrogens in the molecule. As this chain contains an asymmetrically substituted carbon atom, we decided to prepare the enantiomers of compounds I—III in order to study their stereospecificity as oxotremorine antagonists.



I X = CH_2 n = 4
II X = C=O n = 4
III X = C=O n = 6

The enantiomers of I—III were synthesized from the optically active forms of 1-methyl-2-propynylamine. This amine has been resolved and its (−)-enantiomer prepared in pure form by Marszak-Fleury.[7] We resolved the amine into its (+)- and (−)-enantiomers using (+)- and (−)-tartaric acid, respectively. The resolution process was followed by measurements of the optical rotation of the benzoyl derivatives. The optical purity was determined by NMR spectroscopic analysis of the diastereomeric amides formed when optically impure amine is acylated with (−)-*O*-methylmandelyl chloride.[8,9] The anisochronous signals from the *C*-methyl (δ 1.48 and 1.39) and *O*-methyl groups (δ 3.36 and 3.39) were especially suitable for this purpose.

The enantiomers of 1-methyl-2-propynylamine were acylated with 4-chlorobutyryl chloride or succinic anhydride and the amides formed were cyclized to *N*-(1-methyl-2-propynyl)-2-pyrrolidone and *N*-(1-methyl-2-propynyl)succinimide, respectively, from which compounds the enantiomers of I—III were prepared through the Mannich reaction as described for the racemates.[2,5,6] The sign of rotation was unchanged through the reaction sequences.

When the six optically active compounds were tested in mice for antagonism towards tremor induced by oxotremorine, and for mydriatic activity, and on isolated guinea-pig ileum preparations for antagonistic activity towards acetylcholine, it was found that the

* Acetylene Compounds of Potential Pharmacological Value XXIV. Part XXIII: *Acta Pharm. Suec. 12* (1975) 503.

(+)-isomers were considerably more active than their enantiomers. In several tests the (+)-isomers were about twice as active as their corresponding racemates, whereas the (−)-isomers were practically inactive.[10]

As the three (+)-enantiomers were all prepared from (+)-1-methyl-2-propynylamine we found it of great interest to establish its absolute configuration. This was carried out in two independent ways. Upon catalytic hydrogenation, the benzoyl derivatives of (−)-1-methyl-2-propynylamine afforded the benzoyl derivate of (*S*)-(+)-1-methylpropylamine, the absolute configuration of which has been established by the Ingold school [11] and by Kjaer and Hansen.[12] A correlation was also made with (*R*)-alanine (D-alanine). Oxidation of the benzenesulfonyl derivative of (+)-1-methyl-2-propynylamine with potassium permanganate afforded the benzenesulfonyl derivative of D-(−)-alanine.

Thus, (+)-1-methyl-2-propynylamine as well as the (+)-enantiomers of compounds I–III can be assigned the *R* configuration.

EXPERIMENTAL

Melting points were determined in a heated metal block using open capillary tubes and calibrated Anschütz thermometers. IR spectra were run on a Perkin-Elmer 157 G spectrophotometer and ^{1}H NMR spectra on a Perkin-Elmer R 12 B instrument. Unless otherwise stated the optical activity was measured in absolute ethanol with a Perkin-Elmer 141 spectrophotometer. Microanalyses were carried out at the Microanalytical Laboratory, Royal Agricultural College, Uppsala.

Resolution of 1-methyl-2-propynylamine. Racemic 1-methyl-2-propynylamine (79 g, 1.1 mol) was added in small portions to a solution of (+)-tartaric acid (171 g, 1.1 mol) in water (170 ml) and the solution was left for a few days at room temperature. The salt formed (89 g) was recrystallized from a concentrated aqueous solution containing about 60 % of salt. After each recrystallization, amine was liberated from a small sample of the salt and converted to the benzoyl and (−)-*O*-methylmandelyl derivatives. After four recrystallizations constant physical properties of the salt and the derivatives were obtained. Yield 10.9 g of the resolved (+)-tartrate, m.p. 151.5–154 °C, $[\alpha]_D^{22}$ +24.4° (*c* 1.1, H_2O). (Found: C 41.8; H 6.0; N 6.0. Calc. for $C_8H_{13}NO_6 \cdot \frac{1}{2}H_2O$: C 42.1; H 6.2; N 6.1).

The combined mother liquors from the above resolution were saturated with K_2CO_3 and the amine was extracted with ether. The ether solution was dried and fractionated through a helix-packed column. The amine fraction was added to an aqueous solution of (−)-tartaric acid and the salt formed was purified as described above for the enantiomeric salt. The product had m.p. 153.5–155 °C, $[\alpha]_D^{22}$ −24.1° (*c* 1.3, H_2O). (Found: C 42.1; H 6.0; N 6.2. Calc. for $C_8H_{13}NO_6 \cdot \frac{1}{2}H_2O$: C 42.1; H 6.2; N 6.1).

(R)-(+)-1-Methyl-2-propynylamine. The resolved (+)-tartrate (9.0 g, 0.04 mol) was dissolved in a small amount of water, the solution was saturated with K_2CO_3 and extracted with ether. After drying, the ether solution was fractionated through a helix-packed column. The amine fraction was redistilled, affording the pure amine (1.3 g, 47 %), b.p. 82–84 °C, $[\alpha]_D^{22}$ +53.2° (neat, *d* 0.8175).

(S)-(−)-1-Methyl-2-propynylamine was obtained similarly in 51 % yield from the (−)-tartrate, b.p. 82–84 °C, $[\alpha]_D^{22}$ −52.7° (neat, *d* 0.8175). Marszak-Fleury [7] reports $[\alpha]_D^{20}$ −52.2° (neat, *d* 0.822).

(R)-(+)-N-(1-Methyl-2-propynyl)benzamide. Benzoylation of the amine liberated from the resolved (+)-tartrate under customary Schotten-Baumann conditions afforded the benzamide, m.p. 90–91 °C (from ligroin), $[\alpha]_D^{22}$ +45.3° (*c* 1.1). NMR ($CDCl_3$): δ 1.49 (3 H, d, J = 7.02 Hz, CH_3), 2.28 (1 H, d, J = 2.36 Hz, ≡C*H*), 5.02 (1 H, m, C*H*CH$_3$), 6.70–7.20 (1 H, m, N*H*), 7.20–7.95 (5 H, m, Ar*H*). (Found: C 76.2; H 6.4; N 8.2. Calc. for $C_{11}H_{11}NO$: C 76.3; H 6.4; N 8.1).

(S)-(−)-N-(1-Methyl-2-propynyl)benzamide was prepared similarly, m.p. 90–91 °C, $[\alpha]_D^{22}$ −45.5° (*c* 0.7). (Found: C 76.0; H 6.1; N 8.3. Calc. for $C_{11}H_{11}NO$: C 76.3; H 6.4; N 8.1).

N-[(R)-1-Methyl-2-propynyl]-(R)-O-methylmandelamide. O-Methylmandelic acid was resolved *via* the (−)-ephedrine salt [13] to give (*R*)-(−)-*O*-methylmandelic acid, $[\alpha]_D^{22}$ −148.7° (*c* 0.6). This acid was converted to the acid chloride and (*R*)-(+)-1-methyl-2-propynylamine was acylated according to a method described in the literature.[9] The amide was obtained in 40 % yield, m.p. 104–105 °C (from ligroin), $[\alpha]_D^{22}$ −9.6° (*c* 0.8). NMR ($CDCl_3$): δ 1.39 (3 H, d, J = 7.0 Hz, CHC*H*$_3$), 2.29 (1 H, d, J = 2.4 Hz, ≡C*H*), 3.39 (3 H, s, OC*H*$_3$), 4.60 (1 H, s, C*H*OCH$_3$), 4.50–5.10 (1 H, m, C*H*CH$_3$), 6.70–7.20 (1 H, m, N*H*), 7.38 (5 H, s, Ar*H*). (Found: C 71.5; H 6.8; N 6.7. Calc. for $C_{13}H_{15}NO_2$: C 71.9; H 7.0; N 6.5).

N-[(S)-1-Methyl-2-propynyl]-(R)-O-methylmandelamide was prepared as described above for the diastereomeric compound from (*S*)-(−)-1-methyl-2-propynylamine and (*R*)-(−)-*O*-methylmandelic acid, m.p. 87–88 °C (from ligroin), $[\alpha]_D^{22}$ −186.5° (*c* 0.6). NMR ($CDCl_3$): δ 1.48 (3 H, d, J = 7.0 Hz CHC*H*$_3$), 2.26 (1 H, d, J = 2.4 Hz, ≡C*H*), 3.36 (3 H, s, OC*H*$_3$), 4.60 (1 H, s, C*H*OCH$_3$), 4.50–5.10 (1 H, m, C*H*CH$_3$), 6.70–7.20 (1 H, m, N*H*), 7.38 (5 H, s, Ar*H*). (Found: C 71.9; H 6.9; N 6.5. Calc.

for $C_{13}H_{15}NO_2$: C 71.9; H 7.0; N 6.5).

Hydrogenation of (S)-(−)-N-(1-methyl-2-propynyl)benzamide. (*S*)-(−)-*N*-(1-methyl-2-propynyl)benzamide (0.6 g), $[\alpha]_D^{22}$ −45.1° (*c* 0.8), was dissolved in ethanol (25 ml) and hydrogenated at room temperature over-night under a hydrogen pressure of 3 atm using Pd−C as catalyst. The catalyst was filtered off and the solvent removed. The residue was recrystallized first from ligroin and then from ethanol-water affording crystals (0.35 g, 57 %) of (*S*-(+)-*N*-(1-methylpropyl)benzamide, m.p. 95−96 °C, $[\alpha]_D^{22}$ +32.9° (*c* 1.0). The highest values reported [12] for this compound are m.p. 97 °C, $[\alpha]_D^{22}$ +34.9° (*c* 1).

(R)-(+)-N-(1-Methyl-2-propynyl)benzenesulfonamide. Resolved (+)-bitartrate of 1-methyl-2-propynylamine (6.9 g, 0.031 mol) was dissolved in water (10 ml). (The benzoyl derivative of the amine liberated from this salt had $[\alpha]_D^{22}$ +42.6° (*c* 1)). After addition of 2 M NaOH (25 ml), benzenesulfonyl chloride (5.6 g, 0.032 mol) was added dropwise with vigorous shaking and occasional cooling with tap-water. The mixture was then shaken for 3 h and acidified with 1 M HCl. The product separated as an oil which rapidly solidified. It was filtered off, washed with water and recrystallized 3 times from 60 % EtOH. Yield 5.3 g (82 %), m.p. 99−100 °C, $[\alpha]_D^{22}$ +76.1° (*c* 1). (Found: C 57.5; H 5.5; N 6.5; S 15.3. Calc. for $C_{10}H_{11}NO_2S$: C 57.4; H 5.3; N 6.7; S 15.3).

(R)-(+)-N-(Benzenesulfonyl)alanine. A. Acylation of D-(−)-*alanine.* D(−)-Alanine (2.7 g, 0.03 mol), $[\alpha]_D^{22}$ −14.6° (*c* 5, 1 M HCl) was acylated with benzenesulfonyl chloride according to a method described by Wiley, Smith and Johansen [14] to give 3.4 g (50 %) of product, m.p. 126−127 °C. (from benzene-ethyl acetate), $[\alpha]_D^{22}$ +9.9° (*c* 1). $[\alpha]_D^{22}$ +30.0° (*c* 0.4, Na salt in H_2O), $[\alpha]_{546}^{22}$ +36.1° (*c* 0.4, Na salt in H_2O). Gibson and Levin who prepared this compound by resolution of the racemate,[15] report m.p. 126−127 °C, $[\alpha]_{546}^{20}$ +33.4° (*c* 0.4, Na salt in H_2O).

B. Oxidation of (R)-(+)-N-(1-methyl-2-propynyl)benzenesulfonamide. A saturated (~6 %) solution of $KMnO_4$ in water was added dropwise to a stirred suspension of the above sulfonamide (3.14 g, 0.015 mol), $[\alpha]_D^{22}$ +76.1° (*c* 1), in water (50 ml) at 0−5 °C. When no more $KMnO_4$ was consumed, the reaction mixture was filtered. The filtrate was washed twice with ether and acidified. Sodium pyrosulfite was added to remove excess permanganate and the clear solution was evaporated to dryness under reduced pressure. From the residue, the product was extracted with boiling ethyl acetate. Evaporation of the solvent yielded (*R*)-(+)-*N*-(benzenesulfonyl)alanine, which after three recrystallizations from benzene-ethyl acetate afforded crystals (1.9 g, 55 %) of m.p. 126−127 °C, $[\alpha]_D^{22}$ +9.9° (*c* 1), $[\alpha]_D^{22}$ +28.6° (*c* 0.4, Na salt in H_2O), $[\alpha]_{546}^{22}$ +35.0° (*c* 0.4, Na salt in H_2O). The product was in every respect (mixed m.p., IR, NMR) identical with that obtained by method A.

(R)-(+)-N-(1-Methyl-2-propynyl)-4-chlorobutyramide. (*R*)-(+)-1-Methyl-2-propynylamine was acylated with 4-chlorobutyryl chloride as previously described for the racemic amine,[6] m.p. 75−76 °C (from cyclohexane), $[\alpha]_D^{22}$ +88.2° (*c* 0.8); yield 61 %. (Found: C 55.0; H 6.9; N 8.1; Cl 20.4. Calc. for $C_8H_{12}ClNO$: C 55.3; H 7.0; N 8.1; Cl 20.4).

(S)-(−)-N-(1-Methyl-2-propynyl)-4-chlorobutyramide was prepared similarly, m.p. 75−76 °C, $[\alpha]_D^{22}$ −91.7° (*c* 0.9); yield 61 %.

(R)-(+)-N-(1-Methyl-2-propynyl)-2-pyrrolidone. Ring closure of the (*R*)-(+)-4-chlorobutyramide prepared above with sodium ethoxide in ethanol [16] afforded the title compound, b.p. 65−67 °C (0.4 mmHg), n_D^{22} 1.4863; yield 55 %. The oily product gradually crystallized, m.p. 36−38 °C (from petroleum ether), $[\alpha]_D^{22}$ +111.2° (*c* 1.2). (Found: C 69.9; H 8.0; N 10.0. Calc. for $C_8H_{11}NO$: C 70.0; H 8.1; N 10.2).

(S)-(−)-N-(1-Methyl-2-propynyl)-2-pyrrolidone was prepared similarly, b.p. 75 °C (0.6 mm Hg), n_D^{22} 1.4883; yield 73 %. M.p. 37−39 °C, $[\alpha]_D^{22}$ −112.4° (*c* 0.9).

(R)-(+)-N-(1-Methyl-4-pyrrolidino-2-butynyl)-2-pyrrolidone [*(R)-(+)-I*] was prepared by the Mannich reaction from (*R*)-(+)-*N*-(1-methyl-2-propynyl)-2-pyrrolidone, formaldehyde and pyrrolidine in dioxan in the presence of small amounts of CuCl as described for the racemate,[6] b.p. 120−125 °C (0.2 mmHg), n_D^{22} 1.5095, $[\alpha]_D^{22}$ +112.3° (*c* 1.1); yield 68 %.

*Oxalate.** m.p. 125−125.5 °C (from ethanol-ether), $[\alpha]_D^{22}$ +86.2° (*c* 0.5). (Found: C 57.9; H 7.0; N 9.2 Calc. for $C_{15}H_{20}N_2O.(CO_2H)_2$: C 58.0; H 7.2; N 9.0).

(S)-(−)-N-(1-Methyl-4-pyrrolidino-2-butynyl)-2-pyrrolidone [*(S)-(−)-I*] was prepared similarly, b.p. 140−142 °C (0.4 mmHg), n_D^{22} 1.5100, $[\alpha]_D^{22}$ −113.5° (*c* 1.0); yield 63 %.

*Oxalate:** m.p. 125−125.5 °C, $[\alpha]_D^{22}$ −88.5° (*c* 1.0).

(R)-(+)-N-(1-Methyl-2-propynyl)succinamic acid. (*R*)-(+)-1-Methyl-2-propynylamine was acylated with succinic anhydride in acetone as previously described for the racemic amine,[2] m.p. 120.5−121.5 °C (from acetone-light petroleum), $[\alpha]_D^{22}$ +93.9° (*c* 0.9); yield 74 %.

(S)-(−)-N-(1-Methyl-2-propynyl)succinamic acid was prepared similarly, m.p. 120−121°C, $[\alpha]_D^{22}$ −96.2° (*c* 0.7); yield 80 %. (Found: C 56.5; H 6.4; N 8.4. Calc. for $C_8H_{11}NO_3$: C 56.8; H 6.6; N 8.3).

(R)-(+)-N-(1-Methyl-2-propynyl)succinimide. (*R*)-(+)-*N*-(1-Methyl-2-propynyl)succinamic acid was cyclized with the aid of Ac_2O and anhydrous NaOAc,[2] b.p. 90−93 °C (0.6

* In a preliminary note on this work [10] this salt has erroneously been reported to be a sesquioxalate like the racemate.[6]

mmHg), n_D^{22} 1.5039, $[\alpha]_D^{22}$ +31.6° (*c* 0.3); yield 90 %.

(S)-(−)-N-(1-Methyl-2-propynyl)succinimide was prepared similarly, b.p. 75 °C (0.3 mmHg), n_D^{22} 1.5020, $[\alpha]_D^{22}$ −34.3° (*c* 1.0); yield 92 %. (Found: C 63.7; H 6.2; N 9.3. Calc. for $C_8H_9NO_2$ C 63.6; H 6.0; N 9.3).

(R)-(+)-N-(1-Methyl-4-pyrrolidino-2-butynyl)succinimide [*(R)-(+)-II*] was prepared by the Mannich reaction from *(R)*-(+)-*N*-(1-methyl-2-propynyl)succinimide, formaldehyde and pyrrolidine as described for the racemate,[2] m.p. 75–75.5 °C (from ligroin), $[\alpha]_D^{22}$ +53.1° (*c* 0.9); yield 75 %. (Found: C 66.9; H 7.8; N 11.8. Calc. for $C_{13}H_{18}N_2O_2$: C 66.6; H 7.7; N 12.0).

(S)-(−)-N-(1-Methyl-4-pyrrolidino-2-butynyl)succinimide [*(S)-(−)-II*] was prepared similarly, m.p. 75–75.5 °C, $[\alpha]_D^{22}$ −54.0° (*c* 0.7); yield 55 %.

(R)-(+)-N-(1-Methyl-4-perhydroazepino-2-butynyl)succinimide perchlorate [*(R)-(+)-III*] was prepared similarly by the Mannich reaction using perhydroazepine as the cyclic secondary amine, m.p. 192–193 °C (from ethanol), $[\alpha]_D^{22}$ +49.7° (*c* 0.3); yield 58 %.

(S)-(−)-N-(1-Methyl-4-perhydroazepino-2-butynyl)succinimide perchlorate [*(S)-(−)-III*] was prepared similarly, m.p. 193–194 °C, $[\alpha]_D^{22}$ −51.1° (*c* 0.4); yield 65 %. (Found: C 49.5; H 6.3; N 7.8; Cl 9.8. Calc. for $C_{15}H_{23}N_2O_2 \cdot HClO_4$: C 49.7; H 6.4; N 7.8; Cl 9.8).

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Allenic Alcohols from the Reaction of Organolithium Reagents with 4-Alkoxy-2-butyn-1-ols. Addition of Alkyllithiums to 3-Phenylpropargyl Alcohol*

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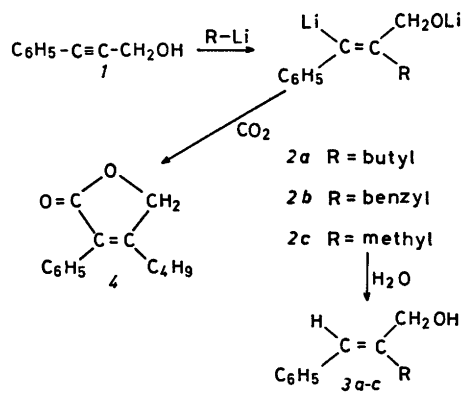
Butyl- and phenyllithium react with 4-alkoxy-2-butyn-1-ol derivatives to give C2 substituted α-allenic alcohols in an over-all displacement (*via* a propargylic rearrangement) of the alkoxy group. Allenic alcohols with a tetra-substituted allene group are obtained in yields > 70 %, whereas alcohols with a hydrogen at C4 suffer an over-all 1,4-elimination of LiOH giving alkenynes as by-products (30 – 40 %). In one case, when the leaving group was a *tert*-butoxy group, a vinylic organometallic intermediate was indicated in the allene-forming reaction. Butyl- and benzyllithium react with lithium 3-phenylpropargyl alcoholate by addition to the triple bond to give, after hydrolysis, 2-substituted (*E*)-3-phenyl-2-propen-1-ol derivatives; methyllithium reacts sluggishly. Tetramethylethylenediamine proved to be an essential co-reagent in these latter addition reactions.

It is well-known that organolithium compounds add to isolated carbon-carbon double bonds.[1] They also add readily to conjugated dienes and styrenes. Alkynes, in contrast to alkenes, are generally prone to undergo α-metallation [2] and additions of organolithium compounds to unconjugated carbon-carbon triple bonds are rare.[1] The additions to diarylacetylenes [1,3,4] and poly-ynes,[1] however, proceed smoothly and so does the allene-forming addition of organolithiums to alkenynes.[1]

In several of the above reactions the solvent plays a major role; electron-donating solvents such as ethers and amines favour the addition, especially the chelate-forming diamine *N,N,N′,N′*-tetramethylethylenediamine [1] (TMEDA).

Assistance by intramolecular coordination of organolithiums with polar groups in the substrate molecule is also a promoting factor in addition reactions. Neighbouring groups such as ether and amino functions, and, pertinent to the present work, hydroxyl groups facilitate addition.[1,5–7]

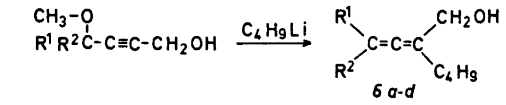
Prior to our preliminary communication [8] the addition reaction of organolithium compounds with propargylic alcohols had not been reported. However, it is known that Grignard reagents add to the triple bond of several acetylenic alcohols when it is relatively proximate to the hydroxyl group.[9] Nearby ether and amino functions also promote this addition.[10]



Scheme 1.

* Allenes and Acetylenes XIV. Part XIII: *Acta Pharm. Suec. 12* (1975) 435.

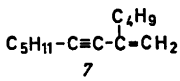
We have reported [8] that butyllithium smoothly adds to the triple bond of 3-phenylpropargyl alcoholate (from *1*) in the presence of 0.2 equiv. of TMEDA in an ether-hexane mixture (Scheme 1). Hydrolysis of the organometallic intermediate *2a* gives the cinnamyl alcohol derivative *3a* [11] in 90 % yield and treatment with CO_2 gives the lactone *4* in 77 % yield (GLC). The acetylenic methoxy compounds *5a* and *5b* [12] react with butyllithium (eqn. 1) by an over-all displacement of the methoxy groups to give the allenic alcohols *6a* and *6b* in yields of around 80 and 40 %, respectively. The lower yield of *6b* is caused by its further reaction with butyllithium to the alkenyne *7*, which is formed in 35 % yield.[8] In these two reactions with butyllithium TMEDA has no significant influence



6 a-d

5a $R^1 = C_3H_7$, $R^2 = CH_3$
5b $R^1 = C_5H_{11}$, $R^2 = H$
5c $R^1 = C_5H_{11}$, $R^2 = CH_3$
5d $R^1 = tert\text{-}C_4H_9$, $R^2 = CH_3$ (eqn. 1)

on the reaction rate or the formation of minor amounts of by-products.



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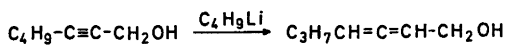
The above reactions with organolithium reagents have been studied in more detail and the results are presented here.

RESULTS AND DISCUSSION

3-Phenylpropargyl alcohol and 2-heptyn-1-ol. It has been reported that butyllithium adds to the triple bond of diphenylacetylene to give a stilbene derivative with *E* configuration,[3] whereas *tert*-butyllithium gives a similar compound with predominant *Z* configuration.[4] The present 2-butylcinnamyl alcohol *3a* was homogeneous on OV-25 and Carbowax 20 M GLC columns, thus indicating a single geometric isomer. The C1 protons of *3a* appear as a single doublet (*J* 1.3 Hz) in the ^1H NMR spectrum, further indicating homogeneous stereochemistry. Treatment of the intermediate *2a* with CO_2 gave the lactone *4* in 77 % yield (GLC), which must arise from the *E* intermediate *2a*. However, it is not absolutely clear whether this *E* intermediate is a direct result of the addition of butyllithium or is a product from subsequent isomerization of an initially formed *Z* intermediate. The stilbene derivatives of Mulvaney [3,4] obviously result from rapid thermodynamic equilibration of the vinylic organometallic intermediates, as it has been shown by other authors [13,14] that vinylic lithium compounds having α-aryl substituents are configurationally instable, especially in the presence of TMEDA.[14] The present *E* intermediates *2* should therefore also be the result of quick thermodynamic equilibration and it is impossible to make a statement regarding the type of addition (*cis* or *trans*).

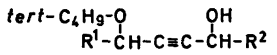
Benzyllithium also reacted easily with 3-phenylpropargyl alcoholate under the above conditions (0 – 20 °C) to give the alcohol *3b*, isolated in 70 % yield by preparative TLC. The stereochemistry of the product *3b* was not proved, but its NMR spectrum as well as GLC analysis on an OV-25 column indicated the presence of a single isomer. In contrast to the above reactions, the addition reaction of 3-phenylpropargyl alcohol with methyllithium required 2 equiv. of TMEDA to proceed with a noticeable rate. Work-up and distillation after a reaction time of 30 h at room temperature gave only 15 % of the alcohol *3c* [15] and a heavy residue was left, indicating polymerizing side-reactions. The analogous reaction of methyllithium with 1-methyl-3-phenylallyl alcohol likewise requires enough TMEDA to combine with all the lithium salts present in solution.[6]

The only observed reaction of 2-heptyn-1-ol (*8*) with butyllithium (eqn. 2) in the presence of 0.2 or 1.5 equiv. of TMEDA (reflux 5 h in ether) was, except for some polymerization, proton abstraction at C4; hydrolysis afforded the allenic alcohol *9* [16] (60 % of the volatile products; 40 % starting material).



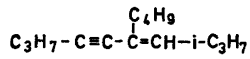
8 *9* (eqn. 2)

Alkoxy compounds. The reaction of the methoxy compound *5a* according to eqn. 1 seemed a promising route to α-allenic alcohols sub-



10a $R^1 = C_3H_7$, $R^2 = i\text{-}C_3H_7$

10b $R^1 = H$, $R^2 = C_2H_5$



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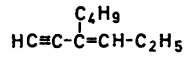


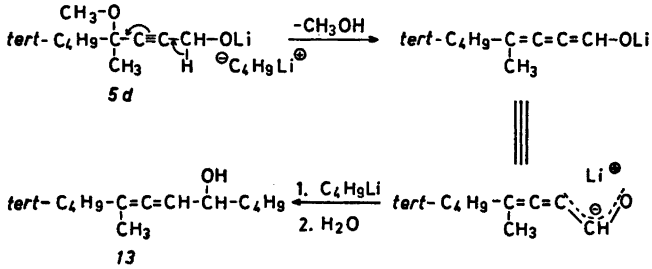
12

stituted at C2, which are otherwise obtainable from the reaction of appropriately substituted acetylenic oxiranes with organocuprates,[17] from dihalocyclopropanes and butyllithium, and by other methods.[18] Quite recently another method, in which acetylenic methoxy compounds of the type *5a–d* are treated with a combination of CuI and a Grignard reagent, was reported.[19] The present synthetic method, however, seems to be limited to the preparation of α-allenic alcohols with a tetrasubstituted allene group, because alcohols having one or two C4 hydrogens suffer an over-all loss of LiOH to give alkenynes, *e.g.* *7* from *6b*. In the reactions according to eqn. 1 we used 2.5 equiv. of butyllithium, but attempts to raise the yield of *6b* by using only 2 equiv. were disappointing. The 1,4-elimination reaction of *6b* proceeded so rapidly that the ratio of *6b*:*7* was practically unchanged. We did not try to improve the yield of the alkenyne *7*, since it is well-known that organolithium reagents easily add to such compounds.[1]

When the *tert*-butoxy compound *10a* was allowed to react with butyllithium for 8 h at room temperature, the alkenyne *11* (30 %, GLC) was the only product formed (> 4 %); much of the starting material was recovered unchanged. The allenic alcohol formed from the analogous acetylene *10b* and butyllithium was also quickly consumed to give the corresponding alkenyne *12* (33 %, GLC) as main product.

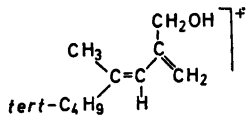
Other authors have reported that 4-bromo-2,3-butadien-1-ol also undergoes 1,4-elimination to give vinylacetylene when treated with butyllithium.[20] In that case the alcoholate may be thought to undergo a bromine-lithium exchange with subsequent loss of Li_2O. Certain α-allenic alcohols also form minor amounts of alkenynes upon treatment with $LiAlH_4$ in refluxing THF.[12,21] Several other allenic derivatives, such as 2,3-alkadien-1-yl alkyl ethers,[22] likewise undergo facile 1,4-elimination reactions upon basic treatment; this reaction is reversible in the presence of a suitable base.

In the reaction products from *5a*, *5c* and *5d* with butyllithium, 8–15 % of a compound having a retention time very close to each of the corresponding allenic main products *6* (formed in yields around 80 %) was observed on GLC (OV-25 and Carbowax 20 M). In the case of *5d* this product was isolated by preparative GLC (OV-25) and by comparison with authentic material, prepared according to Claesson *et al.*,[23] identified as the secondary allenic alcohol *13* (Scheme 2). A plausible route to this product is depicted in Scheme 2. The mass spectra (70 eV) of the isomers *6d* and *13* exhibit important differences, for instance the secondary alcohol *13* having β-hydrogens easily loses water (M − 18, 16 %) whereas *6d* does not (< 1 %). The alcohol *6d* has a peak at *m/e* 154 (20 %), which is lacking for *13*. A possible explanation of this peak is the ion *14*, which could have been formed in a McLafferty



Scheme 2.

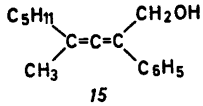
type rearrangement with loss of propene. The corresponding rearrangement of the alcohol *13*, would result in a loss of butene to give *m/e* 140 and this ion is actually present (13 %), while it is small for the isomer *6d*. The other allenic alcohols *6a–c* also exhibit corresponding peaks



14

in their spectra [*m/e* 140 (28 %), *m/e* 154 (8 %) and *m/e* 168 (10 %), respectively]. Hydrocarbon McLafferty rearrangements are implicit in the mass spectra of other allenes [24] but have never been proven.

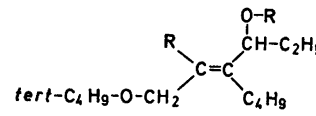
Other organolithium compounds also react with the acetylenic methoxy compounds *5*; phenyllithium with *5c* gave the allenic alcohol *15* in 85 % yield (GLC) accompanied by about 6 % of the allene *6c* from unreacted butyllithium. For the isolation of *15*, the crude material was chromatographed on preparative TLC plates.



15

Although methyllithium adds to the double bond of 1-methyl-3-phenylallyl alcohol [6] and to the triple bond of 3-phenylpropargyl alcohol (slowly) in the presence of an excess of TMEDA, this reagent did not affect the acetylene *5c* to give a detectable amount of an allenic alcohol even in the presence of 3 equiv. of TMEDA (20 °C for 3 days). Fortunately, 2-methyl-2,3-alkadien-1-ols are available by other synthetic procedures.[17–19]

The reaction of the acetylene *10b* was undertaken in an attempt to detect a vinylic organometallic intermediate in the formation of the α-allenic alcohol. In analogous formations of α- and β-allenic alcohols from $LiAlH_4$ reductions we have earlier proved the existence of such vinylic intermediates.[25] Indeed, the existence of the intermediate *16* after 15 h at room temperature was indicated by hydrolysis to compound *17* (19 %; GLC); the stereochemistry of *16* and *17* was not proved. In the case of the *tert*-butoxy compound *10a* no intermediate was detected (< 4 %; GLC). The stabilities of similar β-alkoxy vinylic organolithiums have been discussed as well as their application in syntheses.[26]



16 R = Li
17 R = H

EXPERIMENTAL

General. The general IR and NMR instrumentation has been described.[25] These spectra were routinely recorded and are in full agreement with the proposed structures. Mass spectra were run on an AEI MS-30 spectrometer connected to a Pye 104 gas chromatograph or on a LKB 9000 instrument using the GLC inlet. The ionizing energy was maintained at 70 eV. Correct mass spectral data were obtained for all products.

GLC analyses were run on a Varian 1700 instrument equipped with flame ionization detectors. Columns: 3 m long glass columns packed with 5 % Carbowax 20 M on Chromosorb W (80–100 mesh) or 5 % OV-25 on Gas-Chrom Q (100–120 mesh). The preparative columns were of aluminium, 300 × 0.96 cm and packed with 20 % Carbowax 20 M, 20 % OV-25 or 20 % SE 30 on Chromosorb W (60–80 mesh). "Yield (GLC)" refers to the relative peak areas of the whole reaction mixture in a gas chromatogram run in a temperature range of about 100 °C on an OV-25 column; peak areas were not corrected for detector response. On distillation of several different worked-up reaction mixtures about 10 % of non-volatile residues were obtained, which can be regarded a representative figure for all the present reactions unless otherwise indicated.

For preparative TLC 0.5 mm thick layers of silica gel PF on 20 × 20 cm plates were used.

All reactions with Grignard or lithium reagents were performed under nitrogen or argon.

Organolithium reagent used. Butyllithium was either of commercial origin (*ca.* 20 % in hexane) or prepared from butyl bromide and lithium in ether, in the presence of traces of $HgBr_2$. After complete reaction hexane was added to give a *ca.* 8 % solution of butyllithium. Methyllithium was purchased as a *ca.* 2 M solution in diethyl ether. Phenyllithium and benzyllithium were prepared by warming benzene and toluene respectively, with butyllithium solution and TMEDA under argon.[5]

Addition reactions. General procedure. One equivalent of the propargylic alcohol (1.5 – 3 g) in 30 ml of dry ether was treated with 0.2 equiv. of TMEDA. The mixture was cooled to −30 °C and 2.5 equiv. of the organolithium reagent were added dropwise while stirring. The reaction mixture was allowed to reach room temperature during 0.5 h. Samples were withdrawn periodically for GLC analysis. The reaction was stopped by adding water, the solution was extracted several times with ether and the combined ethereal extracts were washed with water and dried. The products were isolated by preparative GLC or preparative TLC [for *3b* chloroform was used as eluent, and for *15* a benzene-ether mixture (4:1)] and identified by IR, NMR and MS. The allenic products showed the typical "allene band" at *ca.* 1960 cm^{-1} which was of low intensity when the allene group was tetrasubstituted.

(E)-*2-Butyl-3-phenyl-2-propen-1-ol* (*3a*).[11] ^1H NMR ($CDCl_3$): δ 7.47 – 7.18 (5 H, m), 6.66 – 6.50 (1 H, m), 4.20 (2 H, d), 2.50 – 2.14 (2 H, m), 1.80 (1 H, s), 1.65 – 1.13 (4 H, m), 0.87 (3 H, t).

(E)-*2-Benzyl-3-phenyl-2-propen-1-ol* (*3b*). ^1H NMR ($CDCl_3$): δ 7.55 – 6.94 (10 H, m), 6.83 – 6.64 (1 H, m), 4.02 (2 H, d), 3.62 (2 H, d), 2.80 (1 H, s).

(E)-*2-Methyl-3-phenyl-2-propen-1-ol* (*3c*).[14] ^1H NMR ($CDCl_3$): δ 7.53 – 7.20 (5 H, m), 6.64 – 6.47 (1 H, m), 4.15 (2 H, d), 2.84 (1 H, s), 1.87 (3 H, d).

4-Methoxy-4-methyl-2-heptyn-1-ol (*5a*) was prepared as described for similar compounds[23] from 3-(tetrahydro-2-pyranyloxy)propyne[27] (70.0 g; 0.50 mol), 2-pentanone (38.7 g; 0.45 mol) and dimethyl sulfate (100.0 g; 0.79 mol). Yield 70 %. B.p. 66 °C/0.5 mmHg. Found: C 69.0; H 10.2. Calc. for $C_9H_{16}O_2$: C 69.02; H 10.32.

4-Methoxy-4-methyl-2-nonyn-1-ol (*5c*). Prepared as above from 3-(tetrahydro-2-pyranyloxy)propyne (22.6 g; 0.16 mol), 2-heptanone (16.8 g; 0.15 mol) and dimethyl sulfate (33.4 g; 0.27 mol). Yield 68 %. B.p. 78 °C/0.1 mmHg. Found: C 71.7; H 10.9. Calc. for $C_{11}H_{20}O_2$: C 71.70; H 10.94.

4-Methoxy-4,5,5-trimethyl-2-hexyn-1-ol (*5d*) was prepared according to standard procedures,[16] *i.e.* 16.5 g (0.12 mol) of 3-methoxy-3,4,4-trimethyl-1-pentyne[28] was converted to the corresponding acetylenic Grignard reagent and allowed to react with 4.6 g (0.15 mol) of gaseous formaldehyde. Yield 75 %. B.p. 107 °C/10 mmHg. Found: C 70.6; H 10.6. Calc. for $C_{10}H_{18}O_2$: C 70.55; H 10.66.

2-Butyl-4,5,5-trimethylhexa-2,3-dien-1-ol (*6d*). MS, *m/e* (%): 196 (M^+, 1), 181(1), 179(2), 178(0.5), 167(1), 165(1), 163(2), 155(3), 154(20), 149(3), 140(3), 139(13), 137(3), 135(3), 125(3), 123(10), 121(17), 111(7), 109(9), 107(27), 98(4), 97(8), 96(9), 95(9), 93(14), 91(11), 83(17), 81(20), 80(97), 79(18), 77(11), 69(23), 65(7), 59(7), 58(5), 57(100), 56(9), 55(33), 53(15), 51(4), 44(5), 43(20), 42(4), 41(67), 39(17).

6-tert-Butoxy-2-methyl-4-nonyn-3-ol (*10a*) was prepared in analogy with similar compounds[23] from 3-*tert*-butoxy-1-hexyne (80.0 g; 0.52 mol) and isobutyraldehyde (33.6 g; 0.47 mol). Yield 66 %. B.p. 87 °C/0.3 mmHg. Found: C 74.0; H 11.6. Calc. for $C_{14}H_{26}O_2$: C 74.29; H 11.58.

6-tert-Butoxy-4-hexyn-3-ol (*10b*) was prepared similarly from *tert*-butyl propargyl ether[29] (22.0 g; 0.19 mol) and propionaldehyde (10.6 g; 0.18 mol). Yield 64 %. B.p. 95 °C/2 mmHg. Found: C 70.4; H 10.5. Calc. for $C_{10}H_{18}O_2$: C 70.55; H 10.66.

8,9,9-Trimethyl-6,7-decadien-5-ol (*13*) was prepared as described[20] from $LiAlH_4$ reduction of 8-methoxy-8,9,9-trimethyl-6-decyn-5-ol (9.0 g; 0.04 mol). Yield 73 %. B.p. 76 °C/0.2 mmHg. Found: C 79.4; H 12.2. Calc. for $C_{13}H_{24}O$: C 79.53; H 12.32. 8-Methoxy-8,9,9-trimethyl-6-decyn-5-ol was prepared from 3-methoxy-3,4,4-trimethyl-1-pentyne (10.0 g; 0.071 mol) and pentanal (5.6 g; 0.065 mol). Yield 77 %. B.p. 85 °C/0.3 mmHg. Found: C 74.7; H 11.6. Calc. for $C_{14}H_{26}O_2$: C 74.29; H 11.58. MS, *m/e* (%): 196 (M^+, 1), 181(4), 179(5), 178(16), 163(9), 152(9), 151(2), 149(2), 141(1), 140(13), 139(5), 137(6), 135(5), 122(8), 121(13), 119(3), 112(3), 111(33), 109(4), 107(15), 105(6), 95(7), 94(4), 93(25), 91(12), 85(5), 84(7), 83(6), 81(4), 80(3), 79(18), 78(3), 77(11), 69(14), 68(3), 67(6), 66(5), 65(5), 58(5), 57(100), 56(3), 55(31), 53(8), 51(4), 43(19), 41(47), 39(13).

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Anodically Promoted $2\pi + 2\pi$ Cycloaddition of Certain Indene Derivatives

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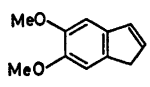
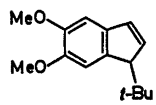
The anodic oxidation of a dilute solution of 5,6-dimethoxyindene or 1-*t*-butyl-5,6-dimethoxyindene in acetic acid/potassium acetate results in the addition of two acetoxy groups across the double bond besides predominant polymer formation. No steric influence from the electrode surface on product composition was found. In concentrated solutions of these substrates the reaction takes an unexpected course, in that $2\pi + 2\pi$ cycloaddition dimers are formed in low yields, again with polymer formation predominant. The mechanism of the cycloaddition process is probably similar to that suggested for iron(III) salt catalyzed $2\pi + 2\pi$ cycloadditions.

The stereochemistry of the anodic addition of acetoxy groups to indene [1] and 1-alkylindenes [2] has been studied previously in order to elucidate possible effects of adsorption on the distribution of diastereomeric diacetates. During the course of this investigation Thompson and Naipawer showed by studies on another heterogeneous process, catalytic hydrogenation over a 5 % Pd/C catalyst, that the methoxy substituent has a particularly strong adsorption-enhancing effect.[3] Hence it became desirable to investigate the anodic oxidation of methoxy substituted indenes, 5,6-dimethoxyindene (*1*) and 1-*t*-butyl-5,6-dimethoxyindene (*2*) being selected as representative substrates. This report shows that anodic addition of acetoxy groups is not a favored reaction with these compounds, but that they undergo an anodically promoted $2\pi + 2\pi$ cycloaddition under certain conditions.



1



2

RESULTS

Synthesis of substrates 1 *and* 2. The synthesis of *1* has been described earlier.[4,5] A somewhat modified procedure, *viz.* ring closure of 3-(3,4-dimethoxyphenyl) propanoic acid (*3*) in polyphosphoric acid followed by sodium borohydride reduction of 5,6-dimethoxy-1-indanone (*4*) and eventual elimination of water from 5,6-dimethoxy-1-indanol (*5*) was developed and gave *1* in an overall yield of *49* % from *3*. It was found to be imperative that the work-up procedure for *5* did not involve any treatment with acid, since otherwise blue polymeric material was rapidly formed.

The synthesis of *2* was performed in essentially the same manner except that 3-*t*-butyl-5,6-dimethoxy-1-indanone was obtained in one step from veratrole and β-*t*-butylacrylic acid by polyphosphoric acid treatment [7] and the final elimination step had to be carried out as a Chugaev reaction.[6] In pyridine/acetic anhydride *2* rearranged to its isomer, *3*-*t*-butyl-5,6-dimethoxyindene. The overall yield of purified *2* from veratrole was 34 %.

Anodic oxidation of 1 *and* 2. The introduction of the two 5,6-methoxy groups into the indene system lowered the half peak potential ($E_{p/2}$) by more than 500 mV.[8] Thus, $E_{p/2}$ values for indene, 1-*t*-butylindene, *1*, and *2* were found to be 1.47, 1.39, 0.81, and 0.81 V *vs.* SCE,

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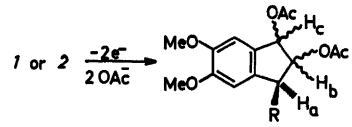
respectively, in HOAc/KOAc (1 M) at the platinum anode. Preparative experiments with *1* and *2* were performed at 1.0 V *vs.* SCE.

Preparative anodic oxidation of *1* and *2* in HOAc/KOAc (1 M) at platinum or graphite electrodes gave polymeric material predominantly under all conditions tried. This made a detailed study of the stereochemistry of the addition reaction along the lines described earlier[1] impossible. From dilute solutions of *1* or *2* small amounts of diacetoxy products *6a* or *6b* (10 and 15 % yield, respectively) were indeed obtained (eqn. 1), but the low yields precluded any meaningful interpretation of the results.

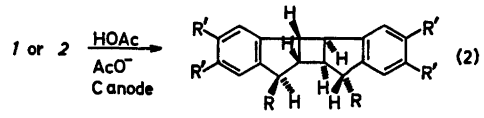


(1)

6a R=H

6b R= *t*-Bu

From *1* at least two diacetates (*6a*) were detectable by GLC/MS but could not be isolated due to experimental difficulties. From *2* two diacetates in a ratio of 60:40 (*6b*) were isolated. They were assigned the *trans* arrangement of H_A and H_B (*6b*) by comparison of their ^1H NMR spectra with the corresponding isomers of 3-*t*-butyl-1,2-diacetoxyindan (60 % isomer: δ 3.07 (H_a), 5.64 (H_b), 6.26 (H_c), J_{ab} 2.0 and J_{bc} 5.4 Hz; 40 % isomer: δ 2.94 (H_a), 5.44 (H_b), 5.96 (H_c), J_{ab} 2.0 and J_{bc} 1.5 Hz; 3-*t*-butyl-1,2-diacetoxyindan: *cis* (ab) – *cis* (bc), δ 3.0 (H_a), 5.8 (H_b), 6.1 (H_c), J_{ab} 4.4 and J_{bc} 4.4 Hz; *cis* (ab) – *trans* (bc), δ 3.3 (H_a), 5.6 (H_b), 6.3 (H_c), J_{ab} 5.8 and J_{bc} 7.0 Hz; *trans* (ab) – *trans* (bc), δ 3.0 (H_a), 5.4 (H_b), 6.1 (H_c), J_{ab} 3.2 and J_{bc} 2.2 Hz; *trans* (ab) – *cis* (bc), δ 3.1 (H_a), 5.7 (H_b), 6.3 (H_c), J_{ab} 1.7 and J_{bc} 5.4 Hz).



(2)

7a R=H, R'= OMe

7b R= *t*-Bu, R'=OMe

The anodic oxidation of concentrated solutions of *1* and *2* in HOAc/KOAc gave an unexpected product, shown (see below) to be a cyclobutane derivative (*7*) formally resulting from a $2\pi+2\pi$ cycloaddition reaction (eqn. 2). This product was isolated in 30 and 10 % yield from *1* and *2*, respectively.

The structural proof for *7* was based on the following evidence. Elemental analysis of *7a* and *7b* was the same as for the starting materials but their GLC retention times were considerably longer. The mass spectrum of *7a* at 70 eV had its 100 % peak at *m/e* 176 and the mass of the molecular ion at *m/e* 352 (0.1 %). At 11 eV the mass spectrum of *7a* had only two peaks, at *m/e* 176 (100 %) and 177 (12 %). This fragmentation pattern is characteristic for cyclobutane derivatives which are known to cleave predominantly according to a reverse cycloaddition mechanism.[9] The mass spectral behavior of *7* was similar, the ion with m/e half that of the parent ion being the 100 % fragment at 70 eV.

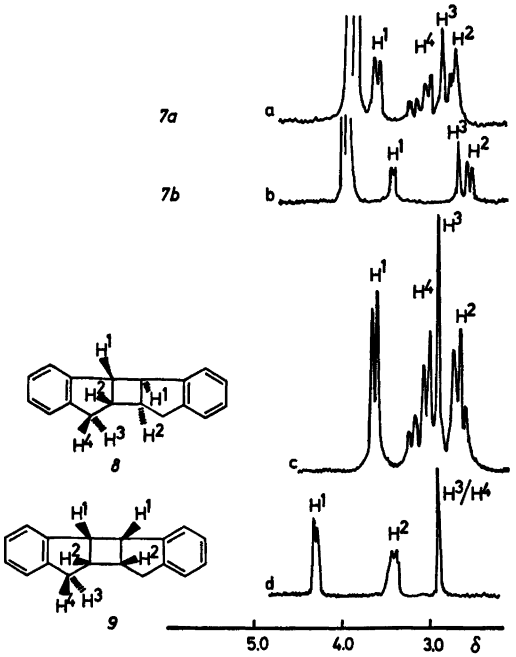


Fig. 1. ^1H NMR spectrum for the δ 2.4 – 4.6 region of *7a*, *7b*, *8*, and *9*. The remaining shifts are for *7a*: δ 3.92 and 3.88 (d, 6 H, CH_3O) and 6.78 and 6.88 (d, 2 H, Ar); for *7b*: δ 3.92 and 3.96 (d, 6H, CH_3O), 0.76 (d, 9 H, *t*-Bu), and 6.92 and 6.94 (d, 2 H, Ar). The spectrum of *9* was taken from Ref. 10.

The ^{1}H NMR spectra of *7a* and *7b* were compared with those of the known [10] indene cyclodimers *8* and *9* (see Fig. 1). In selecting these compounds for comparison, we tacitly assumed that the dimerization process takes place *via* a radical cation mechanism similar to the iron(III) nitrate catalyzed $2\pi + 2\pi$ cycloaddition of *N*-vinylcarbazole.[11] For indene itself it is known [1] that the initial coupling between the radical cation and the parent compound takes place in a head-to-head fashion, thus limiting the possible types of cyclodimer structures to the one with methylene groups in the 1,2-position of the cyclobutane system. Thus, of the four possible cyclodimers of indene only *8* and *9* were of interest here.

Fig. 1 shows the region of δ 2.4 – 4.6 for these four compounds. The similarity between the spectra of *7a* and *8* is striking, as indeed would be expected considering the small structural difference between them. The introduction of the methoxy groups would not be expected to have any significant effect on the shifts of the alicyclic hydrogens. In Fig. *1a* couplings are seen between H^1 and H^2, H^2 and H^4, but not between H^2 and H^3. Substitution of H^4 for a *t*-butyl group will give a spectrum with coupling between H^1 and H^2, whereas H^3 will appear as a singlet (Fig. 1b). This is valid only if the steric arrangement around the cyclobutane ring is as shown for *7b* in eqn. 2. The ^{1}H NMR spectrum of the other possible isomer (*9*) has the H^3 signal at a lower field than that of H^2, contrarily to what is found for *7a*, *7b* and *8*. Taken together, structure *7b* for the cyclodimer from *2* is strongly indicated from the ^{1}H NMR spectral studies.

Table 1 is a summary of ^{13}C NMR spectral data for *7a*, *7b* and *8*. The influence of the methoxy groups upon the shifts of the alicyclic carbons is negligible. The appearance of the spectrum shows that the molecule must possess a C_2 axis as symmetry element.

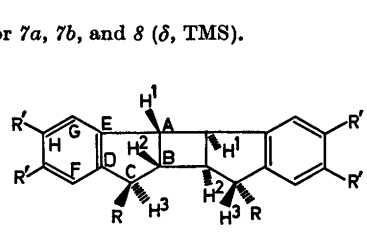
Several attempts to cyclodimerize *1* by treatment with iron(III) nitrate in acetic acid or methanol [11] were unsuccessful.

DISCUSSION

It was previously concluded [2] that the anode surface has no steric influence upon the anodic addition of acetoxy groups to 1-*t*-butylindene. The substitution of the 5,6-hydrogens for methoxy groups was made in order to see if the adsorption-enhancing properties of the methoxy group, strongly exhibited in catalytic hydrogenation, might drastically change the diastereomer distribution. Moreover, the lowering of the oxidation potential caused by the methoxy groups should help to bring the working anode potential closer to the potential of zero charge, at which maximum adsorption of neutral molecules takes place.[12]

It is, however, seen that there is no change in diastereomer distribution from the anodic addition of acetoxy groups to 1-*t*-butylindene [2] (62:38) or to 1-*t*-butyl-5,6-dimethoxyindene

Table 1. ^{13}C NMR chemical shifts for *7a*, *7b*, and *8* (δ, TMS).



Compound	A	B	C	D	E	F	G	H	J^a	K^b	L^c
8	43.1	53.9	39.3	1146	144	125	125	127	55.		
7a	44.1	54.0	39.3	138	135	108	108	148	55.9		
7b	46.8	53.1	62.4[d]	140	137	111	107	149[e]	55.9	34.4	27.4

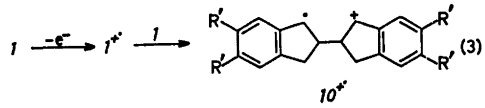
[a] Methoxy carbons. [b] Quaternary *t*-butyl carbon. [c] Methyl carbons in *t*-butyl group. [d] A shift of 20 ppm is reasonable when H is substituted for *t*-butyl. [e] The other aromatic carbon 147.

(60:40). Thus the conclusion from the previous study [2] that no steric effect from the anode is indicated in this type of reactions is reinforced here.

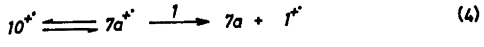
The formation of cyclodimers from runs in concentrated solution is unexpected, since formally no electrochemistry need be involved in the overall formation of *7* from *1* or *2*. A precedent for this type of process has been found earlier during the anodic oxidation of *N*-vinylcarbazole in acetonitrile [13] which gave an 8 % yield of 1,2-bis-(carbazol-9-yl)cyclobutane. No mechanism was suggested.

Certain metal ion catalysts, *e.g.* iron(III) nitrate, affect the same reaction with suitably substituted alkenes.[11] The mechanism was recently [14] proposed to be a radical chain reaction, initiated by the formation of the radical cation in a 1-e transfer from the substrate to the metal ion. Also indene itself can be cyclodimerized by treatment with iron(III) chloride in acetonitrile,[15] again assumed to take place *via* the radical cation.

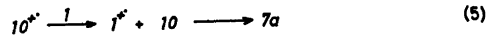
The anodic cyclodimerization reaction reported here is most likely a radical chain reaction similar to that proposed for the iron(III) catalyzed reaction (exemplified by *1* in eqns. 3 and 4 or 3 and 5). In concentrated solutions the radical cation is preferentially trapped by *1*, whereas in dilute solution reaction with acetate ion will predominate.



(3)



(4)



(5)

EXPERIMENTAL

All controlled potential electrolyses were carried out in waterjacketed cells of either 30 or 150 cm³ volume. For electrodes, see Ref. 2. The potential was kept at 1.0 V *vs.* SCE by a potentiostat, built by the Electronics Service Division of this Department.

3-(3,4-Dimethoxyphenyl)propanoic acid. A solution of 3,4-dimethoxycinnamic acid [16] (50 g) and sodium hydroxide (10 g) in water (500 ml) was hydrogenated over 0.5 g of palladium on carbon (5 %) in a Parr low pressure hydrogenation apparatus. The pressure dropped to a constant value in 24 h. The catalyst was filtered off and the aqueous solution was extracted with ether (3 × 100 ml) to remove neutral components. Acidification and extraction with ether/ethyl acetate (5:1) gave a sample which was recrystallized from toluene (81 %), m.p. 99 – 100 °C (lit:[17] 102 °C).

5,6-Dimethoxy-1-indanol. To a solution of sodium borohydride (6 g) in ethanol (200 ml) was added 5,6-dimethoxy-1-indanone [18] (40 g). The mixture was stirred for 24 h at room temperature. Water (800 ml) was added and the reaction mixture extracted with ethyl acetate (5 × 300 ml). The combined extracts were evaporated and the residue was used without further purification (96 %).

5,6-Dimethoxyindene. To 5,6-dimethoxy-1-indanol (38.3 g) was added pyridine (10 ml) and acetic anhydride (100 ml) and the reaction mixture was boiled for 30 min. Then water (500 ml) was added and the mixture was extracted with methylene chloride (2 × 100 ml). The combined extracts were washed with water (3 × 200 ml), saturated potassium hydrogen carbonate solution (50 ml), and water (100 ml). After addition of acetone (100 ml) the solvent was evaporated and the residue distilled. The fraction boiling between 120 and 130°C/11 mmHg was collected. The crude product was contaminated with acetic acid which was removed by washing a methylene chloride solution of the substance with saturated $KHCO_3$ solution. Recrystallization from a mixture of ethanol/water (1:1) gave 20.8 g (60 %) of the pure compound, m.p. 70 – 71 °C (lit:[4,5] 71 – 72 °C), NMR ($CDCl_3$): δ 3.23 (p, 2 H, $-CH_2^a-$), 3.80 (s, 6 H, CH_3O-), 6.36 (p, 1 H, $-CH^b=$), 6.72 (p, 1 H, $-CH^c=$), and 6.88 and 6.92 (d, 2 H, Ar), J_{ab} 1.9, J_{bc} 5.7, J_{ac} 1.9, and J_{c-Ar} 0.9 Hz.

3-t-Butyl-5,6-dimethoxy-1-indanone. A mixture of *β-t*-butylacrylic acid [19] (0.22 mol), veratrole (0.22 mol) and polyphosphoric acid (300 g) was kept at 60 – 70 °C for 1 h. The cooled reaction mixture was mixed with water (100 ml), the temperature being kept below 50 °C. Then the reaction mixture was poured into water (1000 ml) and partly neutralized with sodium hydroxide (100 g; *care: a violent reaction takes place*) whereafter the mixture was extracted with ethyl acetate (5 × 200 ml). The combined extracts were washed with sodium hydroxide solution (200 ml, 5 M) and with water and were then evaporated. The residue was distilled, the fraction boiling at 160 – 165 °C/0.9 mmHg being collected. The yield was 86 %. NMR ($CDCl_3$: δ 0.96 (s, 9 H, *t*-Bu),

2.66 (tr, 2 H, $-CH_2-$), 3.0–3.3 (p, 1 H, $-CH-$), 3.93 and 3.99 (d, 6 H, CH_3O-), and 6.92 and 7.20 (d, 2 H, Ar).

3-t-Butyl-5,6-dimethoxy-1-indanol. To a homogeneous solution of sodium borohydride (4 g) in absolute ethanol (150 ml) was added 5,6-dimethoxy-3-*t*-butyl-1-indanone (27 g). The reaction mixture was allowed to stand for 48 h and then poured into water (1000 ml). Extraction with ethyl acetate (5 × 200 ml) gave after evaporation 27 g (99 %) of the crude hydroxy compound. An IR spectrum of this material shows that all starting material had disappeared. The product was used in the next step without any further purification. NMR ($CDCl_3$): δ 1.04 (s, 9 H, *t*-Bu), 1.7–3.2 (p, 4 H, $-OH$, $-CH-CH_2-$), 3,88 (s, 6 H, CH_3O-), 4.9–5.2 (q, 1 H, $-CH(OH)-$), and 6.9 (s, 2 H, Ar).

1-t-Butyl-5,6-dimethoxyindene. To a slurry of sodium hydride (5 g; 50 % in oil) in ether (200 ml) was added crude 3-*t*-butyl-5,6-dimethoxy-1-indanol (27 g). The mixture was refluxed with stirring for 24 h. Carbon disulfide (9.2 g) was added to the cooled solution and the reaction mixture refluxed once more for 24 h. The solution was cooled again and methyl iodide (18 g) was added and the mixture refluxed for an additional 24 h. Water (200 ml) was added to this solution. The ether phase was separated from the water solution and the water phase once more extracted with ether (200 ml). The combined ether extracts were dried with magnesium sulfate and the ether was evaporated. The residue was distilled until a constant pressure of 11 mmHg was obtained. The temperature of the oil bath used was kept below 150 °C during this procedure. The product was then distilled at oil pump pressure, and the fraction boiling at 130–140 °C/1–1.5 mmHg was collected. Redistillation gave a yield of 10 g (40 %) of pure material which boiled at 130–132 °C/1 mmHg and solidified on standing. NMR ($CDCl_3$): δ (s, 9 H, *t*-Bu), 3.2 (q, 1 H, $-CH^a-$), 3.92 (s, 6 H, CH_3O-), 6.4 (q, 1 H, $-CH=CH^b-$), 6.75 (q, 1 H, $-CH^c=CH-$), and 7.16–6.90 (d, 2 H, Ar). $J_{ab}=J_{ac}=1.8$, $J_{bc}=5.8$, and $J_{c-Ar}=J_{a-Ar}=0.7$ Hz.

Anodic oxidation of 1-t-butyl-5,6-dimethoxyindene in concentrated solution. A solution of 1-*t*-butyl-5,6-dimethoxyindene (13.6 mmol) in HOAc/KOAc(1 M, 10 ml) was electrolysed with carbon electrodes until 0.85 F/mol of substrate had passed through the solution. To avoid severe filming the current direction was reversed every 25 s. The current density during the electrolysis was in the range of 1.0–2.0 mA/cm^2.

The electrolyte was poured into water (50 ml) and extracted with methylene chloride (2 × 20 ml). The combined extracts were washed with water (50 ml), saturated $KHCO_3$ solution (25 ml), and water (50 ml). Then acetone (100 ml) was added and the solvent was evaporated to give 2.9 g of crude product (oil).

GLC analysis (3 % OV-25, 2 m × 3 mm, 140–300 °C/10 °C/min) of this oil revealed five main peaks. Peak 1 (30 %, 200 °C) was starting material, peak 2 (8 %, 220 °C) had a molecular weight of 248 (one oxygen atom added to *2*; probably a hydroxy derivative), peaks 3 and 4 (2 %, 250 °C) were the 5,6-dimethoxy-3-*t*-butylindan-1,2-diol diacetates, and peak 5 corresponded to the dimer *7b* (60 %, 300 °C). The total amount which passed the GLC detector was estimated to be 60 % of the sample.

The separation was done on a silica gel column with a mixture of $CCl_4/CHCl_3$ as eluent. The purest cyclodimer was obtained if the oil first was washed with cold (0 °C) hexane. Thus from 1.5 g of oil was 0.8 g washed off with 10 ml of hexane. The residue which consisted of 5 % starting material and 95 % dimeric and polymeric material, was eluted with a (9:1) mixture to give almost pure *7b* (0.20 g).

Anodic oxidation of 1-t-butyl-5,6-dimethoxyindene (2) in dilute solution. A solution of 1-*t*-butyl-5,6-dimethoxyindene (4.31 mmol) in 25 ml of HOAc/KOAc (1 M) was electrolysed as described above until 1.66 F/mol of indene had passed through the solution. The electrode material was either carbon or platinum. The work-up procedure was the same as the one described above. The yield of crude product was 0.9 g.

GLC analysis of this showed very little starting material to be left (5 %) and two diacetates to be the major products together with a small proportion of dimeric compounds. The total amount which passed the GLC detector was estimated at 30 % of the sample.

The separation of the two diacetates from the residue was done on a silica gel column with a mixture of $CCl_4/CHCl_3$ as eluent. When the starting material and a small fraction of dimer *7b* had been eluted with a 9:1 mixture the two diacetates were eluted with an 8:2 mixture. NMR ($CDCl_3$): δ 1.04 (s, 9 H, *t*-Bu), 2.08 (s, 6 H, $-OCOCH_3$), 3.87 (s, 6 H, CH_3O-), and 6.84 (s, 2 H, Ar). Shifts and coupling constants for the cyclopentane ring of diacetates *6b*: δ 3.07 (H_a), 5.64 (H_b), and 6.26 (H_c), J_{ab} 2.0 and J_{bc} 5.4 Hz; δ 2.94 (H_a), 5.44 (H_b), and 5.96 (H_c), J_{ab} 2.0 and J_{bc} 1.5 Hz. From 1 g of starting material 0.15 g of diacetates was obtained. The residue was eluted with $CHCl_3$. The material obtained showed no peak on GLC analysis and the NMR spectrum had only very broad signals indicating its polymeric nature.

A small fraction of the crude oil was boiled with acetic anhydride and pyridine before the separation procedure but no new diacetates were detectable (^{1}H NMR).

Anodic oxidation of 5,6-dimethoxyindene (1). A solution of 5,6-dimethoxyindene (42 mmol), KOAc (12 g), and HOAc (125 ml) was electro-

lysed as described for the *t*-butyl compound until 0.62 F/mol of substrate had been passed through the solution. The work-up procedure was the same as before. The yield was 8.3 g of a semi-crystalline product.

Dimeric and polymeric material was separated from unreacted starting material by washing with ether. The residue, which was almost pure dimer *7a*, was further purified by passing it through a silica gel column with $CHCl_3/CCl_4$ (1:9) as the eluent. The identification is described in the text.

Anodic oxidation of 5,6-dimethoxyindene (1) in dilute solution. A solution of 5,6-dimethoxyindene (2 mmol), KOAc (1 g), and HOAc (30 ml) was electrolysed as described above until 1.4 F/mol of substrate had passed through the solution. The work-up procedure was the same as before. The products were shown to be the two diacetates by GLC and MS (*m/e* 234; 2 %; 192, 100 %; in line with earlier experience [1,2]). Attempts to purify the diacetates by silica gel chromatography were unsuccessful.

3-t-Butyl-5,6-dimethoxyindene. To a solution of 1-*t*-butyl-5,6-dimethoxyindene (100 mg) in ethanol (10 ml) potassium hydroxide (0.05 g) was added whereby a homogeneous solution was obtained. The mixture was allowed to stand for 48 h and then evaporated to dryness. To the solid residue water (10 ml) was then added and the solution extracted with ether (5 × 10 ml). The combined extracts together with dry acetone (50 ml) were evaporated to dryness. No work-up was necessary due to the completeness of the rearrangement. NMR ($CDCl_3$): δ 1.36 (s, 9 H, *t*-Bu), 3.21 (d, 2 H, $-CH_2-$), 3.87 (s, 3 H, CH_3O-), 3.92 (s, 3 H, CH_3O-), 6.09 (q, 1 H, $-CH=$), and 7,18 – 7.04 (d, 2 H, Ar), J_{CH-CH_3} 1.9 Hz.

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The Crystal Structures of 3-Hydroxybiuret and 3-Hydroxybiuret Hemihydrate

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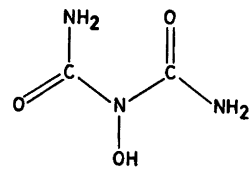
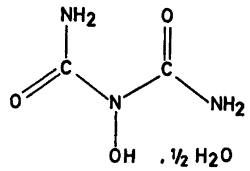
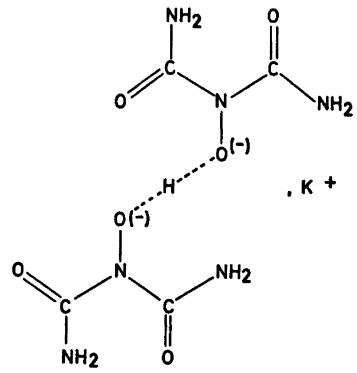
The crystal structures of 3-hydroxybiuret $HON(CONH_2)_2$ and 3-hydroxybiuret hemihydrate $HON(CONH_2)_2 \cdot \frac{1}{2}H_2O$ have been determined using three-dimensional diffractometer-collected X-ray data. The structures were solved by direct phasing techniques and Patterson methods and refined by full-matrix least-squares calculations to give a final R-value of 0.040 for 3-hydroxybiuret and 0.035 for 3-hydroxybiuret hemihydrate. The conformations of the molecules were found to be the same in the two crystal structures, *i.e.* with an *intra*-molecular NH···O=C bond. This conformation was also found in the crystal structure of the potassium acid salt of the compound.[1] The molecules are connected by three-dimensional systems of hydrogen bonds in both structures. The pK_A value of 3-hydroxybiuret was determined to 9.18 ± 0.06.



3-HB



3-HB.½ H_2O



3-HBK

Scheme 1.

The structure determination of 3-hydroxybiuret, $HON(CONH_2)_2$, was undertaken as part of an X-ray study of hydroxylamine derivatives, which are inhibitors of DNA synthesis in several cell systems. The crystal structure of the potassium acid salt of the compound (3-HBK) has been described earlier.[1]

In this paper the crystal structures of the hemihydrate (3-HB.$\frac{1}{2}H_2O$) and the anhydrous compound (3-HB) are reported. Knowledge of the molecular structure of 3-HB in three different crystal structures provides the opportunity to observe the effect of different environments on the dimensions and conformation of the molecule.

As earlier proposed by Zinner and Hitze,[2] it now seems to be evident that the compound prepared by Losee and Bernstein [3] is 3-hydroxybiuret and not the isomeric compound

N,O-dicarbamoylhydroxylamine as postulated. The 3-HB used in the present study was prepared by the same method as that used by Losee and Bernstein, and the melting point reported by these authors is identical with that found for 3-HB (158–160 °C). No other physical data were reported.[3]

EXPERIMENTAL

3-Hydroxybiuret was obtained by treatment of the potassium acid salt[1] with a strongly acidic ion-exchange resin [Amberlite IR-120 (H)], using hydrochloric acid (4 M) as an eluent. Recrystallization of the crude product from aqueous ethanol gave thin, colourless crystals, m.p. 158–160 °C. The pK_A value of the compound was determined to 9.18 ± 0.06 by electrometrical titration in aqueous solution at 22 °C, according to the method described by Albert and Serjeant.[4] The pH values were measured on a Radiometer pH meter 26. Crystals suitable for X-ray work of 3-HB and 3-HB.$\frac{1}{2}H_2O$ were obtained by diffusion (at room temperature) of ether into saturated solutions of the compound in ethanol and aqueous ethanol, respectively.

Some crystal data of 3-HB and 3-HB.$\frac{1}{2}H_2O$ are given in Table 1. The density of 3-HB was measured by flotation in a mixture of bromobenzene and methyl iodide, and of 3-HB.$\frac{1}{2}H_2O$ in a mixture of carbon tetrachloride and methyl iodide. The melting points were determined with a hot stage microscope (Mikroskop Heiztisch Ernst Leitz G.m.b.H., Wetzlar).

The lattice parameters of both compounds were calculated from series of diffractometer-measured θ-values. The intensity data sets were collected on a NONIUS three-circle automatic diffractometer by the moving crystal-stationary detector technique, using graphite monochromatized Mo$K\alpha$-radiation ($\lambda = 0.71069$ Å). The scan speed was 1.2 °/min and each reflexion was scanned over a range of 1.2 °. Background counts were taken for half the scanning time at each of the scan range limits. One standard reflexion was measured for every 25 reflexions.

The crystal of 3-HB chosen for data collection was of the size $0.10 \times 0.14 \times 0.60$ mm³, and was mounted in a glass capillary, oriented with the *c*-axis parallel to the ϕ-axis of the goniostat. Intensities of reflexions were measured in the range $2.5° < \theta < 25.0°$, and each of the 467 independent reflexions were measured twice; 662 of the 934 reflexions (*hkl* and $\bar{h}kl$) had $I_{net} \geq 2.5\ \sigma(I)$ and were considered observed. The reflexions from both octants were used independently in the least-squares refinement.

The crystal of 3-HB.$\frac{1}{2}H_2O$ chosen for data collection was of the size $0.16 \times 0.32 \times 0.80$ mm³.

Table 1. Crystal data for 3-HB and 3-HB.$\frac{1}{2}H_2O$.

	3-HB	3-HB.$\frac{1}{2}H_2O$
Mol. formula	$C_2H_5N_3O_3$	$C_2H_5N_3O_3.\frac{1}{2}H_2O$
Mol. weight	119.1	128.1
Melting point, °C	156–157	158–160
Space group	$P2_12_12_1$	$C2$
a, Å	10.868(7)	19.821(5)
b, Å	11.698(7)	4.093(1)
c, Å	3.603(2)	6.286(2)
β, °		92.96(3)
V, Å³	458.06	509.14
Z	4	4
D_x, g cm^{-3}	1.73	1.67
D_m, g cm^{-3}	1.73	1.68
μMo$K\alpha$, cm^{-1}	1.72	1.69

The hemihydrate of 3-HB has a great tendency to form double crystals, which usually could be observed on the Weissenberg films (double spots). The crystal chosen for data collection also proved to be imperfect in spite of the fact that the Weissenberg films seemed to be normal. The imperfection of the crystal was observed only on the recorder of the diffractometer (double maxima of the peaks within the θ scan range of 1.2 °). The crystal was mounted in a glass capillary and oriented with the *b*-axis parallel to the ϕ-axis of the goniostat. Intensities of reflexions were measured in the range $2.5 < \theta < 25.0°$; 492 of the 511 independent reflexions had $I_{net} \geq 2.5\ \sigma(I)$ and were considered observed.

The data of 3-HB and 3-HB.$\frac{1}{2}H_2O$ were corrected for Lorentz and polarization effects, but no corrections for absorption or extinction were made.

STRUCTURE DETERMINATION

3-HB. The structure was solved by direct methods in a straightforward way using the programs of the X-RAY system.[5] An *E*-map based on 133 $E(hkl)$'s with $|E(hkl)| \geq 1.4$ revealed the positions of the eight non-hydrogen atoms of the molecule. A structure factor calculation based on these eight positions resulted in a conventional *R*-value of 0.24. Two cycles of full-matrix least-squares refinement in which positional as well as individual atomic, isotropic thermal parameters were varied, reduced the *R*-value to 0.087. The five hydrogen atoms were located in the difference Fourier map, and two cycles of anisotropic least-squares refinement with all H-atom parameters fixed

Table 2. Fractional coordinates and thermal parameters for 3-HB and 3-HB.$\frac{1}{2}H_2O$. The thermal parameters are $\times 10^3$ and of the form $T = \exp[-2\pi^2(U_{11}h^2a^{*2} + \ldots + 2U_{12}hka^*b^* + \ldots)]$.

	x	y	z	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
3-HB									
N(1)	0.6226(3)	0.4816(3)	1.0744(11)	3.4(2)	2.7(1)	3.9(2)	−0.1(1)	0.1(2)	−0.7(2)
C(2)	0.5400(3)	0.4117(3)	0.9156(12)	3.1(2)	2.8(2)	2.9(3)	−0.4(1)	0.5(2)	0.5(2)
O(2)	0.4347(2)	0.4383(2)	0.8381(9)	2.3(1)	3.8(1)	5.8(2)	0.1(1)	−0.2(2)	0.4(2)
N(3)	0.5812(2)	0.3008(2)	0.8333(10)	2.2(1)	2.6(1)	3.8(2)	−0.3(1)	−0.6(2)	−0.3(2)
O(3)	0.4963(2)	0.2313(2)	0.6522(9)	2.5(1)	4.2(2)	4.3(2)	−0.7(1)	0.1(2)	−0.8(2)
C(4)	0.6950(3)	0.2516(3)	0.9077(12)	2.8(2)	2.5(2)	3.3(2)	0.0(1)	0.4(2)	0.4(2)
O(4)	0.7794(2)	0.3116(2)	1.0323(8)	2.6(1)	3.2(1)	5.2(2)	−0.3(1)	−1.3(1)	−0.4(1)
N(5)	0.7048(3)	0.1428(3)	0.8293(13)	2.7(2)	2.9(2)	6.7(3)	0.2(1)	−1.1(2)	−0.4(2)
3-HB.$\frac{1}{2}H_2O$									
N(1)	0.0735(1)	0.3286(14)	0.8521(4)	2.84(12)	5.83(18)	3.73(18)	0.51(14)	−0.64(9)	−0.82(16)
C(2)	0.1332(1)	0.2074(13)	0.9134(4)	2.85(13)	3.05(16)	2.73(12)	−0.44(13)	0.06(10)	0.13(13)
O(2)	0.1635(1)	0.0043(12)	0.8108(3)	3.67(10)	4.08(13)	3.31(9)	0.58(12)	−0.53(7)	−1.02(12)
N(3)	0.1635(1)	0.3277(12)	1.1037(3)	2.45(10)	3.92(14)	3.07(11)	0.15(11)	−0.27(8)	−0.16(12)
O(3)	0.2241(1)	0.1707(12)	1.1668(3)	2.75(9)	3.67(12)	3.61(10)	0.06(10)	−0.65(7)	0.46(10)
C(4)	0.1341(1)	0.5206(13)	1.2573(4)	3.62(14)	3.27(15)	2.85(12)	−0.42(16)	0.59(10)	0.02(15)
O(4)	0.0797(1)	0.6569(13)	1.2183(3)	4.36(13)	5.84(16)	3.89(11)	1.23(12)	0.47(9)	−0.85(13)
N(5)	0.1700(2)	0.5457(13)	1.4418(4)	5.38(15)	5.05(18)	2.68(11)	0.22(16)	−0.01(11)	−0.55(14)
O(6)	0.0	0.0	0.5	3.63(15)	4.57(19)	3.39(13)	0.0	−0.12(11)	0.0

Table 3. Fractional coordinates and thermal parameters ($\times 10^3$) for the hydrogen atoms of 3-HB and 3-HB.$\frac{1}{2}H_2O$.

	x	y	z	U
3-HB				
H(11)	0.697(4)	0.452(4)	1.140(15)	4.05
H(12)	0.596(4)	0.552(4)	1.134(17)	4.05
H(3)	0.428(5)	0.226(4)	0.767(17)	4.56
H(51)	0.641(4)	0.101(4)	0.744(17)	4.81
H(52)	0.772(5)	0.110(4)	0.891(19)	4.81
3-HB.$\frac{1}{2}H_2O$				
H(11)	0.055(2)	0.508(16)	0.934(7)	4.09
H(12)	0.052(2)	0.227(14)	0.732(7)	4.09
H(3)	0.259(2)	0.304(13)	1.126(6)	3.29
H(51)	0.205(2)	0.470(16)	1.456(7)	4.12
H(52)	0.157(2)	0.689(16)	1.545(7)	4.12
H(6)	0.025(2)	−0.156(16)	0.431(7)	3.75

reduced the R-value to 0.049. In the remaining two cycles of refinement the positional parameters of the H-atoms were also varied, but the isotropic thermal parameters were set equal to the parameters of the atoms to which they are bonded, and were still fixed. The final R-value is 0.040.

3-HB.$\frac{1}{2}H_2O$. Several attempts to solve this structure by the direct phasing method failed, and the structure was finally solved from the sharpened Patterson map in combination with packing considerations. Least-squares refinement of a first trial structure converged at a false minimum with an R-value of 0.30. The right solution was achieved after moving the positions of all the atoms of the molecule about 0.5 Å in the x-direction. Full-matrix least-squares refinement of the scale factor, atomic positions, and isotropic thermal parameters yielded an R-value of 0.073. The six hydrogen atoms of the structure were located in the difference map. The hydrogen atoms of the water molecule are symmetry related because the water oxygen atom was found in the special position (0, y, $\frac{1}{2}$). In the succeeding anisotropic least-squares refinement the positional parameters of the H-atoms were also varied, but the isotropic thermal parameters (set equal to the parameters of the atoms to which they are bonded) were fixed. The final R-value is 0.035.

All the refinements were based on F, minimizing the function $\sum w(|F_o|-|F_c|)^2$, where the weights were initially taken as unity but later changed as follows: $w=1/\{1+[(F_o-B)/A]^2\}$ with $A=10.0$ and $B=15.0$ for 3-HB, and A= B=8.0 for 3-HB.$\frac{1}{2}H_2O$. The scattering factors were taken from *International Tables for X-Ray Crystallography*.[6] Final parameters for the heavy atoms of 3-HB and 3-HB.$\frac{1}{2}H_2O$ are given in Table 2, while the hydrogen atom parameters are given in Table 3. The notation of the atoms is given in Figs. 1 and 3. The observed and calculated structure factor data are available

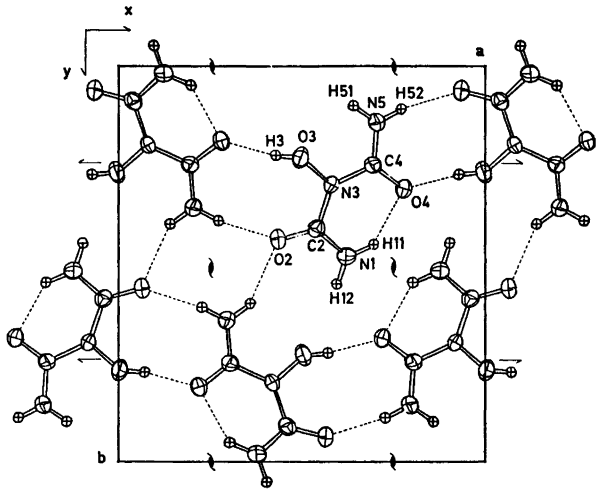


Fig. 1. The structure of 3-HB viewed along the c axis.

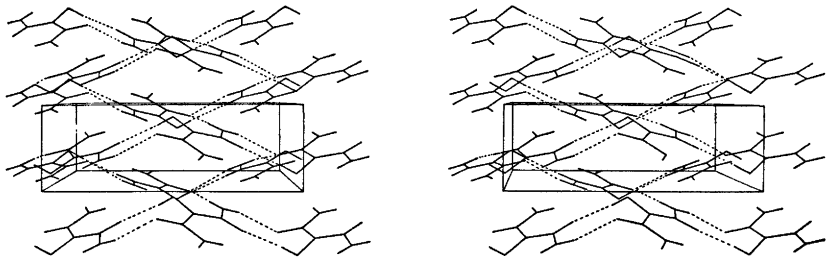


Fig. 2. Stereoscopic diagram of the molecular packing of 3-HB. The view axis is *b*, the *a* axis is →, and the *c* axis is ↓.

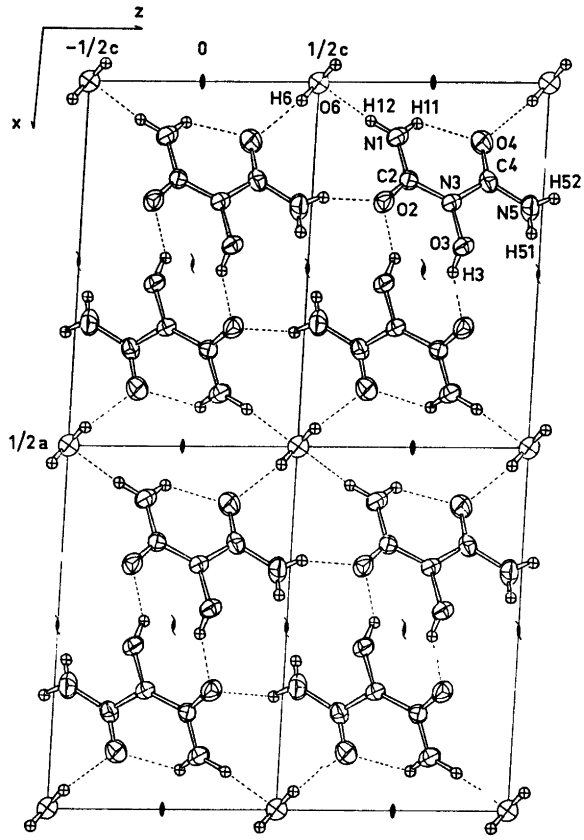


Fig. 3. The structure of 3-HB.$\frac{1}{2}H_2O$ viewed along the *b* axis.

from the author on request. The calculations were performed on a GIER computer and an IBM 360/75 computer, using the following programs: *INDIFF*,[7] a local version of the *N.R.C 2 A Picker Data Reduction Program*,[8] *The X-Ray System*,[5] and *ORTEP*.[9]

DISCUSSION

The molecular arrangement in the crystal of 3-HB is illustrated in Figs. 1–2, and of 3-HB.$\frac{1}{2}H_2O$ in Figs. 3–4. A common feature of the structures is the intensive hydrogen

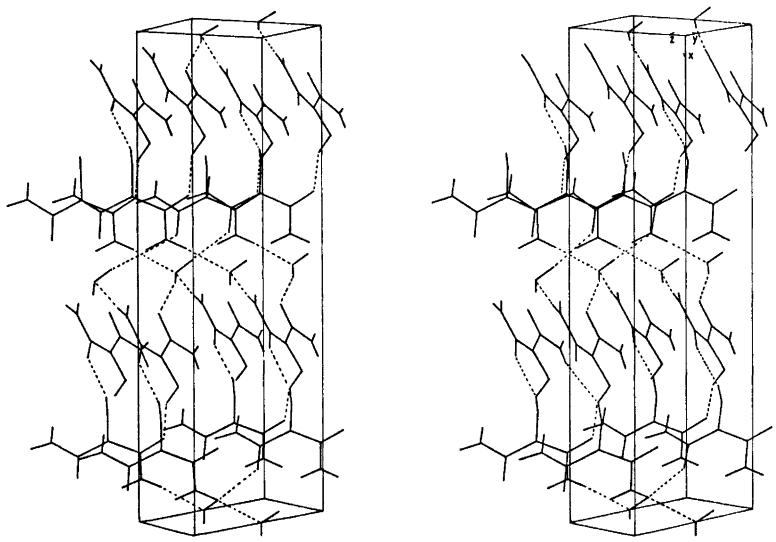


Fig. 4. Stereoscopic diagram of the molecular packing of 3-HB.$\frac{1}{2}H_2O$.

Table 4. Distances and angles concerning the hydrogen bonding systems of 3-HB and 3-HB.$\frac{1}{2}H_2O$.

X–H···Y	Distances (Å) X···Y	H···Y	Angle (°) X–H···Y
3-HB			
N(1)–H(11)···O(4)	2.624(4)	1.91(4)	134(4)
N(5)–H(51)···O(2)$_{1-x,-\frac{1}{2}+y,1\frac{1}{2}-z}$	2.897(4)	2.09(5)	148(4)
N(5)–H(52)···O(2)$_{\frac{1}{2}+x,\frac{1}{2}-y,2-z}$	2.929(4)	2.10(5)	165(6)
O(3)–H(3)···O(4)$_{-\frac{1}{2}+x,\frac{1}{2}-y,2-z}$	2.664(4)	1.82(5)	169(5)
3-HB.$\frac{1}{2}H_2O$			
N(1)–H(11)···O(4)	2.663(5)	1.93(4)	129(4)
N(1)–H(12)···O(6)	2.915(4)	1.97(5)	174(4)
N(5)–H(52)···O(2)$_{x,1+y,1+z}$	2.991(5)	2.11(5)	159(4)
O(3)–H(3)···O(2)$_{\frac{1}{2}-x,\frac{1}{2}+y,2-z}$	2.611(4)	1.77(5)	150(4)
O(6)–H(6)···O(4)$_{x,-1+y,-1+z}$	2.810(3)	1.93(5)	158(5)

bonding, involving all hydrogen atoms except H(12) of 3-HB and H(51) of 3-HB.$\frac{1}{2}H_2O$. The dimensions of the hydrogen bonding systems are given in Table 4. The carbonyl oxygen atoms, O(2) and O(4), are acceptors for two hydrogen bonds in both structures. The hydroxyl oxygen atom, O(3), is donor for one rather strong OH···O bond in each structure, with a carbonyl oxygen atom as the acceptor in both cases. The water oxygen atom of 3-HB.$\frac{1}{2}H_2O$ is donor for two and acceptor for two symmetry related hydrogen bonds. The packing of the molecules is quite different in the two crystal structures of 3-HB, *cf.* Figs. 1 and 3, while the packing of the molecules in 3-HB.$\frac{1}{2}H_2O$ is rather similar to the packing of the 3-HB residues in the crystal structure of the potassium acid salt of 3-hydroxybiuret, 3-HBK.[1]

3-HB. In the crystal structure of 3-HB the molecules are linked into chains in the x-direction by two hydrogen bonds, O(3)–H(3)···O(4)$_{-\frac{1}{2}+x,\frac{1}{2}-y,2-z}$ and N(5)–H(52)···O(2)$_{\frac{1}{2}+x,\frac{1}{2}-y,2-z}$. Each chain is linked to the

Table 5. Bond lengths (Å) and angles (°) for 3-HB, 3-HB.$\frac{1}{2}H_2O$. The dimensions found for the two 3-HB residues in crystal structure of the potassium acid salt of 3-hydroxybiuret,[1] 3-HBK(1) and 3-HBK(2), are given for comparison.

	3-HB	3-HB.$\frac{1}{2}H_2O$	3-HBK(1)	3-HBK(2)
N(1)–C(2)	1.342(5)	1.322(4)	1.335(4)	1.344(4)
C(2)–O(2)	1.218(4)	1.228(5)	1.222(4)	1.229(3)
C(2)–N(3)	1.404(4)	1.401(4)	1.405(4)	1.399(4)
N(3)–O(3)	1.393(4)	1.401(4)	1.390(3)	1.394(3)
N(3)–C(4)	1.390(4)	1.397(5)	1.386(4)	1.388(4)
C(4)–O(4)	1.239(4)	1.228(5)	1.245(4)	1.241(4)
C(4)–N(5)	1.308(5)	1.333(4)	1.334(4)	1.330(4)
N(1)–H(11)	0.91(4)	0.98(6)	0.88(4)	0.86(4)
N(1)–H(12)	0.90(5)	0.95(5)	0.87(4)	0.86(4)
O(3)–H(3)	0.85(5)	0.92(5)	1.31(4)	1.13(4)
N(5)–H(51)	0.91(5)	0.76(5)	0.89(4)	0.93(4)
N(5)–H(52)	0.85(5)	0.92(5)	0.93(4)	0.88(4)
O(6)–H(6)		0.92(6)		
N(1)–C(2)–O(2)	124.8(3)	123.7(3)	123.7(3)	122.6(3)
N(1)–C(2)–N(3)	116.1(3)	117.1(4)	116.1(2)	117.4(2)
O(2)–C(2)–N(3)	119.1(3)	119.1(3)	120.2(2)	120.0(2)
C(2)–N(3)–O(3)	115.3(3)	113.9(3)	115.8(2)	115.4(2)
C(2)–N(3)–C(4)	128.7(3)	127.8(2)	127.7(2)	126.5(2)
O(3)–N(3)–C(4)	116.0(3)	116.4(2)	116.5(2)	117.3(2)
N(3)–C(4)–O(4)	119.6(3)	120.8(2)	122.1(2)	121.2(2)
N(3)–C(4)–N(5)	115.7(3)	115.0(3)	114.6(2)	115.6(3)
O(4)–C(4)–N(5)	124.7(3)	124.2(4)	123.3(3)	123.3(3)
H(11)–N(1)–H(12)	125(4)	125(4)	117(4)	123(4)
H(11)–N(1)–C(2)	118(3)	119(2)	121(3)	118(3)
H(12)–N(1)–C(2)	116(3)	115(3)	120(3)	117(3)
H(3)–O(3)–N(3)	113(4)	107(3)	109(2)	109(2)
H(51)–N(5)–H(52)	120(5)	118(5)	121(4)	123(4)
H(51)–N(5)–C(4)	123(3)	121(4)	118(3)	119(2)
H(52)–N(5)–C(4)	116(4)	120(3)	121(2)	117(3)
H(6)–O(6)–H(6′)		93(5)		

next (antiparallel) chain by the N(5)–H(51)···$O(2)_{1-x,-\frac{1}{2}+y,1\frac{1}{2}-z}$ bond. In this way a three dimensional network of hydrogen bonds is formed, *cf.* Fig. 2.

3-HB.$\frac{1}{2}H_2O$. In the crystal structure of the hemihydrate of 3-HB the molecules form hydrogen bonded pairs by symmetry related OH···O bonds, *cf.* Fig. 3, which are arranged in rows along the long *a*-axis. Each pair is connected with the next pair in the row and the next row of pairs by means of the water molecules. As the water oxygen atoms accept the NH···O bonds from one layer of 3-HB molecules and are donors for the OH···O bonds to another layer, the water molecules also provide for connection in the direction of the short *b*-axis.

The bond lengths and angles for 3-HB and 3-HB.$\frac{1}{2}H_2O$ are given in Table 5, and in addition the dimensions found for the two crystallographically non-equivalent 3-HB residues, 3-HBK(1) and (2), in the crystal structure of the potassium acid salt of 3-hydroxybiuret.[1] Only small differences were found between corresponding bonds and angles (involving non-hydrogen atoms) in the three structures. The negative charge distributed on the two 3-HB residues in the structure of the potassium acid salt does not influence significantly on the bond lengths and angles. The N–O bonds are of equal lengths in the ionized and non-ionized molecules. Greater deviations are observed on the lengths of the carbonyl bonds, *e.g.* C(4)–O(4), and the C(4)–N(5) bonds, but the varia-

Table 6. Least-squares planes (I-III) and angles between them. The angles II:III for 3-HBK(1) and (2) [1] are given for comparison. The equations of the planes are in direct (unit cell) space. Distances (Å) to atoms defining the plane are asterisked.

Plane	
3-HB	
I	$3.3961x + 3.3979y - 3.2586z - 0.2670 = 0$
II	$3.2634x + 3.5929y - 3.2537z - 0.2654 = 0$
III	$3.2210x + 2.6020y - 3.3465z + 0.1362 = 0$
3-HB.½H_2O	
I	$9.5416x + 3.1311y - 2.8400z + 0.6688 = 0$
II	$9.2428x + 2.9831y - 3.2969z + 1.1528 = 0$
III	$9.7349x + 3.2794y - 2.3022z - 0.1237 = 0$

	3-HB			3-HB.½H_2O		
Atom	I	II	III	I	II	III
N(1)	−.017*	.001*		−.021*	.003*	
C(2)	−.018*	−.003*		−.005*	−.009*	
O(2)	−.032*	.001*		−.061*	.003*	
N(3)	.014*	.001*	.002*	.120*	.003*	.002*
O(3)	.079*			.028*		
C(4)	−.009*		−.008*	.008*		−.005*
O(4)	.075*		.003*	.026*		.002*
N(5)	−.091*		.003*	−.095*		.002*
H(11)	−.08			.13		
H(12)	−.06			−.20		
H(3)	−.55			.89		
H(51)	−.17			−.04		
H(52)	−.17			−.07		
Angle II:III	5.1°			10.2°		
	3-HBK(1)			3-HBK(2)		
Angle II:III	3.6°			13.2°		

tions are too small to merit any explanation.

The conformations of the 3-HB molecules (and ions) were found to be roughly the same in the three crystal structures, *i.e.* the conformation with an *intra*-molecular NH···O bond. This conformation was also found in the crystal structures of biuret [10–11] and triuret.[12] None of the 3-HB molecules (or ions) were found to be exactly planar, *cf.* Table 6, but the atoms of each half of the molecules, the "urea-parts", are coplanar in all cases. The angles between the planes of the "urea-parts" are given in Table 6 (angle II:III). The maximum deviation between these angles is about 10°. The standard deviations on the angles are about 0.5°.

The result of the comparison of the molecular geometry of 3-hydroxybiuret in the three crystal structures is as follows: Bond lengths and angles are not affected significantly by the different environments of the molecules (or ions). Even ionization has no significant effect on these molecular dimensions. The conformations of the molecules (and ions) are roughly the same in the three crystal structures and are probably determined by *intra*- rather than by *inter*-molecular forces. Some variations were found in the torsional angles of the molecules (and ions) as reflected in the angles between the "urea-parts". These variations are probably due to the different environments of the molecules (or ions), *e.g.* different hydrogen bonding systems.

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X-Ray Crystallographic and Proton Magnetic Resonance Spectroscopic Investigations of Nipecotic Acid, a Potent Inhibitor of γ-Aminobutyric Acid (GABA) Uptake

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The conformations of (±)-nipecotic acid in the solid state and in aqueous solution have been investigated by X-ray crystallographic methods and by ^{1}H NMR spectroscopy, respectively. Nipecotic acid exists in the crystalline state as well as in aqueous solution in a chair conformation with the carboxylate group in an equatorial position. The crystal structure is stabilized by hydrogen bonds. Nipecotic acid appears to be a better substrate than γ-aminobutyric acid (GABA) for the GABA transport system in rat brain slices. On the basis of the structure of nipecotic acid, it is suggested that GABA is transported by this system in a somewhat folded conformation with less than 5 Å between the zwitterionic centres.

A "high affinity" uptake system may play an important role in the function of GABA as an inhibitory transmitter in the brain.[1–5] Several structural analogues of GABA are potent inhibitors of this uptake system,[6–7] but these compounds have in addition "GABA-like" depressant actions. Specific inhibitors of the GABA uptake process *in vivo* have pharmacological interest and may furthermore provide information regarding the physiological role of the uptake process. Thus, considerable effort has been made to evaluate such compounds and recently (±)-nipecotic acid (piperidine-3-carboxylic acid) has been shown to be a powerful inhibitor of GABA uptake in rat brain slices.[8] Nipecotic acid administered electrophoretically to feline spinal neurones reversibly enhanced the depressant action of similarly administered GABA, but nipecotic acid did not significantly depress the firing of spinal neurones *per se*.[9] Nipecotic acid appears to be able to replace GABA in both the uptake system and the calcium-dependent, potassium-stimulated release system in rat brain slices.[10,11] This uptake system seems to be characterized by pronounced conformational specificity as revealed by investigations of a series of conformationally restricted analogues of GABA.[6,8,9,12]

EXPERIMENTAL

Nipecotic acid was prepared from nicotinic acid according to directions by Freifelder [13] and was identified by infrared and ^{1}H NMR spectroscopy supported by elemental analysis. The crystals used for the X-ray examination were crystallized from a water/*N*,*N*-dimethylformamide solution. Colourless prismatic crystals were formed.

The computations in connection with the X-ray structure determination were performed on an IBM 1130 and an IBM 370/165 computer, using *INDIFF*,[14] a local version of the *NRC 2A Picker Data Reduction Program*,[15] *MULTAN*,[16] The Program System X-Ray 72,[17] and *ORTEP*.[18] The X-ray atomic scattering factors used were those of Cromer and Mann [19] for oxygen, nitrogen, and carbon, and of Stewart, Davidson, and Simpson [20] for hydrogen.

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The ^{1}H NMR spectra were recorded with a Varian HA-100 spectrometer at 100 MHz on the δ-scale with tetramethylsilane as an external standard at 32 °C using a 0.1 M solution of nipecotic acid. The INDOR experiments [21] were performed using a modified HA-100 spectrometer with crystal locked digital frequency generation, and a Varian Spectro system 100 for accumulation of 20 scans.

X-Ray analysis

Crystal data. (±)-Nipecotic acid, $C_6H_{11}NO_2$, M = 129.16. Orthorhombic, $a = 11.177(3)$, $b = 6.123(1)$, $c = 9.539(4)$ Å, $U = 652.8$ Å^3, D_m (flotation) = 1.31 g cm^{-3}, $Z = 4$, $D_c = 1.314$ g cm^{-3}; $\mu(\lambda(\text{Mo}K\alpha) = 0.7107$ Å$) = 1.06$ cm^{-1}; $F(000) = 280$. Systematically absent reflections: $0kl$ when $k+l$ odd, $h0l$ when h odd; thus possible space groups are $Pna2_1$ and $Pnam$. The non-centrosymmetric $Pna2_1$ was indicated by intensity statistics and confirmed by successful refinement of the structure.

The unit-cell parameters were refined by least-squares techniques from the diffractometer measured 2θ angles observed for 35 reflections.

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 0.20 × 0.30 × 0.22 mm. The crystal was mounted with [010] along the ϕ axis of the goniostat.

X-ray diffraction intensities within one quarter of the reciprocal sphere were measured in the range $2.5° \leq \theta \leq 28°$; averaging of equivalent reflections led to 832 unique reflections, 711 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 correction was made owing to the low absorption coefficient.

Structure determination. The structure was determined by direct methods with the multiple solution technique using the automatic phasing program MULTAN.[16] An E map computed from the set of phases with the highest combined figure of merit [22] revealed all the non-hydrogen 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 0.073. The quantity minimized was $\sum w(|F_o| - |F_c|)^2$ where weights were taken as unity. A difference map at this point revealed the positions of all eleven hydrogen atoms (0.3 − 0.5 e Å^{-3}). These were included in all subsequent refinements with fixed isotropic temperature factors equal to the values of the attached non-hydrogen atoms. The least-squares refinement was completed with the introduction of a weighting scheme of the form $w = 1/\{1 + [(|F_o| - 5.75)/6.00]^2\}$.[17] On the last cycle of least-squares refinement the values of maximum and average shift/error were 0.03 and 0.009, respectively. The final R value is 0.033 ($R_w = 0.041$) for all observed independent reflections. A final difference synthesis

Table 1. Final positional and thermal (× 10^4 Å^2) parameters for non-hydrogen atoms. The standard deviations of positional and thermal parameters (× 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}
N(1)	0.4246(2)	0.2319(3)	0.9442 [a]	249(8)	314(9)	252(8)	47(7)	12(7)	22(8)
C(2)	0.3356(2)	0.2929(4)	0.8352(3)	283(9)	306(10)	235(10)	13(8)	−17(8)	0(9)
C(3)	0.2529(2)	0.4730(3)	0.8862(3)	243(8)	302(9)	231(9)	−2(7)	−6(8)	16(9)
C(4)	0.3264(2)	0.6687(4)	0.9365(3)	401(12)	306(11)	397(12)	37(9)	−111(12)	−58(11)
C(5)	0.4191(3)	0.6003(5)	1.0461(4)	435(14)	411(13)	416(13)	23(11)	−165(11)	−111(12)
C(6)	0.4989(2)	0.4215(4)	0.9902(4)	264(9)	438(12)	408(12)	−15(10)	−86(10)	−18(11)
C(7)	0.1698(2)	0.5446(3)	0.7677(3)	253(9)	238(9)	282(9)	−8(8)	−35(8)	24(8)
O(1)	0.0594(1)	0.5487(3)	0.7890(3)	241(7)	573(11)	451(10)	17(7)	16(7)	177(9)
O(2)	0.2184(2)	0.5977(3)	0.6548(3)	334(8)	622(11)	285(8)	11(8)	−1(7)	144(9)

[a] This parameter was held constant to define the origin.

Table 2. 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(11)	0.387(2)	0.166(4)	1.021(3)	27
H(12)	0.475(2)	0.135(4)	0.909(3)	27
H(21)	0.375(2)	0.337(4)	0.759(3)	27
H(22)	0.292(2)	0.169(4)	0.810(3)	27
H(31)	0.205(2)	0.410(4)	0.973(3)	26
H(41)	0.368(2)	0.724(5)	0.860(3)	37
H(42)	0.269(2)	0.776(5)	0.973(3)	37
H(51)	0.377(2)	0.555(5)	1.126(3)	42
H(52)	0.473(2)	0.725(5)	1.073(4)	42
H(61)	0.550(2)	0.478(5)	0.904(3)	37
H(62)	0.553(2)	0.366(4)	1.062(3)	37

showed no peaks or depressions greater than 0.2 e Å^{-3}. Tables 1 and 2 list the final positional and thermal parameters for the non-hydrogen and hydrogen atoms, respectively. The terminal set of structure factors listed with the observed data are available by request.

Description of the structure. The X-ray diffraction analysis of (±)-nipecotic acid confirms the expected zwitterionic structure. The conformation of the molecule is shown in Fig. 1 in which the numbering of the atoms and major bond distances and angles are also indicated. The piperidine ring of nipecotic acid is in the chair form with the carboxylate group in an equatorial position. The least-squares plane through the carboxylate group and C(3) makes an angle of 82.6° with that of the puckered ring. The torsion angle O(1)−C(7)−C(3)−C(2) is ±127.6(2)°. The *intra*-molecular distances N(1)···O(1) and N(1)···O(2) are 4.756(3) and 4.237(3) Å, respectively.

The bond lengths and angles are in agreement with those of the equivalent bond lengths and angles found in several piperidinium derivatives, *e.g.* piperidinium *p*-hydroxybenzoate.[23] The mean values of the piperidine ring C−C and C−N bonds are 1.522 and 1.490 Å, respectively, and the internal bond angles in the piperidine ring are slightly larger than the regular tetrahedral angle. The geometry of the carboxylate group is quite normal. No significant differences are observed between the two C−O bond lengths, in accordance with the fact that each oxygen atom is an acceptor in a hydrogen bond.

The packing of the molecules in the crystals is illustrated in Fig. 2. The crystal structure is stabilized by hydrogen bonds, one for each hydrogen atom covalently bonded to nitrogen. Table 3 lists hydrogen bond distances and angles and van der Waals contacts between non-hydrogen atoms less than 3.4 Å.

Analysis of proton magnetic resonance spectrum

The ^{1}H NMR spectrum of nipecotic acid in deuterium oxide solution is shown in Fig. 3. The spectrum can be divided into 3 sections: the four protons on C(2) and C(6) are assigned to section I, the proton on C(3) to section II, and the four protons on C(4) and C(5) to section III. Section I contains the AB part of an ABX system, the AB coupling constant being characteristic of a pair of geminal protons. The AX and BX coupling constants should describe the interaction between the geminal protons and a single vicinal proton. Section II appears as a symmetrical multiplet of 7 bands. In section I of the spectrum the signals due to the H(6) protons are expected to be present as a very complex many line pattern, each line having only a small intensity. The most prominent lines would therefore intuitively

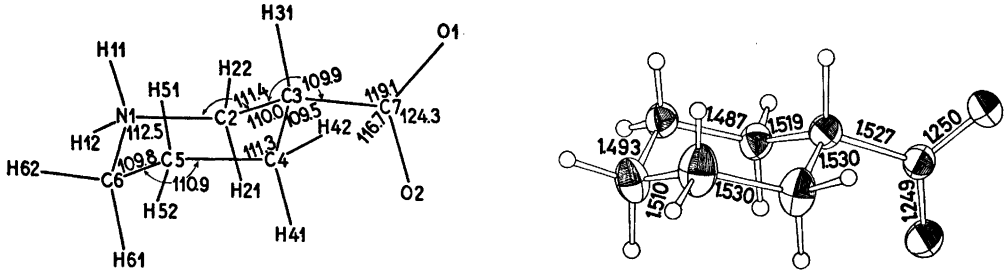

Fig. 1. Perspective drawings of nipecotic acid. (a) The numbering of the atoms and bond angles involving only non-hydrogen atoms. All e.s.d.'s are 0.2°. Bond angles involving hydrogen atoms range from 106 to 112°. E.s.d.'s are 1−3°. (b) The thermal ellipsoids for the non-hydrogen atoms, scaled to 50 % probability; hydrogen atoms are represented as spheres of arbitrary radius. Bond lengths between non-hydrogen atoms. E.s.d.'s are 0.003−0.004 Å. Bond lengths involving hydrogen atoms range from 0.89 to 1.06 Å. All e.s.d.'s are 0.03 Å.

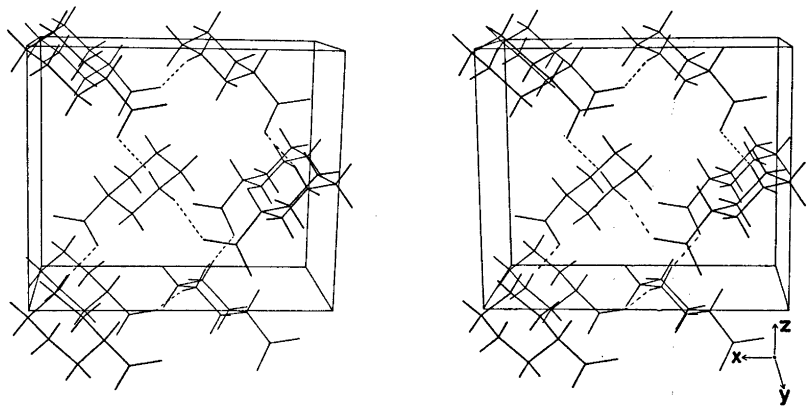

Fig. 2. Stereo diagram illustrating the molecular packing of nipecotic acid. Hydrogen bonds are drawn with broken lines.

Table 3. Hydrogen bond lengths and angles and van der Waals contacts between non-hydrogen atoms less than 3.4 Å.

A———H	B	B equipoint	A–H (Å)	A···B (Å)	H···B (Å)	∠AHB (°)
N(1)–H(12)···O(1)		$(\frac{1}{2}+x,\frac{1}{2}-y,z)$	0.89(3)	2.723(3)	1.86(3)	164(3)
N(1)–H(11)···O(2)		$(\frac{1}{2}-x,-\frac{1}{2}+y,\frac{1}{2}+z)$	0.93(3)	2.695(3)	1.79(3)	163(2)
C(2)	O(1)	$(\frac{1}{2}+x,\frac{1}{2}-y,z)$		3.290(3)		
C(2)	O(2)	$(\frac{1}{2}-x,-\frac{1}{2}+y,\frac{1}{2}+z)$		3.330(4)		
C(4)	O(2)	$(\frac{1}{2}-x,\frac{1}{2}+y,\frac{1}{2}+z)$		3.389(4)		

be ascribed to the two protons at C(2). Based on this a first assignment is made as shown in Fig. 3. The approximate values of the coupling constants would be in agreement with a geminal coupling constant of 12.4 Hz and vicinal coupling constants of 4.0 Hz and 8.6 Hz for the equatorial-axial and axial-axial configurations, respectively. No virtual coupling is expected to contribute to the line separation in the AB part of the ABX system since δ(H-3) $-$ δ(H-4) is of the order of ten times the vicinal couplings between H(3) and H(4).

To verify the assumption above Homo INDOR experiments were undertaken. One of the small lines marked with an asterisk was irradiated, and the remaining part of section I was scanned with the H_2 field. Under appropriate conditions the INDOR spectra corresponding to an AB case were observed. This permitted the accurate location of the lines belonging to each of the AB subspectra in section I. No analysis was attempted of section II in the spectrum, but the approximate position of the chemical shift can be taken as δ 3.1. The available data were used in the iterative program Laocoon III,[24] which yielded chemical shifts and coupling constants. The actual values of the coupling constants δ_A and δ_B depend on the chosen value of δ_X The error limits indicated for the coupling constants result from varying δ_X within ± 3 Hz, which is considered a conservative measure. The computed coupling constants were: $J_{AB} = -12.78 \pm 0.07$ Hz, $J_{AX} = 3.94 \pm 0.05$ Hz (equatorial-axial), and $J_{BX} = 9.32 \pm 0.02$ Hz (axial-axial).

In simple piperidine derivatives the equatorial proton on C(2) is found downfield from its axial counterpart.[25] Estimating the vicinal coupling constants for the protons on C(4) to be $J_{a,e} = 4.4$ Hz and $J_{a,a} = 8.6$ Hz, the multiplet at δ 3.09 assigned to the proton on C(3) could be analyzed as illustrated in Fig. 3, suggesting that this proton is axial and the carboxylate group is equatorial. The vicinal coupling constants used in this assignment are in general agreement with those found in most 6-membered rings [26] as is the geminal coupling constant in piperidines.[27] The spectrum as assigned is consistent with the piperidine ring of nipecotic acid in aqueous solution being predominantly in the chair form with the carboxylate group in an equatorial position.

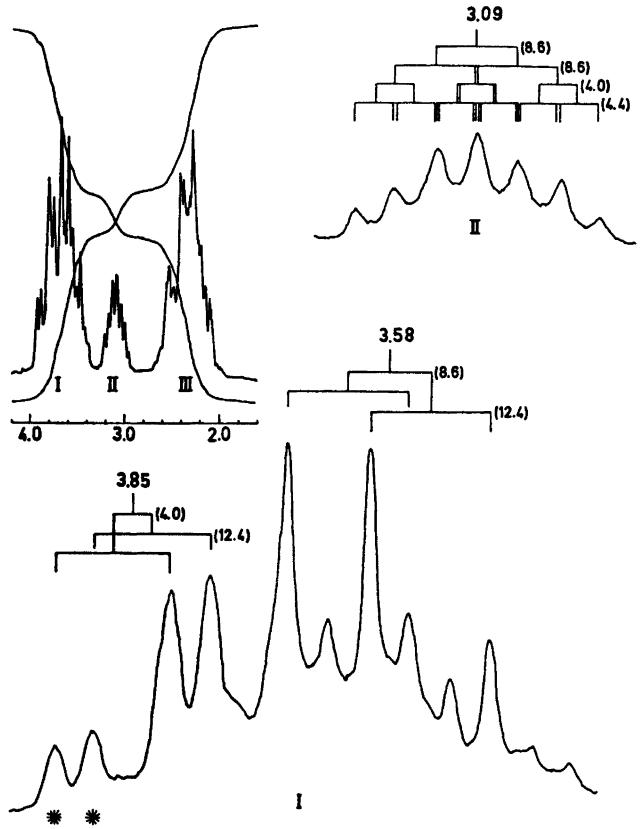

Fig. 3. Proton magnetic resonance spectrum of nipecotic acid. The spectrum is measured at 100 MHz on the δ-scale. Sections I and II of the spectrum are expanded and have been subjected to an approximate first order partial analysis in terms of the chemical shifts of the protons on C(2), δ 3.85 and 3.58, and C(3), δ 3.09, and their respective coupling constants (Hz) as indicated. The asterisks indicate the lines monitored during INDOR experiments.

DISCUSSION

The GABA molecule has considerable flexibility and the ability to adopt different conformational modes may well be essential to its physiological activity.[9] Different conformations of GABA may be required for the initial attraction to postsynaptic receptors and for the subsequent modification of the conductance properties of the postsynaptic membrane.[28] Furthermore, GABA may be transported across membranes in a conformation different from that required for initial binding to the uptake system.[6] Problems concerning the "active conformations" of GABA are approached indirectly by examination of structure-activity correlations of conformationally restricted GABA analogues.

Compared to the GABA molecule, nipecotic acid is a relatively bulky and inflexible molecule. Nonetheless, studies with radioactive nipecotic acid indicate that GABA and nipecotic acid can be counter-transported in brain slices *via* the same mobile carrier and that nipecotic acid appears to have a higher affinity than GABA for this carrier.[11] The X-ray study of nipecotic acid revealed a chair conformation with the carboxylate group in an equatorial position. The torsion angle O(1)−C(7)−C(3) −C(2) is ±127.6° and the *intra*-molecular distances between the nitrogen atom and the two oxygen atoms are 4.756 and 4.237 Å,

respectively. The proton magnetic resonance study indicates that in aqueous solution nipecotic acid exists predominantly in a chair conformation with the carboxylate group equatorial. In solution the carboxylate group can rotate so that the distance between the nitrogen atom and one oxygen atom ranges from 4.3 to 4.9 Å (Dreiding stereomodel). From a study of some conformationally restricted GABA analogues which were competitive inhibitors of GABA uptake it was concluded that the zwitterionic centres should be between approximately 5 and 6 Å apart in order to bind to the transport carrier.[6] The results obtained with nipecotic acid indicate that GABA is transported in a somewhat folded conformation with less than 5 Å between the zwitterionic centres. Analogues of restricted conformation with a greater minimum charge separation than 5 Å may inhibit the transport system by binding to the carrier to form a complex which does not penetrate the membrane.

The β-alanine molecule can assume the same distance between the zwitterionic centres as does nipecotic acid, but it does not inhibit GABA uptake in rat brain slices.[29] Factors other than charge separation may be important for interaction with the GABA transport carrier. The pronounced difference between β-alanine, which has two methylene groups, and nipecotic acid, which has four methylene groups, with respect to GABA uptake may reflect the importance of hydrophobic bonding in interactions with the transport carrier. It has been argued previously that conformational adaptability may be important in interactions with the carrier.[6] Data pertinent to the conformational adaptability of nipecotic acid might be obtained from magnetic resonance relaxation studies and from molecular orbital calculations.

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The Crystal Structure of Morphine Hydrate

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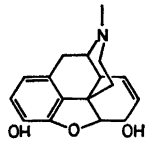
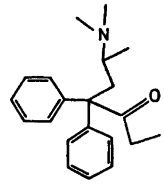
The crystal and molecular structure of the monohydrate of morphine has been determined by X-ray methods. The compound crystallizes in space group $P2_12_12_1$, with $a=7.438(1)$, $b=13.751(3)$, $c=14.901(3)$ Å. The structure was determined by direct methods and refined by full-matrix least-squares methods to an R-value of 0.045 for 1150 observed reflections. The e.s.d.'s for non-hydrogen atoms are 0.005 – 0.006 Å and 0.3 – 0.4° in bond lengths and angles, respectively.

The morphine molecule has the usual T-shape with an angle of 86.6° between the two "planar" parts. The water molecule bridges the phenolic and hydroxylic oxygen atoms through two O–H···O bonds acting as a donor in both. There is an extensive network of hydrogen bonds throughout the molecular crystal, including chains of morphine molecules parallel to the b-axis formed by a strong O–H···N hydrogen bond of 2.635 Å. A common structural feature of narcotic analgetics and phenethylamines is discussed.

Morphine (*1*) is one of the most familiar narcotic analgetics and is widely used as a medicinal agent against severe pain. It has been the central compound in the synthesis and the development of other analgetics, for example methadone (*2*).



1



2

Intensive structural and conformational studies have been performed on analgetics during the last 25 years to elucidate the mechanism of analgesic action. Analgetics, their receptor(s) and structure activity relationships between these compounds have been reviewed recently,[2] however, a complete understanding of the mechanism of analgesia is still not achieved.

The constitution of the alkaloid (*1*) has been known for 50 years [1] although not confirmed until 1952.[3] The structure of morphine was verified by Mackay *et al.*[4] in 1955 and the absolute configuration established in 1962 by Kartha *et al.*[5] Morphine is a rigid molecule and may serve as a model compound for a large class of narcotic agonists and their antagonists acting on the central nervous system. The model may give valuable information about the receptor topography assuming stereospecific receptor interactions.[3,6]

The present crystallographic investigation was part of a research program on analgetics * particularly for comparison with the structure of (–)-morphine.HCl.$3H_2O$ [7] and methadone base,[8] which has recently been studied in this laboratory.

EXPERIMENTAL

Commercial (–)-morphine hydrate was obtained as transparent, colourless single crystals and a specimen of dimensions $0.2\times0.2\times0.4$ mm[3] was used for the experiments. Photographic investigations indicated orthorhombic symmetry; systematic absences are compatible with space group $P2_12_12_1$. Unit cell dimensions were determined on a SYNTEX $P\bar{1}$ diffractometer with graphite crystal monochromated Mo$K\alpha$-radiation ($\lambda=0.71069$ Å).

Intensities were collected with the $2\theta-\theta$ autocollection program using variable scan rate (2 – 8° min^{-1}). The scan range was from

* Previous paper in this series: E. Bye, *Acta Chem. Scand. B 30* (1976) 323.

0.9° below $2\theta(\alpha_1)$ to 0.9° above $2\theta(\alpha_2)$ and the background was counted for 0.35 times the scan time at each end of the scan range. Three periodically measured reflections showed no systematic variation. E.s.d.'s in the intensities were taken as the square root of the total counts with a 2 % addition (of the net counts) for instrumental instability.

1593 independent reflections with $\sin\theta/\lambda \leq 0.60$ were recorded; 1150 with $I \geq 2\sigma_I$ were considered as observed. All calculations were performed in a CYBER-74 computer utilizing the programs in Ref. 9, except for the phase determination.[10] Atomic form factors were those of Hanson *et al.*[11] for O, N, C and of Stewart *et al.*[12] for H.

CRYSTAL DATA

(−)-Morphine hydrate, $C_{17}H_{19}NO_3.H_2O$, orthorhombic.

$a = 7.438(1)$ Å, $b = 13.751(3)$ Å, $c = 14.901(3)$ Å.

$V = 1524.1$ Å^3, $M = 303.17$, $Z = 4$.

$D_o = 1.31$ g cm^{-3} (flotation), $D_c = 1.32$ g cm^{-3}.

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

STRUCTURE DETERMINATION

The structure was determined by direct methods using the program MULTAN.[10] The most probable phase-set based on the 194 highest normalized structure factors ($E \geq 1.42$) gave an E-map which revealed all the 22 non-hydrogen atoms. Isotropic full-matrix least-squares refinement gave $R = 0.10$, and approximate positional parameters of all the hydrogen atoms were calculated from stereochemical considerations, except for the four hydroxyl hydrogen atoms. These were localized in a difference Fourier-map and subsequently all the hydrogen positional parameters were refined. The hydrogen atoms were ascribed B-values equal to the isotropic temperature factors of the heavy atoms to which they are bonded.

The refinement converged at $R = 0.045$ ($R_w = 0.032$) for the 1150 observed reflections and the final parameters are listed in Table 1.

A list of the observed and calculated structure factors may be obtained from this Department.

DISCUSSION

Molecular structure. Bond distances, angles and torsional angles are listed in Tables 2 and 3 with e.s.d.'s as calculated from the correlation matrix. A schematic view of the morphine hydrate unit is given in Fig. 1 which shows the correct absolute configuration of (−)-morphine

Table 1. Final atomic parameters (e.s.d.'s in parenthesis). The anisotropic temperature factors are given by $\exp[-2\pi^2(U_{11}h^2a^{*2} + U_{22}k^2b^{*2} + U_{33}l^2c^{*2} + U_{12}hka^*b^* + U_{13}hla^*c^* + U_{23}klb^*c^*)]$.

ATOM	X	Y	Z	U11	U22	U33	U12	U13	U23
C1	.3869(7)	.2821(3)	.0811(3)	.0556(33)	.0427(26)	.0326(24)	-.0013(27)	.0132(27)	-.0085(22)
C2	.3663(6)	.2934(3)	.1266(3)	.0411(29)	.0355(25)	.0561(30)	.0039(25)	.0107(28)	-.0144(24)
C3	.3472(6)	.1777(3)	.2134(3)	.0336(24)	.0288(22)	.0475(26)	-.0045(24)	-.0048(24)	-.0027(23)
C4	.1795(5)	.2401(3)	.2492(3)	.0264(23)	.0301(22)	.0417(26)	-.0027(21)	.0023(23)	-.0033(21)
C5	.2434(6)	.3344(3)	.3554(3)	.0297(24)	.0318(24)	.0344(26)	.0056(22)	.0070(22)	.0019(21)
C6	.1747(6)	.3979(3)	.4078(3)	.0337(27)	.0451(25)	.0294(23)	.0020(25)	.0021(23)	-.0054(22)
C7	.2974(6)	.4569(3)	.3484(3)	.0288(26)	.0430(25)	.0408(28)	-.0047(23)	.0043(24)	-.0093(22)
C8	.2366(6)	.4099(3)	.2759(3)	.0335(25)	.0333(22)	.0343(25)	-.0062(22)	-.0007(22)	.0026(21)
C9	.0131(6)	.5068(3)	.1473(3)	.0400(27)	.0298(24)	.0353(26)	-.0002(23)	-.0003(23)	.0048(23)
C10	.1324(7)	.4442(3)	.0840(3)	.0493(34)	.0534(29)	.0332(25)	.0036(28)	.0040(28)	.0083(25)
C11	.1796(6)	.3439(3)	.1196(3)	.0424(29)	.0497(26)	.0302(24)	-.0011(25)	.0020(24)	-.0066(24)
C12	.1139(6)	.3179(3)	.2023(3)	.0268(23)	.0278(21)	.0337(23)	-.0019(22)	-.0042(20)	-.0046(21)
C13	-.0472(5)	.3773(3)	.2623(3)	.0243(22)	.0276(20)	.0361(26)	.0033(20)	-.0004(21)	-.0043(20)
C14	.0456(5)	.4843(3)	.2468(3)	.0293(23)	.0331(24)	.0332(25)	-.0045(20)	-.0000(22)	-.0079(21)
C15	-.2079(6)	.3648(3)	.2395(3)	.0357(27)	.0338(24)	.0516(30)	-.0042(22)	.0029(25)	-.0016(23)
C16	-.2404(7)	.3998(4)	.1449(4)	.0385(29)	.0462(29)	.0543(33)	-.0064(26)	-.0175(27)	.0074(24)
C17	-.2332(8)	.5345(4)	.0485(4)	.0609(39)	.0521(31)	.0522(33)	.0110(32)	-.0137(31)	.0031(26)
O1	.3838(4)	.1943(2)	.2647(2)	.0355(17)	.0347(16)	.0718(22)	.0110(17)	.0044(19)	.0113(16)
O2	.2792(4)	.3451(2)	.4783(2)	.0467(23)	.0695(23)	.0324(19)	-.0006(18)	-.0036(18)	.0102(16)
O3	.1231(4)	.2385(2)	.3374(2)	.0451(19)	.0349(16)	.0323(16)	.0033(16)	.0062(15)	.0055(13)
O4	.5544(5)	.2269(3)	.4047(2)	.0478(25)	.0809(32)	.0606(28)	.0054(21)	-.0074(21)	.0051(21)
N	-.1620(5)	.4995(2)	.1305(2)	.0398(21)	.0365(19)	.0495(21)	.0010(21)	-.0108(19)	.0007(19)

ATOM	X	Y	Z	B	ATOM	X	Y	Z	B
HC1	.358(5)	.299(2)	.021(2)	4.5	HC2	.474(5)	.157(2)	.108(2)	3.5
HC5	-.078(5)	.321(2)	.386(2)	2.5	HC6	.080(5)	.444(2)	.440(2)	3.0
HC7	.426(5)	.465(2)	.368(2)	3.0	HC8	.319(5)	.548(2)	.243(2)	3.0
HC9	.042(5)	.583(2)	.137(2)	3.0	H1C10	.084(5)	.442(2)	.027(2)	4.0
H2C10	.259(6)	.478(3)	.074(2)	4.0	HC14	-.027(5)	.529(2)	.279(2)	3.0
H1C15	-.276(5)	.405(2)	.284(2)	3.0	H2C15	-.249(4)	.289(2)	.247(2)	3.0
H1C16	-.386(5)	.402(2)	.136(2)	3.0	H2C16	-.204(5)	.347(2)	.099(2)	3.0
H1C17	-.190(5)	.493(2)	-.010(2)	4.5	H2C17	-.368(6)	.548(3)	.044(2)	4.5
H3C17	-.192(6)	.601(3)	.034(2)	4.5	HO1	.292(5)	.063(2)	.381(2)	4.0
HO2	.224(5)	.328(3)	.508(2)	4.0	H104	.529(7)	.195(3)	.366(3)	5.0
H204	.481(6)	.264(3)	.428(2)	5.0					

Table 2. Bond lengths (Å) and angles (°) for morphine hydrate.

DISTANCE	(A)	DISTANCE	(A)	DISTANCE	(A)
C1 - C2	1,385(6)	C1 - C11	1,396(6)	C2 - C3	1,402(5)
C3 - O1	1,359(5)	C3 - C4	1,387(5)	C4 - C12	1,367(5)
C4 - O3	1,381(4)	C5 - O3	1,478(4)	C5 - C6	1,526(6)
C5 - C13	1,553(5)	C6 - O2	1,413(5)	C6 - C7	1,589(6)
C7 - C8	1,313(6)	C8 - C14	1,500(6)	C9 - C10	1,556(6)
C9 - C14	1,535(5)	C10 - C11	1,519(6)	C11 - C12	1,373(5)
C12 - C13	1,509(5)	C13 - C14	1,541(5)	C13 - C15	1,535(6)
C15 - C16	1,526(7)	N - C9	1,476(5)	N - C16	1,475(5)
N - C17	1,476(6)				

ANGLE	( )	ANGLE	( )
C1 - C2 - C3	122,2(4)	C2 - C3 - C4	115,5(4)
C2 - C3 - O1	120,9(4)	O1 - C3 - C4	123,3(4)
C3 - C4 - O3	124,3(4)	C3 - C4 - C12	122,2(4)
C12 - C4 - O3	113,8(3)	C11 - C12 - C4	122,4(4)
C13 - C12 - C4	109,5(3)	C11 - C12 - C13	127,1(4)
C10 - C11 - C12	117,9(4)	C1 - C11 - C12	116,9(4)
C1 - C11 - C10	124,4(4)	C11 - C1 - C2	120,6(4)
C4 - O3 - C5	106,3(3)	O3 - C5 - C6	110,4(3)
C13 - C5 - O3	106,8(3)	C13 - C5 - C6	113,3(3)
C5 - C13 - C12	100,3(3)	C5 - C13 - C14	115,7(3)
C5 - C13 - C15	112,8(4)	C12 - C13 - C14	106,8(3)
C12 - C13 - C15	112,5(4)	C14 - C13 - C15	109,1(3)
C5 - C6 - C7	113,2(3)	C5 - C6 - O2	113,3(3)
O2 - C6 - C7	109,3(4)	C6 - C7 - C8	121,2(4)
C7 - C8 - C14	119,9(4)	C8 - C14 - C13	109,6(4)
C8 - C14 - C9	113,5(4)	C13 - C14 - C9	107,3(3)
C9 - C10 - C11	115,8(4)	C10 - C9 - C14	112,6(4)
N - C9 - C10	114,9(4)	N - C9 - C14	107,7(3)
C13 - C15 - C16	111,5(4)	C15 - C16 - N	111,3(4)
C16 - N - C9	111,9(3)	C17 - N - C9	112,6(4)
C17 - N - C16	110,3(4)		

Table 3. Torsional angles (°).

DIHEDRAL ANGLE	( )	DIHEDRAL ANGLE	( )
O1 - C3 - C2 - C1	-171,9(4)	O1 - C3 - C4 - O3	3,9(6)
O1 - C3 - C4 - C12	175,2(4)	C3 - C4 - C12 - C13	-174,1(3)
C1 - C11 - C12 - C13	172,5(4)	C4 - C12 - C13 - C15	-105,9(4)
C11 - C12 - C13 - C15	85,8(5)	C4 - C12 - C13 - C5	14,2(4)
C11 - C12 - C13 - C5	-154,1(4)	C4 - C12 - C13 - C14	135,0(3)
C11 - C12 - C13 - C14	-33,4(5)	C12 - C13 - C15 - C16	-63,1(5)
C12 - C13 - C14 - C9	60,5(4)	C12 - C13 - C14 - C8	63,1(4)
C12 - C13 - C5 - O3	-21,8(4)	C12 - C13 - C5 - C6	100,2(4)
C2 - C1 - C11 - C10	167,7(4)	C1 - C11 - C10 - C9	-169,4(4)
C11 - C10 - C9 - N	-93,2(5)	C11 - C10 - C9 - C14	30,6(6)
C10 - C9 - N - C17	-62,6(5)	C10 - C9 - N - C16	62,4(5)
C10 - C9 - C14 - C8	58,7(5)	C10 - C9 - C14 - C13	-62,5(4)
C13 - C14 - C9 - N	65,2(4)	C13 - C14 - C8 - C7	-39,1(5)
C4 - O3 - C5 - C13	21,1(4)	C4 - O3 - C5 - C6	-102,8(4)
C14 - C9 - N - C16	-64,0(4)	C14 - C9 - N - C16	64,0(4)
C14 - C9 - N - C17	171,0(3)	C9 - N - C16 - C15	56,6(5)
C13 - C15 - C16 - N	-51,5(5)	C15 - C16 - N - C17	-176,9(4)
C8 - C7 - C6 - O2	168,9(4)	C8 - C7 - C6 - C5	41,5(6)
C7 - C6 - C5 - O3	89,6(4)	C14 - C13 - C5 - O3	-134,5(3)
C11 - C10 - C4 - C5	-176,9(6)	C12 - C11 - C10 - C9	,2(6)
C12 - C11 - C10 - C9	,2(6)		

along with the conventional atomic numbering. The average C–N bond length of 1.475 Å is normal for unprotonated amino groups. The three C–N bond distances are equal in the present case, contrary to the somewhat different $C-N^+$ distances reported for the crystal structures of morphine.HCl.$3H_2O$[7] and naloxone HCl.$2H_2O$.[13,14] There are, however, no obvious chemical reasons for any variation in these C–N bonds.

The C6–O2 distance of 1.415 Å is normal for such a C–O bond as compared to the value 1.457(8) Å found in morphine.HCl.$3H_2O$.[7] Other bond lengths and interbond angles have expected values including the lengthening of the C13–C single bonds and the large variation of the C–C13–C tetrahedral angles (105–116°). This clearly demonstrates the non-bonded interactions around C13, in agreement with earlier reports on acyclic compounds containing a quaternary carbon atom.[15] The C3–O1 bond length (1.361 Å) is slightly shorter than the value most frequently found in phenols. This may be due to the strong O–H···N hydrogen bond (see below).

Strain in the molecular skeleton of morphine is evident from the distortion of the aromatic ring A (Fig. 1). Deviations from some least-squares planes are listed in Table 4. This shows that C12 is displaced by 0.03 Å from plane I.

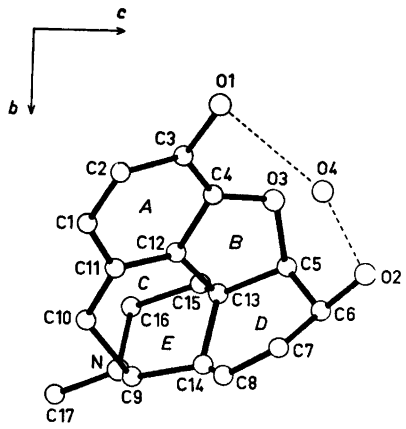


Fig. 1. The morphine hydrate unit.

Rings C and D both contain an unsaturated bond but differ markedly in conformations. The normal halfchair conformer is observed for ring C whereas D has adopted the boat form due to the oxygen-bridge in ring B. The overall conformation may be described by the interplanar angle between the least-squares planes through the atoms of rings A, B, C and D, E, respectively, (planes II and III in Table 4). The angle is 86.6°, as compared to 90.9 and 82.6° in the hydrochlorides of morphine [7] and naloxone,[13] respectively, giving the usual T-shaped molecule.

Table 4. Deviations (Å) of individual atoms from some least-squares planes.

Plane I, Benzene ring[a]			
C1	0.004	C11	0.022
C2	−0.021	C12	−0.031
C3	0.012	C10*	0.316
C4	0.014	C13*	0.130
O1*	0.151		
Plane II, Rings A, B, C			
C1	0.019	O3	−0.097
C2	0.079	C10	0.023
C3	0.008	C11	−0.161
C4	−0.186	C12	−0.315
C5	0.129	C13	−0.366
C9	0.020	C14	0.569
O1	0.233		
Plane III, Rings D, E			
C5	−0.130	C13	−0.342
C6	0.354	C14	0.255
C7	−0.111	C15	0.131
C8	−0.110	C16	−0.189
C9	−0.261	C17	0.043
O2	0.014	N	0.296

[a] Atoms with an asterisk do not define the plane.

It is of interest to compare some conformational features of the bases of morphine and methadone. With a near "cyclic" conformation as observed in methadone [8] the dimethylamino group is (−)-*syn-clinal* relative to the quaternary carbon atom C4, denoted C13 in this paper. A similar conformation is observed in morphine, properly described by the torsional angles C11−C12−C13−C15 (85.8°), C12−C13−C15−C16 (−63.1°), C13−C15−C16−N (−51.5°) and C14−C13−C15−C16 (54.3°). These are close to the corresponding values found in methadone (94.2, −61.5, −68.5, and 64.2°)[8] and the molecular geometry resembles the particular conformation proposed by Beckett *et al.*[6] based on the assumption that methadone interacts with the same receptor as morphine.

Molecular packing. The crystal structure of morphine hydrate is shown in Fig. 2. The alkaloid molecules are linked in chains along the *y*-direction through a strong O1−H···N hydrogen bond of 2.635 Å. This is close to the shortest value listed by Pimentel *et al.*,[16] although somewhat larger than those observed in *N*-(5-chlorosalicylidene)aniline [17] (2.584 Å) and in the alkaloid gerradine [18] (2.59 Å). These are, however, *intramolecular* bridges in donor-acceptor systems with rigid molecular structures. The strong character of the present O−H···N hydrogen bond agrees with the HO1···N distance of 1.57 Å and the O1−HO1 distance of 1.07 Å, which is long for such bonds.

An interesting feature of the present hydrate is the water molecule bridging the hydroxyl and phenolic oxygen atoms, O4 being the donor-atom in both hydrogen bonds. The distance O4···O2 of 2.798 Å is normal for an O···O interaction [16] whereas O4···O1 (3.004 Å) is a rather weak interaction. However, the localization of the H1O4 hydrogen atom in a difference map and the distances and angles given in Table 5 support the assumption of O4···O1 as a hydrogen bond. The interbond angle H1O4−O4−H2O4 of 122° is large compared with the values given by Ferraris *et al.*[20] for hydrates. (H−O−H bond angles are in the range 103−115°.) The water oxygen is

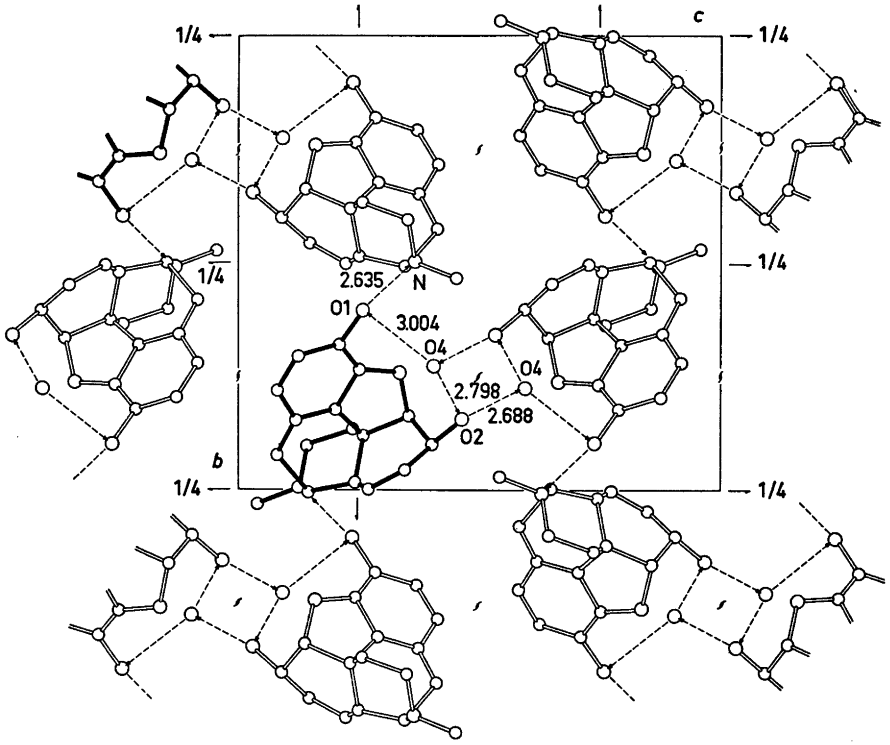


Fig. 2. The crystal structure of morphine hydrate as seen along the *a*-axis.

Table 5. Distances (Å) and angles (°) of the A—H···B hydrogen bonds. The letters (i) and (ii) give the symmetry code of the acceptor, whereas R and R′ denote the atoms which the donor and acceptor are bonded to, respectively.[a]

Atoms	Distances A···B	A—H	H···B	Angles ∠A—H···B
O1—HO1···N(i)	2.635	1.07	1.57	172
O2—HO2···O4(ii)	2.688	0.79	1.91	170
O4—H1O4···O1	3.004	0.74	2.28	166
O4—H2O4···O2	2.798	0.84	1.97	170
σ(average)	0.005	0.04	0.05	4

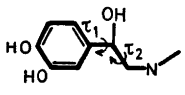
Atoms	Angles ∠R—A—H	∠H···B—R′
C3—O1—HO1···N—C17	115	106
C3—O1—HO1···N—C16		104
C3—O1—HO1···N—C9		111
C6—O2—HO2···O4—H1O4	114	120
C6—O2—HO2···O4—H2O4		110
H2O4—O4—H1O4···O1—C3	122	99
H2O4—O4—H2O4···O2—C6		120

[a] Reference molecule: x,y,z; (i) $-x,y-\frac{1}{2},\frac{1}{2}-z$; (ii) $x-\frac{1}{2},\frac{1}{2}-y,1-z$.

acceptor in a O – H···O hydrogen bond of 2.688 Å from a hydroxyl oxygen (O2) related by a 2-fold screw axis. The water oxygen atom is connected to two different morphine molecules in a hydrogen bond system between O2 and O4 to form a helix running along the *a*-axis (see Fig. 2).

CONCLUDING REMARKS

During the last twenty years extensive studies have been performed on structure activity relationships between narcotic analgetics. However, one point seems to have received little attention; there is a phenethylamine framework in the skeleton of morphine. This is exemplified below with adrenaline (*3*) and the heavy lines in morphine (*4*). It is still not known whether the phenethylamines have a maximally extended chain or not when interacting with their receptors. In a fully extended chain, the dihedral angles τ_1 and τ_2 (see *3*) are 90 and 180°, respectively, which is usually observed in crystal structures.[19,21] Apomorphine (*5*) is known to interact with dopamine receptors but lacks any analgesic activity. Giesecke [22] recently reported $\tau_1 =$



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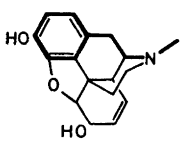

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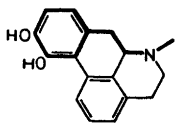

5

$-40°$ and $\tau_2 = 180°$ for apomorphine which means an extended chain, nearly coplanar with the aromatic nucleus. The present corresponding dihedral angles are 0 and $-93°$, respectively, and the two different τ_2 values of morphine and apomorphine, both being quite rigid molecules, may explain the inactivity of the latter as an analgetic.

Acknowledgement is made to Prof. A. Nordal for supplying the crystals.

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A Base-promoted 1,4-Elimination Reaction. Influence of Solvent and Base on Rate and Deuterium Isotope Effect

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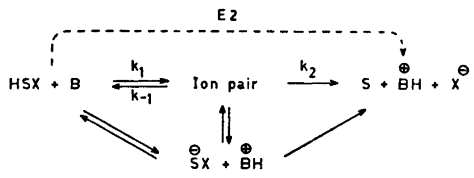
The 1,4-elimination reactions of 3-(2-acetoxy-2-propyl)indene (*B*) promoted by sodium methoxide and tertiary amines have been studied in various protic solvents. We found unusually small deuterium isotope effects ($k_H/k_D = 0.9-2$) with tertiary amines as the promoting bases in solvents ranging in polarity from *t*-BuOH to a mixture of methanol/water. Even in a mixture of 82.5 % DMSO in methanol the deuterium isotope effect did not change significantly. These observations suggest that the 1,4-elimination with these amines takes place by a mechanism in which ion pairs are reversibly formed.

The isotope effect on the 1,4-elimination reaction with NaOMe in methanol was determined to be 7.6, a value consistent with an irreversible *E1cB*-mechanism. The isotope effect for a closely related 1,2-elimination, *i.e.* the reaction of 1-(2-acetoxy-2-propyl)indene (*A*) with NaOMe in methanol, was 6.5.

Stepwise base-promoted elimination reactions (*E1cB*) can be classified mechanistically on the basis of: (a) the extent of the dissociation of the proton from the substrate, and (b) the degree to which the dissociation is reversible under the reaction conditions. According to this view there exists a spectrum of mechanisms ranging from the one extreme with a non-steady-state concentration of the intermediate anion to the other extreme with SX^- reacting irreversibly to form products.[1]



Scheme 1.

Mechanisms in which the anion is reversibly formed in steady-state concentration can be placed between these extremes. As in proton transfer reactions, the intermediate has been suggested in some elimination reactions to be a tightly solvated anion or an ion pair. Strong indications for the existence of the ion-pair mechanism, $(E1cB)_{ip}$, have been obtained recently.[1-5]

Criteria of the $(E1cB)_{ip}$-mechanism have been a low deuterium isotope effect in combination with the absence of significant deuterium exchange and the absence of a negative salt effect. Miller and coworkers [3] used these criteria in their mechanistic interpretation of the elimination of HBr from *cis*-1,2-dibromoethene with triethylamine in dimethylformamide. The isotope effect found was 1.00, and there was neither rate retardation nor hydrogen exchange when triethylammonium or triethylammonium-*d* bromide was present during the reaction.

The above mentioned ion pairs should not be confused with two other types of ion pairs sometimes discussed in connection with elimination reactions: (a) the promoting agent is an ion pair, *e.g.* *t*-BuOK, (b) the substrate and leaving group form a carbonium ion ion-pair. Rappoport and coworkers [6] have proposed ion pairs as intermediates in reactions involving tertiary-amine promoted elimination of HCN from acidic substrates (tri- and tetra-cyano-substituted ethanes) but in these reactions the intermediates are rather stable and are formed in non-steady-state concentrations.

The above discussion applies to 1,4- as well as 1,2-elimination reactions. Very few examples of 1,4-elimination reactions are described in the literature. No conclusive evidence for such

a one-step reaction has been published.[5,7–11] Cristol and coworkers [7,8] have studied 1,4-elimination from *cis*- and *trans*-9,10-di-X-dihydroanthracene derivatives (X = halogen, OH, OAc, and OCOPh) with sodium hydroxide in ethanolic dioxane or pure ethanol. *syn*-1,4-Elimination was found to be preferred. The reactions are presumably stepwise, at least the reactions with the diols. Naphthalene tetrachlorides with sodium methoxide in methanol/acetone gave 1,2- and 1,4-elimination of HCl.[9] 1,4-Elimination was also found in reactions of 1-chloro-2-alkylperfluorocyclobutene and -pentene in KOH/EtOH or MeOH.[10] These two substrates have free rotation around the $C_\gamma - C_\delta$ bond and mixtures of *cis*- and *trans*-olefin were formed. No incorporation of deuterium in the recovered butene-substrate was observed when the reaction was performed in MeOD.

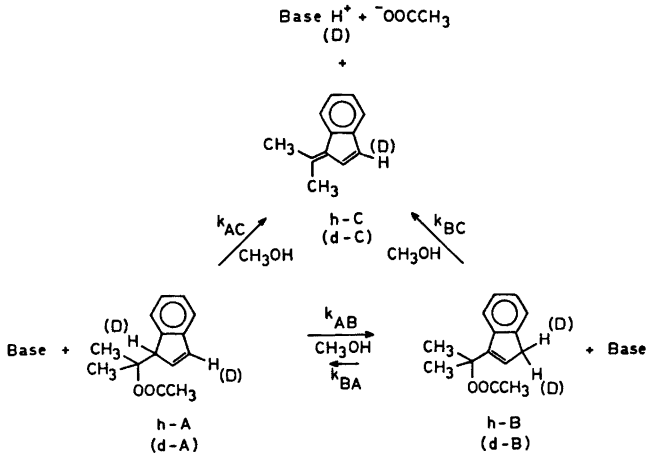
We have recently reported methoxide-promoted 1,4-elimination reactions in methanol from 3-(1-acetoxyethyl)indene and compared the product composition (of *cis*- and *trans*-olefin) and the deuterium isotope effect with the results obtained with non-stereospecific 1,2-elimination from *erythro*- and *threo*-1-(1-acetoxyethyl)indene.[11]

Our laboratories have also reported base-catalysed 1,3-proton transfer competing with 1,2- and 1,4-elimination reactions (Scheme 2) and found not only unusually small but also unusually large isotope effects.[5] The 1,3-proton transfer reaction, which has been indicated to proceed *via* at least one ion-pair intermediate, was used as an ion-pair probe. The results with *N*-ethylpiperidine (EP) as the catalytic and elimination promoting base suggested that all three reactions had at least one ion pair in common.

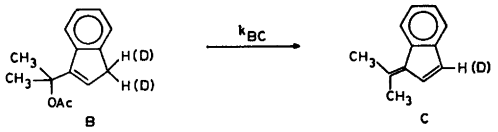
The goal of the present work has been to get further indications for (or against) the suggestion that *B* and tertiary amines react by an ion pair mechanism. Mechanistically interesting questions are, *e.g.*, how the base structure and solvent affect the reversibility of the ion-pair formation and the rate of the dissociation step. Thus the 1,4-elimination of HOAc from *B* was studied in various protic solvents and with several bases.

RESULTS AND DISCUSSION

The 1,4-elimination reactions of 3-(2-acetoxy-2-propyl)indene (*B*) with the tertiary amines triethylamine (TEA) and *N*-ethyl-*N*,*N*-diisopropylamine (EDIPA) have been studied in different protic solvents in the presence of the corresponding ammonium acetate. *B* has also been reacted with sodium methoxide in methanol. The only product of these reactions was 1-isopropylideneindene (*C*) (Scheme 3). The kinetics of the reactions were studied using a sampling – quench – extraction – ^{1}H NMR procedure and/or UV-spectrophotometry. The reactions are shown to be "true" 1,4-elimination reactions, *i.e.* the reactions are not mixtures



Scheme 2.



Scheme 3.

of 1,3-proton transfer and 1,2-elimination. This publication includes a discussion of results obtained recently with *B* in methanol with the bases TEA, *N*-ethylpiperidine (EP) and 1,4-diazabicyclo[2.2.2]-octane (DABCO).[5]

I. 1,4-Elimination reactions with tertiary amines. When a methanol solution of *B* was reacted with tertiary amines of varying base strength and causing different degrees of steric hindrance, the product *C* was obtained (Table 1, run 1 – 4). The weakest base, DABCO, gave the lowest elimination rate and TEA the highest. Large steric hindrance reduced the rate and increased the small isotope effect (Tables 1 and 2, run 4 compared with runs 2 and 3). All deuterium isotope effects are small and consistent with mechanisms involving reversible formation of intermediates (Table 2). However, an alternative explanation of the small isotope effects is the *E2*-mechanism that has a transition state which is highly asymmetric and/or non-linear with respect to proton transfer.[12] The fact that DABCO, the weakest base, was the worst promoting agent is consistent with the ion-pair mechanism, because protonated DABCO is a stronger acid than the other protonated amines and therefore is expected to yield faster ion-pair collapse to starting material (k_{-1} in Scheme 1).[13]

Buffers were added to the reaction solutions to lower the methoxide ion concentration. As the rate data (Table 1) show, the methoxide ion is not the active base when enough buffer is added. Another reason for adding trialkylammonium acetates was to allow for detection of dissociated states.

Lowering the polarity of the solvent is predicted to decrease the elimination rate because the rate determining transition state is expected to be more polar than the initial state. It is also reasonable to predict a larger ratio of ion-pair collapse/ion-pair elimination (*i.e.* k_{-1}/k_2 in Scheme 1) causing the deuterium isotope effect to decrease. Experimentally this behaviour was also found when *B* was reacted with TEA in *t*-BuOH (Tables 1 and 2, run 7) instead of methanol. The rate was decreased about six times and the isotope effect from 1.3 to 0.9. When the polarity of the solvent was increased by addition of water to the methanol, the rate and the isotope effect were increased from 1.3 to 2.0.

An often used method for increasing the basicity of a protic base solution is to add DMSO.[14] When the reaction with TEA was carried out in a mixture of 82.5 % DMSO in methanol, the rate was increased by a factor of 24 but the isotope effect was only slightly enhanced. This experiment illustrates the difficulty in predicting isotope effects when a mechanism with more than one transition state is involved.

The isotope effect on the dissociation step (k_2 in Scheme 1) is expected to be very small since the major bond-breaking and bond-forming process involving the proton ought to be passed once the ion pair is formed. Corrections for the secondary isotope effects expected (see part II) would result in somewhat smaller primary isotope effects. This means that not only the primary isotope effect on the rate of the reaction with TEA in *t*-BuOH, but also the effect with DABCO in methanol ought to be significantly < 1. Thus the ionization transition states for the reaction with these amines ought to be ion-pair-like because inverse isotope effects are commonly interpreted with very product-like transition states involving stronger bonding of the hydrogen in products than in reactants.[15]

The above mentioned ion pairs are expected to be contact ion pairs. An interesting question concerning Scheme 1 is whether the ion pairs eliminate by a one- or multistep process. The incorporation of protium in *d-C* (at the 3 position) when using TEA and EP in methanol was 7.4 ± 3 % and 2.0 ± 3 %, respectively. This could be due to exchange with solvent-separated and/or free ions. However, even if the existence of ions could be shown, this does not necessarily prove that the elimination from the ion pair(s) is stepwise.[16] No incorporation, *i.e.* 0 ± 2 %, of protium in *d-B* could be detected for any of the reactions after 40 – 60 % reaction to *d-C* as determined by ^{1}H NMR spectroscopy.

Table 1. Reactions with *B* and tertiary amines; kinetic data. Initial substrate conc. in the UV-cell was 8×10^{-5} M, otherwise 0.03 M.

Run No.	Initial concentration Base/M	Buffer/M	k_{BC}[a] 10^{-5} M^{-1} s^{-1}	Rel. rate[e]
DABCO in MeOH at 20.00(3) °C; ^{1}H NMR				
1-h[b,c]	0.500	0.030	$0.78(^{+10}_{-14})$[d]	1.0
1-d[c]	0.500	0.030	$0.87(^{+4}_{-12})$[d]	
EP in MeOH at 30.00(3) °C; ^{1}H NMR				
2-h[c]	1.000	0.030	8.88(22)	5.7
2-d[c]	1.000	0.030	6.52(16)	
TEA in MeOH at 29.99(3) °C; ^{1}H NMR				
3-h[c]	1.00	0.030	13.5(3)	8.7
3-d[c]	1.00	0.030	10.7(3)	
EDIPA in MeOH at 29.91(7) °C; UV				
4-h	1.035	0	8.11(41)	
4-h	1.036	0.003	3.67(18)	
4-h	1.019	0.015	2.88(14)	
4-h	1.038	0.030	2.88(14)	1.8
4-d	1.038	0.030	1.65(8)	
EDIPA in MeOH at 30.00(7) °C; ^{1}H NMR				
4-h[f]	1.036	0.030	3.06(31)	
4-h[f]	0.8288	0.030	3.6(4)	
4-d[f]	0.8288	0.030	2.18(22)	
TEA in MeOH/DMSO (82.5 wt % DMSO) at 29.91(7) °C; UV				
5-h	0.1018	0	434(22)	
5-h	0.08684	0.013	466(23)	300
5-d	0.08684	0.013	320(16)	
5-d	0.1241	0.018	321(16)	
TEA in MeOH/DMSO (80.5 wt % DMSO) at 19.99(7) °C; ^{1}H NMR				
5-h[f]	0.0674	0.028	304(30)	390
5-d[f]	0.0674	0.028	217(22)	
TEA in MeOH/H_2O (33.5 wt % H_2O) at 29.91(7) °C; UV				
6-h	0.9792	0	84.9(40)	
6-h	0.9693	0.015	62.5(31)	
6-h	0.9856	0.029	62.9(32)	41
6-h	0.9856	0.029	63.7(32)	
6-d	0.9856	0.029	31.8(16)	
6-d	0.9856	0.029	32.0(16)	
TEA in MeOH/H_2O (33.5 wt % H_2O) at 29.91(7) °C; ^{1}H NMR				
6-h[f]	0.9395	0.058	65.5(66)	
6-d[f]	0.9395	0.058	34.9(35)	
TEA in *t*-BuOH at 30.00(7) °C; ^{1}H NMR				
7-h	1.025	0.015	2.0(2)	
7-h	1.010	0.030	2.17(13)	1.4
7-d	1.010	0.030	2.40(13)	

[a] Estimated errors are considered as maximum errors. [b] Bengtsson, S. unpublished results. [c] Ref. 5. [d] When calculating the rate constants with DABCO, the rates have been divided by the statistical factor 2. [e] When calculating relative rates, the factor 2 has been used to convert rate constants from 20 to 30 °C. [f] One-point-kinetics.

Table 2. Reactions with *B* and tertiary amines; deuterium isotope effects (calculated from the data in Table 1) and protium incorporation.

No.	Analytical method	Solvent	Base	Initial base conc. /M	Initial buffer conc. /M	Temp./°C	k_{BC}^{H}/k_{BC}^{D}	Incorporation of protium
1	^{1}H NMR	MeOH	DABCO	0.5	0.030	20	0.9(2)	
2	^{1}H NMR	MeOH	EP	1.0	0.030	30	1.36(7)	2 ±3 % in *d-C*
3	^{1}H NMR	MeOH	TEA	1.0	0.030	30	1.26(7)	7.4±3 % in *d-C*
4	UV	MeOH	EDIPA	1.0	0.030	30	1.75(10)	
4	^{1}H NMR	MeOH	EDIPA	0.8	0.030	30	1.7(2)	0 ±2 % in *d-B*
5	UV	MeOH/DMSO	TEA	0.1	0.013	30	1.45(10)	
5	^{1}H NMR	MeOH/DMSO	TEA	0.1	0.028	20	1.4(2)	0 ±2 % in *d-B*
6	UV	MeOH/H_2O	TEA	1.0	0.029	30	1.98(10)	
6	^{1}H NMR	MeOH/H_2O	TEA	0.9	0.058	30	1.9(2)	0 ±2 % in *d-B*
7	^{1}H NMR	*t*-BuOH	TEA	1.0	0.030	30	0.9(1)	0 ±2 % in *d-B*

Table 3. Reactions with *A* and *B* in NaOMe/MeOH; kinetic data and deuterium isotope effects.

Substrate	Initial base conc./M	k_{BC} (or k_{AC})/ 10^{-3} M^{-1} s^{-1}	k_H/k_D
0.02654 M;[a] 19.97(7) °C; ^{1}H NMR			
h-B	0.02642	13.6(8)	7.9(10)
0.02699 M;[a] 19.97(7) °C; ^{1}H NMR			
d-B	0.02642	1.73(10)	
0.03327 M;[a] 30.00(7) °C; ^{1}H NMR			
h-B	0.02905	39.6(24)	7.6(10)
0.02615 M;[a] 30.00(7) °C; ^{1}H NMR			
d-B	0.02905	5.20(30)	
0.8×10^{-4} M;[a] 29.91(7) °C; UV			
h-B[b]	0.01053	41.3(20)	7.6(4)
h-B	0.01053	39.8(20)	
d-B	0.01052	5.45(27)	
d-B	0.01321	5.26(27)	
h-A	0.01053	15.9(8)	6.5(3)
h-A	0.01322	16.0(8)	
h-A	0.01322	16.1(8)	
d-A	0.01322	2.46(12)	
d-A	0.01322	2.48(12)	
d-A	0.01053	2.43(12)	

[a] Initial substrate concentration. [b] Initial substrate concentration 1.8×10^{-4} M.

II. Reactions with sodium methoxide. When sodium methoxide is used instead of tertiary amines in methanol, the rate is increased drastically. The large isotope effect (7.6) shows that the transfer of the proton from the substrate to the base is involved in the rate determining step. The 1,2-elimination from 1-(2-acetoxy-2-propyl)indene (*A*) has also been studied. The elimination rate was about 20 % smaller with this substrate (per proton) and the isotope effect was 6.5 (Table 3). No competing 1,3-proton transfer was involved in the reactions of either *A* or *B*, because no trace of *A* or *B* was observed when a deficiency of base was used. Thus *A* gives exclusively 1,2-elimination and *B* exclusively 1,4-elimination.

As *d-B* is a 1,1-di-*d*-substituted compound, a secondary isotope effect >1 is expected.[17] Correction for this secondary isotope effect should result in a somewhat smaller primary isotope effect for *B* than observed. The magnitude of secondary isotope effects of this type has been determined for hydroxide catalysed ionization of nitroalkanes. At 25 °C the value was 1.18 for $CH_3CD_2NO_2$ and 1.14 for the corresponding reaction with $PhCD_2NO_2$.[18] If the isotope effect obtained with *B* (7.6) is corrected by a factor of this magnitude, a primary isotope effect of about 6.5 is obtained 7.6/1.16 = 6.5). Thus the primary isotope effects for the 1,2- and 1,4-elimination are similar.

The value of the equilibrium constant, $[B]_{eq}/[A]_{eq}$ is 19 in methanol at 30 °C.[5a] Thus the acidity of *A* and *B* is of about the same magnitude. The pK_a of NaOMe in methanol (= 18.1)[19a] should not differ much from the pK_a's of the substrates *A* and *B*. Large isotope effects are therefore expected if the reactions are stepwise.[15,19] On the other hand, *E2*-reactions

on the borderline *E1cB–E2* probably have rather small isotope effects.[20,21] Furthermore, some incorporation of protium in *d-C* (5.4 ± 4.0 %) has been observed when a 0.05 M solution of *d-B* was reacted with NaOMe.

The small difference in rate between the 1,2- and 1,4-elimination (per proton) and the large and similar primary isotope effects in connection with the small incorporation of protium in *d-C* suggest that *A* and *B* react by irreversible *E1cB*-mechanisms. The hypothesis that ions are involved in the reactions is also supported by the fact that the *A* and *B* resembling substrates, *erythro-* and *threo*-1-(1-acetoxyethyl)-indenes and 3-(1-acetoxyethyl)indene, react non-stereospecifically with NaOMe in methanol.[11]

Study of the stereochemistry of the reactions discussed under I and II is not possible with the substrates *A* and *B*. Work is in progress in our laboratories on the stereochemistry and other mechanistic aspects of *E1cB*-reactions.

EXPERIMENTAL

Syntheses

3-(2-Acetoxy-2-propyl)indene (*h-B*) and the corresponding 1,1-di-*d* compound (*d-B*) were prepared according to methods published previously.[5a] ^{1}H NMR spectra of *h-* and *d-B* showed no impurities. The deuterium content of *d-B* was 98.2 ± 1.0 atom-% in the 1 position as determined by ^{1}H NMR spectroscopy.

Kinetics

General. All kinetic runs were made at constant temperature in a thermostat (Heto 01 PT 623) or in a jacketed cell of quartz (Hellma 160 B) coupled to the thermostat with an insulated tubing. The temperature of the bath was measured with a calibrated mercury thermometer with an absolute accuracy of ± 0.05 °C. The deviation of the temperature during all runs was < 0.02 °C. Thus the absolute temperature of the bath was t ± 0.07 °C. When the deuterium isotope effects were measured, the temperature deviation was smaller, < 0.02 °C, because the settings of the thermostat were not changed between the runs.

The NMR spectra were recorded with a Varian A 60 D ^{1}H NMR spectrometer. The time was measured with a chronometer or, in the UV-kinetics, with the time scale of the recorder. All glassware, except the UV-cell, was cleaned with chromic acid and rinsed with water, dilute ammonium hydroxide, and distilled water before drying at 150 °C at least over night.

Solvents and bases. Methanol (Fluka spectrograde quality) stored over 3 Å molecular sieves was used without further purification. Dimethyl sulfoxide (Merck spectrograde quality) was distilled at reduced pressure and the centercut stored in a dry nitrogen atmosphere over 3 Å molecular sieves. *t*-BuOH (Merck *p.a.*) was purified by blowing dry oxygen for 24 h through the liquid in which pure-cut pieces of sodium had been dissolved. After distillation using a Widmer column, the center cut was dried and distilled from calcium hydride and then stored under dry nitrogen over molecular sieves. Distilled water was boiled before use. Triethylamine (BDH, > 99 %) was first dried over KOH and then purified by two methods. Distillation through a "spinning-band" column (Nester-Faust) and distillation from *p*-bromobenzoyl chloride in a dry nitrogen atmosphere using a Vigreux column produced amine of equal purity (< 0.009 % diethylamine in the distillate). The former method was also used to purify *N*-ethyl-*N*,*N*-diisopropylamine (Fluka, > 98 %). After distillation the amine contained < 0.003 % diisopropylamine. The analysis were made by GLC on a 2.5 m × 3 mm steel column packed with 20 % Ucon LB-550-X, 20 % KOH on Chromosorb P 80/100, 180 kPa N_2 at 80 °C. The amines were stored over 3 Å molecular sieves under dry nitrogen. A stock solution of NaOMe was prepared by adding methanol-washed pure-cut pieces of sodium to dry methanol. The concentration was determined by titration of aliquots of this stock solution with 0.1 M HCl.

The substrates, bases and solvents (except the methanol) were stored in the freezer.

Quench-extraction-^{1}H NMR procedures. (a) TEA as base in t-*BuOH.* The substrate was weighed into the reaction flask and dissolved in thermostated acetic acid-*t*-BuOH solution (10 ml), and a thermostated solution of TEA in *t*-BuOH (20 ml) was added with a pipette. When sampling, the reaction solution (10 ml) was withdrawn with a pipette and rapidly transferred to a 60 ml stop-cocked centrifuge tube containing CCl_4 (1 ml), 1 M HCl (30 ml) and ice (10 g). The mixture was shaken for 1 min, centrifuged and the organic layer transferred to an NMR tube. The methyl region was integrated and the mol-% of each of the two components of the mixture was evaluated. The analytical procedure has previously been calibrated.[5a]

(b) NaOMe as base in methanol. Thermostated methanol (30 ml) was transferred with a pipette to one of the two compartments of the reaction vessel. The other compartment was filled with thermostated base solution (10 ml). After 10 min in the thermostat the solutions were mixed by shaking the flask. The sampling-quench-extraction-^{1}H NMR procedure

was the same as in (a), but only one extraction with CCl_4 (0.6 ml) was necessary.

(c) One-point kinetics. The substrate was weighed into a 10 ml narrow-necked flask. This flask was then filled with thermostated base solution (containing buffer) and the chronometer was started The same base solution was used both in the reactions with *h-B* and *d-B*. The procedure was otherwise the same as in (b).

Kinetics studied with UV-spectrophotometry. The UV-spectra of *B* and *C* in methanol solution and in MeOH/DMSO were recorded using a Pye Unicam 1800 A spectrophotometer. *C* had absorption maximum at 306.5 nm in pure methanol and at 308 nm in MeOH/DMSO. *B* had no absorption at these wavelengths.

The reactions were run in a thermostated jacketed cell. The absorbance at the absorption maximum was followed as a function of time using a recorder. The reactions were started outside the spectrophotometer in a water thermostat. Solutions of substrate and base were thermostated and transferred with pipettes to a flask. Reaction solution was transferred to the cell with a pasteur pipette.

Evaluation of rate constants and estimation of errors. (a) Quench–extraction–^1H NMR-procedure. The elimination reactions consumed base, but at infinite time the base concentration had diminished by only 3 %. Thus the reactions are expected to show near pseudo-first-order behaviour, and the rate constants were obtained from the slopes of plots ln (mol-% *B*) *versus* time.

(b) UV-kinetics. The reactions are expected to show near first order behaviour, because $[\text{base}]_0/[\text{substrate}]_0 > 10^4$ and 10^2, respectively. Thus ln $(A_\infty - A)$ was plotted *versus* time and the rate constants were evaluated from the slopes. The estimated errors are considered as maximal errors including random errors and maximal systematic errors.

Determination of protium incorporation in d-C. To attain a value of the protium incorporation in *d-C* with NaOMe as base, a 0.05 M solution of *d-B* was run $10t_{\frac{1}{2}}$ (otherwise the same conditions as in the kinetic runs). This solution (20 ml) was then quenched and prepared as in the kinetic experiments. The area under the 2 and 3 protons was compared with the area of the methyl protons. The 2 position was assumed to contain no deuterium. As ^1H NMR reference a solution of *h-C*, obtained as above, was used. The protium content in *d-C* obtained from *d-B* was 5.4 ± 4.0 % in the 3 position.

No incorporation of protium in the 1 position in *d-B* could be detected during the kinetic runs with amines and NaOMe after 40–60 % reaction as determined by ^1H NMR (*i.e.* 0 ± 2 %).

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The Influence of Borate and Calcium on the Gel Formation of a Sulfated Polysaccharide from *Ulva lactuca*

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The water-soluble, sulfated polysaccharide isolated from *Ulva lactuca*, forms a soft gel when dialysed against sea-water, provided the polysaccharide concentration is 1 % or higher and the pH above 7.5. The components of sea-water needed for this gel formation are boric acid and calcium ions. At concentration normally found in sea-water, neither borate nor calcium ions alone produce a gel.

It is assumed that the gel formation involves the formation of borate-polysaccharide complexes leading to intermolecular linkages which are stabilized by calcium ions in an unknown manner. This assumption is supported by experiments with extraction of this polysaccharide from the dry seaweed and by the results obtained with the isolated polysaccharide. Agreement between these experiments leads to the conclusion that the sulfated polysaccharide (*in vivo*) most probably occurs as a gel stabilized by the borate and calcium ions in sea-water.

The chemical structure of sulfated polysaccharides of green algae has been extensively investigated.[1] Little is known, however, about the form in which they occur in the plant. These polysaccharides are usually described as water-soluble, as they can be extracted by distilled water, and they are apparently located outside the cell membrane, possibly as the matrix substance of the cell wall. However, their solubility in sea-water does not seem to have been investigated, in spite of the fact that this might give some indications about their physical state in the plant and, thus, their biological function.

This work is an investigation of the solubility properties and gel formation of the sulfated polysaccharide of *Ulva lactuca*. This polysaccharide represents one of the main groups of sulfated polysaccharides of green algae, containing rhamnose, xylose, glucose, and glucuronic acid in a highly branched molecule without any well-defined backbone structure.[1]

EXPERIMENTAL

Ulca lactuca was collected at Drøbak in the Oslofjord in September. The polysaccharide was prepared by dialysing the dried weed contained in a dialysis bag against a solution of EDTA (0.1 M) at pH 7, followed by dialysis against water, centrifugation and freeze-drying of the clear supernatant. The solubility of the polysaccharide was investigated by dissolving it in distilled water and dialysing against salt solutions of the desired composition. The polysaccharide solution (2.5 ml) was dialysed against four batches of 50 ml salt solution, and each period of dialysis lasted for at least 3 h. After dialysis, the contents of the bags were centrifuged, the precipitate washed with salt solution and dissolved in distilled water. The amounts of soluble and insoluble polysaccharide were determined by the phenol-sulfuric acid method using glucose as a standard.

Extraction experiments were carried out by suspending dry algal material in water and dialysing against the salt solution. After thorough dialysis, the content of the dialysis bag was centrifuged and the amount of carbohydrate in the supernatant determined by the phenol-sulfuric acid reaction.

Optical rotation was measured in a Zeiss polarimeter, using a 10 cm cell.

RESULTS

Polysaccharide solutions of different concentrations were dialysed against natural sea-water of a salinity of 31.5. When the concentra-

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Table 1. Gel formation by dialysis against sea-water at different polysaccharide concentrations, (*C*).

C % (W/V)	Soluble % (W/W)	Insoluble % (W/W)
0.5	100	0
1.0	10	90
2.0	3	97
3.0	3	97

tion of polysaccharide was 1 % or more, the solution became a soft gel which could be washed with sea-water without dissolving. At a concentration of 0.5 % the polysaccharide remained completely soluble and no signs of gel formation was observed. (Table 1). In all subsequent experiments, solutions containing 2 % polysaccharide have been used.

In order to investigate whether the gelling of this polysaccharide from *Ulva* was caused by the presence of specific ions, or the high ionic strength of sea-water, dialysis against some simple salt solutions were carried out. As shown in Table 2, none of these salt solutions led to gel formation.

These results indicated that components of sea-water other than the main cations were essential for gel formation, and experiments with artificial sea-water or components thereof were carried out. The composition of the artificial sea-water (a slightly modified Kester medium [8]) is given in Table 3. Some results are given in Table 4 suggesting that the presence of boric acid is essential for gel formation. Other experiments using artificial sea-water buffered at pH 8.0 with Tris were carried out as shown in Table 5. These clearly indicate that boric acid and calcium ions are the only components in sea-water necessary for gelation. Boric acid alone or calcium ions alone do not lead to gel formation.

The formation of borate complexes with carbohydrates (and diols in general) depends upon the presence of borate ions, *i.e.* the pH of the solution. Dialysis experiments were carried out against solutions containing boric acid, a simplified sea-water medium and tris buffer with different pH values. The results are given in Table 6.

Some extraction experiments were carried out by dialysing a suspension of dry weed against the extraction medium. The results are given in Table 7, showing that distilled water and unbuffered salt solutions removed roughly the same amount of material, while at pH 8 the extraction was incomplete without the presence of a calcium complexing agent. In natural sea-water and in a simplified Kester medium containing borate, an amount of carbohydrate corresponding to only 5 % of the dry weed was removed.

In order to determine whether the amount of borate bound to the polymer was sufficient to cause an appreciable change in the optical rotation of the polymer, $[\alpha]_D$ was determined for the polysaccharide, using a 2 % solution in tris buffer without borate and in the presence of 260 mg boric acid/l. The observed values of $[\alpha]_D$ were -61 and -59.5, respectively. Thus, no effect of the presence of borate was observed.

As shown in Table 5, calcium ions are needed in addition to borate to obtain a gel. No gel formation took place when a polysaccharide solution was dialysed against a solution of borate in tris buffer pH 8, followed by tris buffer solution without borate, and finally tris buffer containing calcium ions. Both borate and calcium ions were present in sea-water

Table 2. Dialysis against some simple salt solutions.

Composition of solution	Soluble %	Insoluble %
1 M KCl	100	0
0.3 M $CaCl_2$	100	0
1 M NaCl	100	0
0.3 M $MgCl_2$	100	0
0.055 M $MgCl_2$, 0.012 M $CaCl_2$, 0.55 M NaCl	100	0
Sea-water	5	95

Table 3. Composition of solutions and of artificial sea-water.

Solution	Compound	Conc. g/l	Artificial sea-water (amount in 1 l)
	NaCl	22.4	22.4 g
A I	Na_2SO_4	41.00	
	KCl	6.93	
A II	KBr	1.00	91.4 ml
	NaF	0.030	
A III	H_3BO_3	0.273	
B	$MgCl_2.6H_2O$	203	49.8 ml
C	$CaCl_2.2H_2O$	191	9.7 ml
D	$SrCl_2.6H_2O$	26.7	0.82 ml
E	$NaHCO_3$	33.6	5.00 ml
F	$NaHPO_4.12H_2O$	10	0.5 ml
G	$NaNO_3$	50	0.5 ml
H	Trace element solution [2]		1.0 ml
I	Vitamin solution [3]		1.0 ml
	Distilled water to 1 l		

Table 4. Dialysis against components of synthetic sea-water medium.

	Soluble %	Insoluble %
Complete medium	4	96
NaCl, Solution A I, B, C, E	100	0
NaCl, Solution A I, II, III, B, C, D, E	3	97
NaCl, Solution A I, III, B, C, E	4	96

concentrations. A similar experiment with glycine buffer pH 9 replacing the tris buffer gave the same result.

DISCUSSION

The results clearly show that the water-soluble sulfated polysaccharide isolated from *Ulva lactuca* forms a gel in sea-water and that only two of the components of the sea-water are needed for the gel formation; boric acid and calcium ions. The gel is very soft and does not retain any fixed shape. It is thus very different from both a typical alginate and carrageenan gel.[1] Even if little is known about the correlation between gel properties and the molecular structure of the gel, it seems probable that the *Ulva*-polysaccharide gel only contains very few intermolecular linkages per molecule, *i.e.* that it is near to the critically branched state.[3]

Table 5. Dialysis against tris buffer (pH 8) containing various sea-water components.

	Soluble %	Insoluble %
A III	100	0
A III, NaCl	100	0
A I, II, III, NaCl	100	0
A III, B	100	0
A III, C	1.5	98,5
C	100	0

Table 6. Dialysis against a simplified sea-water medium (NaCl, Solution A I, A III, B and C) in tris-buffers with varying pH.

pH	Soluble %	Insoluble %
8.02	2	98
7.86	2	98
7.50	2	98 [a]
7.25	100	0

[a] Gel softer than usual.

Table 7. Extraction experiments.

Extraction medium	Carbohydrate extracted, % of dry algae
Distilled water	18.2
0.1 M EDTA followed by distilled water	19.5
0.1 M $CaCl_2$	16.7
0.7 M NaCl	16.8
Tris-buffer, pH 8	9.9
Tris-buffer, pH 8, containing 0.1 M EDTA	18.1
Tris-buffer, pH 8, NaCl, Solution A I, II, B, C	9.0
Tris-buffer, pH 8, NaCl, Solution A I, II, III, B, C	4.5
Natural sea-water	4.7

The *Ulva*-polysaccharide gel dissolves in distilled water in contrast to alginate gels formed by dialysis of sodium alginate against sea-water, which needs exchange of calcium with monovalent ions to dissolve. This agrees well with the extraction results. *Ulva*-polysaccharide may be extracted by distilled water, while in order to extract alginate from brown algae, calcium ions must be replaced with monovalent ions, *e.g.* by acid pre-extraction and extraction with sodium hydroxide or sodium carbonate.[4] In solutions buffered at pH 8 only incomplete extraction of *Ulva*-polysaccharide was obtained. Gels formed by isolated polysaccharide, however, dissolved slowly upon dialysis against media buffered at pH 8 only if the media did not contain borate ions.

The results thus indicate that the formation of gel depends upon an equilibrium reaction between borate and polysaccharide, and it is likely that the intermolecular linkages leading to gel formation involve the formation of borate-polysaccharide complexes. In agreement with this is also the observation that no gel formation took place at pH 7.25 and a markedly softer gel than normal was formed at pH 7.50 (Table 6). That no change in optical rotation in presence of borate was observed, indicates that only small amounts of borate are bound, as should be expected from the gel structure. It is likely that the borate complex is formed with the vicinal hydroxyl groups of the 1,4-linked rhamnose units. These units, however, carry usually half-ester sulfate at C(2) (Ref. 1) and it is probably only a few units in the molecule with the free vicinal hydroxyl groups necessary for formation of a borate complex.

The effect of calcium ions is less clear. No gel is formed without borate and calcium ions being present simultaneously. The calcium ions may form bridges between borate complexes of the type shown in Fig. 1A, or they may be necessary for stabilizing borate linkages of the type shown in Fig. 1B. Both explanations are in agreement with the observation that complete extraction of the polysaccharide takes place also at pH 8 when calcium ions are removed by EDTA (Table 7).

The structures in Fig. 1 are only meant to indicate two possibilities, and neither of them gives a satisfactory explanation for the observation that the presence of magnesium ions do not give gel formation, *i.e.* that the formation of junctions is specific with respect to the cation.

If we assume, as the present results strongly suggest, that the sulfated polysaccharide from *Ulva lactuca* occurs in the plant as a gel stabilized by borate linkages, this is, as far as the author is aware, the first example of a borate-polysaccharide complex in Nature, and further investigation into the nature of the gels obtained with the polysaccharide from *Ulva*

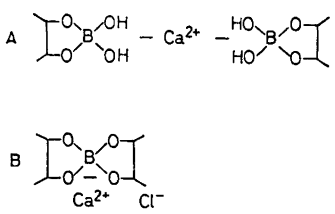

Fig. 1. Possible interactions between borate-polysaccharide complexes and calcium ions.

lactuca seems to be of direct importance for the understanding of the biological function of this complex.

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Synthesis of Some 4-Aminoalkyl-5-methyl-3-isoxazolols Structurally Related to Muscimol and γ-Aminobutyric Acid (GABA)

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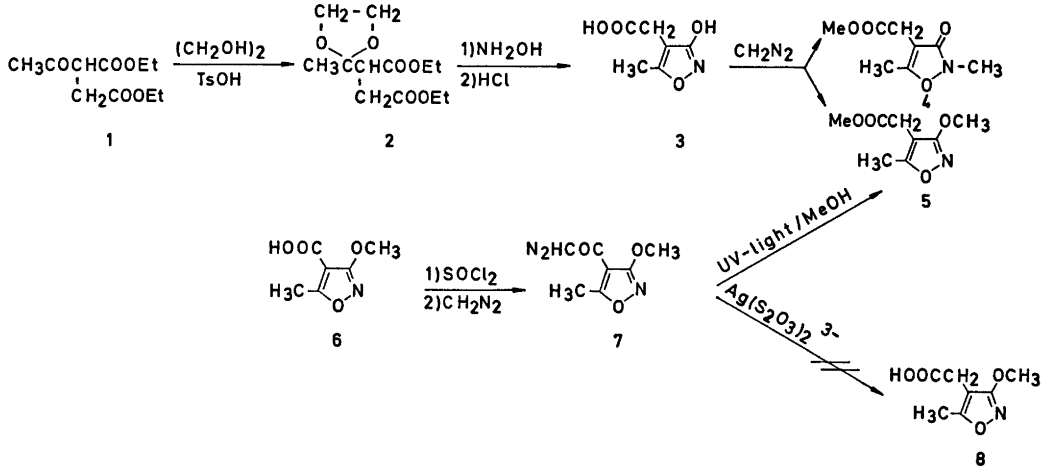
The synthesis of 4-(2-aminoethyl)-5-methyl-3-isoxazolol zwitterion (*16a*) and 4-(2-aminopropyl)-5-methyl-3-isoxazolol zwitterion (*16b*) which are structural analogues of 5-aminomethyl-3-isoxazolol (Muscimol) and of γ-aminobutyric acid (GABA) is described. The key reaction of the sequences leading to these compounds involves hydroxylamine treatment of the appropriately substituted five-membered cyclic enamides (*13a* and *b*). Furthermore the preparation of the β-alanine analogues 4-aminomethyl-5-methyl-3-isoxazolol zwitterion (*19*) and 4-(1-aminoethyl)-5-methyl-3-isoxazolol zwitterion (*20*) is described. The pK_A values of all four compounds have been determined.

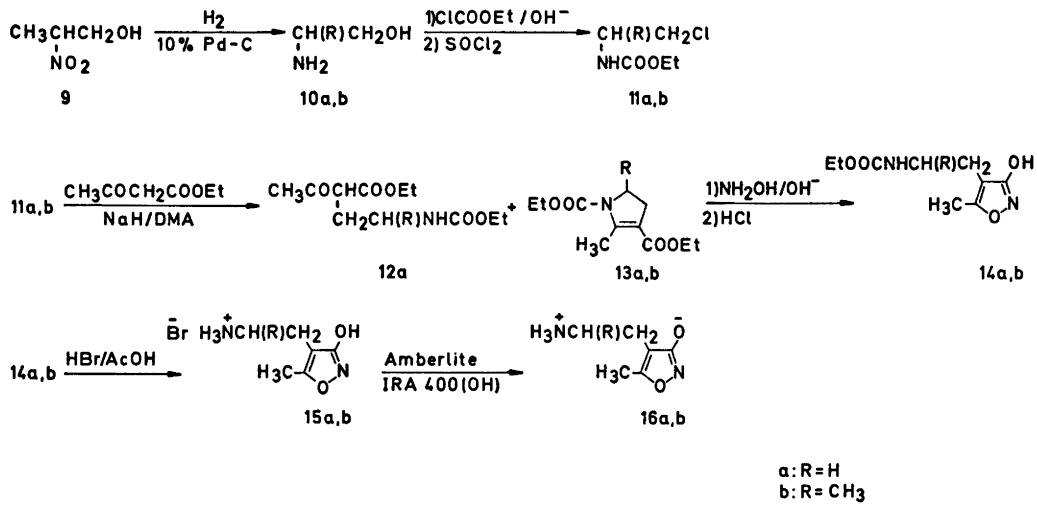
As part of the investigations of the biological properties of conformationally restricted analogues of γ-aminobutyric acid (GABA) structurally related to muscimol (5-aminomethyl-3-isoxazolol) [1,2] a series of bicyclic [3,4] and 5-aminoalkylsubstituted [5–7] 3-isoxazolols has been prepared. This paper presents the synthesis of some 3-isoxazololes with an aminoalkyl moiety in the 4-position.

As an approach to the synthesis of 4-(2-aminoethyl)-5-methyl-3-isoxazolol (*16a*), the method of preparation of 3-isoxazololes *via* ethylene acetals of β-oxoesters [4,8,9] was extended to the preparation of 3-hydroxy-5-methylisoxazol-4-ylacetic acid (*3*). Reaction of the ethylene acetal *2* with hydroxylamine, however, gave *3* in a poor yield and since *5* could only be obtained in a 30 % yield by treatment of *3* with diazomethane further transformations in the planned reaction sequence were considered of limited value.

The synthesis of the 3-methoxyisoxazole *5* *via* the diazoketone *7*, which was readily obtained from the acid *6* [10] upon treatment with



Scheme 1.



Scheme 2.

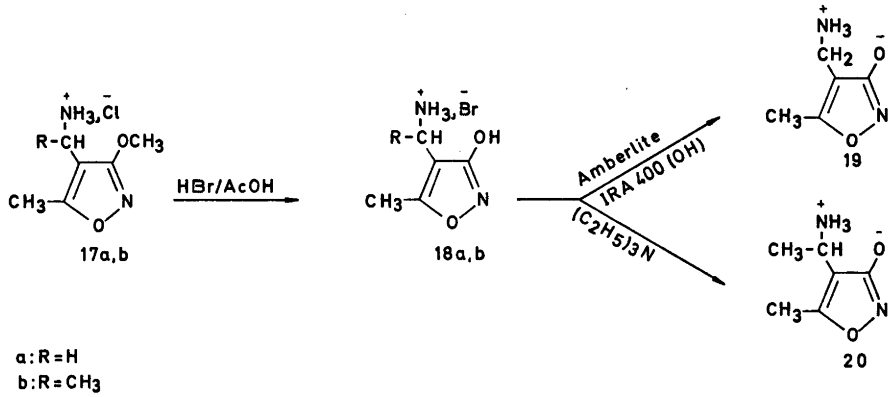
thionyl chloride followed by diazomethane, was then examined. A methanolic solution of the diazoketone *7* was irradiated with unfiltered UV-light to give *5* in a good yield. However, several experiments revealed the limit of the synthetic scale under the used conditions to be about 200 mg of *7*. Attempts to rearrange the diazoketone *7* to *8* using silver thiosulfate according to the method of Wiberg and Hutton [11] led to complex mixtures from which no compound was isolated.

Finally *16a* was successfully prepared by the sequence outlined in Scheme 2. Ethyl acetoacetate was alkylated by the urethane *11a* to give a mixture of two compounds. Column chromatography of the mixture gave the compound with the smaller R_F value in a pure state, whereas the other compound during this procedure was partly transformed into the compound with the smaller R_F value and could not be isolated in a pure state. Based on IR, UV, and ^{1}H NMR spectroscopy and supported by chemical evidence the compound with the smaller R_F value was shown to be the cyclic enamide *13a*. On the basis of spectroscopic and TLC examinations the other compound was assigned the β-oxoester structure *12a*. Treatment of the above-mentioned mixture of *12a* and *13a* in boiling toluene for 8 h using 4-toluenesulfonic acid as a catalyst completed the cyclization of *12a* into *13a* which was obtained in a good yield.

The cyclic enamide *13a* was treated with hydroxylamine to give a reasonable yield of the isoxazole *14a*. Investigation of the reaction mixture on TLC plates, however, revealed the presence of trace amounts of a compound which gave a violet spot on TLC using iron(III) chloride as a spraying reagent. The compound may be the corresponding 5-isoxazolone [3-methyl-4-(2-ethoxycarbonylaminoethyl)-5-isoxazolone] but several attempts to isolate the compound in a pure state were unsuccessful. The observed reaction course is remarkable as various β-oxoesters protected at the oxo group as benzyl-enamines, upon treatment with hydroxylamine exclusively yield 5-isoxazolones.[13]

In the sequence leading to the isoxazolol zwitterion *16b* the starting material 2-nitropropan-1-ol (*9*) was low-pressure hydrogenated using a 10 % Pd-C catalyst to give 2-aminopropan-1-ol (*10b*). The desired compound *16b* was obtained by a reaction sequence analogous to that described above including the cyclic enamide *13b*. All attempts to obtain *16b* in a crystalline state failed, and the compound could only be isolated as a glassy substance.

The synthesis of 4-aminomethyl-5-methyl-3-isoxazolol zwitterion (*19*) was achieved from 3-methoxy-4-aminomethyl-5-methylisoxazole hydrochloride (*17a*) prepared according to the directions of Bowden *et al.*[10] *17a* was readily cleaved by hydrogen bromide in glacial acetic



Scheme 3.

acid to give the 3-isoxazolol *18a* which by use of a strongly basic ion exchange resin gave the zwitterion *19*. This compound has previously been prepared by Bowden *et al.* by hydrolytic cleavage of 4-trifluoroacetamidomethyl-5-methyl-3-isoxazolol but was isolated as the 4-toluenesulfonate by these authors.[10]

A strong UV absorption at 281 nm and an absorption band at 1630 cm^{-1} in the IR spectra indicated the presence of conjugation in the compounds *13a* and *b*. This together with the ^1H NMR spectra and elemental analyses confirmed the cyclic enamide structure of these compounds.

The IR, UV, and ^1H NMR data obtained from the 3-oxygenated isoxazole moieties of *3*, *5*, *7*, *14a,b*, *15a,b*, and from the 3-isoxazolone moiety of *4* are in accordance with the general findings described by Jacquier *et al.*[8] The spectroscopic data of the zwitterions *16a*, *19*, and *20* are in accordance with those published for other isoxazole zwitterions.[3-7] The IR absorption bands at 2100, 1620, and 1360 cm^{-1} in the spectrum of *7* are in accordance with the results published for various diazocarbonyl compounds by Yates *et al.*[14] Some IR, UV, and ^1H NMR data of the starting materials *6* and *17a,b* are given in the experimental part as no spectroscopic data of these compounds are available in the literature.

The pK_A values of *16a,b*, *19*, and *20* are given in the experimental part. The pK_A values of the compounds are in accordance with those published for other isoxazole zwitterions.[3-7]

EXPERIMENTAL

Unless otherwise stated 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.[13] 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. pH values were measured on a Radiometer pH meter 26. The pK_A values were determined according to the method described by Albert and Serjeant[15] as described in a previous paper.[5]

Diethyl acetosuccinate acetal (2). A solution of 54.0 g (0.25 mol) of diethyl acetosuccinate (*1*),[16] 25.0 g (0.40 mol) of ethylene glycol, and 1 g of 4-toluenesulfonic acid in 500 ml of benzene was refluxed for *ca.* 40 h using a Dean-Stark water separator. The solution was washed with two 200 ml portions of water, dried (K_2CO_3), and distilled to give 40.9 g (62 %) of *2* as a colourless oil, b.p. 155–158 °C/7 mmHg. An analytical sample was further purified by column chromatography using CH_2Cl_2 as an eluent. (Found C 55.30; H 7.84. Calc. for $C_{12}H_{20}O_6$: C 55.37; H 7.75). IR data (neat) cm^{-1}: 1735(s). ^1H NMR data ($CDCl_3$): δ 4.13 and 4.07 [2 × q (J = 7 Hz in both cases), 4 H, 2 × CH_2-CH_2-O]; 3.95 (s, 4 H, $O-CH_2-CH_2-O$); 3.3–3.0 (m, 1 H, $CH-CH_2$); 2.9–2.2 (m, 2H, $CH-CH_2$); 1.38 (s, 3 H, CH_3-C); 1.27 and 1.23 [2 × t (J = 7 Hz in both cases), 6 H, 2 × CH_3-CH_2-O].

3-Hydroxy-5-methylisoxazol-4-ylacetic acid (3). To a solution of 15.2 g (0.22 mol) of hydroxylamine hydrochloride in 400 ml of methanol was added 41.4 g (0.30 mol) of potassium carbonate. After stirring for 15 min 52.0 g (0.20 mol) of *2* was added and the suspension was refluxed for 12 h. The filtered reaction mixture was evaporated *in vacuo* to give an oil which was dissolved in 250 ml of water.

The aqueous solution was extracted with three 100 ml portions of methylene chloride which were discarded. To the aqueous phase was added 250 ml of concentrated hydrochloric acid and the solution was boiled for 1 h. The solution was extracted continuously for 2 h by ether-methylene chloride 4:1 to give 11.1 g of an oily product which was submitted to column chromatography (silica gel: 500 g; eluent: benzene-ethyl acetate-formic acid 10:20:0.3 to which increasing amounts of ethyl acetate were added) to yield 1.60 g (5.2 %) of yellowish crystals. An analytical sample was recrystallized from ethyl acetate to give colourless crystals, m.p. 150–152 °C. (Found: C 45.95; H 4.52; N 8.80. Calc. for $C_6H_7NO_4$: C 45.86; H 4.49; N 8.97). $\lambda_{max}(C_2H_5OH)$: 211 nm ($\varepsilon = 5.92 \times 10^3$). IR (KBr) cm^{-1}: 3600–2200(s); 1700(s); 1670(s); 1560–1520 (s, several bands). ^1H NMR (CD_3COCD_3): δ 9.68 (s, 2 H, O*H* and COO*H*); 3.33 (s, 2H, OC–C*H*$_2$–C); 2.27 (s, 3 H, C*H*$_3$–C=).

Methyl 2,5-dimethyl-3-oxoisoxazolin-4-yl-acetate (4) and methyl 3-methoxy-5-methylisoxazol-4-ylacetate (5). To a solution of 750 mg (4.8 mmol) of 3-hydroxy-5-methylisoxazol-4-ylacetic acid (*3*) in 50 ml of ether was added with stirring a solution of *ca.* 0.5 g (*ca.* 15 mmol) of diazomethane (prepared from 4.30 g (20 mmol) of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide)[17] in 40 ml of ether. Stirring was continued for 2 h and the remaining diazomethane was destroyed by addition of excess of formic acid. The solution was evaporated to dryness *in vacuo* to give 950 mg of a colourless oil which was submitted to column chromatography (silica gel: 30 g; eluent: ether-petroleum ether 1:1). Distillation of the fractions containing *4* in a "Kugelrohr" at 1 mmHg (oven temp. 160 °C) gave 432 mg (49 %) of a colourless oil (Found: C 51.10; H 6.17; N 7.39. Calc. for $C_8H_{11}NO_4$: C 51.88; H 5.99; N 7.56). $\lambda_{max}(CH_3OH)$ 233 nm ($\varepsilon = 6.74 \times 10^3$). IR (neat) cm^{-1}: 3600–2700 (m); 1740(s); 1660(s). ^1H NMR ($CDCl_3$): δ 3.69 (s, 3 H, –COOC*H*$_3$); 3.47 (s, 3 H, N–C*H*$_3$); 3.27 (s, 2 H, =C–C*H*$_2$–C); 2.22 (s, 3 H, =C–C*H*$_3$). Distillation of the fractions containing *5* in a "Kugelrohr" at 1 mmHg (oven temp. 125 °C) gave 273 mg (30 %) of a colourless oil which solidified to colourless crystals, m.p. 41.5–42.5 °C. (Found: C 51.80; H 6.07; N 7.58. Calc. for $C_8H_{11}NO_4$: C 51.88; H 5.99; N 7.56). $\lambda_{max}(CH_3OH)$: 211 nm ($\varepsilon = 6.02 \times 10^3$). IR (KBr) cm^{-1}: 3700–3200 (m); 3050–2800(m); 1730(s); 1665(m); 1530(s); 1480(s). ^1H NMR ($CDCl_3$): δ 3.83 (s, 3 H, =C–OC*H*$_3$); 3.57 (s, 3 H, –COOC*H*$_3$); 3.15 (s, 2 H, =C–C*H*$_2$–C); 2.17 (s, 3 H, =C–C*H*$_3$).

3-Methoxy-5-methylisoxazole-4-carboxylic acid (6). *6* was prepared as described by Bowden *et al.*[10] to give crystals, m.p. 195–196 °C (Ref. 10: 195–197 °C). $\lambda_{max}(CH_3OH)$: 215 nm ($\varepsilon = 7.42 \times 10^3$). IR (KBr) cm^{-1}: 3600–2300(m); 1680(s); 1620(s); 1530(s); 1490(s). ^1H NMR ($CDCl_3$): δ 13.5–11 (broad band, 1 H, –COO*H*); 3.86 (s, 3 H, –OC*H*$_3$); 2.53 (s, 3 H, =C–C*H*$_3$).

3-Methoxy-4-diazoacetyl-5-methylisoxazole (7). A solution of 1.57 g (10 mmol) of 3-methoxy-5-methylisoxazole-4-carboxylic acid (*6*)[10] in 12 g (100 mmol) of thionyl chloride was refluxed for 40 min. Excess of thionyl chloride was removed *in vacuo* and the residual oil was dissolved in 25 ml of dry ether. The ethereal solution was added dropwise with stirring to a potassium hydroxide dried solution of *ca.* 1 g (*ca.* 24 mmol) of diazomethane (prepared from 7.17 g (33 mmol) of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide)[17] in 75 ml of ether. Stirring was continued for 3 h and after addition of an excess of formic acid in order to destroy the remaining diazomethane the solution was evaporated to dryness *in vacuo* to give 1.79 g of crude product as yellow crystals. Recrystallization from ether-petroleum ether afforded 900 mg (50 %) of yellow crystals, m.p. 107.5–108.5 °C. (Found: C 46.35; H 4.02; N 23.10. Calc. for $C_7H_7N_3O_3$: C 46.41; H 3.90; N 23.20). $\lambda_{max}(CH_3OH)$: <210 nm; 246 nm ($\varepsilon = 9.07 \times 10^3$); 291 nm ($\varepsilon = 16.1 \times 10^3$). IR (neat) cm^{-1}: 3700–3200(w); 3120(w); 2950(w); 2110(s); 1620(s); 1595(s); 1520 (m); 1470(m); 1360(s). ^1H NMR ($CDCl_3$): δ 5.97 (s, 1 H, C*H*–N$_2$); 4.02 (s, 3 H, =C–OC*H*$_3$); 2.62 (s, 3 H, C*H*$_3$–C=).

Methyl 3-methoxy-5-methylisoxazol-4-ylacetate (5). A solution of 200 mg (0.11 mmol) of *7* in 50 ml of methanol was irradiated in a quartz-tube with unfiltered UV-light (Philips HPK 125 W, BA 15 D, Typ 57203B/00) for 2 h at *ca.* 10 °C. The solution was filtered and evaporated to dryness *in vacuo* and the residue was submitted to column chromatography (silica gel: 20 g; eluent: methylene chloride-ethyl acetate 3:1) to give 157 mg of crude product. Recrystallization from ether-petroleum ether (–70 °C) gave colourless crystals 87 mg (43 %) of which the IR-spectrum was identical with that of *5* prepared as described above.

Diethyl 2-methyl-2-pyrroline-1,3-dicarboxylate (13a). To a suspension of 21.9 g (0.5 mol) of a 55 % dispersion of sodium hydride in mineral oil and 250 ml of DMA was added with stirring 65.0 g (0.5 mol) of ethyl acetoacetate. When the hydrogen evolution had ceased, 15 g of sodium iodide (dried at 140 °C for 24 h) and 83.5 g (0.55 mol) of *11a*[12] was added and the stirred solution was heated to 80 °C for 4 h. After cooling to room temperature 750 ml of water and 125 ml of hydrochloric acid (4 M) was added and the solution was extracted with three 200 ml portions of methylene chloride. The pooled organic phases were washed with 200 ml of water, dried ($MgSO_4$), and evaporated *in vacuo* to give an oil which was distilled at 144–146 °C/0.5 mmHg to give a colourless oil which solidified upon standing. TLC showed the distillate to consist of two compounds (spraying reagent DNP) of which the less polar compound was shown to be *13a* whereas the other compound according to the spectro-

scopic investigations is assumed to be the corresponding β-oxoester *12a*. IR (neat) cm^{-1}: 3700 – 3100(m); 3050 – 2800(m); 1740 – 1690(s, several bands); 1530(m). To complete the cyclization the mixture was dissolved in 400 ml of toluene and after addition of 1 g of *p*-toluenesulfonic acid the solution was refluxed for *ca.* 8 h using a Dean-Stark water separator, dried (K_2CO_3), and evaporated *in vacuo* to give an oil. Distillation at 126 – 135 °C/0.5 mmHg gave 73.7 g (66 %) of *13a* as a colourless oil which solidified upon standing. 0.5 g of *13a* was purified by column chromatography (silica gel: 25 g; eluent: benzene-ethyl acetate 4:1) to give crystals of *13a*. Recrystallization from methanol yielded colourless crystals, m.p. 56 – 57 °C. (Found: C 58.25; H 7.34; N 6.11. Calc. for $C_{11}H_{17}NO_4$: C 58.13; H 7.54; N 6.16). λ_{max}(C_2H_5OH): 281 nm ($\varepsilon = 19.5 \times 10^3$). IR data (KBr) cm^{-1}: 3600 – 3100(m); 3050 – 2800(s); 1740 – 1720(s); 1690(s); 1630(s). ^1H NMR ($CDCl_3$): δ 4.15 and 4.12 [2 × q ($J = 7$ Hz in both cases), 4 H, 2 × O – CH_2 – CH_3]; 4.0 – 3.5 (m, 2 H, CH_2 – C=C); 2.54 (s, 3 H, CH_3 – C=C); 1.27 and 1.25 [2 × t ($J = 7$ Hz in both cases), 6 H, 2 × O – CH_2 – CH_3].

*4-(2-Ethoxycarbonylaminoethyl)-5-methyl-3-isoxazolol (*14a*)*. To a solution of 2.1 g (30 mmol) of hydroxylamine hydrochloride in 50 ml of ethanol was added a solution of 6.3 g (*ca.* 90 mmol) of potassium hydroxide in 30 ml of ethanol. The mixture was stirred at room temperature for 5 min and cooled to −5 °C. To the cooled solution was added 3.7 g (15 mmol) of *13a* and after stirring for 5 min the reaction mixture was placed at 5 °C for 8 days. After evaporation to dryness *in vacuo* 15 ml of water and 15 ml of concentrated hydrochloric acid was added with cooling and stirring. The mixture was left at room temperature for 1 h and was subsequently extracted continuously for 1 h with ether-methylene chloride 4:1. The extract was dried ($MgSO_4$) and evaporated *in vacuo* to give 1.45 g of crude product which was purified by column chromatography (silica gel: 75 g; eluent: methylene chloride-ethyl acetate-formic acid 50:50:2) to give 700 mg of yellow crystals. Recrystallization from ether gave colourless crystals 373 mg (12 %) m.p. 138 – 139 °C. (Found: C 50.50; H 6.65; N 13.19. Calc. for $C_9H_{14}N_2O_2$: C 50.46; H 6.59; N 13.08). λ_{max}(C_2H_5OH): 213 nm ($\varepsilon = 5.76 \times 10^3$). IR (KBr) cm^{-1}: 3600 – 3200(s); 3200 – 2200(m); 1690(s); 1660(m); 1560 – 1520(s, several bands). ^1H NMR ($CDCl_3$-DMSO-d_6 6:1): δ 8.20 (s, *ca.* 1 H, O*H*); 5.9 – 5.5 (broadened signal, 1 H, N*H*); 4.00 [q ($J = 7$ Hz), 2 H, O – CH_2 – CH_3]; 3.5 – 3.0 (broadened q, 2 H, CH_2 – CH_2 – NH); 2.40 [t ($J = 6$ Hz), 2 H, =C – CH_2 – CH_2]; 2.19 (s, 3 H, CH_3 – C=); 1.18 [t ($J = 7$ Hz), 3 H, O – CH_2 – CH_3].

*4-(2-Aminoethyl)-5-methyl-3-isoxazolol hydrobromide (*15a*)*. A solution of 400 mg (1.87 mmol) of *14a* in glacial acetic acid (5 ml) containing 43 % of hydrogen bromide was refluxed for 90 min. After reflux for 60 min an additional amount of 3 ml of glacial acetic acid containing 43 % of hydrogen bromide was added. After cooling to room temperature the solution was evaporated to dryness *in vacuo* to give brownish crystals. Recrystallization from methanol-ether gave 343 mg (77 %) of colourless crystals, m.p. 205 – 206 °C (decomp.). (Found: C 32.45; H 5.01; Br 35.58; N 12.63. Calc. for $C_6H_{11}BrN_2O_2$: C 32.31; H 4.97; Br 35.82; N 12.56). λ_{max}(CH_3OH): < 210 nm. IR (KBr) cm^{-1}: 3600 – 2200(s); 1660(s); 1595(m); 1575(m); 1540 – 1520(s, several bands); 1500(s); 1370(w). ^1H NMR (DMSO-d_6): δ 8.3 – 7.6 (broad signal, 3 H, NH_3^+); 3.2 – 2.3 (broad signal, 4 H, CH_2 – CH_2 – NH_3^+); 2.25 (s, 3 H, CH_3 – C=). The OH-proton could not be detected.

*4-(2-Aminoethyl)-5-methyl-3-isoxazolol zwitterion (*16a*)*. A solution of 1.80 g (8.1 mmol) of *15a* in water (12 ml) was passed through a column containing an ion exchange resin [Amberlite IRA 400 (OH), 40 ml] using acetic acid (1 M) as an eluent. Recrystallization from water-ethanol gave 339 mg (30 %) of colourless crystals, m.p. 159.5 – 161.5 °C (decomp.). (Found: C 50.85; H 7.21; N 19.98. Calc. for $C_6H_{10}N_2O_2$: C 50.69; H 7.09; N 19.71). λ_{max} (CH_3OH): 213 nm ($\varepsilon = 5.83 \times 10^3$). IR (KBr) cm^{-1}: 3600 – 2200(s); 2090(w); 1655(s); 1570 – 1550(s, several bands); 1500 – 1460(s, several bands). pK_A-Values (H_2O, 17 °C): 5.12 ± 0.05, 10.42 ± 0.06. ^1H NMR {D_2O [sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard]}: δ 4.79 (s, *ca.* 4 H, DO*H*); 3.10 [t ($J = 6$ Hz), 2 H, CH_2 – NH_3^+]; 2.55 [t ($J = 6$ Hz), 2 H, CH_2 – CH_2 – C=]; 2.15 (s, 3 H, CH_3 – C=).

*2-Aminopropan-1-ol (*10b*)* was prepared by low pressure (3 atm.) hydrogenation of an ethanolic solution of 2-nitropropan-1-ol (*9*) in a PARR hydrogenation apparatus using 500 mg of a 10 % Pd-C catalyst and 500 ml of ethanol per 0.5 mol of starting material. Yield 70 %, b.p. 71 – 74 °C/11 mmHg. (Ref. 18: b.p. 80 – 86 °C/20 mmHg. Ref. 19: b.p. 76 – 78 °C/15 mmHg).

*1-Chloro-2-ethoxycarbonylaminopropane (*11b*)* was prepared *via* 1-hydroxy-2-(ethoxycarbonylamino)propane as described by Najer *et al.*[20] using *10b* as starting material.

*Diethyl 2,5-dimethyl-2-pyrroline-1,3-dicarboxylate (*13b*)*. To a suspension of 17.9 g (0.41 mol) of a 55 % dispersion of sodium hydride in mineral oil, and 200 ml of DMA was added with stirring 53.0 g (0.41 mol) of ethyl acetoacetate. When the hydrogen evolution had ceased, 61.5 g (0.41 mol) of sodium iodide (dried at 140 °C for 24 h) and 66.2 g (0.45 mol) of *11b* was added and the solution was stirred for 3 h at 100 °C. After cooling to room temperature the crude product was isolated as described for *13a*. Treatment with *p*-toluenesulfonic acid in toluene under reflux for 20 h and isolation as described for *13a* gave 33.7 g of crude product as a brownish oil which was

used without further purification for the preparation of *14b*. An analytical sample of the crude product was purified by column chromatography (silica gel: 25 g; eluent: methylene chloride) to give a colourless oil which was distilled in a "Kugelrohr" at 0.5 mmHg (oven temperature 160 °C) to give pure *13b* as an oil. (Found: C 59.65; H 7.85; N 5.72. Calc. for $C_{12}H_{19}NO_4$: C 59.73; H 7.94; N 5.81). λ_{max} (CH_3OH): 281 nm($\varepsilon = 17.2 \times 10^3$). IR (neat) cm^{-1}: 3700 – 3100(w); 3050 – 2800(s); 1725(s); 1690(s); 1630(s). ^1H NMR (CCl_4): δ 4.07 and 4.00 [2 × q ($J = 7$ Hz in both cases), 4 H, $2 \times O-CH_2-CH_3$]; 4.5 – 3.8 (m, 1 H, $N-CH-CH_3$); 3.2 – 1.9 (m, 2 H, $=C-CH_2-CH$); 2.44 (t, 3 H, $CH_3-C=$); 1.6 – 1.0 (m, 9 H, CH_3-CH and $2 \times O-CH_2C-CH_3$).

4-(2-Ethoxycarbonylaminopropyl)-5-methyl-3-isoxazolol (14b) was prepared as described above for *14a*. 7.23 g (30 mmol) of crude *12b* gave 4.55 g of crude product which was submitted to column chromatography [silica gel: 200 g; eluent: methylene chloride-ethyl acetate-formic acid (60:40:1)] to give 2.9 g of product. Recrystallization from ethyl acetate-benzene gave 820 mg (14 %) of colourless crystals, m.p. 170 – 170.5 °C. (Found: C 52.50; H 6.97; N 12.11. Calc. for $C_{10}H_{16}N_2O_4$: C 52.62; H 7.07; N 12.27). λ_{max}(CH_3OH): 216 nm ($\varepsilon = 6.70 \times 10^3$). IR (KBr) cm^{-1}: 3340(m); 3200 – 2200(m); 1680(s); 1660(m); 1560 – 1520(s, several bands). ^1H NMR ($CDCl_3$ – DMSO-d_6 5:3): δ 7.2 – 6.5 (broad s, *ca.* 1 H, O*H*); 6.5 – 6.0 (broadened signal, 1 H, N*H*); 3.95 [q ($J = 7$ Hz), 2 H, $O-CH_2-CH_3$]; 4.2 – 3.3 (m, 1 H, $-CH-NH$); 2.35 [d ($J = 7$ Hz), 2 H, $=C-CH_2-CH$]; 2.20 (s, 3 H, $CH_3-C=$); 1.18 and 1.05 [2 × t ($J = 7$ Hz in both cases), 6 H, $2 \times O-CH_2-CH_3$].

4-(2-Aminopropyl)-5-methyl-3-isoxazolol hydrobromide (15b). A solution of 250 mg (1.1 mmol) of *14b* was treated with glacial acetic acid containing 43 % of hydrogen bromide as described for the preparation of the compound *15a* to give an oil which crystallized from methanol-ether to give 219 mg (85 %) of *15b* as colourless crystals, m.p. 198 – 199 °C (decomp.). (Found: C 35.25; H 5.78; Br 33.70; N 11.82. Calc. for $C_7H_{13}BrN_2O_2$: C 35.46; H 5.52; Br 33.71; N 11.82). λ_{max}(CH_3OH): 211 nm ($\varepsilon = 5.88 \times 10^3$). IR (KBr) cm^{-1}: 3600 – 3300(w); 3300 – 2300(s); 2050 – 1900(w); 1660(m); 1595(m); 1530(s); 1500(s). ^1H NMR {D_2O [sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard]}: δ 4.90 (s, *ca.* 4 H, DO*H*); 3.8 – 3.2 (m, 1 H, $CH-CH_3$); 2.60 [d ($J = 6$ Hz), 2 H, $=C-CH_2-CH$]; 2.30 (s, 3 H, $CH_3-C=$); 1.28 [d ($J = 6$ Hz), 3 H, CH_3-CH].

3-Methoxy-4-aminomethyl-5-methylisoxazole hydrochloride (17a) was prepared as described by Bowden *et al.*[10] to give crystals, m.p. 222 – 223 °C (decomp.). Ref. 10: m.p. 229 °C (decomp.). λ_{max}(CH_3OH): 210 nm ($\varepsilon = 5.93 \times 10^3$). IR (KBr) cm^{-1}: 3600 – 3300(m); 3300 – 2500(s); 1655(m); 1525(s); 1480(s). ^1H NMR (DMSO-d_6): δ 9.0 – 8.2 (broadened s, 3 H, NH_3^+); 3.87 (s, 3 H, OCH_3); 3.66 (s, 2 H, $CH_2-NH_3^+$); 2.42 (s, 3 H, $=C-CH_3$).

4-(2-Aminopropyl)-5-methyl-3-isoxazolol zwitterion (16b). pK_A Values (H_2O, 21 °C): 4.87 ± 0.05, 10.06 ± 0.06.

4-Aminomethyl-5-methyl-3-isoxazolol hydrobromide (18a) was prepared as described above for *15a* using 200 mg (1.12 mmol) of *17a* as starting material. The crude product obtained as reddish yellow crystals was recrystallized from methanol-ether to give 125 mg (53 %) of colourless crystals, m.p. 209 – 209.5 °C (decomp.). (Found: C 28.90; H 4.46; Br 38.10; N 13.57. Calc. for $C_5H_9BrN_2O_2$: C 28.72; H 4.34; Br 38.23; N 13.40). λ_{max}(CH_3OH): < 210 nm. IR (KBr) cm^{-1}: 3600 – 3300(m); 3300 – 2300(s); 1655(s); 1585(m); 1560 – 1500(s, several bands). ^1H NMR (DMSO-d_6): δ 8.6 – 7.4 (broad signal, 4 H, O*H* and NH_3^+); 4.0 – 3.4 (broadened s, 2 H, $CH_2-NH_3^+$); 2.35 (s, 3 H, $=C-CH_3$).

4-Aminomethyl-5-methyl-3-isoxazolol zwitterion (19). A solution of 330 mg (1.58 mmol) of *18a* in water (2 ml) was passed through a column containing an ion exchange resin [Amberlite IRA 400, (OH), 5 ml] using acetic acid (1 M) as an eluent. Recrystallization of the crude product (methanol-ether) gave 102 mg (50 %) as colourless crystals, m.p. 163.5 – 165 °C (decomp.). (Found: C 43.00; H 6.56; N 19.90. Calc. for $C_5H_8N_2O_2 \cdot 2/3H_2O$: C 42.85; H 6.71; N 19.99). [Found after drying of *19* over P_2O_5 (24 h; 75 °C; 0.1 mmHg): C 44.95; H 6.76; N 20.91. Calc. for $C_5H_8N_2O_2 \cdot 1/3H_2O$: C 44.77; H 6.51; N 20.89]. λ_{max}(CH_3OH): < 210 nm. IR (KBr) cm^{-1}: 3600 – 3300(m); 3300 – 2300(s); 2180(m); 1655(m); 1520 – 1480(s, several bands). pK_A Values (H_2O, 21 °C): 4.74 ± 0.05, 9.95 ± 0.03. ^1H NMR data [D_2O (sodium 3-(trimethylsilyl)-propanesulfonate was used as an internal standard)]: δ 4.73 (s, 4 H, DO*H*); 4.1 – 3.6 (broad signal, 2 H, $CH_2-NH_3^+$); 2.5 – 2.0 (broad signal, 3 H, $CH_3-C=$).

3-Methoxy-4-(1-aminoethyl)-5-methylisoxazole hydrochloride (17b) was prepared as described by Bowden *et al.*[10] to give crystals, m.p. 199.5 – 200.5 °C (decomp.). (Ref. 10: m.p. 203 °C). λ_{max}(CH_3OH): 212 nm ($\varepsilon = 4.57 \times 10^3$). IR (KBr) cm^{-1}: 3600 – 3300(w); 3300 – 2400(s); 2050 – 1900(w); 1640(s); 1610 – 1580(m, several bands); 1535(s); 1505(s); 1470(s). ^1H NMR (DMSO-d_6): δ 9.0 – 8.1 (broadened s, 3 H, NH_3^+); 3.87 (s, 3 H, OCH_3); 4.5 – 4.0 (m, 1 H, $CH_3-CH-NH_3^+$); 2.40 (s, 3 H, $=C-CH_3$); 1.48 [d ($J = 6$ Hz), 3 H, CH_3-CH].

4-(1-Aminoethyl)-5-methyl-3-isoxazolol hydrobromide (18b) was prepared as described above for *15a* using 500 mg (2.6 mmol) of *17b* as starting material. The crude oily product was crystallized from methanol-ether to give 432 mg (75 %) of colourless crystals, m.p. 193 – 195 °C (decomp.). (Found: C 32.25; H 5.03; Br 36.05; N 12.71. Calc. for $C_6H_{11}BrN_2O_2$: C 32.30; H 4.97; Br 35.82; N 12.56). λ_{max} (CH_3OH): 211 nm ($\varepsilon = 5.10 \times 10^3$). IR (KBr)

cm^{-1}: 3600–3300(m); 3300–2300(s); 1660–1640(s); 1540(s); 1495(s). ^1H NMR (DMSO-d_6): δ 8.7–7.3 (broad signal, 3 H, NH_3^+); 4.5–3.7 (m, 1 H, $CH_3-CH-NH_3^+$); 2.33 (s, 3 H, $CH_3-C=$); 1.47 [d ($J=6$ Hz), 3 H, CH_3-CH]. The OH proton could not be detected.

4-(1-Aminoethyl)-5-methyl-3-isoxazolol zwitterion (20). To a solution of 39 mg (0.17 mmol) of *18b* in 2 ml of ethanol was added 50 µl (5 mmol) of triethylamine at 40 °C, and the reaction mixture was kept at room temperature to complete crystallization. 19 mg (80 %) of colourless crystals were obtained. Recrystallization from water-ethanol-ether gave crystals, m.p. 204.5–205 °C (decomp.). (Found: C 50.40; H 7.27; N 19.92. Calc. for $C_6H_{10}N_2O_2$: C 50.69; H 7.09; N 19.71). $\lambda_{max}(CH_3OH)$: <210 nm. IR (KBr) cm^{-1}: 3600–3300(m); 3300–2300(m); 2200(m); 1640(m); 1520–1490(s). pK_A Values (H_2O, 20 °C): 4.74±0.03, 9.73±0.03. ^1H NMR {D_2O [sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard]}: δ 4.75 (s, 3 H, DO*H*); 4.28 [q ($J=7$ Hz), 1 H, CH_3-CH]; 2.23 (s, 3 H, $CH_3-C=$); 1.58 [d ($J=7$ Hz), 3 H, CH_3-CH].

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Short Communications

On the Reaction of Epoxides with Trimethylchlorosilane

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The *N*-acetyl derivative of pimaricin, a polyene macrolide antibiotic containing an epoxy function, has been mass spectrometrically investigated as a persilyl derivative to distinguish between the molecular compositions $C_{33}H_{47}NO_{14}$ [1] and $C_{33}H_{47}NO_{13}$ [2,3] which differ by one hydroxyl group. The persilylated derivative was formed by reacting *N*-acetylpimaricin with a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine.[2] This solution is one of the more widely used silylating agents and it is commercially available as Trisil ®. The reactions of epoxides with silylating agents does not seem to have been studied extensively. Epoxides are known to react with pure trimethylchlorosilane by addition, forming the silyl ether of a chlorohydrin.[4] (Fig. 1). Pierce states [5] that "the epoxy group is more reactive than the primary hydroxyl group" under silylating conditions, apparently referring to the work of Romantsevich and Malinovskii.[6]

Although mass spectrometric as well as chemical evidence [2,3] seems to favour the $C_{33}H_{47}NO_{13}$ formula for pimaricin, we were disturbed by the conflict between the reported molecular formula for the persilyl derivative of *N*-acetylpimaricin and Pierce's statement concerning the high reactivity of the epoxy function when treated with silylating agents. We therefore felt it desirable to investigate the behaviour of some simple epoxides when treated with (a) pure trimethylchlorosilane (TMCS) and (b) Trisil ®. This was done in sealed glass tubes at 100 °C, and the progress of the reaction was followed by taking samples at different intervals and analysing them by GLC. All

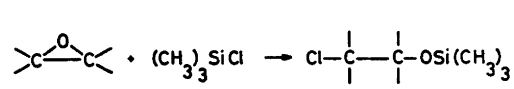

Fig. 1. The reaction between an epoxide and trimethylchlorosilane.

Table 1. The times needed for complete derivatisation of the various epoxides with TMCS and Trisil ® at 100 °C.

Epoxide	TMCS	Trisil
1,2-Epoxycyclohexane	2 h	2 h
1,2-Epoxycyclooctane	24 h	36 h
1,2-Epoxycyclododecane	16 h	150 h
trans-2,3-Epoxybutane	15 min	—
1,2-Epoxy-3,3,3-trichloropropane	24 h	24 h
1,2-Epoxydecane	5 min	16 h
(1,2-Epoxyethyl) benzene	150 h	16 h

epoxides investigated gave derivatives and their GLC retention times were in all cases longer than those of the original epoxides. The reactivity, however, varied considerably among the epoxides. The times for complete reaction for the various epoxides are listed in Table 1.

Exact mass determinations and the fragmentation patterns in the mass spectra of the derivatives (details of which will be discussed below) clearly show that the epoxides react with the silylating agents used by addition of the elements of trimethylchlorosilane over the epoxy function. Two products were formed when the epoxy ring could open in two ways. [7] The predominant product was always formed by opening at the primary carbon atom to give the silyl ether function on the secondary carbon atom and the chlorine atom added to the primary carbon atom (Fig. 2). The two silylation methods (a) and (b) gave the same product distribution, only differences in the reaction rates being observed. These rates are, however, in all cases lower than the normal reaction rate for a hydroxy function. The example chosen by Pierce [5] to illustrate the high reactivity of the epoxy function under silylating conditions in comparison to the reactivity of the hydroxyl function is evidently an exception from the general trend that we have observed. One difference was observed between the products obtained by the two different methods; the silyl ethers formed by pure trimethylchlorosilane were much more sensitive to hydrolysis than the same ethers formed with Trisil ®. The hydrolysed products showed up as new peaks in the gas chromatograms when the reaction tubes had been exposed to

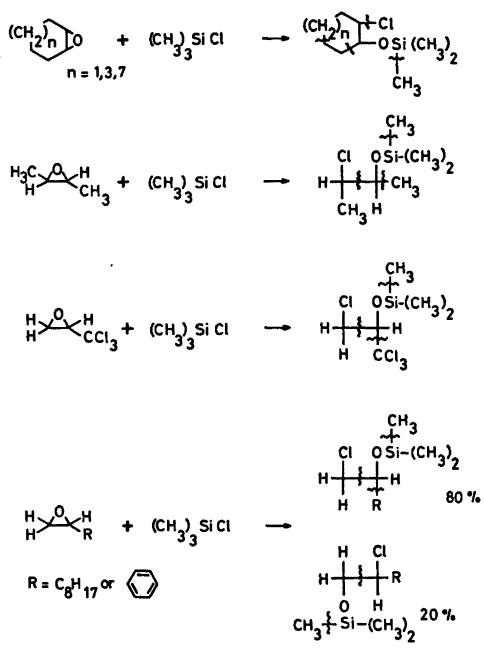

Fig. 2. Reaction schemes for the various epoxides with trimethylchlorosilane and prominent fragmentations in the mass spectra of the silyl ethers formed.

the atmosphere for a few hours. These new peaks had retention times that were shorter than that of the corresponding silyl ether but longer than that of the original epoxide. Mass spectra of these new compounds showed that they were the free chlorohydrins without the silyl ether group.

The epoxides were also treated with other silylating agents for comparison. Treatment with the second component in Trisil ®, pure hexamethyldisilazane, gave no reaction whatsoever. When epoxycyclohexane was treated with bis(trimethylsilyl)trifluoroacetamide, BSTFA, and with trimethylsilylimidazole in pyridine, Trisil-Z ®, it was obvious that some kind of reaction occurred since the epoxide disappeared from the gas chromatogram, but the products formed could not be analysed by GLC and MS.

The mass spectra of the silylated epoxides all display weak molecular-ion peaks in contrast to the M − 15 peaks which are prominent. Both peaks display a P + 2 peak of about 30 % intensity which is the typical isotope pattern for an ion containing one chlorine atom. This statement is obviously not valid for 1,2-epoxy-3,3,3-epoxypropane. The strong peak at M − 15 is characteristic for the mass spectra of all silyl ethers and represents one way of α-cleavage to lose one of the methyl groups in the trimethylsilyl group (Fig. 2). The two other ways of α-cleavage also give rise to ions that are observed in the different spectra.

In the silylated epoxides of the alicyclic compounds the ring opening by α-cleavage is followed by the expulsion of one or more ethylene units. Prominent peaks corresponding to the carbocyclic skeleton of these molecules are also present. The loss of chlorine or hydrogen chloride from the molecular ions, as well as from the M − 15 ions, gives peaks lacking the chlorine isotope pattern in all observed spectra.

The unsymmetrical epoxides can give two different derivatives. This is observed for (1,2-epoxyethyl) benzene (styrene oxide) and 1,2-epoxydecane while 1,2-epoxy-3,3,3-trichloropropane gives just one product. The mass spectrum of the latter derivative displays prominent peaks at M − 15, M − CH_2Cl and M − CCl_3, all representing α-cleavage in the structure where the epoxide ring has opened in the normal way at the primary carbon atom (Fig. 2). For (1,2-epoxyethyl) benzene, as well as 1,2-epoxydecane, two products are observed in the gas chromatogram after silylation. Mass spectra were recorded for both compounds in each case, and the spectra displayed molecular ions at the same mass number but different fragmentation patterns indicating that the two components were isomers. The more abundant isomers gave mass spectra with strong peaks at M − 15 and M − CH_2Cl which corresponds to α-cleavage in the silyl ether originating from epoxide-ring opening at the primary carbon atom (Fig. 2). The mass spectra of the less abundant isomers are dominated by strong peaks at 103 mu. This ion is formed by α-cleavage to give $CH_2OSi(CH_3)_3^+$ which is typical of the mass spectra of the isomers formed by abnormal epoxide-ring opening at the secondary carbon atom. The exact mass of the strong M − 15 peak in the mass spectrum of silylated 1,2-epoxydecane was determined to be 249.1447 while the calculated value for $C_{13}H_{29}ClOSi - CH_3 = C_{12}H_{26}ClOSi$ is 249.1441. This together with the fragmentation patterns in the recorded spectra proves that the reaction taking place when the epoxides are treated with trimethylchlorosilane is an addition over the epoxy function.

Experimental. GLC analyses were performed on a Perkin-Elmer Modell 900 Gas Chromatograph equipped with 2 m × 3 mm steel columns with 3 % SE-30 on 80/110 mesh Chromosorb W AW DMCS as the stationary phase. Combined GLC-MS measurements were performed on an LKB 9000 instrument and exact mass determinations were obtained with an AEI 902 instrument at the Department of Medical Biochemistry, University of Göteborg. All the investigated epoxides except 1,2-epoxydecane were commercially available and were used without further purification after having been found pure by GLC analysis. 1,2-Epoxydecane was prepared using the method of Prileschajev.[8]

Trisil®, trimethylchlorosilane, and hexamethyldisilazane were purchased from Pierce Chemical Company, Rockford, Illinois.

Preparation of derivatives The epoxides were derivatised in the following way with pure trimethylchlorosilane or Trisil® (trimethylchlorosilane-hexamethyldisilazane in pyridine). Mixtures of 25 μl of the epoxide and 0.2 ml of trimethylchlorosilane (or 1 ml of Trisil®) were kept in sealed glass tubes at 100 °C for different time intervals. The tube was then cooled and opened, and the reaction mixture was transferred to a micro round flask and the volatile components were removed under vacuum. Dry pentane (2 ml) was added and solid by-products were separated by centrifugation. The clear pentane solution was concentrated under vacuum and the oily residue was directly used for GlC and MS analysis.

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Nitrodiphenyl Ethers from 1,2- or 1,4-Dinitrobenzenes and Copper in Pyridine

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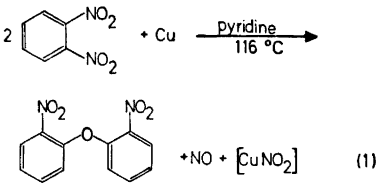
Copper and copper salts have been used in a variety of organic reactions, including Ullmann's biaryl and diaryl ether syntheses[1-3] and various copper-promoted arylations.[3,4]

Symmetrical dinitrophenyl ethers are formed when 1,2- or 1,4-dinitrobenzenes are treated with copper in boiling pyridine [Reaction (1)].



(1)

1,2-Dinitrobenzene gave bis(2-nitrophenyl) ether (*1*) (65 %), and 1,4-dinitrobenzene bis(4-nitrophenyl) ether (77 %). Some 2-nitrophenol (*2*) or 4-nitrophenol, respectively, was also obtained.

Various copper compounds were tried (Table 1). Copper(0) was found to be most suitable. Both copper and copper(I) oxide dissolved during the reactions. The molar ratio of copper to 1,2-dinitrobenzene in reaction 1 was varied from 0.5 to 5. The yields of *1* and *2* were essentially the same in all cases. We believe the stoichiometric ratio to be 0.5 (*cf.* Scheme 1). A lower ratio gave lower yields and longer reaction times. Reaction 1 can be carried out in the presence of glacial acetic acid or water with essentially unchanged yields.

Table 1. Yields of products from reaction 1 with different copper sources.

Copper compound	*1*/%	*2*/%
Cu	65	19
Cu_2O	25	38
CuO	0	9
None	0	8

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Preparation of derivatives The epoxides were derivatised in the following way with pure trimethylchlorosilane or Trisil® (trimethylchlorosilane-hexamethyldisilazane in pyridine). Mixtures of 25 μl of the epoxide and 0.2 ml of trimethylchlorosilane (or 1 ml of Trisil®) were kept in sealed glass tubes at 100 °C for different time intervals. The tube was then cooled and opened, and the reaction mixture was transferred to a micro round flask and the volatile components were removed under vacuum. Dry pentane (2 ml) was added and solid by-products were separated by centrifugation. The clear pentane solution was concentrated under vacuum and the oily residue was directly used for GlC and MS analysis.

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Nitrodiphenyl Ethers from 1,2- or 1,4-Dinitrobenzenes and Copper in Pyridine

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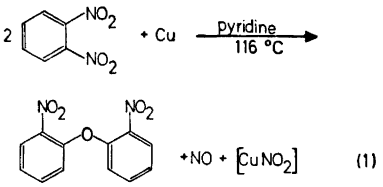
Copper and copper salts have been used in a variety of organic reactions, including Ullmann's biaryl and diaryl ether syntheses[1-3] and various copper-promoted arylations.[3,4]

Symmetrical dinitrophenyl ethers are formed when 1,2- or 1,4-dinitrobenzenes are treated with copper in boiling pyridine [Reaction (1)].



(1)

1,2-Dinitrobenzene gave bis(2-nitrophenyl) ether (*1*) (65 %), and 1,4-dinitrobenzene bis(4-nitrophenyl) ether (77 %). Some 2-nitrophenol (*2*) or 4-nitrophenol, respectively, was also obtained.

Various copper compounds were tried (Table 1). Copper(0) was found to be most suitable. Both copper and copper(I) oxide dissolved during the reactions. The molar ratio of copper to 1,2-dinitrobenzene in reaction 1 was varied from 0.5 to 5. The yields of *1* and *2* were essentially the same in all cases. We believe the stoichiometric ratio to be 0.5 (*cf.* Scheme 1). A lower ratio gave lower yields and longer reaction times. Reaction 1 can be carried out in the presence of glacial acetic acid or water with essentially unchanged yields.

Table 1. Yields of products from reaction 1 with different copper sources.

Copper compound	*1*/%	*2*/%
Cu	65	19
Cu_2O	25	38
CuO	0	9
None	0	8

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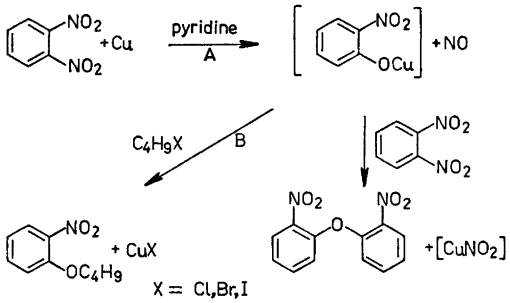
Apparently, pyridine is essential for the reaction. No ether could be detected when the reaction was carried out in quinoline or diglyme or without a solvent. In the presence of triphenylphosphine the yield of *1* was reduced to 10 %. Triphenylphosphine oxide was also formed.

When 1 mol of 1,2-dinitrobenzene reacted with copper in boiling pyridine, about 0.5 mol of nitric oxide (NO) was evolved. This led us to consider that the reaction proceeds *via* an intermediate resembling 2-nitrophenoxycopper(I), *cf.* Scheme 1. A pyridine solution of 2-nitrophenoxycopper(I) was therefore prepared from methylcopper(I) and 2-nitrophenol according to the method given by Whitesides *et al.*[5] To this solution 1,2-dinitrobenzene was added and the mixture heated to 116 °C for 2 h. A high yield of *1* was obtained.



Scheme 1.

Samples were withdrawn at intervals from a boiling mixture of pyridine, copper and 1,2-dinitrobenzene and hydrolysed and analysed by GLC. The formation of *2*, which might be a hydrolytic product of 2-nitrophenoxycopper(I), starts earlier than the formation of *1*. However, some *2* could arise from the attack of nitrite ion on 1,2-dinitrobenzene.[6] Effecting reaction 1 in the presence of sodium nitrite doubled the yield of *2*.

Butyl 2-nitrophenyl ether, *3*, was formed together with *1* when the reaction was carried out in the presence of 1-halobutanes (see Table 2). The yields of *3* do not differ much for the three butyl halides but the rate of disappearance of 1,2-dinitrobenzene varies with the halogen (I ~ Br > Cl). This indicates that paths A and B in Scheme 1 may not be mutually independent and that Scheme 1 may give a simplified picture of the reaction paths. No *3* was observed in the absence of copper, or after an attempted reaction of 2-nitrophenol and butyl bromide in pyridine.

An intermediate such as 2-nitrophenoxycopper(I) may be important in the reactions leading to both symmetrical and unsymmetrical ethers (*cf.* Scheme 1). Copper(I) phenolates react with organic halides in diglyme at 120 °C to give moderate yields of ethers[5,7]. It has been suggested that aryloxycopper(I) compounds are intermediates in the Ullman diaryl ether synthesis[8] and in oxidative couplings of phenols catalysed by copper(I) chloride.[9] The first step on the route from 1,2-dinitrobenzene and copper to an intermediate resembling 2-nitrophenoxycopper(I) might be the formation of a dinitrophenyl anion radical and copper(I), *cf.* Saegusa *et al.*[10] Once formed, the phenoxycopper(I) complex attacks a molecule of dinitrobenzene. One of the nitro groups then leaves as a nitrite anion. The reaction between 1,2- or 1,4-dinitrobenzenes and potassium cyanide gives a small yield of symmetrical

Table 2. Yields of products from reactions between 1,2-dinitrobenzene, copper and 1-halobutanes (C_4H_9X) in pyridine.

X	*3*	*1*	*2*	$t_{\frac{1}{2}}(h)$[a]
I	16	15	47	0.4
Br	22	17	41	0.6
Cl	23	31	31	5
Br	50(37)[b]	2	7	

[a] $t_{\frac{1}{2}}$ = time for 50 % reaction; see Experimental. [b] Isolated yield. Three mol of butyl bromide.

Table 3. Isolated yields of products from reactions of dinitrobenzenes, copper and phenols in boiling pyridine. A, unsymmetrical diaryl ether; B, symmetrical diaryl ether; C, 2-nitrophenol.

Substituent on the phenol	A	B	C
1,2-Dinitrobenzene			
None	67	0.5	22
4-Methyl-2-nitro-	63	6[a]	12
4-Nitro-	73	2[a]	15
2,4,5-Trinitro-	0	62	
4-Bromo-	74	0	18
2-Bromo-	72	0.5	18
4-Iodo-	92[b]	0	2
1,4-Dinitrobenzene			
None	86	0	0
4-Iodo-	87[c]	0	0

[a] Isolated as a mixture of diphenyl ethers. Relative amounts determined by GLC. [b] 75 °C, 2.3 h, 20 % excess iodophenol. [c] 80 °C 2.5 h, 20 % excess iodophenol.

diphenyl ethers,[11] perhaps by a related reaction path.

Unsymmetrical nitrophenyl ethers were formed when 1,2- or 1,4-dinitrobenzenes were treated with copper and phenols in pyridine. The results are summarized in Table 3. Apparently, reactions with phenols proceed faster than the one leading to symmetrical ethers. About ten times more of the unsymmetrical product was obtained from 1,2-dinitrobenzene and 2-nitro-4-methylphenol, as shown in Table 3. More strongly nucleophilic phenols yielded larger amounts of unsymmetrical diphenyl ethers. In the absence of copper, about 9 % of 2,4'-dinitrophenyl ether was formed from 1,2-dinitrobenzene and 4-nitrophenol after prolonged boiling with pyridine.

The reactions given in Table 3 could be of synthetic use since they occur under mild conditions and permit the presence of bromo and iodo substituents in the substrates.

Experimental. A finely powdered copper of electrolytic purity was used. Products were identified by spectral methods and melting points. A general procedure for similar reactions has been reported earlier.[4]

Bis(2-nitrophenyl) ether (1). A mixture of 1,2-dinitrobenzene (5 mmol), copper (25 – 2.5 mmol) and pyridine (25 ml) was boiled (116 °C) under nitrogen for 2 h to yield *1*, 0.42 g (65 %) and 2-nitrophenol, 0.13 g (19 %).

The gas evolved from the reaction mixture was collected (56 ml corresponding to a molar ratio of 0.47 between gas and 1,2-dinitrobenzene) and injected into a GLC-MS equipped with a Linde Molecular Sieve 5A column (80 °C). The MS (70 eV) showed the gas to be almost pure nitric oxide (except for nitrogen).

Reaction with 2-nitrophenoxycopper(I). Dry pyridine (4 ml) was added to methylcopper(I)[5] (about 1.2 mmol) under N_2 and at 0 °C followed by a solution of 2-nitrophenol (1.2 mmol) in pyridine (2 ml). The solution became reddish brown and gas evolved. After 3 h at 0 °C 1,2-dinitrobenzene (1,2 mmol) in a little dry pyridine was injected through a septum and the solution was heated to 116 °C for 2 h. After workup[4] 2-nitrobiphenyl was added as an internal standard. The yield of *1* (76 %, based on 2-nitrophenol or 1,2-dinitrobenzene) was determined by GLC.

Bis(4-nitrophenyl) ether. 1,4-Dinitrobenzene (5 mmol) and copper (5 mmol) were boiled in pyridine (25 ml) for 10 h. Yield after recrystallisation from ethanol 0.50 g (77 %). 4-Nitrophenol 0.02 g (3 %) was also isolated.

Reactions with 1-halobutanes. A mixture of distilled 1-halobutane (6 mmol), 1,2-dinitrobenzene (5 mmol), copper (25 mmol) and pyridine (20 ml) was stirred under N_2 at 75 °C 2-Nitrobiphenyl was added as an internal standard and samples withdrawn at intervals were analysed by GLC. The results were plotted and times when 50 % of the 1,2-dinitrobenzene remained were determined.

Reactions of 1,2- and 1,4-dinitrobenzene with phenols. A dinitrobenzene (5 mmol), copper (10 mmol) and a phenol (5 mmol) were boiled in pyridine (25 ml) for 0.5 – 2 h. Yields based on dinitrobenzene were determined by GLC using an internal standard and have been optimized only in the reactions with 4-iodophenol. Most of the diphenyl ethers were purified by dissolving in dichloromethane or dichloromethane:tetrachloromethane 1:1 and filtering under suction through a 6 × 2.5 cm column of silica gel moistened with solvent. Fresh solvent was added until the diphenyl ether had passed into the suction flask. Evaporation of the solvent gave the diphenyl ether, now free of more polar impurities. Since the reactions were terminated when no dinitrobenzenes remained, this purification was usually adequate.

2-Nitro-4'-iododiphenyl ether from 1,2-dinitrobenzene (5 mmol) and 4-iodophenol (6 mmol) at 75 °C. Reaction time 2.3 h. Yield 1.57 g, 92 %. A higher reaction temperature or a longer time results in the formation of nitrobenzene and 2-nitrodiphenyl ether together with the title compound. Excess 4-iodophenol is needed as otherwise a little *1* will be formed.

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Palladium(II) Catalysed Aromatic Acetoxylation. IX. * An Insoluble Poly-4-vinylpyridinepalladium(II) Complex as Catalyst for the Acetoxylation of Aromatic Compounds

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The palladium(II) catalyzed acetoxylation of aromatic compounds is of synthetic interest due to its anomalous *meta* selectivity.[1-4] The problem of catalyst separation and recovery, common to many metal ion catalyzed reactions in the homogeneous phase, however, limits its usefulness to small-scale laboratory runs.

One way to achieve a better catalyst function is to anchor the metal complex to an insoluble polymeric support.[5,6] We now report results on the acetoxylation of aromatic compounds by a Pd(II) complex with poly-4-vinylpyridine occupying two of the available ligand sites (Fig. 1). Nitrate ion is used as a cooxidant and oxygen as the primary oxidant. The reaction is thus analogous to the previously reported homogeneous acetoxylation with amine acetatonitratopalladium(II) complexes as catalysts.[1]

The results are shown in Table 1. The *meta* selectivity was retained with the heterogeneous catalyst and the reaction gave yields of the same magnitude as the corresponding homogeneous process using acetatonitrato-(2,2′-bipyridine)palladium(II) as a catalyst. The yield increases to a limiting value with increasing amounts of catalyst in the system. Compounds with activating substituents gave nitration products predominantly, as was also found in the homogenous reaction.[7,8] Experiment No. 9, in which a homogeneous solution obtained by extracting the polymer-anchored complex was used as the acetoxylation medium, shows that the experiments with the supported complex must have both a homogeneous and a heterogeneous component. The polymer complex could be used several times with little loss of catalytic activity in each run.

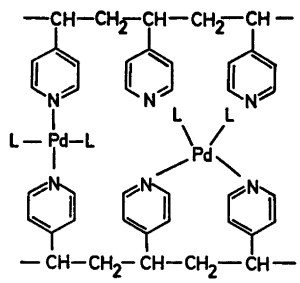

Fig. 1. Poly-4-vinylpyridinepalladium(II) complex.

The reaction rate was determined as a function of total volume, V, of the reacting system in order to confirm the heterogeneity of the process. For a heterogeneous system eqn. 1 can be applied,[9] in which S is the area of the catalyst interface (assumed to be constant within a given batch of catalyst), c concentrations of dissolved reaction components, and α and

* Part VIII, see Ref. 4.
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Table 1. Acetoxylation of some aromatic compounds catalyzed by polymer-anchored Pd(II) complex.[a]

Compound (Exp. No.)	Catalyst	Acetoxy derivatives Yield, %[b]	Isomer distribution *ortho*	*meta*	*para*	Byproducts, %[b]
Anisole (1)[c]	Het.	24	21	13	66	Nitroanisoles, 76
Anisole (2)[d]	Hom.	low	10	41	49	Nitroanisoles, main products
Toluene (3)[e]	Het.	60	43	28	29	Nitrotoluenes, 44
p-Xylene (4)	Het.	33[f]	—	—	—	Different products
Chlorobenzene (5)[d]	Hom.	70	2	60	38	
Chlorobenzene (6)	Het.	25	19	42	39	
Chlorobenzene (7)	Het.	38	19	42	39	
Chlorobenzene (8)	Het.	44	19	42	39	
Chlorobenzene (9)	Hom.	13	19	42	39	

[a] Reaction conditions, see the Experimental part. [b] GLC yield based on Pd(II). [c] Trace of acetoxymethyl phenyl ether. [d] See Ref. 1. [e] Nuclear 90 %, α-acetate 10 %. [f] α-Acetate.

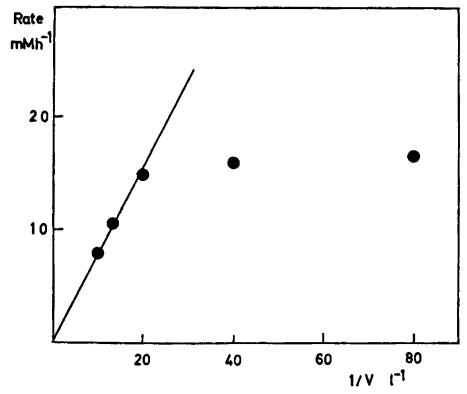

Fig. 2. Rate of reaction *versus* $1/V$.

β constants. For constant S and c, eqn. 1 transforms into eqn. 2. Fig. 2 shows a plot of rate *vs.* $1/V$, which at least for high total

$$-dc/dt = k'(S/V)c_a^{\alpha}c_b^{\beta} \quad \text{eqn. (1)}$$

$$-dc/dt = k''(1/V) \quad \text{eqn (2)}$$

volumes is a straight line through the coordinates origin. The large deviations at low V values are presumably due to the fact that further oxidation of products (formation of diacetates) takes place in these experiments. Thus we have shown in this report that the Pd(II) catalyzed acetoxylation of aromatic compounds is possible with polymer-supported Pd(II) catalysts.

Experimental. Most chemicals used were of the quality and origin described in earlier work.[1-3] Solutions of nitrogen dioxide were prepared by heating a known amount of lead(II) nitrate and dissolving the gas in glacial acetic acid. 4-Vinylpyridine was purchased from Aldrich Europe and polymerized according to a literature method.[10]

Preparation of the polymer-anchored complex. Poly-4-vinylpyridine (2.0 mmol based on monomer) was dissolved in ethanol (25 ml) and mixed with a benzene (25 ml) solution of palladium(II) acetate (1.0 mmol). The precipitate formed was stirred for 15 min and then collected by filtration and washed with ether.

Acetoxylation procedure for exps. 1–9. A mixture of substrate (10 mmol), NO_2 (10 mmol), $Pd(OAc)_2$-poly-4-vinylpyridine (1 mmol) in glacial acetic acid (50 ml) was stirred for 4 h at 110 °C under oxygen atmosphere. In exps. 5 and 6, 2 and 4 mmol, respectively, were used of Pd(II)-PVP. In exp. 7, 50 ml catalyst solution was prepared by extraction of 1 mmol of Pd(II)-PVP in 50 ml of glacial acetic acid at 110 °C for 2 h.

Kinetic experiments. A 0.2 M glacial acetic acid solution (V ml) of chlorobenzene was stirred with NO_2 (10 mmol), $Pd(OAc)_2$-poly-4-vinylpyridine (1 mmol) for 4 h at 110 °C under oxygen atmosphere. Samples were taken at 30 min intervals and analyzed by GLC. Yield and isomer distribution were determined using a Varian 1400 gas chromatograph, equipped with a disc integrator (2 m × 3 mm neopentylglycol succinate, 5 % on Chromosorb W column). The yield was determined using an internal standard calibrated against authentic samples.

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The Crystal and Molecular Structure of 1,4-Dihydro-2,3-quinoxalinedione

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The crystal and molecular structure of 1,4-dihydro-2,3-quinoxalinedione, $C_8H_6N_2O_2$, has been determined using single crystal X-ray diffractometer data. The crystals are monoclinic, $P2_1/n$, with a unit cell $a=8.068(1)$, $b=4.275(1)$, $c=20.911(2)$ Å, $\beta=100.42(1)°$, $Z=4$. The final R-factor was 0.043 for 1028 observed reflexions. In the crystal the molecules exist as diones connected in two dimensions *via* N−H···O hydrogen bonds.

In solution 1,4-dihydro-2,3-quinoxalinedione is in equilibrium with the tautomers with one and two hydroxyl groups. This amide-iminol tautomerism involves a fast hydrogen transfer between nitrogen and oxygen. It has generally been found that compounds capable of this tautomerism usually exist predominantly in the amide form. This is so even in cases where the iminol has larger aromatic stabilization, *e.g.* 2-pyridone.[1,2] Kvick [3] has recently studied the crystal structures of some substituted 2-pyridones and found the iminol in two cases, *viz.* 6-chloro-2-hydroxypyridine and 6-bromo-2-hydroxypyridine. Cheeseman *et al.*[4,5] have studied the UV- and IR-spectra of the title compound and its *O*- and *N*-methyl derivatives. They found that the dione is the predominant tautomer both as solid and in chloroform solution. This X-ray crystallographic study confirms their results and gives a more detailed knowledge of the bonding situation.

EXPERIMENTAL

Commercial 2,3-dihydroxyquinoxaline (Fluka) in the form of long needles was recrystallized from ethanol. The crystals obtained were colourless prisms. Weissenberg photographs showed monoclinic symmetry and, from systematic extinctions, space group $P2_1/c$, or the equivalent $P2_1/n$, could be deduced. A crystal of approximate dimensions $0.12\times0.20\times0.05$ mm^3 was mounted on an automatic Enraf-Nonius CAD4 diffractometer with Cu$K\alpha$ radiation. The unit-cell dimensions were determined from the θ-values of 36 intensities measured in the range $\theta=38$ to 48°, using $\lambda(\mathrm{Cu}K\alpha_1)=1.54051$ Å.

Three-dimensional intensity data were collected at 22 °C by the ω-2θ scan technique with $\Delta\omega=0.8°+0.5°\tan\theta$. The background was measured with stationary crystal-stationary counter for 1/4 of the time spent on the scan. Double scans were made for each reflexion for a net count of 2000 within a maximum time of 4 minutes. Two standard reflexions were measured at regular intervals. Their intensities showed no systematic variation. Data were collected to $\theta=70°$. After data reduction, including Lp-correction but no absorption correction, 1028 observed structure amplitudes were left. 419 intensities with $I<3\sigma(I)$, where $\sigma(I)$ was based on counting statistics, were considered unobserved.

Phases for the structure amplitudes were determined by the symbolic addition method using program GAASA.[6] The coordinates of all atoms except the hydrogens were found from a subsequent E-map. Minimizing $\sum w_i(|F_o|-|F_c|)^2$, the positional parameters and anisotropic temperature factors together with an overall scale factor were refined. The weights were calculated from $w_i^{-1}=\sigma^2(|F_o|^2)/4|F_o|^2+0.001|F_o|^2$. The resulting R-factors were $R=0.072$ and $R_w=0.115$. A difference map now revealed all hydrogen atom positions. Including them with isotropic temperature factors in a refinement resulted in $R=0.043$ and $R_w=0.067$. The form factors were those of Stewart, Davidson and Simpson [8] for hydrogen and of Hansson, Herman, Lea and Skillman [7] for the other atoms. The final parameters are listed in Table 1. Lists of observed and calculated structure amplitudes can be obtained on request.

Table 1. Positional and thermal parameters. The form of the anisotropic temperature factor is exp $[-(h^2\beta_{11}+k^2\beta_{22}+l^2\beta_{33}+2\ hk\beta_{12}+2\ hl\beta_{13}+2\ kl\beta_{23})]$. The β_{ij}-values have been multiplied by 10^5. Standard deviations are given in parentheses. R_1, R_2 and R_3 are the root-mean-square displacements (Å) along the principal axes of the thermal vibration ellipsoids.

Atom	x	y	z	B (Å^2)
O(1)	−0.17951(18)	1.03404(33)	0.43634(6)	For β_{ij}, see below
O(2)	−0.30928(17)	0.81867(36)	0.31338(6)	
C(1)	−0.10232(23)	0.83765(42)	0.41013(8)	
C(2)	−0.17287(23)	0.72138(43)	0.34182(8)	
N(1)	0.04666(19)	0.71670(35)	0.43861(7)	
N(2)	−0.07729(20)	0.51915(35)	0.31589(7)	
C(3)	0.14291(23)	0.50837(39)	0.40948(8)	
C(4)	0.08028(23)	0.40821(39)	0.34638(8)	
C(5)	0.29926(25)	0.40414(47)	0.44148(9)	
C(6)	0.17454(27)	0.20471(47)	0.31528(9)	
C(7)	0.39322(28)	0.20719(49)	0.41023(11)	
C(8)	0.33074(29)	0.10932(51)	0.34705(11)	
H(N1)	0.0875(34)	0.7730(50)	0.4802(12)	4.0(5)
H(N2)	−0.1133(32)	0.4453(54)	0.2767(12)	3.5(5)
H(C5)	0.3385(28)	0.4803(49)	0.4872(11)	3.0(5)
H(C6)	0.1254(30)	0.1307(47)	0.2700(12)	3.6(5)
H(C7)	0.5032(38)	0.1363(50)	0.4302(13)	4.3(5)
H(C8)	0.3974(38)	−0.0437(59)	0.3262(14)	5.4(6)

Atom	β_{11}	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}	R_1	R_2	R_3
O(1)	1197(24)	6290(97)	148(3)	756(38)	−7(7)	−249(14)	0.267	0.166	0.177
O(2)	1104(25)	7180(108)	129(3)	769(37)	−46(7)	51(13)	0.268	0.145	0.201
C(1)	927(31)	4358(109)	110(4)	63(44)	24(9)	9(16)	0.201	0.151	0.177
C(2)	912(29)	4269(111)	103(4)	−51(44)	9(8)	71(16)	0.201	0.144	0.176
N(1)	995(27)	4217(95)	104(4)	194(37)	−39(8)	−92(13)	0.210	0.137	0.182
N(2)	1015(27)	4399(94)	94(3)	−22(39)	−23(7)	−56(14)	0.203	0.134	0.191
C(3)	933(29)	3289(97)	133(4)	−51(43)	11(9)	−31(16)	0.186	0.158	0.176
C(4)	869(29)	3459(101)	125(4)	−116(42)	15(8)	28(15)	0.186	0.155	0.171
C(5)	1012(34)	4763(121)	180(5)	104(48)	−74(10)	−92(19)	0.231	0.156	0.204
C(6)	1288(36)	4344(117)	153(5)	−27(49)	89(10)	−90(17)	0.205	0.176	0.202
C(7)	1007(36)	5042(129)	258(6)	344(52)	−34(12)	−91(22)	0.254	0.169	0.212
C(8)	1247(38)	4907(130)	240(6)	422(53)	147(12)	−112(21)	0.234	0.178	0.222

CRYSTAL DATA

1,4-Dihydro-2,3-quinoxalinedione, $C_8H_6N_2O_2$
F.W. 162.2 g mol^{-1}

Monoclinic, space group $P2_1/n$

$a = 8.068(1)$, $b = 4.275(1)$, $c = 20.911(2)$ Å, $\beta = 100.42(1)°$

$V = 709.3$ Å^3

$Z = 4$, $F(000) = 336$

$D_x = 1.518$ g cm^{-3}

$\mu(\mathrm{Cu}K\alpha) = 8.4$ cm^{-1}

DISCUSSION

Bond distances and angles for a molecule of 1,4-dihydro-2,3-quinoxalinedione in the crystal are given in Fig. 1, which also shows the labelling of the atoms. The question of which tautomer is predominant in the solid state is clearly answered by the C−O distances of 1.23 Å and the localization of the hydrogen atoms bonded to nitrogen. Obviously the molecule is a dione. There is no evidence that more than one tautomer is present in the crystal.

Chemically equivalent distances and angles are very similar and so are the thermal vibrations of corresponding atoms (Table 1). In the

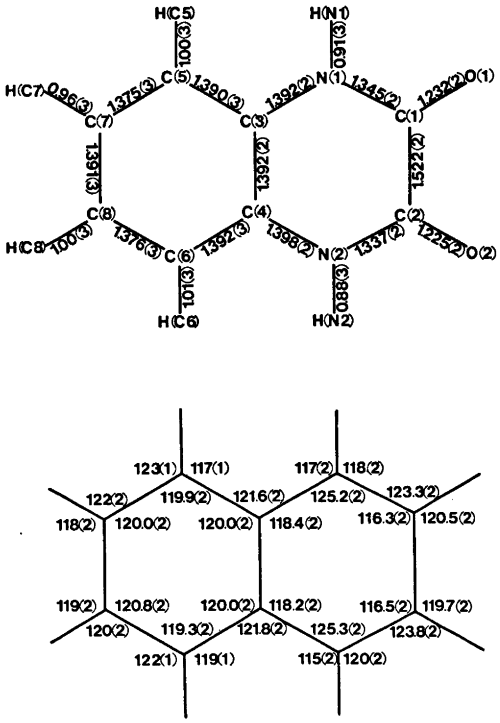

Fig. 1. Bond distances (Å) and angles (°) in 1,4-dihydro-2,3-quinoxalinedione.

Table 2. Deviations (Å) from a least-squares plane through the molecule. All atoms except hydrogens were included and given equal weight in the calculation.

Atom	Deviation (Å)	Atom	Deviation (Å)
O(1)	0.070	C(6)	0.011
O(2)	0.004	C(7)	0.024
C(1)	0.004	C(8)	0.050
C(2)	−0.015	H(N1)	−0.085
N(1)	−0.040	H(N2)	−0.040
N(2)	−0.025	H(C5)	−0.055
C(3)	−0.036	H(C6)	−0.001
C(4)	−0.019	H(C7)	0.062
C(5)	−0.028	H(C8)	0.030

crystalline form the molecule is not completely planar, *cf.* Table 2. Atom O(1) has the largest deviation, 0.07 Å, from the least-squares plane fitted to all non-hydrogen atoms. The conformation can be described as a very flattened boat as is the case for one of the two independent molecules in the crystal structure of 2,3-*t*-butyl-quinoxaline.[9] Probably the molecule is planar, of symmetry C_{2v}, in solution and distorted by intermolecular forces in the solid state.

Tinland [10] has calculated bond distances in quinoxaline using a modified semi-empirical Pariser-Parr-Pople method. His values are in

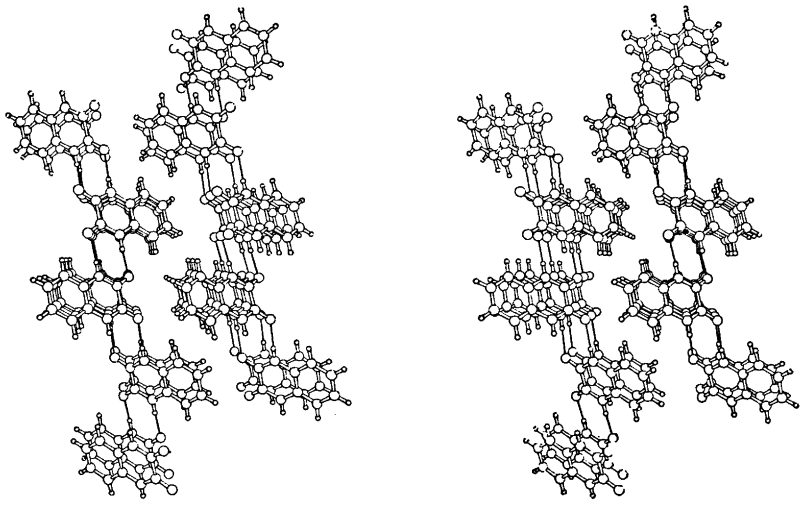

Fig. 2. A stereoview down [010] of the crystal structure showing the packing of the molecules and the hydrogen bonding. Hydrogen bonds are drawn as single lines.

Table 3. Intermolecular distances and angles describing the hydrogen bonding.

Atoms	Distance (Å)	Atoms	Angle (°)
O(1)–N(1)	2.847(2)	C(1)–O(1)–H(N1)	123.0(8)
O(1)–H(N1)	1.95(2)	O(1)–H(N1)–N(1)	170(2)
O(2)–N(2)	2.826(2)	C(2)–O(2)–H(N2)	131.7(8)
O(2)–H(N2)	1.95(2)	O(2)–H(N2)–N(2)	175(2)

good agreement with the bond distances found in 2,3-*t*-butyl-quinoxaline [9] except the distance C(1)–C(2) (numbering according to Fig. 1), which is 0.06 Å longer, possibly because of the bulky *t*-butyl groups. Tinland's calculation predicted a shortening of 0.02 Å in the distances C(5)–C(7) and C(6)–C(8) and a similar elongation of the other bonds in that ring relative to the benzene carbon-carbon distances. In 1,4-dihydro-2,3-quinoxalinedione the shortening of the two bonds prevails while the other distances in the aromatic ring, around 1.39 Å, are unaffected. The distances in the hetero atom ring (*cf.* Fig. 1) are approximately 1.52, 1.34 and 1.40 Å compared to 1.48, 1.32 and 1.36 Å, respectively, in 2,3-*t*-butyl-quinoxaline. The distances C(3)–N(1) and C(4)–N(2) are only slightly shorter than the C–N distances in 1,2-diaminobenzene (1.41 Å).[11]

The diamide part of the molecule has dimensions very similar to those of oxamide, $(NH_2CO)_2$, with the distances C–C 1.542(6), C–O 1.243(4), and C–N 1.315(4) Å.[12] Oxamide is planar and in the *trans* conformation in the crystals. The distance C(1)–C(2) of 1.522(2) Å in the present compound indicates essentially σ-bonding. On the other hand there is a considerable double bond character in the C–N bonds just as in oxamide. This π-overlap is only possible with an approximately planar hetero atom ring.

Fig. 2 shows the packing of the molecules. The only short intermolecular contacts are *via* hydrogen bonds N–H···O, *cf.* Table 3. One of the amide parts in a molecule is hydrogen bonded over an inversion centre to another molecule. The other amide part is hydrogen bonded to two other molecules along a 2_1-axis. The hydrogen bonds connect the molecules to infinite zig-zag layers, parallel to [010], which are stacked with normal van der Waals contacts.

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Partition of Cell Particles in Three-phase Systems

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Three-phase systems consisting of water and three polymers, dextran, Ficoll and poly(ethylene glycol), have been used for partition of some cell particles. Particles of biological origin are stable in these systems due to the high water content, 60 – 75 %. The partition behaviour of a particle is strongly affected by introducing charged polymers in the three-phase system. By varying the amount of charged polymer the affinity of particles for the different phases can successively be changed. In this way *Chlorella pyrenoidosa*, *Saccharomyces cerevisiae* and subchloroplast particles have each been shown to consist of more than one fraction. These fractions can be separated from each other by a single partition step. By introducing hydrophobic groups to one of the polymers, the partition of cell particles can in some cases be affected. Subchloroplast particles and mitochondria have been resolved into several fractions, differing in hydrophobicity, with such three-phase systems.

One of the methods used for studying the composition of biological materials is liquid-liquid extraction with biphasic systems obtained by mixing aqueous solutions of two suitable polymers.[1] Such dextran-poly(ethylene glycol)-water two-phase systems have been used for separation of biological macromolecules and cell particles.[1,2] The homogenity of cell particles such as chloroplasts,[3,4] red blood cells,[5] mitochondria,[6] and *Chlorella pyrenoidosa* [7] has been studied mostly by the technique of counter-current distribution.[1] These investigations have shown that such particles consist of several subpopulations.

The partition of a substance between the two phases depends on pH as well as type and concentration of salt included in the system.[8,9] The partition can also be adjusted by using a polymer, which bears a few covalently bound groups, *e.g.* trimethylamino-poly(ethylene glycol), poly(ethylene glycol)-sulfonate or palmitoyl-poly(ethylene glycol).[10,11] The two former polymers are ionic while the latter one is hydrophobic.

By mixing aqueous solutions of three different polymers a system with three phases can be obtained.[1,12,13] The study of several proteins in these systems has shown that due to the high water content they can partition between all the phases without losing their biological activity. The three isoenzymes of enolase have by this method been enriched each in one of the phases in a single partition step.[13]

The three-phase systems ought to have advantages compared to two-phase systems also for the analysis of heterogeneous populations of particles. In this work the partition of some particles of biological origin such as cells, chloroplast fragments, and mitochondria has been investigated in three-phase systems consisting of water, dextran, Ficoll, and poly(ethylene glycol).

EXPERIMENTAL

Materials

Dextran T 500, batch No. 5996 ($M_w = 5 \times 10^5$) and Ficoll, batch No. 4720 ($M_w = 4 \times 10^5$) were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. M_w = weight average molecular weight.

Poly(ethylene glycol) (PEG) with $M_n = 4000$ and $M_n = 6000$ were obtained from Union Carbide, New York, as Carbowax. M_n = number average molecular weight.

Trimethylamino-PEG (TMA-PEG) and PEG-sulfonate (S-PEG) prepared from Carbowax 4000 [14] were gifts from Dr. G. Johansson. Deoxycholate-PEG (DC-PEG), the ester between PEG and deoxycholic acid, was prepared from Carbowax 6000 by Dr. G. Johansson (internal communication).

Microorganisms. *Sarcina lutea* was cultivated in a medium containing Nutrient Broth, pH 7.3 and 0.5 % sodium chloride. *Chorella pyrenoidosa* was cultivated as described by Albertsson and Baird.[15] Bakers' yeast, *Saccharomyces cerevisiae*, standard yeast and special yeast, was obtained from Jästbolaget, Sollentuna, Sweden.

Before partition all these particles were washed twice by centrifugation and resuspension in 0.010 M potassium phosphate buffer, pH 6.8.

Subchloroplast particles. Class II chloroplasts were fragmentated just before use with the help of a Yeda press by a method described by Andersson and Åkerlund.[16]

Mitochondria from rat liver were prepared according to Ericson [6] and the mitochondria were kept in a 0.32 M sucrose solution.

All further chemicals were of analytical grade and the water was double distilled in a quartz apparatus.

Methods

The three-phase system. The three-phase system used contained 7 % dextran, 12 % Ficoll, and 12 % PEG with $M_n = 4000$ (including substituted PEG) and 0.5 mM potassium phosphate buffer pH 6.8.

4.00 g systems were prepared by mixing sample, water solutions of dextran 20 %, Ficoll 40 % and/or a solution containing both charged and uncharged PEG, the total amount of PEG being 40 %. The last solution was prepared from water solutions of PEG and substituted PEG, both 40 %. The solutions were then mixed in proportions required to yield the desired amount of charged PEG in the system. To minimize the variations in composition from time to time the highest concentration of charged PEG in the system was chosen as the concentration of charged PEG in the stock solution. For example, if the maximal concentration of TMA-PEG to be used in the system was 20 %, the solutions of TMA-PEG and PEG, both 40 %, were mixed in the proportion of 1:4. Thus a series of systems with the same polymer concentration but differing in percentage of substituted PEG were obtained.

All concentrations are given in per cent weight per weight. The systems were well mixed by turning them upside down 40 times and allowed to settle at room temperature for 30 min. After that, they were mixed again and now allowed to settle for 90 min.

Each of the three phases is enriched in one of the polymers, the bottom phase in dextran, the middle phase in Ficoll and the top phase in PEG. The ratio between the volumes of the top phase, the middle phase and the bottom phase in these systems is 1.60:1.50:0.70. The composition of each phase (Table 1) was determined by a method described in a previous paper.[13]

Determination of the concentration of cells in each phase. After separation of the three phases 0.200 ml from the top phase and 0.200 ml from the middle phase were withdrawn and each aliquot was diluted with 1.50 ml of distilled water. The material at the interface between the top phase and the middle phase ($interface_{TM}$) was collected in a test tube and 1.50 ml of distilled water was added. The high viscosity of the bottom phase makes withdrawal from this phase difficult. After removing the excess of the two upper phases the bottom phase was diluted with 5.25 ml of distilled water and mixed well to ensure a homogeneous suspension. The material at the interface between the middle phase and the bottom phase ($interface_{MB}$) was included in the bottom phase suspension, also this depending on the high viscosity (when cells from bakers' yeast were partitioned the bottom phase had to be further diluted). The concentration of particles in these suspensions was determined by measuring the absorbance at a suitable wavelength (Table 2) using a Zeiss spectrophotometer, PMQ II.

The distribution of particles is given as the amount of particles in each phase expressed as the percentage of the total amount of particles in the system. The amount of particles in a phase was calculated from the concentration of particles and the volume of the phase. The amount of material at the $interface_{TM}$ is obtained as the difference between the total amount and the sum of amounts in the three phases, including the $interface_{MB}$. These values agree quite well with the measured concentrations at the $interface_{TM}$.

The percentage in each phase was plotted *versus* the amount of substituted PEG with a separate curve for each phase.

Table 1. Composition of the phases in a three-phase system in which 10 % of the PEG was replaced by TMA-PEG. All concentrations are given in per cent weight by weight.

Phase	PEG+TMA-PEG/%	Ficoll/%	Dextran/%	Water/%
Top	22	3	0	75
Middle	7	25	1	67
Bottom	0	3	37	60

Table 2. Wavelength used for absorption studies

Type of cell particle	Wavelength/nm
Sarcina lutea	400
Yeast cells	400
Chlorella pyrenoidosa	465
Subchloroplast particles	680
Mitochondria	529

RESULTS

Partition of a relatively homogeneous particle

Sarcina lutea.. The distribution of the coccus *Sarcina lutea* in a three-phase system containing varying amounts of TMA – PEG is shown in Fig. 1. The total amount of cells in a system corresponded to an absorbance of A_{400} (1 cm) = 8.0. In a system containing no TMA-PEG 87 % of the material is found in the bottom phase and 13 % is found in the middle phase. When PEG is successively replaced by TMA-PEG the affinity of the coccus for the three phases changes so that the cells successively move from the bottom phase over the middle phase to the $interface_{TM}$ and the top phase. The bottom phase curve decreases linearly while the middle phase curve goes through a maximum when the concentration of TMA-PEG is 0.20 % of the total amount of PEG. When 1.00 % of TMA-PEG is used in the system, 34 % of the material is found in the top phase and 59 % is found at the $interface_{TM}$. At higher contents of positively charged PEG in the system, *Sarcina lutea* partition only between the top phase and the $interface_{TM}$. It is noticeable that a minute variation in the concentration of TMA-PEG has a great effect on the partition of this organism.

It has earlier been shown that a suspension of *Sarcina lutea* consists of relatively homogeneous cells.[2] The extraction profile of the coccus (Fig. 1) is therefore characteristic for

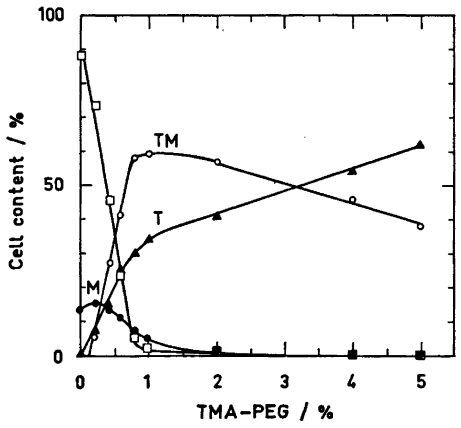

Fig. 1. The effect of TMA-PEG on the partition of *Sarcina lutea*. Cell content in the bottom phase □, in the middle phase ●, in the top phase ▲ and at the interface between the top and middle phase ○.

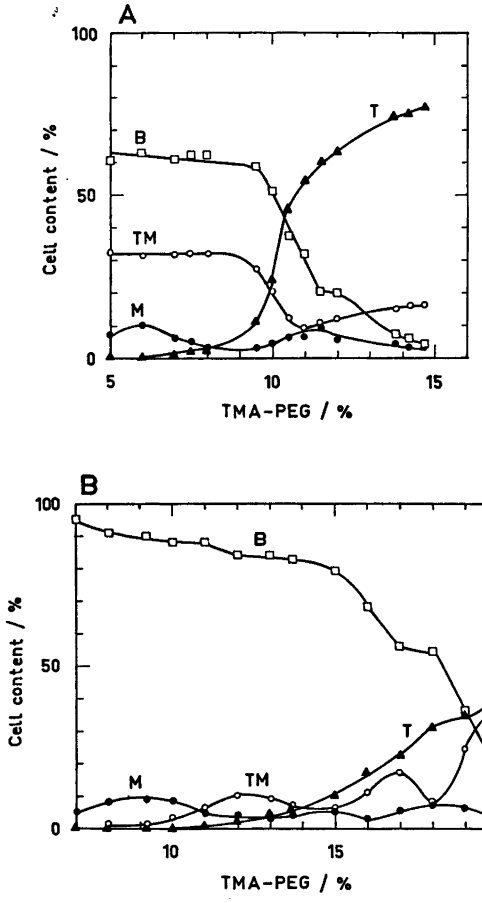

Fig. 2. The partition of two types of bakers' yeast in three-phase systems containing TMA-PEG. Cell content in the bottom phase □, in the middle phase ●, in the top phase ▲ and at the interface between the top and middle phase ○. (A) Standard yeast; (B) Special yeast.

a suspension of homogeneous particles. Furthermore, this extraction profile is analogous to that of a single protein.[13]

Partition of heterogeneous particles

Yeast cells. Two types of bakers' yeast, *Saccharomyces cerevisiae*, were investigated: so called standard yeast and special yeast. These two kinds of yeast are known to consist of more than one strain.[2] In these partition experiments the concentration of cells in the system was 0.05 g/kg.

The partition pattern of a cell-suspension from standard yeast is shown in Fig. 2 A. It is seen that the cells are extracted from the bottom phase in two distinct steps by the upper phases when the amount of TMA-PEG in the system is increased. The middle phase curve has two different maxima: one at 6 % TMA-PEG and another at 11 % TMA-PEG. This indicates the presence of at least two types of cells in the standard yeast.

Cells from special yeast (Fig. 2 B) partition quite differently from cells of standard yeast. In systems containing 15 % TMA-PEG 75 % of the cells of standard yeast are found in the top phase while most of the material from special yeast, 80 % of the cells, still is in the bottom phase. On the other hand the cells of standard yeast move from the bottom phase *via* the middle phase to the top phase within a smaller interval. This indicates that the cells of special yeast are more heterogeneous and the diagram also shows a more complex partition pattern. The material in the bottom phase is extracted into the other phases in three steps and both the middle phase curve and the interface$_{TM}$ curve show three distinct peaks. This means that in a system containing 17 % TMA-PEG one fraction of cells ought to be concentrated in the bottom phase, another at the interface$_{TM}$ and a third one in the top phase.

To verify this assumption the following separation experiment was made. Three 4.00 g systems containing 17 % TMA-PEG were weighed out, two with cell-suspension from special yeast and one with only buffer (blank system). After separation the top phase from one of the yeast-containing systems and the top phase from the blank system were interchanged. The three systems were gently mixed again and after separation each phase was analyzed (Fig. 3). The fraction from the original top phase remains up to 88 % in the top phase and more than 90 % of the material in the middle phase and the bottom phase is still in these phases. The material at the interface$_{TM}$ is the same. The experiment shows that three rather pure fractions can be obtained in just one single partition step. The above results with yeast cells agree quite well with experiments made in two-phase systems.[2]

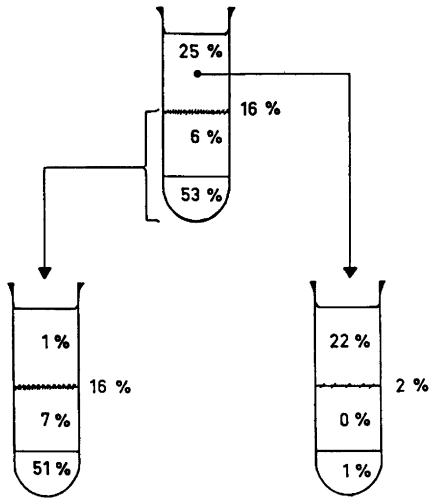

Fig. 3. Separation of the three fractions of cells in special yeast in a three-phase system with 17 % of the PEG replaced by TMA-PEG.

Chlorella pyrenoidosa. It has earlier been shown by partition that normally grown *Chlorella* is composed of two major sub-populations differing in stage of maturation or age. Cells that have just divided differ in their partition from older cells.[7]

When *Chlorella* was distributed in the three-phase systems a complete extraction from bottom to top phase was not achieved by using only positively charged PEG. To complete the extraction a negatively charged polymer, S-PEG, had to be included in the system. The total cell content in a system corresponded to an absorbance of A_{465} (1 cm) = 2.0.

Fig. 4A shows the partition of *Chlorella* taken from a growing culture. Three major fractions are indicated. An older population of *Chlorella*,

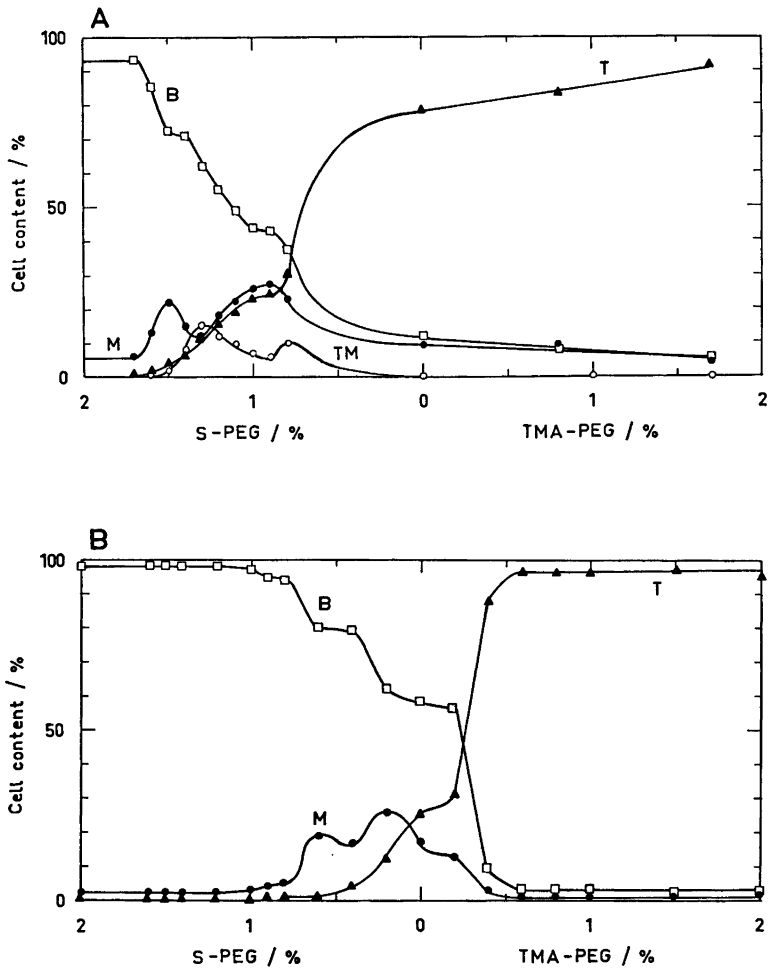

Fig. 4. The effect of TMA-PEG and S-PEG on the partition of *Chlorella pyrenoidosa*. Cell content in the bottom phase □, in the middle phase ●, in the top phase ▲ and at the interface between the top and middle phase ○. (A) *Chlorella* from a growing culture; (B) *Chlorella* which has been stored.

which had been standing in 10 mM potassium phosphate buffer pH 6.8 for three months, was also investigated (Fig. 4B). This three months old population had quite different partition properties. No material at all was found at the interface$_{TM}$ and more of the positively charged PEG was needed for extracting the cells from the bottom phase. Also this aged population of *Chlorella* consists of three major fractions. The proportion of these fractions is, however, different from that in the young population. This indicates that the *Chlorella* changes its surface properties with time.

A one month old population of *Chlorella* was subjected to an extraction experiment, carried out as the one described with yeast cells. Also in the case of *Chlorella* the three main fractions could easily be separated; one in the bottom phase, another at the interface$_{TM}$ and a third one in the top phase.

Subchloroplast particles. Mechanical fragmentation of chloroplasts give rise to a very heterogeneous mixture of particles.[16] When these fragments are partitioned in three-phase systems the extraction curves indicate the presence of three major fractions (Fig. 5). In this respect

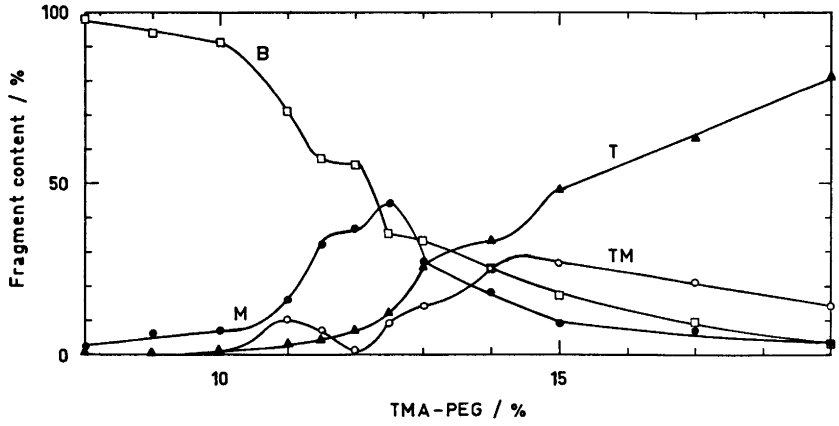

Fig. 5. The effect of TMA-PEG on the partition of fragments from Class II chloroplasts. Fragment content in the bottom phase □, in the middle phase ●, in the top phase ▲ and at the interface between the top and middle phase ○.

the results agree with results from countercurrent distribution of the fragments.[16] The three-phase system contained polymers and 2.5 mM sodium phosphate buffer pH 7.4, 2.5 mM sucrose and 0.5 mM sodium chloride. The amount of fragments in a system corresponded to 50 μg chlorophyll.

Fig. 6 shows a separation of the three major fractions of subchloroplast particles in a system in which 15 % of the PEG is replaced by TMA-PEG. In this experiment each phase was shaken with new pure blank phases. Some biological activities of the fragments in each phase and at the interface$_{TM}$ were controlled: Nicotinamideadenine dinucleotide phosphate reduction, with dichlorophenolindophenol – isoascorbate as electron donor, and dichlorophenolindophenol reduction, with water as electron donor.[16] These investigations show that the subchloroplast particles retain their biological activity in the system.

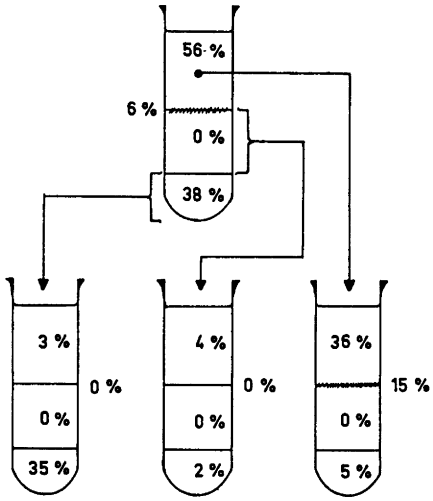

Fig. 6. Separation of the three major fractions of subchloroplast Class II particles in a three-phase system with 15 % of the PEG replaced by TMA-PEG.

Partition with DC-PEG. In all experiments described above the effect of a charged polymer on the partition of different cell particles has been investigated. However, polymers with other covalently bound groups can be used. One group which has been substituted to the PEG is deoxycholate and this group will give the polymer a hydrophobic character. Since the PEG-chain is the dominant part of the molecule, it partitions as PEG itself. In these systems a substance is partitioned under conditions of zero interfacial potential in the system.[3] This means that the electrical charge of a partitioned substance does not influence its partition behaviour.

It has earlier been observed that a hydrophobic PEG can be used to influence the partition of some proteins while the polymer has no effect on others.[11] In analogy with proteins, the partition of some cell particles can be affected by DC-PEG. By a successive replacement of PEG by DC-PEG in the three-phase system the

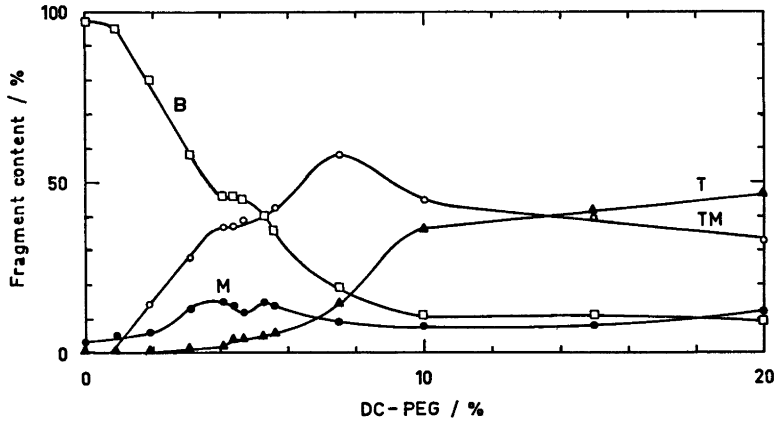

Fig. 7. Partition of chloroplast Class II fragments in three-phase systems containing various amounts of DC-PEG. Fragment content in the bottom phase □, in the middle phase ●, in the top phase ▲ and at the interface between the top and middle phase ○.

distributions of chloroplast fragments and mitochondria from rat liver can be affected while the distributions of cells from bakers' yeast and *Sarcina lutea* are unaffected. In these experiments the materials were partitioned in three-phase systems containing 8 % dextran, 8 % Ficoll and 8 % (PEG+DC-PEG), the latter two with $M_n = 6000$.

The effect of DC-PEG on the partition of chloroplast fragments is shown in Fig. 7. The extraction curves obviously indicate the presence of two major fractions. The three-phase system contained except polymers 50 mM potassium sulfate, 2.5 mM sodium phosphate buffer pH 7.4, 2.5 mM sucrose, and 0.5 mM sodium chloride. The amount of fragments in the system corresponded to 50 μg chlorophyll. The biological activity of the fragments in these systems was controlled as mentioned above. The fragments retained their biological activity, but the activity of the material from the top phase decreased somewhat.

The partition diagram of mitochondria from rat liver (Fig. 8) indicates the presence of three fractions. With higher concentration than 10 % DC-PEG the partition is stabilized, 23 % of the material is found at the interface_{MB}, 43 % of the material is found at the interface_{TM} and

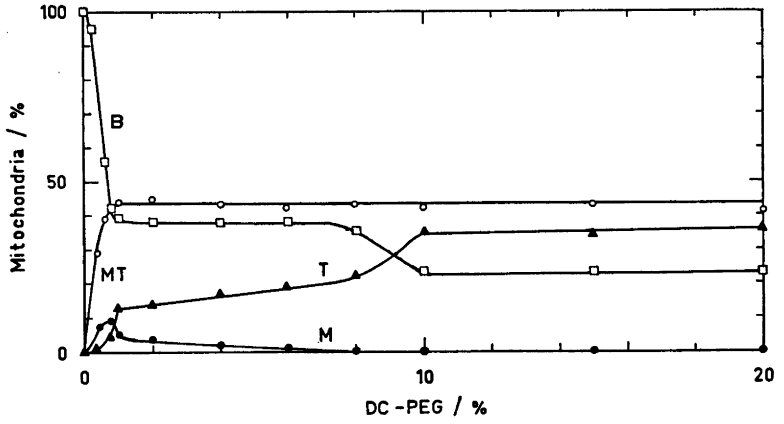

Fig. 8. The effect of DC-PEG on the partition of mitochondria from rat liver. Mitochondria in the bottom phase □, in the middle phase ●, in the top phase ▲ and at the interface between the top and middle phase ○.

the rest of the material, 34 %, is found in the top phase. Different forms of mitochondria from rat liver have been observed earlier.[6] The three-phase system contained except polymers 0.2 M sucrose and the total amount of organelles corresponded to an absorbance of A_{520} (1 cm) = 2.5. The mitochondrial material in each phase and at the interface$_{TM}$ was also controlled for some biological activities: succinate dehydrogenase, an inner membrane-bound enzyme, and fumarase, a matrix enzyme.[6] It was found that also the mitochondria retained their biological activity in these systems.

Each type of cell particle, which was been partitioned in the three-phase systems, was analyzed under light microscope but no broken cells have been observed.

DISCUSSION

The distribution of a substance in a two-phase system can be expressed by its partition coefficient K (= concentration in the upper phase divided with the concentration in the lower phase). The partition coefficient is related to the net charge, Z, of the substance *via* eqn. (1) [1]

$$\ln K = \ln K^0 + Z\psi\gamma \qquad \text{eqn.(1)}$$

where ψ is the electrical interfacial potential, K^0 is the partition coefficient under zero interfacial potential *or* when Z is zero and γ is a constant including thermodynamic constants and temperature. A system with three liquid phases can be treated as a combination of three different two-phase systems. On this basis the quantitative models for two-phase systems [1,9] have been applied to three-phase systems.[17]

In the three-phase system used here, 22 % (w/w) of the PEG + TMA-PEG is found in the top phase, 7 % (w/w) is located in the middle phase and the bottom phase contains less than 0.5 % (w/w). This unequal distribution of the charged PEG gives rise to differences in the electrical potential both between the top- and middle phase and between the middle- and bottom phase.[17] The top phase is therefore positive compared to the middle phase which in turn is positive compared to the bottom phase.

In a system containing uncharged PEG and potassium phosphate buffer the ions of the latter will direct the partition. The two anions of this buffer, $H_2PO_4^-$ and HPO_4^{2-}, differ in their partition properties [9] but they have both higher affinity for the bottom phase than for the other two phases. The interfacial potentials, ψ_{MB} and ψ_{TM}, are in this case working in the same directions as with TMA-PEG, but they have lower absolute values. Replacing the uncharged PEG with charged TMA-PEG in the phosphate containing system will increase the electrical potentials across the interfaces further. The resulting interfacial potential will be a mean of the interfacial potentials coming from TMA-PEG and potassium phosphate, and weighed with respect to the concentration of these species. Due to the unequal distribution of the TMA-PEG between the phases, a small amount of this charged PEG increases ψ_{TM} drastically to a certain stable value. ψ_{MB}, on the other hand, increases slowly with increasing amount of TMA-PEG, but will reach almost the same stable value as ψ_{TM} when the percentage of TMA-PEG in the system is high enough. Thus, increasing the amount of charged PEG in systems with constant concentration of buffer will change the interfacial potential across the interfaces, but to different values.

Since cells have a large number of charges on their surface their partition properties are strongly affected by charged polymers. It has been shown by particle-electrophoresis that most cells are negatively charged.[18] That is why all investigated cells except *Chlorella pyrenoidosa* are extracted from the bottom phase over the middle phase to the top phase with increasing amount of TMA-PEG in the three-phase system. In the case of fresh *Chlorella* the cells are extracted from the top phase to the lower two phases with increasing amount of S-PEG. The reason for this effect of S-PEG on the partition is that the interfacial potentials, $\psi_{TM}$and ψ_{MB}, arisen from the unequal distribution of this negatively charged polymer, are working in the opposite direction of those interfacial potentials arisen from the unequal distribution of the TMA-PEG.

However, the partition properties of *Chlorella* are changed during storage. In a system containing no charged PEG the aged cells have lower affinity for the top phase and this is probably due to a decrease in the value of K^0. This means that there might be a change in

the hydrophobicity of the cell with time so that *Chlorella* in its growing state has a more hydrophobic surface. When the aged *Chlorella* was partitioned the extraction steps were much sharper. This higher sensitivity towards changes in the interfacial potentials of the system can be explained by a change in the number of charges. If so, the cells increase their surface charge by storage. Greater number of charges on the surface of stored cells would decrease the tendency of aggregation and would explain why no material was found at the interfaces when these aged cells were distributed. The above agrees with results from cell electrophoresis of *Chlorella* where it is found that the cells change their surface properties during their life-cycle.[18]

When the three-phase technique is applied for partition of proteins their partition dependence on pH has been investigated.[13] This technique can also be used to investigate the distribution of cell particles. However, the isoelectric points, pI, of particles are often low. Low values of pH may either destroy the particles or increase the possibility of particle aggregation. The method described in this paper, where the interfacial potentials are varied, is thus preferable since it is possible to work at a constant and moderate pH.

By attaching groups other than charged ones to one of the polymers in the three-phase system other specific properties of a particle can be used for separation purposes. The substitution of deoxycholate on PEG makes this polymer hydrophobic. The more hydrophobic a membrane is the greater its affinity for the top phase will be. From a mixture of cell particles fractions can successively be extracted into the top phase by enhancing its hydrophobicity by adding increasing amounts of DC-PEG to the system.

When DC-PEG was used for partition of fragments from Class II chloroplasts two different fractions were found while three different fractions were found when TMA-PEG was used. In the former case the particles were partitioned at constant interfacial potentials and therefore the two fractions differ only in their hydrophobicity. In the latter case, however, the particles distribute both according to their hydrophobicity and to their number of charges. Under these circumstances one of the fractions, which was also obtained using DC-PEG, is probably split up into two fractions. These apparently differ in their charge density but not in their hydrophobic character.

It has been found that cell particles are not damaged and their biological activities are retained in these three-phase systems even at very high polymer concentrations. In spite of the higher viscosity of the three-phase system it has clear advantages compared to the two-phase system. In the former systems particles partition between the three phases as well as the two interfaces. That gives five locations, which under ideal conditions, can yield five fractions from a heterogeneous population of cells. If the partition properties of the different fractions are unequal enough, they can be separated from each other by a single, yet simple and not so time-consuming operation.

The affinity of a cell for a phase can be changed by introducing substituted groups onto any of the three polymers. Thus a certain desired property of a cell can be utilized for separation purposes and the cell can be directed to any of the three phases or two interfaces. So can, for example, one fraction in a cell mixture be directed to the middle phase and "impurities" can at the same time be extracted from the middle phase by the two other phases and the two interfaces. Two subpopulations, on the other hand, can easily be separated from each other by directing them to one phase each, the top phase and the bottom phase. The middle phase will then serve as a spacer phase and can serve as acceptor of "impurities" from the other two phases.

The three-phase technique can also be applied for analytical purposes. By studying the middle phase distribution curve for a partitioned particle suspension the presence of heterogeneity can be discovered. A homogeneous particle distributed in three-phase systems gives rise to only one peak in the middle phase curve. If there is several constituents in a mixture the curve has several maxima. For example a particle suspension consisting of two subpopulations with different partition properties yields a curve with two peaks. Thus, the number of well defined subpopulation fractions is equal to the number of peaks in the middle phase curve.

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Dissociation and Rate Constants of Some Human Liver Alcohol Dehydrogenase Isoenzymes

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ADH from human liver forms binary complexes with NADH, associated with a blue shift of the peak of the fluorescence emission of NADH. The wavelength shift is the same for all isoenzymes but the accompanying intensification of the fluorescence is different. The fluorescence is further increased by the formation of the very tight ternary enzyme-NADH-isobutyramide complexes. These properties are similar to those for the horse liver ADH, as well as the molecular weight of E = 40 000 ** per active site of the dimer molecule (EE). "Stopped-flow" determined velocity constants (ER⇌E + R) were found to be in good agreement with ethanol activity constants previously determined by activity measurement, confirming the validity of the ordered ternary complex mechanism also for the human ADH. No single isoenzyme activity as high as that reported by Mourad and Woronick or Drum has been found.

In 1967 Mourad and Woronick [1] described the isolation and crystallization of a human liver alcohol dehydrogenase (ADH) consisting of a single isoenzyme component and having a specific activity with ethanol equivalent to 60 – 70 % of that of the horse liver enzyme. Drum [2] has reported a human liver ADH isoenzyme with an activity of 120 % of the horse liver enzyme. The specific activity of the human ADH isolated by other investigators [3–6] was never so high. In most cases it was about half or less of the activity reported by Mourad and Woronick. These discrepancies may be attributed to human liver ADH isoenzymes with widely varying activities. However, after the isolation of six human ADH isoenzymes Pietruszko *et al.*[6] were not able to find any isoenzyme whose activity was higher than half of that reported by Mourad and Woronick, and isoenzymes with ethanol activity even lower than that were found. For three of these isoenzymes we have determined the rate constants for the binary complexes of the enzyme and NADH. The dissociation constants for the reduced coenzyme (E + R⇌ER) were determined for all six isoenzymes as well as dissociation constants for the ternary complexes with isobutyramide (EI + R⇌EIR). In the previous paper [6] it was shown that one isoenzyme (1A) was a hybrid. By a "best fit" procedure it was possible to determine the different coenzyme dissociation constants for the ternary complexes of the monomers ($K_{EI,R}$). The corresponding rate constants for this isoenzyme ($k_{EI,R\ on}$ and $k_{EI,R\ off}$) have been determined from a similar analysis of "stopped-flow" curves.

MATERIALS AND METHODS

Chemicals and enzyme preparations were the same as in the previous paper.[6] Separated isoenzymes of human ADH were stored at 0 °C in 10 % saturated ammonium sulfate solutions at pH 7.5.

Enzyme assays were done in 1 cm cuvettes at 340 nm at 23.5 °C in 3 ml total volume using NAD^+, 0.45 mM; ethanol, 8.6 mM, in glycine buffer, 0.062 M, pH 10.0, using a Beckman DU spectrophotometer. Glycine (Sørensen) was obtained from Merck AG, Darmstadt, Germany; isobutyramide from Eastman Organic Chemicals.

Determination of fluorescence curves and dissociation constants. The recording spectro-

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** Abbreviations used: E, Alcohol dehydrogenase (ADH) monomer; R, NADH; I, Isobutyramide.

fluorimeter has previously been described.[7] The excitation wavelength was 330 nm. The fluorescence intensity was recorded at 410 or 430 nm. The fluorimeter was not compensated for the different intensity of the light source at different wavelengths. The dissociation constants were determined, as earlier described, by titrating solutions of human ADH isoenzymes with suitable increments of NADH and recording the fluorescence increase at 23.5 °C in phosphate buffer $\mu = 0.1$, pH 7.0 in the presence or absence of 0.1 M isobutyramide. All solutions were made in water freshly distilled from alkaline permanganate.

Commercial NADH (Grade III), obtained from Sigma (U.S.A.) was used without further purification. Solutions of NADH were standardized by using acetaldehyde and yeast ADH as described previously.[8] The concentration of NADH was determined spectrophotometrically at 340 nm before and after oxidation to NAD by acetaldehyde + yeast ADH. The purity of NADH as judged by the amount of non-oxidizable 340 nm absorption was 96 %. NADH solutions were prepared immediately before use and used only one day.

All dissociation constants are calculated from 2 – 6 experimental curves giving a mean error of ± 20 %, which also applies to the velocity constants.

Determination of velocity constants. A "stopped-flow" fluorescence spectrometer,[9] recording the increase in fluorescence proportional to the formation of the binary complex of ADH with NADH (ER) or ternary complex of ADH with NADH and isobutyramide (EIR) was used. The formation of ER and EIR complex was followed at 23.5 °C in phosphate buffer $\mu = 0.1$. The isobutyramide concentration was 0.1 M. The excitation wavelength was 330 nm and fluorescence was recorded at 410 nm. 3 μN E mixed with an equal volume of 3 μM R in all ER experiments gave 1.5 μN concentration at $t = 0$. In the EIR determinations the concentration at $t = 0$ was [E] = 1.5 μN, [R] = 0.75 μM.

The velocity constants were determined from several points of the experimental curves. k_{on} was calculated from

$$d[ER]/dt = k_{on}[E][R] - k_{off}[ER] \quad (1)$$

explicitly solved.[10] The 1A isobutyramide experiments were treated according to the following section.

Determination of velocity constants for 1A isoenzyme + isobutyramide combining with NADH. In a previous paper [12] it was shown that the fluorescence increase following the coupling of NADH (R) to (i) a mixture of the two isoenzymes (EE and SS) of horse liver alcohol dehydrogenase or (ii) the hybride (ES) gave identical fluorimetric titration curves (Fig. 1). A calculation of the titration curve of ES from known dissociation constants and specific fluorescences of EE and SS gave good agreement with experimental results. This means that dissociation and fluorescence of each monomer are independent of its comonomer.

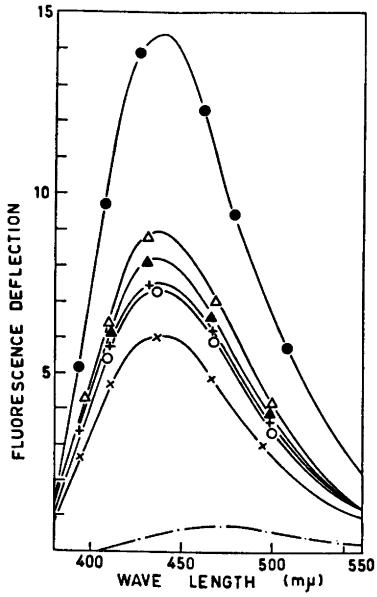

Fig. 1. Fluorescence emission spectra of 1 μN ternary complexes of the isoenzyme with NADH and isobutyramide at the excitation wavelength of 330 nm at pH 7.0 in phosphate buffer $\mu = 0.1$. ●, isoenzyme 1B; △, isoenzyme 3B; ▲, isoenzyme 4; +, isoenzyme 2; ○, isoenzyme 1A; ×, isoenzyme 3A; –.–, 1 μM NADH. For numbering see Ref. 6.

Stopped-flow experiments, mixing a solution of isoenzyme 1A in excess isobutyramide with an equal volume of a coenzyme solution gave an experimental curve quite different from the simple second order "on" and first order "off" reaction curves. Assuming different rate constants for the monomers in 1A isoenzyme (E_1 and E_2) made it possible to obtain a best fit calculated curve, Fig. 2. This is also based on the previously found dissociation constants and specific fluorescence. In these calculations different values of $k_{on,1}$ and $k_{on,2}$ were tested in order to obtain the best fit with the experimental curves.

From the equations

$$\frac{d[E_1IR]}{dt} = k_{on,1}[E_1I][R] - k_{off,1}[E_1IR] \quad (2)$$

$$\frac{d[E_2IR]}{dt} = k_{on,2}[E_2I][R] - k_{off,2}[E_2IR] \quad (3)$$

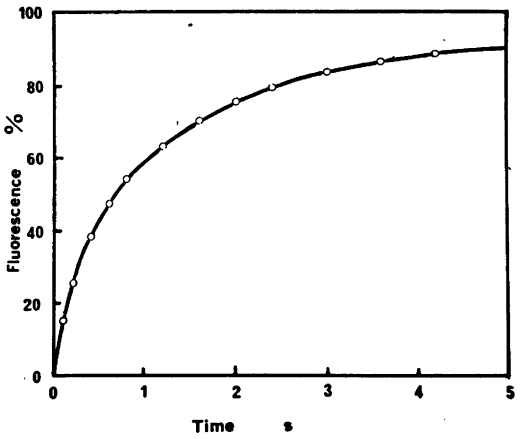

Fig. 2. Stopped-flow. Solid curve, experimental: 0.93 μN of 1A isoenzyme mixed with 0.75 μM NADH at $t=0$. Concentrations after mixture. Open Circles: calculated values for $K_1=2$ nM, $K_2=5$ nM, $F_1=5$ cm/μM, $F_2=29$ cm/μM, $k_{on1}=8$ μM^{-1} s^{-1}, $k_{on2}=1.75$ μM^{-1} s^{-1}.

a total fluorescence

$$F_{tot}=F_1[E_1IR]+F_2[E_2IR]+F_R[R] \quad (4)$$

was calculated with a programmed Canola 164P desk calculator. (Specific fluorescence for $E_1IR=F_1$, for $E_2IR=F_2$ and for $R=F_R$).

The back reaction did not affect the calculations in the treated part of the curve due to the very high affinity between enzyme and coenzyme in isobutyramide.

The corresponding treatment of binary binding in the absence of isobutyramide was not possible due to the lower fluorescence and higher dissociation in this case. The given binary constants thus represent mean values for the two different dimer halves of the 1A hybrid isoenzyme.

RESULTS

The fluorescence peak of NADH at 470 nm is shifted to 430 nm upon binding to human ADH and the intensity is increased. Table 1 gives the increase at 430 nm and at 410 nm. Table 1 also gives the fluorescence increase for the ternary complex with isobutyramide. Fluorescence curves for the ternary complexes are given in Fig. 1 showing identical shape and position of peak but very different fluorescence intensities for all isoenzymes. The value for isoenzyme 1A is the total fluorescence increase for the two different dimer halves found in a previous paper.[6]

The dissociation constants of NADH with several isoenzymes of human ADH determined in the presence of excess isobutyramide are shown in Table 2. The values for different isoenzymes of human ADH vary between 0.002 and 0.007 μM, not much different from that determined for horse liver ADH, 0.0055 μM.[11]

The dissociation constants in the absence of isobutyramide are shown in Table 3. They are about one order of magnitude greater than the corresponding constants in the presence of isobutyramide and two to six times lower than the corresponding constants established for horse liver ADH.[12]

The "on" velocity constants for NADH with isoenzymes 1A, 1B and 3B as determined by "stopped-flow" are also listed in Table 3. k_{off} is calculated as $k_{off}=K_{ER}k_{on}$. The values so obtained for isoenzymes 1A, 1B and 3B are listed in Table 3 and compared with the turnover numbers at V_{max} previously obtained in the same pH, temperature and buffer. It can be seen that the k_{off} values calculated from

Table 1. Human ADH isoenzymes: Specific fluorescence of binary complexes with NADH and ternary complexes with NADH and isobutyramide. The excitation wavelength was 330 nm.

Isoenzyme	Fluorescence increase at 410 nm "Q" values		Fluorescence increase at 430 nm fluorescence maximum	
	1 μM ER/ 1 μM NADH	1 μM EIR/ 1 μM NADH	1 μM ER/ 1 μM NADH	1 μM EIR/ 1 μM NADH
1A	6.9	40[a]	3.3	18
1B	5.1	73	2.1	35
2		41		18
3A		33		15
3B	9.7	46	4.7	22
4		42		20

[a] Total increase: 1A is a hybrid with the two halves giving ternary increase factors 12 and 68, resp.

Table 2. Human liver ADH isoenzymes: Dissociation constants with NADH in the presence of 0.1 M isobutyramide at pH 7.0 and 23.5 °C. Rate constants for 1A and 1B isoenzymes. All values were determined in phosphate buffer $\mu = 0.1$, pH 7.0 containing 0.1 M isobutyramide, 23.5 °C.

Isoenzyme	1A		1B	2	3A	3B	4
$K_{EI,R}$ (μM)	0.002[a],	0.005[b]	0.007	0.005	0.005	0.003	0.007
$k_{on,EI,R}$ (μM)$^{-1}$ s^{-1})	8[a],	1.8[b]	0.8				
$k_{off\ EI,R}$ (s^{-1}) calc.	0.016[a]	0.009[b]	0.006				

[a] $K_{EI,R}$, k_{on} and k_{off} for the monomer of the enzyme with the highest affinity for NADH. [b] $K_{EI,R}$, k_{on} and k_{off} for the other monomer.

Table 3. Dissociation and rate constants for the formation and decomposition of ER complexes of NADH with different human isoenzymes as compared with previously found turnover numbers,[6] obtained with 500 μM NAD^+ and varying concentrations of ethanol. All values were determined in phosphate buffer $\mu = 0.1$, pH 7.0 at 23.5 °C.

Isoenzyme	1A	1B	2	3A	3B	4
$K_{E,R}$ (μM)	0.06	0.13	0.09	0.05	0.06	0.03
k_{on} (μM^{-1} s^{-1})	9	2.5	—	—	5	—
k_{off}, calc. (s^{-1})	0.5	0.3	—	—	0.3	—
Turnover No. (Ref. 6) (act. site)$^{-1}$, s^{-1}	0.41	0.18	—	—	0.28	—

directly determined k_{ER} and k_{on} are very close to the values of turnover numbers determined kinetically. A curve calculated from the velocity constants given in Table 2 was in good agreement with a slow relaxation curve in an earlier experiment with isobutyramide.[6]

DISCUSSION

The rate limiting process in human as well as horse liver ADH ethanol oxidation is the dissociation of the ER complex. It is likely therefore that the ordered ternary complex mechanism [13] is also operative in the ethanol dehydrogenation by the human liver ADH at pH 7.0.

The relative low activity of the human ADH is fully explainable: The "off" velocity constant, k_{off}, in $ER \rightleftharpoons E + R$ is 0.3 s^{-1} in human ADH, as compared with 3 s^{-1} in horse ADH.

When k_{on} values with and without isobutyramide are compared (Table 3 and 2) it can be seen that isobutyramide lowers the "on" velocities.

With the horse ADH it has been established that k_{on} values in the presence and absence of isobutyramide are the same.[14] The considerably lower dissociation constants between human enzyme and NADH (with isobutyramide) are mainly due to the considerably lower (50 times) "off" velocity constants overtaking the smaller decrease of "on" velocity constants.

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Mass Spectrometric Studies of Selenaanalogues of 1,6,6aλ^4-Trithiapentalenes (1,6,6aλ^4-Triselenapentalenes and 1,6aλ^4-Diselena-5,6-diazapentalenes)

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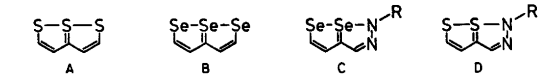
A number of 1,6,6aλ^4-triselenapentalenes and 1,6aλ^4-diselena-5,6-diazapentalenes have together with the corresponding sulfur analogues been subjected to electron impact.

The mass spectra of analogous selenium- and sulfur-containing compounds show great similarities. The main difference results from the more facile loss of selenium compared with sulfur. This gives rise to the formation of abundant hydrocarbon ions corresponding to $[M-Se_3H]^+$ in the spectra of 1,6,6aλ^4-triselenapentalenes. Corresponding ions are not observed in the spectra of 1,6,6aλ^4-trithiapentalenes.

The question whether the unusual type of structure and bonding in 1,6,6aλ^4-trithiapentalenes (A) will also be found in analogous compounds in which the sulfur atoms have been replaced by other heteroatoms has led to the synthesis of 1,6,6aλ^4-triselenapentalenes (B) and 1,6aλ^4-diselena-5,6-diazapentalenes (C).[1-3] Crystallographic studies [4,5] suggest a linear selenium arrangement in the triselena compounds which



A B C D

leads to the assumption that the Se—Se bonds in these compounds may be described as three-center four-electron bonds by analogy with the S—S bonding in trithiapentalenes.[6] It was therefore of interest to study whether this analogy in bonding is also reflected in the electron impact induced fragmentation.

Mass spectra of four 1,6,6aλ^4-triselenapentalenes have been recorded and compared with those of the sulfur analogues. Three of these (*2*,*4* and *8*) have previously been reported [7,8] whereas no mass spectrometric data have so far been published on 1,6aλ^4-dithia-5,6-diazapentalenes (D).

The presence of five relatively abundant selenium isotopes gives rise to rather complex clusters of isotopic peaks for species containing more than one selenium atom, especially pronounced when the mass ranges of two clusters overlap each other. The relative probabilities of occurrence of the various isotopic peaks corresponding to two and three selenium atoms in an ionic species have been calculated and the corresponding isotopic abundance ratios are shown in Fig. 1.

1,6,6aλ^4-Trithiapentalenes and 1,6,6aλ^4-triselenapentalenes. The trithia- and triselenapentalenes studied are listed in Table 1. The mass spectra with indication of the predominant modes of fragmentation are shown in Figs. 2 and 3.

A comparison of the spectra of two analogues, *e.g. 1* and *2* shows closely related behaviour in the two types of compounds upon electron impact. Peaks corresponding to $[M-H]^+$, $[M-CH_3]^+$, $[M-XH]^+$ and $[CH_3CX]^+$ are characteristic features in both spectra.

A remarkable difference is the appearance of two hydrocarbon ion peaks at *m/e* 78 and 77

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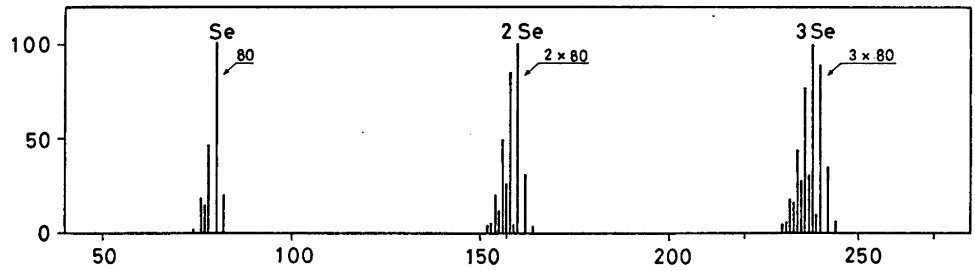


Fig. 1. The isotopic abundance ratios for ions containing 1, 2 and 3 selenium atoms.

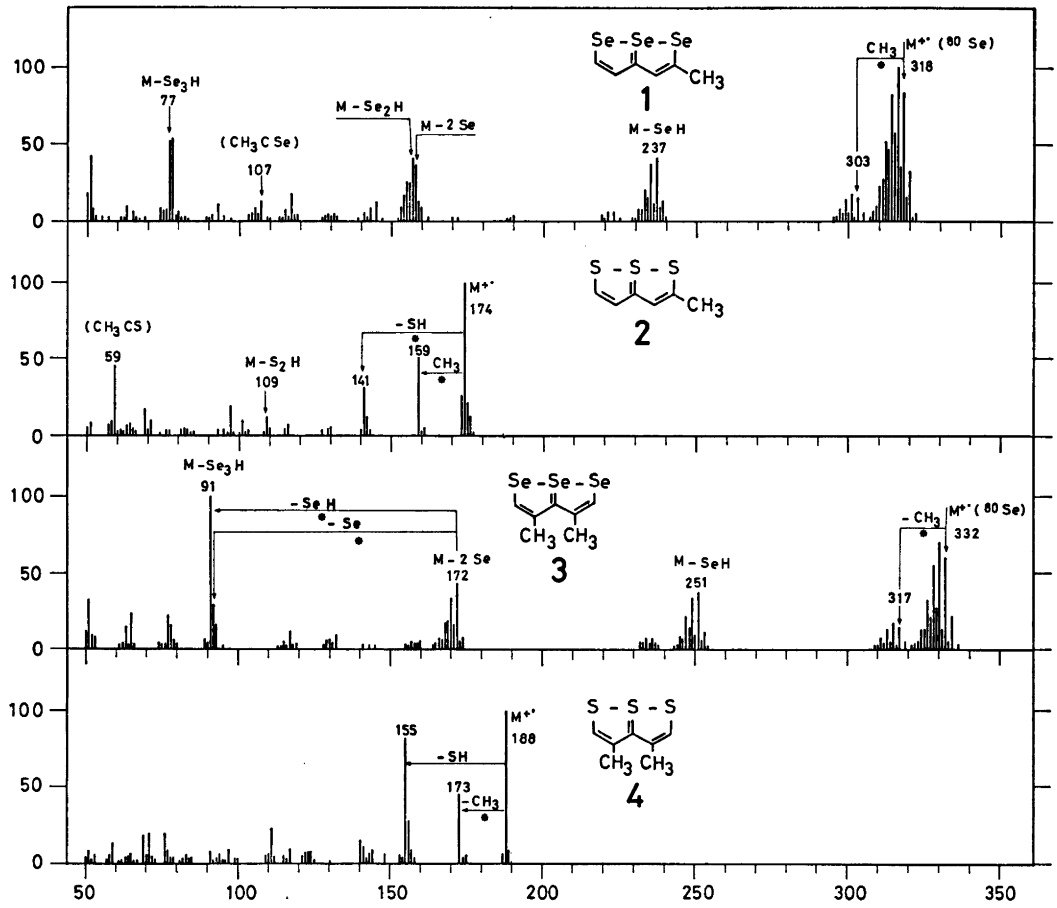
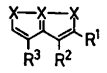


Fig. 2. MS of compounds *1—4*.

Table 1. Trithia- and triselenapentalenes studied.



	X	R^1	R^2	R^3
1	Se	CH_3	H	H
2	S	CH_3	H	H
3	Se	H	CH_3	CH_3
4	S	H	CH_3	CH_3
5	Se	H	$-CH_2-CH_2-CH_2-$	
6	S	H	$-CH_2-CH_2-CH_2-$	
7	Se	C_6H_5	H	H
8	S	C_6H_5	H	H

in the spectrum of compound *1* only. The peak of *m/e* 78 formally corresponds to the loss of all three selenium atoms from the molecular ion. Metastable peaks indicate that a stepwise formation *via* the $[M-Se_2]^+$ ion is possible. The *m/e* 77 ion is formed from the $[M-SeH]^+$ ion by additional losses of two selenium atoms.

Direct loss of Se from the molecular ion appears to be less favourable than the loss of Se_2. The integrated abundances of the two corresponding ions are 5 and 17 %, respectively, of that of the molecular ions. In the sulfur analogues the corresponding $[M-S]^+$ and $[M-2S]^+$ ions are practically absent.

Similar differences appear when comparisons are made between the other pairs of compounds.

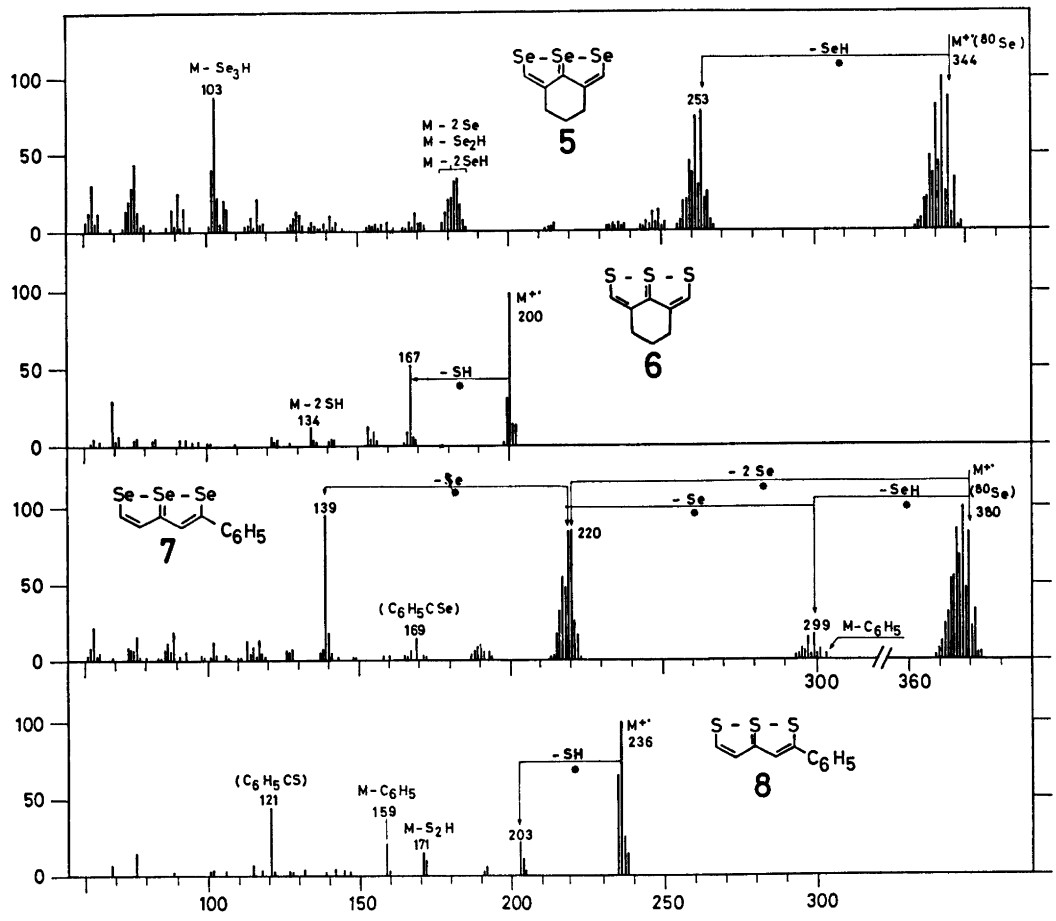


Fig. 3. MS of compounds *5–8*.

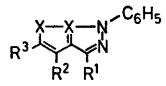
Hydrocarbon ions corresponding to $[M-S_3]^+$ and $[M-S_3H]^+$ are not found whereas $[M-Se_3H]^+$ ions are important in all selenium compounds. (The abundance of the odd electron $[M-Se_3]^+$ ion varies considerably.)

These differences in behaviour of analogous sulfur and selenium compounds, *i.e.* the easier elimination of the selenium, may be ascribed to general differences in influence of sulfur and selenium on the behaviour of such compounds upon electron impact.[9,10]

Apart from differences mentioned above the fragmentation behaviour of a trithiapentalene shows great similarities with that of the corresponding selena analogue. This supports the assumption that the bonding in the Se – Se – Se sequence in 1,6,6aλ^4-triselenapentalenes is much like that in the S – S – S sequence of 1,6,6aλ^4-trithiapentalenes.

1,6aλ^4-Diselena-5,6-diazapentalenes and 1,6aλ^4-dithia-5,6-diazapentalenes. Since only few examples of these types of compounds are available so far, it has not been possible to draw general conclusions concerning their behaviour upon electron impact. The compounds listed in Table 2 are studied.

Table 2. Dithia- and diselena-5,6-diazapentalenes studied.



	X	R^1	R^2	R^3
9	S	CH_3	CH_3	H
10	Se	CH_3	CH_3	H
11	Se	H	H	CH_3

The mass spectrum of *9* exhibits an abundant molecular ion. The fragmentation pattern is to a great extent characterized by the $-N-N-C_6H_5$ function. The predominant process is cleavage of the nitrogen-phenyl bond with charge retention almost exclusively on the phenyl moiety. Also important is the direct elimination of $C_6H_5N_2$ yielding *m/e* 143 and of C_6H_5NH giving rise to the peak *m/e* 156. Loss of CH_3 and SH is much less important than for the corresponding methyl substituted trithiapentalene *4*.

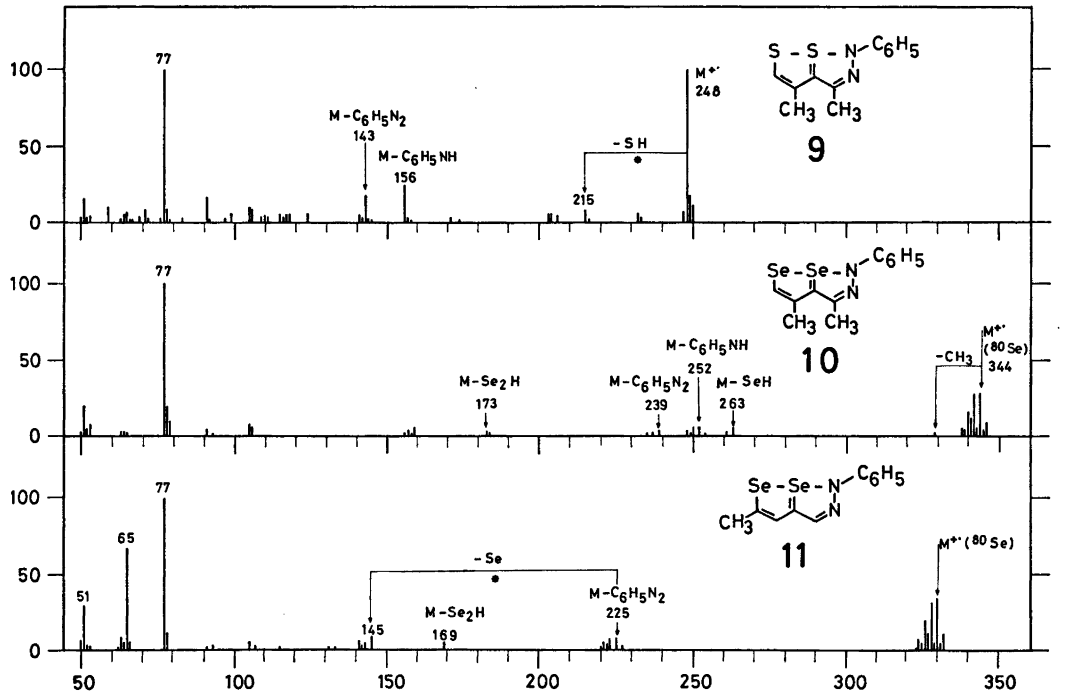


Fig. 4. MS of compounds *9 – 11.*

The mass spectrum of *10* (Fig. 4) is very similar to that of the sulfur analogue *9*. The *m/e* 77 peak appears with approximately the same intensity as the normalized molecular ion peak corresponding to a 100 % content of the ^{80}Se isotope.*

Elimination of two selenium atoms from the molecular ions of *10* and *11* is not a likely process, as it was in the case of the triselenapentalenes, and peaks corresponding to $[M-Se_2H]^+$ appear with relatively low abundance. However, in the corresponding sulfur analogues no $[M-S_2]^+$ and $[M-S_2H]^+$ ions are formed at all, demonstrating the easier elimination of selenium as compared with sulfur.

Peaks corresponding to losses of C_6H_5NH and $C_6H_5N_2$ are exhibited in the spectrum of *10* in accordance with the behaviour of the sulfur analogue, whereas no $[M-C_6H_5NH]^+$ ion is generated in the fragmentation of *11*. Also the $[M-SeH]^+$ ion is practically absent in this case. The hydrogen atoms involved in these elimination processes are probably not accessible in the case of *11*.

The abundant hydrocarbon ion *m/e* 65 in *11* has no counterpart in the spectrum of *10*. It may be generated from the $[M-C_6H_5N_2]^+$ ion by loss of two selenium atoms (*cf.* the abundant hydrocarbon ions in the spectra of the triselenapentalenes). The abundance of the *m/e* 39 ion is 44 % in the spectrum of *11* and only 8 % in that of *10*.

EXPERIMENTAL

Mass spectra were obtained with an MS 902 mass spectrometer using the direct sample insertion system with the lowest feasible ion source temperature (70 – 120 °C). 70eV Electrons were used. Peaks corresponding to doubly charged ions appearing at half mass numbers as well as peaks of abundance lower than 2 % were omitted in the spectra shown.

1,6,6aλ⁴-Triselenapentalenes and 1,6aλ⁴-diselena-5,6-diazapentalenes were prepared according to Refs. 1 – 3.

2-Methyl-1,6,6aλ⁴-trithiapentalene (*2*).[11]

3,4-Dimethyl-1,6,6aλ⁴-trithiapentalene (*4*).[12]

3,4-Trimethylene-1,6,6aλ⁴-trithiapentalene (*6*).[13]

2-Phenyl-1,6,6aλ⁴-trithiapentalene (*8*).[11]

* A reduced spectrum is easily obtained by increasing the intensities of the ions containing one, two and three ^{80}Se atoms by a factor of 2.0, 3.4 and 4.6, respectively, and removing peaks corresponding to other selenium isotopes.

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Nuclear Magnetic Resonance of Aromatic Heterocycles. XII. Synthesis, ^{1}H and ^{13}C NMR Parameters of some 2-Substituted Tellurophenes

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The synthesis of 2-chloro-, 2-bromo-, and 2-iodotellurophene is reported. Di-2-tellurienyl telluride was unexpectedly obtained in two attempts to synthesise 2-nitrotellurophene. The ^{1}H and ^{13}C NMR parameters of the chloro, bromo, iodo, methyl, *N*,*N*-dimethylcarboxamido, and 1-acetylethyl derivatives have been determined. The substituent-induced shifts are discussed in comparison to those of the corresponding furan, thiophene, and selenophene derivatives.

In a previous investigation, the NMR parameters of six 2-substituted tellurophenes (CHO, $COCH_3$, COOH, $COOCH_3$, SCH_3, CH_2OH) were compared with those of the corresponding furans, thiophenes, and selenophenes.[1] However, four of the substituents contained carbonyl groups so this set was not considered to be representative enough for a more detailed discussion of the substituent-induced shifts of the four five-membered chalcogen heterocycles. In particular substituents with $-I+M$ effects (the halogens) and with very strong electron-attracting (nitro) and very strong electron-donating effects (*e.g.* methoxy) were missing.

We have now partially overcome the difficulties in synthesising 2-substituted tellurophenes containing such substituents.

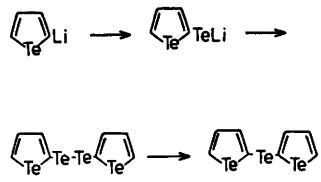
The halo derivatives of tellurophenes cannot be obtained by reactions with halogens as 1,1-addition products are formed.[2]

Recently, very convenient methods for obtaining halo and nitro derivatives of furan, thiophene, and selenophene were reported. The 2- and 3-chloro compounds of these heterocycles were prepared by treating the corresponding lithio derivatives with hexachloroethane.[3] 2-Iodo- and 2-nitrothiophene were obtained by nucleophilic attack of nitrite ion on di-2-thienyliodonium chloride.[4]

Applying this method to 2-lithiotellurophene at low temperature, 2-chloro- and 2-bromotellurophene were obtained in fair yields. The separation from unreacted starting material and tetrahaloethylene could, however, not be achieved by distillation. The pure compound was therefore obtained by treating the crude reaction mixture with a chloroform solution of bromine and by reducing the resulting 1,1-dibromide derivative with sodium hydrogen sulfite.

The 2-fluoro derivatives of thiophene and selenophene have been obtained through the reaction of the 2-lithio derivatives with perchloryl fluoride.[5,6] Unfortunately, attempts to prepare 2-fluorotellurophene with this method were without success.

In order to obtain both 2-iodo- and 2-nitrotellurophene, crude di-2-tellurinyl-iodonium salt, obtained through the reaction of 2-lithiotellurophene with *trans*-1-chloro-2-dichloroiodo ethylene, was treated with sodium nitrite in dimethylformamide. 2-Iodotellurophene was obtained as expected, but no product with the expected properties of 2-nitrotellurophene could be isolated. 2-Iodotellurophene was also obtained, but in lower yield, by decomposing the iodonium salt in dimethylformamide without the presence of sodium nitrite. The only compound besides 2-iodotellurophene that could be isolated was crystalline di-2-tellurienyl tel-



Scheme 1.

luride, whose structure was confirmed both by spectroscopic and analytical data and by an authentic synthesis. This compound was also obtained during another attempt to prepare 2-nitrotellurophene by reacting 2-lithiotellurophene with ethyl nitrate. Probably, 2-lithiotellurophene is partly decomposed under the influence of ethyl nitrate to give tellurium, which reacts with 2-lithiotellurophene, and the resulting intermediate is converted to di-2-tellurienyl telluride *via* the ditelluride. This reaction sequence, outlined in Scheme 1, is in accordance with the reaction route proposed for the synthesis of diaryl telluride.[7]

Di-2-tellurienyl telluride was indeed readily obtained by treating 2-lithiotellurophene with tellurium, whereupon the reaction mixture was hydrolysed with water.

In addition, the NMR spectra of 2-methyl-,[8] 2-(*N*,*N*-dimethylcarboxamido)-,[9] and 2-(α-acetoxy)ethyl-[10] tellurophene were studied. The syntheses of these compounds have been described previously.

^{1}H NMR SPECTRA

The ^{1}H NMR spectra of the 2-substituted derivatives studied in this paper were recorded in deuterioacetone solutions, and the NMR parameters are collected in Table 1. The assignments of the chemical shifts were based on results from previous studies,[1,11] and on the assumption that substituent effects in tellurophene are analogous to those in the other five-membered heterocycles. As shown in Table 1, the coupling constants fall in well-defined intervals with the same relative order as in thiophene and selenophene, but the magnitudes of the coupling constants are somewhat larger in the tellurophene derivatives compared to the sulfur- and selenium-containing heterocycles. Furthermore, the halo-substituted derivatives increase the intervals for the proton-proton couplings in the tellurphene series.

By plotting J_{45} and J_{34} of the halo-substituted derivatives [12–14] against the electronegativity of the heteroatom (3.5, 2.5, 2.4, and 2.1 for O, S, Se, and Te, respectively), good correlations were obtained, while the correlation coefficient for J_{35} was much lower. The coupling constants were taken from Refs. 3, 13, 15, 16, and Table 1.

In attempts to correlate the proton substituent-induced shifts of the halo derivatives [12–14] against the electronegativity of the heteroatom, it was only the 3- and 5-positions of the chloro derivatives that gave acceptable correlation coefficients (0.95 and 0.99, respectively).

Table 1. ^{1}H NMR chemical shifts (δ), coupling constants (Hz), and shifts relative to the α- and β-protons for some 2-substituted tellurophenes, furans, thiophenes, and selenophenes at 60 MHz[a]

Substituent	Hetero atom	H_3	H_4	H_5	J_{34}	J_{35}	J_{45}	ΔH_3	ΔH_4	ΔH_5
Cl	Te	7.33	7.34	8.75	4.26	1.47	7.33	−0.46	−0.45	−0.22
Br	Te	7.72	7.41	8.91	4.27	1.49	7.28	−0.07	−0.38	−0.06
I	Te	8.11	7.32	9.13	4.06	1.54	7.10	+0.32	−0.47	+0.16
CH_3	Te	7.23	7.47	8.64	3.90	1.26	7.14	−0.56	−0.32	−0.33
$CON(CH_3)_2$	Te	7.94	7.87	9.19	4.10	1.95	6.00	+0.15	+0.08	+0.22
”	O	6.97	6.55	7.68	3.45	0.80	1.80	+0.61	+0.19	+0.22
”	S	7.46	7.08	7.60	3.70	1.10	4.80	+0.36	−0.02	+0.20
”	Se	7.59	7.30	8.28	3.80	1.10	5.50	+0.26	−0.03	+0.18
$CH(OCOCH_3)CH_3$	Te	7.60	7.67	8.87	4.10	1.82	6.10	−0.19	−0.12	−0.10
”	O	6.36	6.36	7.46	–	–	–	0.00	0.00	0.00
”	S	7.06	6.93	7.31	3.60	1.90	4.80	−0.04	−0.17	−0.09
”	Se	7.22	7.14	8.00	3.80	2.10	4.60	−0.11	−0.19	−0.10

[a] ^{1}H NMR shifts for the parent compounds are taken from Ref. 5.

In the tellurophene series, good correlations could be obtained between the electronegativity of the halo-substituents and the proton shifts (ΔH) for all positions except position 4. This behaviour was also observed in the other congener systems.[12–14]

^{13}C NMR SPECTRA

The ^{13}C NMR spectra were also recorded in deuterioacetone solutions, because all derivatives were soluble enough to give 20 % solutions and to enable use of the deuterium resonance as internal lock signal for the ^{13}C measurements. All shifts were determined from the proton-decoupled spectra using TMS as an internal standard. The assignments of the ring carbons were made with the same procedure as in Ref. 1, based on the fact that the direct coupling for the α-carbons is larger than that for the β-carbons, and that the long-range couplings have the same relative order as in the 2-substituted thiophene and selenophene series, where the long-range coupling over three bonds (^3J) is larger than that over two bonds (^2J). It has, however, been demonstrated that 3-substituted thiophenes and selenophenes, as well as both 2- and 3-substituted furans, show exceptions to this rule.[14] The resulting shifts are presented in Table 2, and the coupling constants in Table 3. For the iodo and methyl derivatives, the coupling constants are missing because there was a reaction between the substrate and TMS. In Table 4, the relative shifts, which also show the expected substituent effects, are given. As the 2-(*N*,*N*-dimethylcarboxamido) and 2-(α-acetoxy)ethyl derivatives of furan, thiophene, and selenophene were not included in our comparative study of the ^{13}C NMR parameters in furan, thiophene, and selenophene,[14] their NMR parameters and relative shifts for all four congeners are also given in Tables 1, 2 and 4.

In our previous studies, the relative shifts of furan, selenophene, and tellurophene were plotted against those of thiophene for use in the prediction of the shifts of the less common heterocycles in this series, since those of thiophene are known.[14] In equations (1) – (4), these relations for the carbons of tellurophene are presented, and in equations (5) – (7) those for the protons.

$$\Delta C_2^{Te} = 1.18\ C_2^S + 1.73 \quad (r = 0.99) \tag{1}$$

$$\Delta C_3^{Te} = 1.10\ C_3^S - 0.99 \quad (r = 0.95) \tag{2}$$

$$\Delta C_4^{Te} = 1.04\ \Delta C_4^S - 0.86 \quad (r = 0.78) \tag{3}$$

$$\Delta C_5^{Te} = 1.25\ \Delta C_5^S - 0.45 \quad (r = 0.95) \tag{4}$$

$$\Delta H_3^{Te} = 1.23\ \Delta H_3^S - 0.16 \quad (r = 0.98) \tag{5}$$

$$\Delta H_4^{Te} = 1.64\ \Delta H_4^S + 0.04 \quad (r = 0.91) \tag{6}$$

$$\Delta H_5^{Te} = 1.16\ \Delta H_5^S - 0.02 \quad (r = 0.97) \tag{7}$$

By comparing these equations for tellurophene with those in Refs. 1 and 14, where only six substituents, much more similar in character than the twelve in this paper, were included, it is found that the slopes of the carbon

Table 2. ^{13}C NMR chemical shifts (ppm) for some 2-substituted tellurophenes, furans, thiophenes, and selenophenes at 25.142 or 15.0 MHz.

Substituent	Hetero atom	C_2	C_3	C_4	C_5	CH	CO	$COCH_3$	$N(CH_3)_2$	CH_3
Cl	Te	136.4	139.1	136.0	128.7					
Br	Te	110.0	142.6	137.4	131.5					
I	Te	68.9	149.2	139.4	135.0					
CH_3	Te	144.6	137.5	136.8	124.9					22.6
$CON(CH_3)_2$	Te	146.6	138.4	138.4	132.6		168.2		38.4	
"	O	149.0	115.9	111.9	144.8		160.1		37.1	
"	S	139.6	129.8	127.3	129.8		164.0		38.0	
"	Se	146.1	131.6	130.6	135.6		165.2		38.1	
$CH(OCOCH_3)CH_3$	Te	152.7	134.8	137.4	127.1	21.3	170.2	73.4		24.3
"	O	154.6	108.4	111.0	143.4	20.9	170.1	65.3		18.5
"	S	145.6	125.9	127.3	125.9	21.0	170.0	67.8		22.3
"	Se	152.9	127.7	129.6	131.3	21.0	170.0	69.9		23.2

Table 3. ^{13}C NMR coupling constants (Hz) for some 2-substituted tellurophenes.

Substituent	$J(C_3-H_3)$	$J(C_4-H_4)$	$J(C_5-H_5)$	$J(C_5-H_4)$	$J(C_3-H_5)$	$J(C_4-H_3)$	$J(C_4-H_5)$	$J(C_5-H_3)$	$J(C_5-H_4)$
Cl	165.0	164.0	185.0	6.0	10.0	4.2	2.2	9.8	4.8
Br	166.0	164.0	187.0	5.8	10.5	4.6	2.5	10.9	4.3
$CON(CH_3)_2$	163.0	166.0	185.6	5.7	10.7	4.5	2.0	10.7	–
$CH(OCOCH_3)CH_3$	160.0	164.0	185.6	5.7	10.7	5.0	2.0	10.4	4.6

equations have decreased much more than the slopes of the proton equations. Only minor changes in the equations for the furan, thiophene, and selenophene shifts were observed when the 2-(*N*,*N*-dimethylcarboxamido) and 2-(α-acetoxy)ethyl substituents are included instead of methoxy, nitro, and cyano in the set of substituents previously studied.[14]

EXPERIMENTAL

2-Chlorotellurophene. To 4.0 g (0.022 mol) of tellurophene[11] in 30 ml of dry ether 13.6 ml of a 20 % solution of butyllithium in hexane was added dropwise under nitrogen at room temperature. When the addition was complete, the reaction mixture was stirred for 30 min, and then cooled to −70 °C. At this temperature, a solution of 6.7 g (0.028 mol) of hexachloroethane in 34 ml of dry ether was added and the temperature was not allowed to rise above −60 °C. The reaction mixture was kept at −70 °C for 3 h, whereupon the cooling bath was removed. When the reaction mixture had reached room temperature, it was hydrolysed with ice water. Most of the organic layer was separated and the mother liquor was then acidified (HCl) and extracted with ether. The combined organic phases were washed with a solution of sodium hydrogen carbonate and dried over magnesium sulfate. After evaporation, the residual red oil was chromatographed on aluminia using light petroleum ether (b.p. 40–60 °C) as eluent; 2.5 g (53 %) of 2-chlorotellurophene b.p. 124 °C/80 mmHg was obtained. (Found: C 22.34; H 1.39; Cl 16.59; m.wt. 218. Calc. for C_4H_3ClTe: C 22.44; H 1.41; Cl 16.55; m.wt. 214.1).

2-Bromotellurophene was prepared as described above for 2-chlorotellurophene from 2-lithiotellurophene (prepared from 10 g of tellurophene in ether and 28.0 g (0.056 mol) of hexabromoethane in 22 ml of dry ether and 120 ml of tetrahydrofuran. The crude product was treated with 150 ml of light petroleum and the solid material filtered off and the filtrate evaporated *in vacuo*. The resulting oil was distilled at 17 mmHg and the fraction boiling at 93–103 °C was collected. As this fraction did not consist of pure 2-bromotellurophene, it was diluted with 10 ml of chloroform and treated at 0 °C dropwise with a solution of bromine in chloroform until a permanent brown colouring was obtained. The orange precipitate was filtered off, washed with cold chloroform and benzene and then treated with 100 ml of a saturated solution of sodium hydrogen sulfite with vigorous stirring. The mixture was extracted with ether and the combined ethereal phases were washed, dried and evaporated. The remaining oil was chromatographed on aluminia by eluting with light petro-

Table 4. ^{13}C NMR shifts relative to the α- and β-carbons of the parent compounds (ppm) for some 2-substituted tellurophenes, furans, thiophenes, and selenophenes.[a]

Substituent	Hetero atom	ΔC_2	ΔC_3	ΔC_4	ΔC_5
Cl	Te	9.1	1.1	−2.0	1.4
Br	Te	−17.3	4.6	−0.6	4.2
I	Te	−58.4	11.1	1.4	7.8
CH_3	Te	17.3	−0.5	−1.2	−2.4
$CON(CH_3)_2$	Te	19.3	0.4	0.4	5.4
"	O	5.4	5.6	1.4	1.2
"	S	14.0	2.5	0.0	4.2
"	Se	15.1	1.8	0.8	4.7
$CH(OCOCH_3)CH_3$	Te	25.4	−3.3	−0.6	−0.2
"	O	11.0	−2.0	0.7	−0.2
"	S	20.0	−1.4	0.0	0.3
"	Se	21.9	−2.1	−0.2	0.3

[a] ^{13}C NMR shifts for parent compounds, see Ref. 5; however Cβ for selenophene should be 129.8, *cf.* Ref. 13.

leum ether. Yield 5.0 g (44 %), b.p. 100 °C/17 mmHg. (Found: C 18.66; H 1.13; Br 31.10. Calc. for C_4H_3BrTe: C 18.58; H 1.16; Br 30.90).

2-Iodotellurophene. 2-Lithiotellurophene was prepared from 10 g (0.056 mol) of tellurophene [11] in 80 ml of dry ether and 27 ml of a 20 % solution of butyllithium in hexane and cooled to −70 °C, whereupon it was slowly added to 7.12 g (0.030 mol) of *trans*-1-chloro-2-dichloroiodo ethylene [17] in 50 ml of dry toluene at −70 °C. The reaction mixture was stirred at −70 °C for 3 h and then poured into ice-water. The precipitated iodonium salt was filtered off, washed with water, acetone, and ether, dried and immediately used in the next step.

5.6 g (0.011 mol) of ditellurienyl-iodonium salt and 4 g of sodium nitrite in 30 ml of anhydrous *N,N*-dimethylformamide were stirred at 60 °C for 5 h and at 100 °C for 1 h. After cooling, the mixture was diluted with water and extracted with ether. The combined ether phases were washed with water, dried over sodium sulfate and evaporated. The residue was chromatographed on alumina using light-petroleum as eluent. The first fractions contained 1.1 g of pure 2-iodotellurophene according to GLC and TLC analyses. (Found: C 15.23; H 1.10; I 41.03. Calc. for C_4H_3ITe: C 15.72; H 0.99; I 41.52).

Continuing the elution using benzene and more polar solvents such as ether and chloroform, no compounds with the physical properties of 2-nitrotellurophene could be observed. The only product that could be isolated was di-2-tellurienyl telluride (1.2 g).

Di-2-tellurienyl telluride. 2-Lithiotellurophene was prepared from 2.0 g (0.011 mol) of tellurophene in 15 ml of dry ether and 6 ml of 20 % butyllithium in hexane. 0.7 g of tellurium was added and the reaction mixture was stirred at room temperature under nitrogen for 2.5 h, whereupon it was poured into ice-water. The organic material was extracted with ether and the combined ethereal phases dried and evaporated. The residual oil gave after column chromatography using a mixture of light petroleum and ether (7:3) 0.3 g of the product which was recrystallised from ligroin, m.p. 73−74 °C. ^1H NMR spectrum ($CDCl_3$): δ 8.15 (H-3), 7.62 (H-4), 9.16 (H-5); J_{34} 4.0 Hz, J_{35} 1.3 Hz, J_{45} 6.9 Hz. (Found: C 19.96; H 1.26; m.wt. 490. Calc. for $C_8H_6Te_2$: C 19.81; H 1.24; m.wt. 484.9).

2-Methyltellurophene was prepared as described in Ref. 11, *2-(α-acetoxy)ethyltellurophene* in Ref. 10 and *2-(N,N-dimethylcarboxamido)-tellurophene and -selenophene* in Ref. 9. 2-(*N,N*-Dimethylcarboxamido)furan and -thiophene were prepared according to the general procedure described in Ref. 18, giving products identical to those described in Refs. 18 and 19. 2-(α-Acetoxy)ethylfuran, -thiophene, and -selenophene were prepared from the parent compounds *via* the 2-acetyl derivatives in analogy with the procedures in Refs. 11 and 10, giving products identical with those described in Ref. 20.

The ^1H NMR spectra were obtained with a Varian A60 and a Jeol MH100 high resolution spectrometer. The ^{13}C NMR spectra were obtained either at 25.142 MHz on a Varian XL-100-15 spectrometer equipped with frequency sweep, proton wide band decoupler and Fourier transform operation or at 15.0 MHz with a Jeol JNM-60 spectrometer with a built-in Jeol 980A computer with 12 K memory.

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Synthesis of Some Acetylated Alkyl 1-Thio-α-D-glucopyranosides

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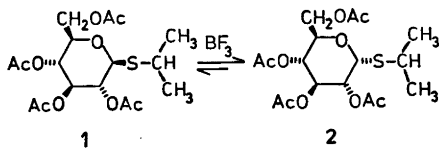
Some alkyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranosides have been prepared by anomerization of the corresponding β-D-glucopyranosides, using boron trifluoride as catalyst.

1-Thioglycosides inhibit enzymic hydrolysis of the corresponding *O*-glycosides and, when attached to a suitable matrix, may be used for the purification of glycosidases by affinity chromatography. Alkyl *trans*-1-thioglycopyranosides (generally the β-glycosides) may be prepared by reaction between a glycosyl halide and a thiol [1] or by S-alkylation of the corresponding 1-thioglycopyranose.[2] Although some alkyl *cis*-1-thioglycopyranosides have been reported, there is no general method for the synthesis of this group of substances.

Acetylated alkyl glycopyranosides are anomerized on treatment with a strong acid, such as sulfuric acid.[3] titanium tetrachloride [4] or boron trifluoride.[5] Because of the anomeric effect,[6,7] the isomer with an axial aglycone in the most stable chair form predominates, the proportion between the two anomers being approximately 9:1 at equilibrium. We have now investigated the analogous anomerization of some acetylated alkyl 1-thio-β-D-glucopyranosides.

The tetraacetates of ethyl, isopropyl and 1-heptyl 1-thio-β-D-glucopyranoside were prepared by alkylation of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside. Preliminary experiments, in which the isopropyl derivative (*1*) was treated with either sulfuric acid in acetic anhydride – acetic acid, titanium tetrachloride in chloroform or boron trifluoride in dichloromethane, demonstrated that anomerization was affected by all three reagents. The reactions with sulfuric acid and titanium tetrachloride were accompanied by discoloration and degradation. With boron trifluoride in dichloromethane at room temperature, equilibrium could be established without noticeable degradation. At equilibrium, the ratio of α- (*2*) to β-glucosides (*1*) was 7:3, and they were readily separated by chromatography on silica gel. The fully acetylated ethyl, isopropyl and 1-heptyl 1-thio-α-*D*-glucopyranosides were consequently prepared by anomerization of the corresponding β-glucosides with boron trifluoride. Separation of all 3 pairs of anomers was achieved by chromatography on silica gel and the α to β ratio was approximately 7:3.

The anomerization reaction for acetylated alkyl O-glucopyranosides [8] was slower than for the corresponding galactosides, xylosides and arabinosides. The same should most probably be valid for 1-thioglycosides, and it should consequently be possible to anomerize acetylated alkyl 1-thioglycopyranosides of all common sugars.



1 2

EXPERIMENTAL

General methods. Concentrations were performed under reduced pressure. Precoated plates with silica gel F_{254} (Merck) and silica gel (230 – 400 mesh, Merck) were used for TLC and for column chromatography, respectively. Light petroleum refers to a fraction with b.p. 60 – 71 °C. ^{1}H NMR spectra were recorded with a Varian A-60 A instrument and optical

rotations determined in chloroform, $c = 1$, with a Perkin-Elmer 141 polarimeter. Melting points are corrected.

Alkyl 2,3,4,6-tetra-O-*acetyl-1-thio-β*-D-*glucopyranosides.* 2-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2-thiopseudourea hydrobromide [9,10] (4 mmol) in water (25 ml) was treated with potassium carbonate (6 mmol) in water (10 ml) at room temperature for 30 min. The mixture was then extracted with chloroform (3 × 20 ml), the chloroform solution washed with water (2 × 25 ml), dried ($CaCl_2$) and concentrated to a syrup. The resulting 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucose (~4 mmol) was dissolved in acetone (4 ml) and M aqueous potassium carbonate (4 ml) was added. The alkyl halide, ethyl bromide, isopropyl iodide or 1-heptyl bromide (4.4 mmol) was added and the mixture stirred, at room temterature, until all starting material had reacted (30 – 60 min, TLC, light petroleum – ethyl acetate, 3:2). The mixture was poured into ice-water (60 ml) and extracted with chloroform (3 × 25 ml). The chloroform solution was washed with water (2 × 25 ml), dried ($CaCl_2$) and concentrated to a syrup. This product was purified by chromatography on a silica gel column (4 × 30 cm), irrigated with light petroleum-ethyl acetate (3:2). The yield of crystalline 1-thio-β-D-glucopyranoside was approximately 50 %.

Ethyl 2,3,4,6-tetra-O-*acetyl-1-thio-β*-D-*glucopyranoside* crystallized from ethanol and showed m.p. 81 – 82 °C, $[\alpha]_{578}^{22}$ −27°, in good agreement with published values.[2,11]

Isopropyl 2,3,4,6-tetra-O-*acetyl-1-thio-β*-D-*glucopyranoside* crystallized from ethanol and showed m.p. 110 – 111 °C, $[\alpha]_{578}^{22}$ −22°.

1-Heptyl 2,3,4,6-tetra-O-*acetyl-1-thio-β*-D-*glucopyranoside* crystallized from hexane and showed m.p. 69 – 70 °C, $[\alpha]_{558}^{20}$ −35°.

NMR spectra of the three 1-thio-β-D-glucopyranosides were in agreement with the proposed structures and almost superimposable, except for the signals from the protons in the aglycones.

Anomerization of alkyl 2,3,4,6-tetra-O-*acetyl-1-thio-β*-D-*glucopyranosides.* A solution of the fully acetylated alkyl 1-thio-β-D-glucopyranoside (1 mmol) in dry dichloromethane (5 ml) was saturated with boron trifluoride. The solution was kept at room temperature and the reaction followed by TLC (light petroleum-ethyl acetate, 3:2). After 2 – 3 h, when equilibrium was established, the solution was washed with M sodium hydrogen carbonate (5 ml), water (2 × 5 ml) dried ($CaCl_2$) and concentrated. The product was fractionated on a silica gel column (30 × 3 cm) irrigated with light petroleum – ethyl acetate (3:2). The yield of pure α-anomer, which was eluted first, was 60 – 65 %, the recovery of the β-anomer was 20 – 25 %.

Ethyl 2,3,4,6-tetra-O-*acetyl-1-thio-α*-D-*glucopyranoside* crystallized from ethanol and showed m.p. 96 – 97 °C, $[\alpha]_{578}^{20}$ +204°, in good agreement with published values.[12]

Isopropyl 2,3,4,6-tetra-O-*acetyl-1-thio-α*-D-*glucopyranoside* crystallized from ethanol and showed m.p. 69 – 70 °C, $[\alpha]_{578}^{20}$ +204°. (Found: C 50.1; H 6.3; S 7.6. $C_{17}H_{69}O_2S$ requires: C 50.2; H 6.4; S 7.9.)

1-Heptyl 2,3,4,6-tetra-O-*acetyl-1-thio-α*-D-*glucopyranoside* crystallized from hexane and showed m.p. 40 – 41 °C, $[\alpha]_{578}^{20}$ +181°.

NMR spectra of the three 1-thio-α-D-glucopyranosides were in agreement with the proposed structures and almost superimposable, except for the signals from the protons in their aglycones.

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The Preparation of 4-Hydroxy-5,6-dihydro-2-pyrones and Their Conversion to Kawa-lactones as well as to Other Precursors of Naturally Occurring 2-Pyrones

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The dianion of ethyl acetoacetate was reacted with a series of aldehydes to give aldol-type products, which after hydrolysis and acidification readily lactonized to 4-hydroxy-5,6-dihydro-2-pyrones. Some of the lactones were methylated to give the Kawa-lactones kawain, marindinin, methysticin, and 5,6-dihydroyangonin. Furthermore, synthetic precursors (and in one case possibly a biogenetic precursor) to radicinin, pestalotin, a pestalotin-related metabolite, and nectriapyrone were prepared.

2-Pyrones constitute an important sub-group of the acetogenins, examples being the Kawa-lactones[1] [*e.g.* kawain (*2a*) and methysticin (*2e*)] and the gibberellin synergist pestalotin (*5*).[2,3]

The Kawa-lactones have earlier been prepared according to the following procedures: Reformatsky-type reactions from 4-bromo-3-methoxycrotonic esters,[4] conjugate addition of methanol to 5-hydroxy-2-alkynoic esters,[5] or by $TiCl_4$-promoted addition of diketene to aldehydes.[6] Recently Seebach and Meyer [7] have published an elegant synthesis of pestalotin (*5*) by acetoacetate dianion addition to an aldehyde, and Carlson and Oyler [8] have reported on the synthesis of the same compound using the propiolic acid dianion as an acyl equivalent.

We here report on the synthesis of some naturally occurring 5,6-dihydro-2-pyrones and their synthetic precursors by modifying the method of Seebach and Meyer,[7] by which the dianion of ethyl acetoacetate reacts with an aldehyde to give an aldol-type product, which upon hydrolysis and acidification readily lactonizes to a 4-hydroxy-5,6-dihydro-2-pyrone (*1*).*

We have found it to be very important that the hydrolysis of the aldol product first formed is carried out at low temperature (0 °C) in order to avoid decarboxylation and retroaldol processes. Following the original procedure,[7] the formation of by-products made isolation difficult. This is shown by the reaction of 4-methoxycinnamaldehyde with ethyl acetoacetate giving a mixture of about equal amounts of the expected dihydropyrone and of 6-(4-methoxyphenyl)-3,5-hexadien-2-one (NMR evidence), only the latter compound being readily isolated. Ether extraction of the alkaline hydrolysate before acidification greatly facilitates the work-up. Taking these precautions, we have prepared compounds *1a*–*1i* and the 7,8-dihydro derivative of *1a*, mostly in good yields, the purity of the crude products usually being good enough for preparative purposes. The NMR-data recorded in Table 1 give evidence for correct structure assignments and reveal that the synthesized 4-hydroxy-5,6-dihydro-2-pyrones exist fully enolized when dissolved in DMSO.

O-Methylation of the appropriate 4-hydroxy-5,6-dihydro-2-pyrones to the naturally occurring compounds *2a*, *2d*, *2e*, and marindinin (7,8-dihydro derivative of *2a*) gave some difficulties, when the phenyl substituent of *1* carried alkoxy groups. While *1a* was smoothly methylated under standard conditions (di-

* *Added in proof. Cf.* also D. Seebach and H. Meyer *Ger. Offenlegungsschrift* P 2400429.3 of 17.7.75.

Table 1. ^{1}H NMR shifts (in ppm downfield from internal TMS) and coupling constants (in Hz) of 4-hydroxy-5,6-dihydro-2-pyrones. Solvent: DMSO-d_6. Observed multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. The assignments are made in accordance with Achenbach and Regel.[18]

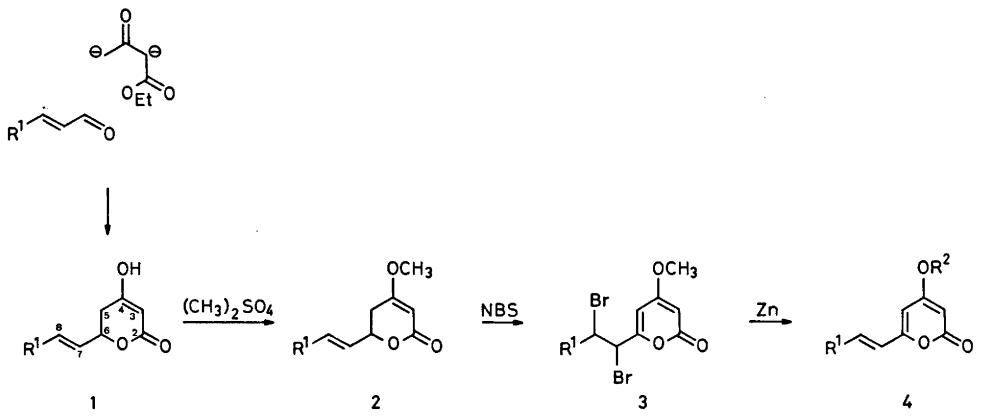
Compound	3-H [a]	5-H	5-H [b]	6-H	7-H [a,c,d]	8-H [c]	R
1a	5.00 s	2.54 s	2.66 d	4.80–5.20 m	6.25 q	6.70 d	7.10–7.50 (aromatic H)
1b	5.08 s	2.56 s	2.68 d	4.87–5.35 m	6.38 q	7.05 d	6.75–7.85 (aromatic H) 3.86 s (OCH_3)
1c	5.05 s	2.55 s	2.66 d	4.90–5.35 m	6.37 q	6.75 q	6.70–7.50 (aromatic H) 3.79 s (OCH_3)
1d	4.96 s	2.50 s	2.63 d	4.65–5.10 m	6.16 q	6.65 q	6.80–7.50 (aromatic H) 3.72 s (OCH_3)
1e	4.97 s	2.50 s	2.62 d	4.80–5.30 m	6.15 q	6.60 d	6.80–7.20 (aromatic H) 5.98 s (OCH_2O)
1f	5.03 s	2.55 s	2.68 d	4.80–5.30 m	6.35 q	covered by aromatic H	6.80–7.30 (aromatic H) 3.72 s (OCH_3) 3.82 s (OCH_3)
1g	5.10 s	2.54 s	2.68 d	4.80–5.25 m	6.27 q	6.67 d	6.40–7.25 (aromatic H) 3.78 s (OCH_3) 3.80 s (OCH_3)
7,8-dihydro derivative of *1a*	5.00 s	2.36 s	*ca.* 2.5	4.5–5.9 m	1.70–2.18 m	2.34–2.92 m	7.25 (aromatic H)
1h	4.85 s	2.45 s	2.58 d	4.70–5.10 m	5.55 q	5.75 m	1.75 d (*J* 5)
1i	4.92 s	2.38 s	2.53 d	4.50–5.00 m	5.48 q	5.86 m	0.87 t (*J* 6.5) 1.30 m (*J* 6.5) 2.00 q (*J* 6.5)
9	1.67 s	2.1–3.1 complex		4.69 q [e]	1.67 s	5.61 q (*J* 6)	1.60 (C-9 CH_3, only one component of d visible)

[a] For compound *9* the value indicates the shift of the methyl group at the given position. [b] $J_{5,6}$ is in the range of 1–2 Hz. [c] $J_{7,8}$ is for all 5,8-unsaturated compounds except for *9* in the range of 15–16 Hz. [d] $J_{6,7}$ is for all 7,8-unsaturated compounds except for *9* in the range of 4–6 Hz. [e] $J_{5ax,6ax}$ = 12 Hz, $J_{5eq,6ax}$ = 5 Hz.

methyl sulfate and potassium carbonate in refluxing acetone) to give kawain (*2a*) (55 % over-all yield from cinnamaldehyde), there was extensive decomposition of the other compounds. When the reaction was performed at room temperature according to Isawa and Mukaiyama,[6] marindinin (7,8-dihydro derivative of *2a*) was obtained in good yield from the corresponding 4-hydroxy-5,6-dihydro-2-pyrone, but *1d* still gave extensive decomposition. Treatment of compounds *1d* and *1e* with diazomethane afforded 5,6-dihydroyangonin (*2d*, 14 % yield) and methysticin (*2e*, 38 % yield), respectively.

The purpose of synthesizing compounds *1b*, *1c*, *1f*, and *1g* was to investigate a possible connection between the substitution pattern of alkoxy groups on the aromatic nucleus and the yield of methylated product. Methylation of the just-mentioned 4-hydroxy derivatives resulted in extensive decomposition of *1f* and *1g*, whereas it was possible to isolate *2b* and *2c* in moderate yields.

The acetoacetate dianion synthesis was also applied to the synthesis of 4-hydroxy-6-(1-propenyl)-2-pyrone (*4b*) a synthetic [9] and possibly biogenetic [10] precursor of radicinin. The two syntheses of *4b* published proceed *via* demethylation of *4a* and involve several steps from either the difficultly accessible tetraacetic acid lactone [9] or from triacetic acid lactone.[11] 4-Hydroxy-6-(1-propenyl)-5,6-dihydro-2-pyrone (*1h*) was prepared in 75 % yield from crotonaldehyde and methylated with dimethyl sulfate and potassium carbonate in refluxing acetone to give *2h* in 82 % yield. Allylic bromination of the methylated product was expected to give either a 5-bromo or a 6-bromo derivative if not the fully unsaturated pyrone derivative. Surprisingly, the only compound which could be isolated was the dibromo derivative (*3a*). The yield isolated was 36 or



a: R^1 = phenyl
b: R^1 = 2-methoxyphenyl
c: R^1 = 3-methoxyphenyl
d: R^1 = 4-methoxyphenyl
e: R^1 = 3,4-methylenedioxyphenyl
f: R^1 = 2,3-dimethoxyphenyl
g: R^1 = 3,4-dimethoxyphenyl
h: R^1 = methyl
i: R^1 = propyl

a: R^1 = methyl

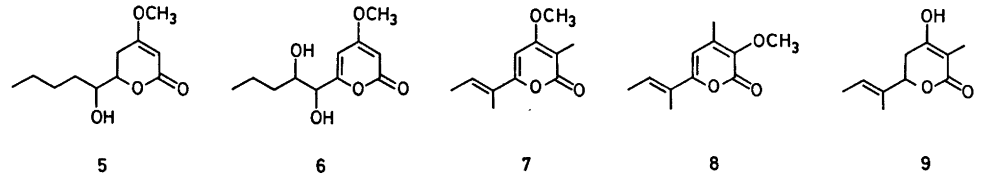
a: R^1 = methyl, R^2 = methyl
b: R^1 = methyl, R^2 = hydrogen

42 %, when one equivalent or two equivalents of *N*-bromosuccinimide, respectively, were used. Dehalogenation with zinc of the dibromo derivative gave 4-methoxy-6-(1-propenyl)-2-pyrone (*4a*) in 93 % yield. Even though the yield in synthesizing *3a* is low, we believe that *4a*, and therefore also *4b*, can be synthesized more easily by our method than by the two above-mentioned.[9,11] Furthermore, the conversion of *2h* to *4a* can be effected without isolation of the intermediate dibromo derivative. The reaction of *2h* to give *3a* is highly unexpected and is accompanied by minor brominated products, which contain vinylic protons in the side chain (NMR evidence). Therefore, we believe that the reaction is best explained by assuming allylic bromination at the 5 as well as the 6 position of the ring followed by two allylic rearrangements giving rise to the more stable pyrone *3a*.

The 6-(1-pentenyl) derivative (*1i*) is of interest seen in the light of the recent isolation of the *Penicillium* metabolite (*6*) [12] and represents an alternative synthon for the synthesis of pestalotin (*5*). Synthetic approaches to *6* with the aim of establishing the relative configuration as well as to *5* are in progress.

Recently, an antibiotic monoterpenoid, nectriapyrone, has been isolated from *Gyrostroma missouriensis* Seeler.[13] Nectriapyrone has been assigned structure *7*, but structure *8* cannot be completely ruled out. We believe that *7* is obtainable from *9*, which is prepared by reacting tiglic aldehyde with the dianion of ethyl 2-methylacetoacetate. Work is in progress for synthesizing *7*.*

* *Added in proof.* Nectriapyrone (*7*) has now been synthesized: T. Reffstrup and P.M. Boll *Tetrahedron Lett.* (1976) 1903.



5 6 7 8 9

EXPERIMENTAL

All m.p.'s are uncorrected. ^{1}H NMR spectra were recorded on a JEOL C-60 HL spectrometer. UV spectra were recorded on a Beckman ACTA III spectrophotometer. For explanation of NMR data *cf.* Table 1. Microanalyses were performed at the Microanalytical Department of the University of Copenhagen. The progression of the methylation reactions were monitored conveniently by TLC with ether or ether/light petroleum as eluent.

General procedure for preparation of 6-substituted 4-hydroxy-5,6-dihydro-2-pyrones (1). The dianion of ethyl acetoacetate (2.6 g, 0.02 mol) is prepared in 50 ml of tetrahydrofuran with sodium hydride (0.02 mol) and butyllithium (0.02 mol) according to the method of Huckin and Weiler.[14] The aldehyde (0.01 mol) is added dropwise (solid aldehydes are dissolved in tetrahydrofuran prior to addition) to the stirred reaction mixture maintained at 0 °C. The stirring is continued for another 10 min and the mixture is then added dropwise to 300 ml of ice water. The strongly alkaline solution is stirred at room temperature for 3.5 h, after which time it is extracted with ether (3 × 75 ml). Cooling of the aqueous phase and careful acidification to pH 1 with conc. HCl under simultaneous addition of ice result normally in the precipitation of crystalline material. If the pyrone separates as oily drops, the aqueous phase is extracted with CH_2Cl_2 to give, after drying and evaporation of solvent, crystalline material.

The compounds synthesized in this manner are quite pure, and give after trituration with ether, often satisfactory elemental analyses. Comparison of NMR spectra of the isolated products and the recrystallized products confirms the statement that the products can be considered as "practical grades" and that they can be used directly for synthetic purposes.

4-Hydroxy-6-(α-trans-styryl)-5,6-dihydro-2-pyrone (1a). Reaction of cinnamaldehyde (2.64 g, 0.02 mol) gave 3.68 g (85 %) of very light yellow crystals, m.p. 109–111 °C (decomp.), which upon recrystallisation from ether/methanol afforded colourless crystals with m.p. 128–130 °C (decomp.) (lit.[5] m.p. 142–144 °C)* UV 256 nm (ε 17 500), 263 nm (ε 17 200) (lit.[5] UV 247–248 nm (ε 29 000)*. (Found: C 72.65; H 5.66. Calc. for $C_{13}H_{12}O_3$: C 72.71; H 5.59).

4-Hydroxy-6-(α-trans-2-methoxystyryl)-5,6-dihydro-2-pyrone (1b). 2-Methoxycinnamaldehyde (5.99 g, 0.044 mol) was reacted to give 7.00 g (76 %) of a light yellow powder, m.p. 109–112 °C (decomp.). On recrystallisation from ether/methanol colourless crystals of m.p. 130–132.5 °C (decomp.) were obtained (Anal. $C_{14}H_{14}O_4$: C, H, O).

* The reported data are more in agreement with those found for kawain.[1]

4-Hydroxy-6-(α-trans-3-methoxystyryl)-5,6-dihydro-2-pyrone (1c). 3-Methoxycinnamaldehyde (5.0 g, 0.034 mol) gave upon reaction 4.47 g (59 %) of a yellow powder, m.p. 106–110 °C (decomp.). Recrystallisation from ether/methanol raised the m.p. to 129–133 °C (decomp.) (Found: C 68.10; H 5.80. Calc. for $C_{14}H_{14}O_4$: C 68.28; H 5.73).

4-Hydroxy-6-(α-trans-4-methoxystyryl)-5,6-dihydro-2-pyrone (1d). 4-Methoxycinnamaldehyde (3.53 g, 0.022 mol) afforded on reaction 3.96 (74 %) of powderlike yellow crystals, m.p. 117–120 °C (decomp.), which upon recrystallisation from ether/methanol gave colourless crystals with m.p. 128–130 °C (decomp.) (Found: C 68.40; H 5.95. Calc. for $C_{14}H_{14}O_4$: C 68.28; H 5.73).

4-Hydroxy-6-(α-trans-3,4-methylenedioxystyryl)-5,6-dihydro-2-pyrone (1e). Reaction of 3,4-methylenedioxycinnamaldehyde (3.90 g, 0.022 mol) afforded 4.78 g (74 %) of a reddish powder, m.p. 119–122 °C, shrinking at 115 °C (decomp.). Recrystallisation from ether/light petroleum gave not quite colourless crystals of m.p. 128–132 °C (decomp.) (Found: C 64.45, H 4.88. Calc. for $C_{14}H_{12}O_5$: C 64.61; H 4.64).

4-Hydroxy-6-(α-trans-2,3-dimethoxystyryl)-5,6-dihydro-2-pyrone (1f). When 2,3-dimethoxycinnamaldehyde (1.26 g, 0.008 mol) was reacted 1.02 g (56 %) of yellow powder, m.p. 110–114 °C (decomp.), was obtained. Recrystallisation from ether/methanol resulted in colourless crystals, m.p. 119–122 °C (decomp.) (Found: C 64.55; H 5.74. Calc. for $C_{15}H_{16}O_5$: C 65.21; H 5.84).

4-Hydroxy-6-(α-trans-3,4-dimethoxystyryl)-5,6-dihydro-2-pyrone (1g). 3,4-Dimethoxycinnamaldehyde (2.16 g, 0.011 mol) afforded upon reaction 1.16 g (37 %) of colourless crystals, m.p. 112–114 °C (decomp.). On recrystallisation from ether/methanol the m.p. was raised to 130–135 °C (decomp.) (Found: C 64.50; H 5.68. Calc. for $C_{15}H_{16}O_5$: C 65.27; H 5.84).

4-Hydroxy-6-phenylethyl-5,6-dihydro-2-pyrone. 3-Phenylpropanal (9.0 g, 0.067 mol) was reacted to give 12.46 g (85 %) of the title compound, m.p. 98–100 °C (decomp.). Trituration with ether afforded analytically pure title compound with m.p. 121–123 °C (decomp.) (Found: C 71.40; H 6.53. Calc. for $C_{13}H_{14}O_3$: C 71.54; H 6.47).

4-Hydroxy-6-(trans-1-propenyl)-5,6-dihydro-2-pyrone (1h). Crotonaldehyde (0.70 g, 0.01 mol) was reacted to give 1.16 g (75 %) of colourless crystals, m.p. 101–103 °C (decomp.). Recrystallisation from ether/methanol afforded 0.75 g of the title compound, m.p. 110–112 °C (decomp.) (Found: C 62.05; H 6.59. Calc. for $C_8H_{10}O_3$: C 62.32; H 6.54).

4-Hydroxy-6-(trans-1-pentenyl)-5,6-dihydro-2-pyrone (1i). *trans*-2-Hexenal (9.8 g, 0.1 mol) gave upon reaction 16.17 g (89 %) of yellow crystals melting at 71–75 °C (decomp.). Recrystallized from ether/light petroleum the m.p. of the title compound was 86–87 °C

(decomp.) (Found: C 65.70; H 7.89. Calc. for $C_{10}H_{14}O_3$: C 65.91; H 7.74).

*4-Hydroxy-3-methyl-6-(*trans*-1-methyl-1-propenyl)-5,6-dihydro-2-pyrone* (*9*). Ethyl 2-methylacetoacetate (5.76 g, 0.04 mol) was converted to the dianion,[14] and to the reaction mixture was added tiglic aldehyde (3.36 g, 0.04 mol). When worked up following the general procedure 5.60 g (77 %) of colourless crystals of m.p. 134–139 °C (decomp.) were isolated. After trituration with ether the title compound melted at 140–141 °C (decomp.) (Found: C 65.70; H 7.60. Calc. for $C_{10}H_{14}O_3$: C 65.91; H 7.74).

6-(4-Methoxyphenyl)-3,5-hexadien-2-one. This synthesis was carried out following the procedure of Seebach and Meyer,[7] but with the purpose of synthesizing a dihydropyrone: To 0.03 mol of the dianion of ethyl acetoacetate in 50 ml of tetrahydrofuran[14] maintained at 0 °C was added solid 4-methoxycinnamaldehyde (2.43 g, 0.015 mol) in one portion. After solution the mixture was stirred for another 10 min and then poured into 300 ml of water at room temp. and stirred for 3.5 h. The mixture was acidified with conc. HCl to pH 1 and the solid formed was filtered off. Yield: 3.37 g, m.p. 108–115 °C. NMR spectra indicated that the crude product was a mixture of 55 % of the dihydropyrone (*1d*) and 45 % of the title compound. Three recrystallisations from ether/methanol afforded the title compound in low yield, m.p. 107–108 °C (lit.[15] m.p. 107–108.5 °C) NMR ($CDCl_3$) 2.50 (s, 3 H), 3.72 (s, 3 H), 6.18 (d, 1 H), 6.57 (d, 1 H), 6.75–7.60 (complex, 6 H).

(R,S)-*Kawain* (*2a*). A solution of *1a* (0.432 g, 0.002 mol) in dry acetone (10 ml) was mixed with dimethyl sulfate (0.315 g, 0.0025 mol) and anhydrous potassium carbonate (1 g). The mixture was refluxed for 7 h, cooled, filtered and the solid washed with dry acetone. Evaporation of the combined filtrate and washings gave a residue, which on trituration with ether deposited slightly yellow crystals (300 mg, 65 %), m.p. 136–138 °C. Recrystallisation from ether/methanol gave colourless crystals with m.p. 144–145 °C (lit.[16] m.p. 142–144 °C) UV 244 nm (ε 24 800) (lit.[17] 245 nm (ε 25 700). The NMR spectrum was in accord with that published.[18]

(R,S)-*Methysticin* (*2e*). Compound *1e* (0.520 g, 0.002 mol) dissolved in methanol/ether was reacted with excess of diazomethane. On evaporation of solvent 0.52 g of a yellow oil was obtained. It could not be induced to crystallize and was thus separated by preparative TLC (silica gel, ether as eluent). The fraction with $R_F = ca.$ 0.5 was isolated to give 210 mg (38 %) of yellow crystals, m.p. 114–124 °C. Recrystallisation from ether/methanol raised the m.p. to 130–131.5 °C with shrinking at 125 °C (lit.[4] m.p. 132–134 °C). The NMR spectrum was in accord with that published.[18]

5,6-Dihydroyangonin (*2d*). Treatment of *1d* (0.492 g, 0.002 mol) in methanol/ether with excess of diazomethane gave, after removal of solvent, 0.50 g of a light orange oil containing a small amount of crystals. Recrystallisation was carried out without success. Separation by preparative TLC (silica gel, ether as eluent, $R_F = 0.45$) afforded 0.075 g (14 %) of crystals, m.p. 103–107 °C. Recrystallisation from ether/methanol raised the m.p. to 120–121 °C (lit.[20] m.p. 123 °C). NMR (DMSO-d_6): δ 2.54 (s, 1 H), 2.67 (d, 1 H, $J_{5,6} = 3$), 3.78 (s, 3 H), 4.80–5.15 (m, 1 H), 5.20 (s, 1 H), 6.19 (q, 1 H, $J_{7,8} = 16$, $J_{6,7} = 5$), 6.70 (d, 1 H, $J_{7,8} = 16$), 6.90–7.60 (aromatic H).

(R.S)-*Marindinin.* 4-Hydroxy-6-phenylethyl-5,6-dihydro-2-pyrone (0.436 g, 0.002 mol) in dry acetone (20 ml) was stirred for 15 h at room temp. with anhydrous potassium carbonate (1 g) and dimethyl sulfate (0.315 g, 0.0025 mol). On work-up as described for (*R,S*)-kawain 0.48 g of a slight yellow oil was obtained. It soon solidified and was recrystallized from ether to give the title compound (0.21 g, 45 %) m.p. 68–70 °C (lit.[19] m.p. 73–74 °C). The NMR spectrum was in accord with that published.[18]

*4-Methoxy-6-(*α-trans*-2-methoxystyryl)-5,6-dihydro-2-pyrone* (*2b*). Compound *1b* (0.49 g, 0.002 mol) was methylated by the method given for (*R,S*)-kawain to yield 0.52 g of brown syrup, which was separated by preparative TLC (silica gel, ether as eluent, $R_F = 0.5$). The title compound was isolated as partly crystalline material (0.260 g, 50 %). Crystallisation was unsuccessful. NMR (DMSO-d_6) 2.52 (m, 1 H), 2.69 (m, 1 H), 3.78 (s, 3 H), 3.85 (s, 3 H), 5.1 (m, 1 H), 5.23 (s, 1 H), 6.35 (q, 1 H, $J = 15$ and 6), 6.8–7.6 (complex, 5 H).

*4-Methoxy-6-(*α-trans*-3-methoxystyryl)-5,6-dihydro-2-pyrone* (*2c*). Compound *1c* (0.49 g, 0.002 mol) was methylated by the method given for (*R,S*)-kawain to yield 0.50 g of light brown syrup. Separation by preparative TLC (silica gel, ether as eluent, $R_F = 0.5$) afforded a nearly colourless syrup (0.220 g, 42 %) of the title compound. When left in ether/methanol for 2 weeks at 4 °C 0.059 g of crystals of *2c* was obtained, m.p. 82–84 °C. NMR (DMSO-d_6) 2.52 (m, 1 H), 2.65 (m, 1 H) 3.78 (s, 6 H), 5.06 (m, 1 H), 5.23 (s, 1 H), 6.35 (q, 1 H, $J =$ 15.5 and 5), 6.1–7.4 (complex, 5 H).

*4-Methoxy-6-(*trans*-1-propenyl-5,6-dihydro-2-pyrone* (*2h*). Compound *1h* (2.5 g, 0.015 mol) was methylated as described for (*R,S*)-kawain to give 2.07 g (82 %) of crystalline material, m.p. 77–79 °C. Recrystallized from ether *2h* melted at 79–80 °C. NMR ($CDCl_3$) 1.70 (d, 3 H, $J_{8,9} = 5$), 2.38 (s, 1 H), 2.50 (d, 2 H, $J_{5,6} =$ 2), 3.70 (s, 3 H), 4.50–5.00 (m, 1 H), 5.08 (s, 1 H), 5.50 (q, 1 H, $J_{7,8} = 16$, $J_{6,7} = 5$), *ca.* 5.85 (m, 1 H, $J_{8,7} = 16$) (Found: C 63.90; H 6.88. Calc. for $C_9H_{12}O_3$: C 64.27; H 7.19).

4-Methoxy-6-(1,2-dibromopropyl)-2-pyrone (*3a*). *N*-Bromosuccinimide (1.96 g, 0.011 mol)

was added to a solution of *2h* (1.68 g, 0.01 mol) in tetrachloromethane (60 ml). The mixture was heated under reflux for 2.5 h while being irradiated with a tungsten lamp. When cooled, the mixture was filtered, the filtrates were washed with water and dried (Na_2SO_4). Removal of solvent *in vacuo* left a brown oil, which on trituration with ether afforded 1.17 g (36 %) of crystals. When recrystallized from ether the title compound melted at 114–116 °C. NMR ($CDCl_3$) *ca.* 1.90 (complex, virtual coupling,[21] 3 H), 3.75 (s, 3 H), *ca.* 4.50 (complex, virtual coupling,[21] 2 H), 5.42 (d, 1 H, $J=2$), 5.94 (d, $J=2$) (Found: C 33.44; H 3.40; Br. 47.99. Calc. for $C_9H_{10}Br_2O_3$: C 33.16; H 3.10; Br 49.02).

When the above reaction was performed with 0.022 mol of *N*-bromosuccinimide the yield was raised to 42 %.

*4-Methoxy-6-(*trans*)1-propenyl)-2-pyrone* (*4a*). Compound *3a* (0.173 g, 0.0005 mol) dissolved in 1 ml of glacial acetic acid and 5 ml of ether was treated with 0.1 g of zinc dust for 10 min. Water (0.5 ml) was added and the solution was decanted from undissolved zinc. More ether was added and the solution was extracted twice, each time with 5 ml of water, and once with 5 ml of 2 N NaOH. After drying and evaporation of the ether 77 mg (93 %) of colourless needles, m.p. 98–99 °C, were left. After recrystallisation from ether/light petroleum the m.p. was 102–103 °C (lit.[9] m.p. 102–103 °C). The NMR and IR spectra were in accord with those published.

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Pyrylium Salts. Part VIII.* Derivatives of 5,13:6,12-Bisepoxydibenzo[*a*,*f*]cyclodecenes

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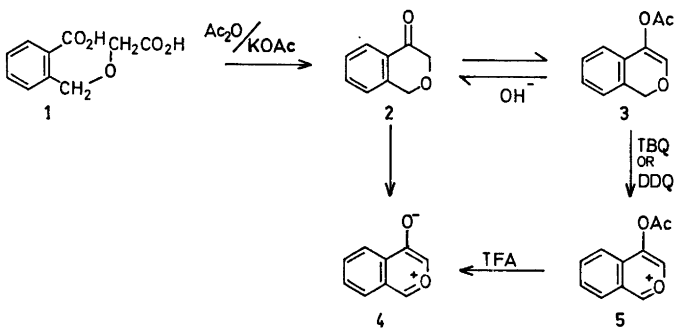
In the reaction between 4-acetoxyisochromene and TBQ or DDQ adducts were formed between the reduced quinone and the generated 4-acetoxy-2-benzopyrylium ion. The adducts in TFA yielded the *syn*- and *anti*-isomers of 5,6,12,13-tetrahydro-5,13:6,12-bisepoxydibenzo[*a*,*f*]-cyclodecene-7,14-dione; NMR showed the presence of intermediate 2-benzopyrylium-4-olate, which was dimerised.

In a recent paper we have described a synthesis of 1a,6a-dihydroindeno[1,2-*b*]azirin-6(1*H*)-ones and their photolytic rearrangements to isoquinolinium derivatives.[2] The oxygen hetero-analogue 2,3-epoxyindanone, however, was rearranged to isocoumarin on photolysis rather than to the analogous 2-benzopyrylium-4-olate.[3] The failure to form the pyrylium salt may be due to insufficient aromatic stabilisation of the pyrylium betaine (*4*). On added stabilisation from phenyl substituents, however, equilibria exist between the pyrylium and the epoxide valence isomers; the equilibrium position is dependent on conditions but is in favour of the epoxide structure.[4,5] In this work we describe a synthesis of the 2-benzopyrylium-4-olate system which is related to the method used in the synthesis of 2-benzothiopyrylium-4-olates.[1,6] The latter were readily dimerised. The oxygen analogue (*4*) was anticipated to be even more reactive in view of the relative stabilities of the thiopyrylium and pyrylium cations.[7]

The synthesis of *4* (Scheme 1) is based on hydride abstraction from isochroman-4-one (*2*). The latter is sensitive to acid as it is a benzyl ether, and it could not be satisfactorily prepared by acid catalysed cyclisation of benzyloxyacetic acid,[8] whereas the thioether analogue can be cyclised to isothiochroman-4-one in acid media.[9] Isochroman-4-one was instead prepared by cyclisation of *o*-carboxybenzyloxyacetic acid (*1*) by means of potassium acetate in acetic anhydride. The optimum yield of cyclic product (50 %) was reached by the use of four equivalents of potassium acetate in which case the major cyclic product was

* Part VII; see Ref. 1.



Scheme 1.

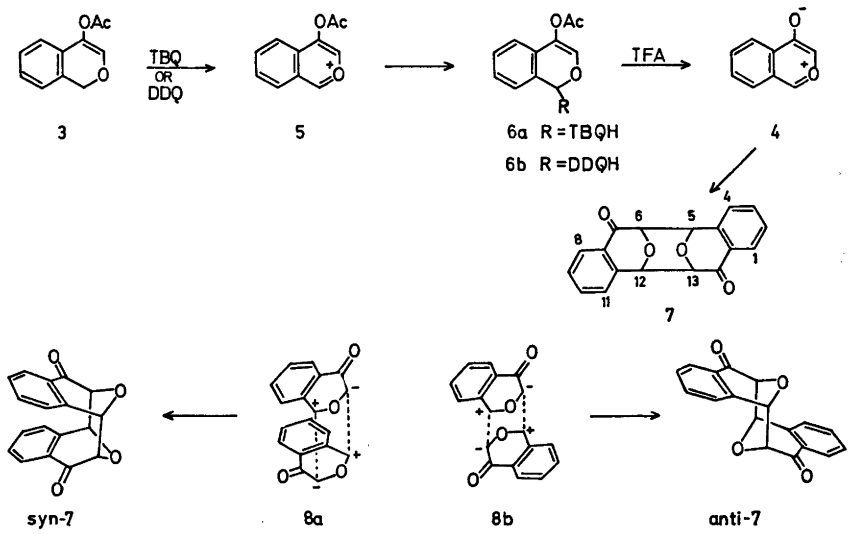
the enol acetate *3*; the latter can be converted to the ketone *2* by mild alkaline hydrolysis.

Triphenylmethyl perchlorate is a useful reagent for hydride abstraction in pyrylium synthesis;[10] it has been applied with success in hydride abstraction from isothiochroman-4-one.[6] No reaction occurred, however, between the trityl salt and the ketone *2* or its enol acetate *3* in liquid SO_2. Triphenylmethane was formed on heating the reagents together in acetonitrile or a mixture of acetic anhydride and acetic acid, but the desired products were largely polymerised. Activated quinones are alternative reagents in hydride abstractions.[11] The reactions are acid catalysed.[11,12] Fortunately the reaction between the acid sensitive 4-acetoxyisochromene (*3*) and tetrachloro-1,2-benzoquinone (TBQ)[12] proceeded readily in cold benzene solution without acid catalyst. Isochroman-4-one (*2*), however, did not react under these conditions. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) could also be used in the hydride abstraction from *3*. The products obtained in the reactions between *3* and TBQ or DDQ are assigned structure *6* which arise by bond formation between the phenolate oxygen atom in the hydroquinone formed and the highly electrophilic pyrylium carbon atom C-1.

The NMR spectra of both adducts in trifluoroacetic acid (TFA) were the same with H-1 at δ 10.4 and H-3 at 9.6; an initial paramagnetic shift for the acetyl protons from δ 2.3 to 2.6 was followed by a gradual diamagnetic shift to δ 2.3. The chemical shifts are similar to those observed for 4-acetoxy-2-benzothiopyrylium perchlorate.[6] The NMR data show that the adduct formation *6* is reversible in acids such as TFA and the diamagnetic shift of the acetyl protons is due to deacetylation of the 4-acetoxy-2-benzopyrylium cation. *Ca.* one equivalent of $TBQH_2$-monoacetate was isolated from the corresponding adduct *6a* in TFA. Attempts to isolate the concurrently formed 2-benzopyrylium-4-olate (*4*) as fluoroboric acid salt failed since *4* reacted further under polymerisation and dimerisation (20 – 27 % yield). The *syn* and *anti* dimeric structures *7* were assigned to the products from spectroscopic data. Only one isomer was isolated from the DDQ-adduct whereas the isomer mixture from the TBQ-adduct contained *ca.* 5 % of this isomer. Both isomers have strong carbonyl absorption at 1685 cm^{-1} (KBr). The NMR spectra contain two non-aromatic types of mutually coupled methine protons. Both products have the molecular ion in the mass spectra at *m/e* 292.

A symmetrical double layer arrangement in the transition state dimerisation is unlikely because of the same regional polarisation in the reactants which will result in charge repulsion. Charge attraction, however, will be operative if the reactants are oriented so that the carbonyl groups point in opposite directions (Scheme 2). The reactants can further be arranged in two ways illustrated by *8a* and *8b* to give a *syn*- and an *anti*-isomer. The *syn*-isomer can be regarded as a 1,4-dioxane derivative locked in the boat conformation, and the *anti*-isomer as a 1,4-dioxane derivative locked in the chair conformation. The coupling between the vicinal methine protons in the isomer from the DDQ-adduct (*6b*) was small (broad singlets at δ 4.4 and 5.3) whereas the coupling in the other isomer was 11 Hz (δ 4.9 and 5.5). The former isomer is assigned the *anti*-structure and the latter the *syn*-structure in accordance with the torsional angles between the vicinal methine protons in the respective locked 1,4-dioxane conformations. The aromatic proton *ortho* to the carbonyl group in the *anti*-isomer (δ 8.0 – 8.1) and in isochroman-4-one (δ 7.8 – 8.0 in CCl_4) is at lower field than the other aromatic protons (δ 7.5 – 7.9 and 7.0 – 7.5), respectively. The aromatic *ortho*-proton in the *syn*-isomer, however, lies above the other benzene ring and is in the aromatic shielding zone which opposes the deshielding effect from the carbonyl group; all the aromatic protons in the *syn*-isomer appear in the region δ 6.9 – 7.8 (CD_3CN). The structure assignments agree with the interpretations of the spectral data of the sulfur hetero-analogues in which case the structure of the parent *syn*-isomer was confirmed by X-ray analysis.[6,13]

The mass spectra of the dimers are similar but differ slightly in relative fragment intensities. The molecular ion (*m/e* 292) intensities were 42 and 10 % (*m/e* 133 base peak) for the *anti*- and *syn*-isomers, respectively. The high intensity (*ca.* 80 %) of the *m/e* 146 species



Scheme 2.

roused our interest since this mass number corresponds to the molecular weight of the betaine *4*; the latter could have arisen by thermal dissociation of the dimer *7* in the mass spectrometer prior to ionisation. The appearance potential for the *m/e* 146 species from the *anti*-isomer was found to be 9.70 eV whereas the ionisation potential of *anti-7* was 8.75 eV. The values are to be compared with the ionisation potentials of 2,3-epoxyindan-1-one (9.10 eV) and *N*-benzylisoquinolinium-4-olate (7.10 eV)[14] The ionisation potential of 2-benzopyrylium-4-olate (*4*) is expected to be no higher than that of the mesoionic *N*-benzylisoquinolinium-4-olate which excludes thermal generation of the former in the mass spectrometer. The appearance and ionisation potential data also exclude transient formation of *4* followed by thermally induced valence isomerisation to 2,3-epoxyindan-1-one; the *m/e* 146 species is formed through electron-impact induced fragmentation routes from the molecular ion of *7*.

1,3-Diphenyl-2-benzopyrylium-4-olate is the minor component in equilibrium mixtures with its valence isomer 2,3-diphenyl-2,3-epoxyindan-1-one.[4,5] A dimeric molecule is formed by the reaction of the former as a 1,3-dipolar reactant with the carbonyl group of the latter.[5] 2-Methyl-3-phenyl-2,3-epoxyindan-1-one on photolysis yielded mainly the corresponding isocoumarin, but the product also contained 6 % of a dimeric material. The latter was assigned the same cyclic skeleton as in *7* but its stereochemistry was not discussed.[15] In the dimerisation of 2-benzothiopyrylium-4-olate there is a clear preference for the *syn*-isomer,[6] and this is the preferential stereochemical course in Diels-Alder reactions and in 1,3-cycloaddition reactions,[16] whereas introduction of a methyl group into the 1-position of the heterocycle resulted in formation of the *anti*-dimer.[1] In the present work only the *anti*-isomer *7* was isolated after dissociation of the DDQ-adduct *6b* whereas *syn-7* was the major isomer formed from the TBQ-adduct.

EXPERIMENTAL

NMR spectra were recorded with a Varian A-60 or A-100 instrument, UV spectra with a Cary 14 spectrophotometer, and mass spectra with an AEI-902 spectrometer. Ionisation and appearance potential values were determined as previously described by semilog plot interpretation of the ionisation efficiency curves.[14] The ionisation and appearance potential values are the average of three determinations, the deviation being ± 0.05 eV.

4-Acetoxyisochromene (3). A solution of *o*-carboxybenzyloxyacetic acid[17] (10.0 g, 0.048 mol) and potassium acetate (20.0 g, 0.211 mol) in acetic anhydride was heated under reflux

for 3 h. The solution was then evaporated at reduced pressure, water was added, and the mixture was extracted with ether. The ether extracts were dried, evaporated and the residue was distilled; the collected material had b.p. 94–96 °C at 0.01–0.05 Torr (5.5 g) and was the title compound admixed with isochroman-4-one. The title compound was isolated by fractional crystallisation from dilute ethanol as the less soluble material and was obtained in 49 % yield (4.4 g), m.p. 48–49 °C (Lit.[8] 49 °C); δ ($CDCl_3$) 2.3 (MeCO), 5.2 (2 H-1), 6.9 (H-3), 7.0–7.5 (4 H-arom); λ_{max} (MeCN) 244 (log ε 3.91), 280 nm (3.77).

Isochroman-4-one (2). 4-Acetoxyisochromene (4.0 g, 0.021 mol) was dissolved in ethanol (20 ml) and 2 N NaOH added dropwise until the base was no longer consumed. The solution was then diluted with water and left in the cold. The title compound was precipitated in 70 % yield (2.2 g), m.p. 53 °C [Lit.[8] 53 °C]; δ (CCl_4) 4.2 (2 H-3), 4.8 (2 H-1), 7.8–8.0 (H-5), 7.0–7.5 (3 H-arom); λ_{max} (MeCN) 245 (log ε 4.01), 288 cm (3.18).

4-Acetoxy-1-(2-hydroxy-3,4,5,6-tetrachlorophenoxy)isochromene (6a). A solution of TBQ (6.6 g, 0.027 mol) in anhydrous benzene (75 ml) was added dropwise to a solution of 4-acetoxyisochromene (5.0 g, 0.027 mol) in anhydrous benzene (25 ml). The precipitated product was filtered off after 5 h at room temperature and the product washed with cyclohexane to remove any TBQ; yield 6.5 g (57 %). The analytical sample was recrystallised from toluene, m.p. 153 °C. (Found: C 46.97; H 2.36 Calc. for $C_{17}H_{10}Cl_4O_5$: C 47.10; H 2.31); δ (acetone-d_6) 2.3 (Ac), 6.3 (H-1), 6.7 (H-3), 7.2–7.4 (4H-arom.); λ_{max} (MeCN) 217 (log ε 4.35), 263 (4.01), 298 nm (3.44).

4-Acetoxy-1-(2,3-dichloro-5,6-dicyano-4-hydroxyphenoxy)-isochromene (6b) was prepared as above with DDQ instead of TBQ. The precipitated adduct after 3 h was slightly contaminated with DDQ and $DDQH_2$ which were difficult to remove due to low solubilities. The crude product (*ca.* 75 % yield) was used in the next reaction step without further purification. δ (acetone-d^6) 2.3 (Ac), 6.6 (H-1), 6.9 (H-3), 7.2–7.5 (4 H-arom.); λ_{max} (MeCN) 220 (log ε 4.5), 255 (4.1), 347 nm 3.8).

syn-*5,6,12,13-Tetrahydro-5,13:6,12-bisepoxydibenzo*[a,f]*cyclodecene-7,14-dione (7).* The TBQ-adduct *6a* (4.3 g, 0.01 mol) was added to anhydrous TFA (100 ml), and the mixture was stirred vigorously at room temperature for 1 h. $HBF_4 \cdot Et_2O$ (1.6 g, 0.01 mol) was added, and the stirring was continued for 48 h. After evaporation the residue was extracted with ether (3 × 75 ml), and the ether solution was washed with 2 N Na_2CO_3 to remove the acid and most of the hydroquinone. The ether solution was then evaporated and the residue dissolved in chloroform, and the solution was passed through a column of basic aluminium oxide in order to remove the residual hydroquinone. The chloroform eluate was slowly concentrated in a stream of nitrogen when most of the *anti*-isomer *7* crystallised out. The remaining solution was chromatographed on a silica gel column. The residual *anti*-isomer was eluted before the *syn*-isomer *7* with chloroform. The *syn*-isomer crystallised from the chloroform eluate on slow concentration in a stream of N_2; m.p. 172–174 °C. The total yield of the *anti*-isomer (*7*) was 1 % (20 mg), and the yield of the *syn*-isomer *7* was 19 % (270 mg). (Found: C 74.22; H 4.18. Calc. for $C_{18}H_{12}O_4$: C 73.97, H 4.11). δ (CD_3CN) 4.9 (H-6 and –13, d, $J_{5,6} = J_{12,13} = 11$ Hz), 5.5 (H-5 and H-12, d), 6.9–7.8 (8 H-arom); λ_{max} (MeCN) 250 (log ε 4.28), 296 nm (3.34); m.s. (*m/e*) 292 (10 %, M), 246 (7), 159 (13), 147 (18), 146 (77), 134 (44), 133 (100), 131 (42).

anti-*5,6,12,13-Tetrahydro-5,13:6,12-bisepoxydibenzo*[a,f]*cyclodecene-7.14-dione (7)* was prepared as above from the DDQ-adduct *6b*. Again a chloroform solution of the reaction product was passed through a column of basic aluminium oxide to remove the residual hydroquinone. The title compound was crystallised from the chloroform eluate by slow concentration of the solution under a stream of N_2; yield 27 %, m.p. 280 °C. (decomp.). (Found: C 73.55; H 4.03. Calc. for $C_{18}H_{12}O_4$: C 73.97; H 4.11). δ (DMSO-d_6) 4.4 (H-6 and H-13, broad singlet), 5.3 (H-5 and H-12, broad s), 7.5–7.9 (6 H-atom), 8.0–8.1 (H-1 and H-8); λ_{max} (MeCN) 240 (log ε 4.45), 291 nm (3.42); m.s. (*m/e*) 292 (42 %, M), 246 (8), 159 (19), 147 (13), 146 (87), 134 (35), 133 (100), 131 (30).

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Reaction of Partially Benzoylated Sugars with Hydrogen Bromide. Preparation of Some Deoxyhexofuranoses

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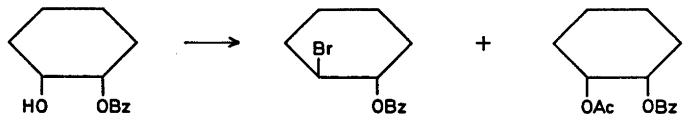
Treatment of methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside with hydrogen bromide in acetic acid followed by reaction with silver benzoate gave tetra-*O*-benzoyl-6-bromo-6-deoxy-β-D-galactofuranose. The latter was reduced to tetra-*O*-benzoyl-β-D-fucofuranose and to 5,6-dideoxy-α-L-*arabino*-hexofuranose derivatives.

Golding *et al.*[1] have found that *cis*-1,2-cyclohexanediol reacts rapidly with hydrogen bromide in acetic acid (HBA) to give *trans*-1-acetoxy-2-bromocyclohexane in high yield. Other 1,2-diols react similarly; the reaction proceeds *via* a monoacetate and a 1,3-dioxolanylium ion, which subsequently undergoes substitution with bromide ions to give the *trans*-acetoxy bromide.[1,2]

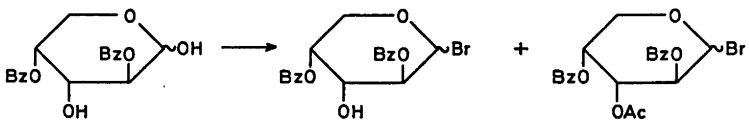
This reaction might be used for the preparation of bromo-deoxy carbohydrates by treatment of partially acylated carbohydrates with HBA. Since partially benzoylated sugars are often more readily prepared, and more stable, than their acetylated counterparts it was first investigated whether *cis*-2-benzoyloxycyclohexanol (*1*) would react with HBA analogous to the acetate. This was actually found to be the case; *trans*-1-benzoyloxy-2-bromocyclohexane (*2*) was formed in high yield together with the acetate (*3*) when *1* was treated with HBA for 2 h.

Attempts to apply this reaction to the preparation of bromo-deoxy sugars were in most cases unsuccessful. Treatment of 2,4-di-*O*-benzoyl-*D*-arabinopyranose (*4*) with HBA rapidly gave the corresponding glycosyl bromide (*5*). On further reaction the latter was slowly acetylated to give the 3-*O*-acetate (*6*). Bromine was not introduced at any position other than C1. Similar results were found when 1,3,5-tri-*O*-benzoyl-α-D-ribofuranose was treated with HBA.

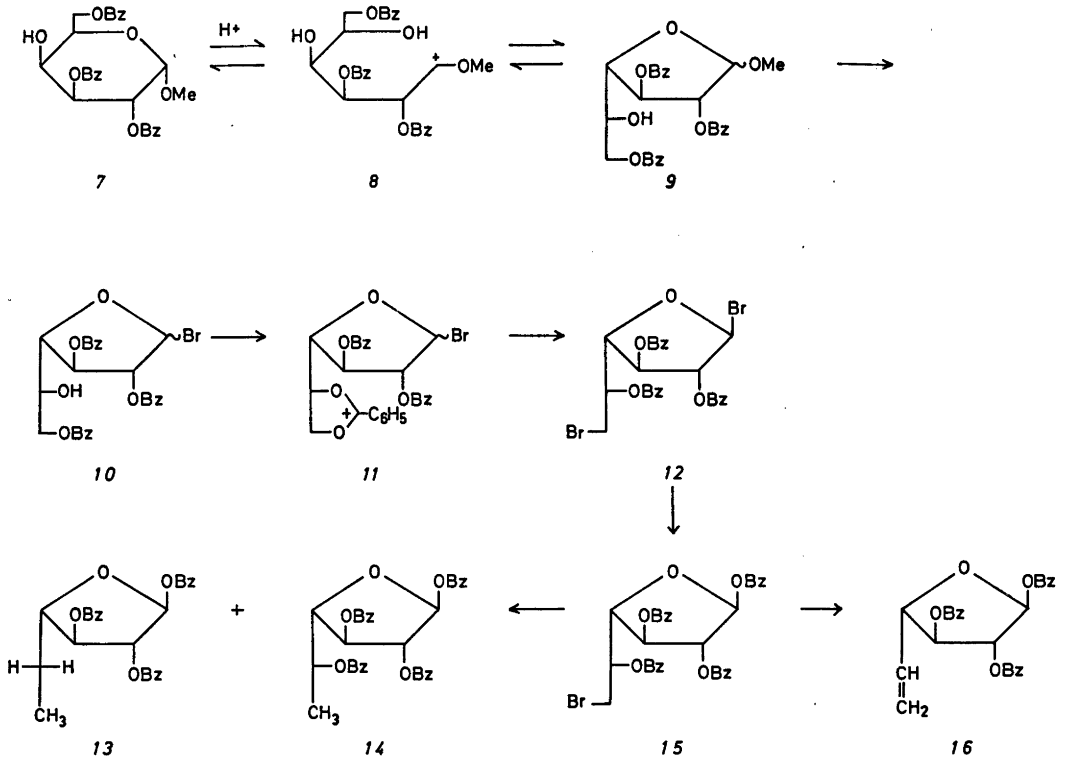
When, on the other hand, methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside (*7*) was treated with HBA for 3 h at room temperature it was converted into tri-*O*-benzoyl-6-bromo-6-deoxy-β-D-galactofuranosyl bromide (*12*). Since *12*



1 *2* *3*



4 *5* *6*



is a rather unstable syrup it was not purified. Treatment of crude *12* with silver benzoate gave the crystalline 1-*O*-benzoate (*15*) in *ca.* 50 % yield based on *7*.

The first step in the reaction of *7* with HBA is probably a ring-contraction, giving the methyl furanoside (*9*) *via* the oxo-carbonium ion (*8*).[3,4] Reaction of *9* with HBr may then give the corresponding bromide (*10*) which yields the benzoxonium ion (*11*). Subsequent reaction of *11* with bromide ions finally gives *12*.

Hydrogenolysis of *15* with palladium on carbon gave tetra-*O*-benzoyl-β-D-fucofuranose (*14*) accompanied by a small amount of tri-*O*-benzoyl-5,6-dideoxy-α-L-*arabino*-hexofuranose (*13*). The latter is probably formed *via* the 5,6-unsaturated furanose (*16*). Reduction of *15* with zinc in acetic acid gave a good yield of *16* which on catalytic hydrogenation yielded *13* as the sole product. Some methyl 5,6-dideoxy-α-L-*arabino*-hexofuranosides have been prepared previously by Ball *et al.*[5]

The structure of *14* was proved through its ^{1}H NMR spectrum and by the fact that it gave crystalline D-fucose on debenzoylation. The structures of *12*, *13*, *15*, and *16* were derived from their NMR spectra only.

The reaction described above, although of limited general applicability, provides a convenient method for the preparation of D-fucofuranose derivatives.

EXPERIMENTAL

Melting points are uncorrected. Preparative thin layer chromatography (TLC) was performed on 20 × 40 cm plates using 1 mm layers of Merck silica gel PF_{254}. ^{1}H NMR spectra were measured at 100 or 90 MHz in deuteriochloroform using tetramethylsilane as internal reference.

trans-*2-Bromo-benzoyloxycyclohexane (2)*. To *cis*-2-benzoyloxycyclohexanol [6] (1.0 g) in dichloromethane (5 ml) was added 5 ml of a 30 % solution of hydrogen bromide in glacial acetic acid (HBA) and the mixture was kept at room temperature for 2 h. It was then diluted with dichloromethane, washed with water and aqueous sodium hydrogen carbonate, dried and evaporated. The residue (1.0 g) was separated into two fractions by column chromato-

graphy on silica gel using ether-pentane (1:3) as eluent. The fast moving fraction gave 765 mg (59 %) of *2*. An NMR spectrum was in agreement with the structure and showed no impurities. Crystallization from ethanol gave 500 mg of a product with m.p. 63 – 64 °C (reported [7] m.p. 64 °C).

The second fraction gave 105 mg (9 %) of *cis*-2-acetoxy-benzoyloxycyclohexane (*3*), which had its NMR spectrum identical with that of a previously described product.[6]

*Tetra-O-benzoyl-6-bromo-6-deoxy-β-*D*-galactofuranose (15).* Methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside [8] (*7*) (5.0 g) was dissolved in dichloromethane (5 ml) and HBA (25 ml) was added. The mixture was kept for 3 h at room temperature. Dichloromethane (50 ml) was added and the solution was washed with ice-water and with aqueous sodium hydrogen carbonate, dried and evaporated. The syrupy residue consisted largely of tri-*O*-benzoyl-6-bromo-6-deoxy-β-D-galactofuranosyl bromide (*12*), which could not be induced to crystallize. ^{1}H NMR: δ 6.64 (H1), 5.88 (H2), 5.59 (H3), 5.05 (H4), 5.98 (H5), *ca.* 3.7 (H6); $J_{12} \simeq 0$ Hz, $J_{23} \simeq 0$, $J_{34} = 4.2$, J_{45} *ca.* 4, J_{56} *ca.* 6.

The crude glycosyl bromide (*12*) was dissolved in acetonitrile (50 ml) and stirred for 3 h with silver benzoate (10 g). The mixture was diluted with dichloromethane, filtered through carbon and evaporated. The residue was dissolved in dichloromethane, filtered through carbon and evaporated. Crystallization from ether gave 3.2 g (49 %) of *15*, m.p. 158 – 162 °C. Recrystallization from ethyl acetate-pentane gave the pure product, 3.04 g (47 %), m.p. 163 – 165 °C, $[\alpha]_D^{20} - 37.0°$ (*c* 5.1, $CHCl_3$). Anal. $C_{34}H_{27}BrO_9$: C, H, Br. ^{1}H NMR: δ 6.81 (H1), 5.79 (H2), 5.70 (H3), 4.97 (H4), 5.94 (H5), 3.80 (H6); $J_{12} = 0.4$ Hz, $J_{23} = 1$, $J_{34} = 4.2$, $J_{45} = 3.5$, $J_{56} = 6.6$.

*Tetra-O-benzoyl-β-*D*-fucofuranose (14).* A solution of *15* (1.0 g) in ethyl acetate (15 ml) and triethylamine (1.2 ml) was hydrogenated overnight at room temperature and 1 atm. pressure in the presence of 400 mg 5 % palladium on carbon. The mixture was then filtered through carbon and the carbon was washed with dichloromethane. The filtrate was washed with 4 N hydrochloric acid and with aqueous sodium hydrogen carbonate and evaporated. The product (925 mg) was separated into two fractions by preparative TLC with ether-pentane as eluent. The fast-moving fraction gave 70 mg (10 %) of tri-*O*-benzoyl-5,6-dideoxy-α-L-*arabino*-hexofuranose (*13*), which was crystallized from methanol, m.p. 81 – 83 °C, $[\alpha]_D^{25} - 23.9°$ (*c* 1.5, $CHCl_3$). Anal. $C_{27}H_{24}O_7$: C, H. ^{1}H NMR: δ 6.24 (H1), 5.78 (H2), 5.44 (H3), 4.48 (H4), 1.99 (H5), 1.12 (H6); $J_{12} \simeq 0$ Hz, $J_{23} = 1.0$, $J_{34} = 3.8$, $J_{45} = 6.4$, $J_{56} = 7.3$.

The next fraction gave 687 mg (78 %) of the fucose derivative (*14*) as a syrup which was pure as seen from an NMR spectrum, $[\alpha]_D^{20} = 52.3°$ (*c* 3.5, $CHCl_3$). Anal. $C_{34}H_{28}O_9$: C, H. ^{1}H NMR: δ 6.78 (H1), 5.5 – 5.9 (H2, H3, and H5), 4.72 (H4), 1.58 (H6); $J_{12} = 0$ Hz, $J_{34} = J_{45} = 4.2$, $J_{56} = 6.6$.

The product could be crystallized with some difficulty from ethanol; m.p. 88 – 93 °C.

*Tri-*O*-benzoyl-5,6-dideoxy-α-*L*-arabino-hex-5-enofuranose (16).* A solution of *15* (3.0 g) in acetic acid (100 ml) and water (25 ml) was stirred at 90 °C. Zinc dust (9 g) was added in the course of 20 min and the mixture was then stirred for an additional 20 min. Dichloromethane was then added and the mixture was washed with water and aqueous sodium hydrogen carbonate, dried and evaporated. The syrupy residue (2.1 g) was crystallized from methanol to give 1.4 g (67 %) of *16*, m.p. 102 – 103 °C. Recrystallization from methanol gave the pure product, m.p. 104 – 106 °C, $[\alpha]_D^{25} - 23.1°$ (*c* 2.2, $CHCl_3$). Anal. $C_{27}H_{22}O_7$: C, H. ^{1}H NMR: δ 6.76 (H1), 5.81 (H2), 5.45 (H3), 5.00 (H4), 6.18 (H5), 5.60 (H6), 5.36 (H6′); $J_{12} \simeq 0$ Hz, $J_{23} = 1.3$, $J_{45} = 5.8$, $J_{46} = J_{46'} = 1.4$, $J_{56} = 17.2$, $J_{56'} = 10.3$, $J_{66'} = 1.4$.

*Tri-*O*-benzoyl-5,6-dideoxy-α-*L*-arabino-hexofuranose (13).* A solution of *16* (1.0 g) in ethyl acetate (10 ml) was hydrogenated with 200 mg of 5 % palladium on carbon. Filtration and evaporation from methanol gave 786 mg (78 %) of *13*, m.p. 78 – 82 °C. After recrystallization the product melted at 81 – 83 °C. A mixed melting point and an NMR spectrum proved the identity with that of the product described above.

Preparation of D*-fucose from 15.* The 6-bromo-derivative (*15*) (2.0 g) was hydrogenated as described above to give a mixture of *13* and *14*. The crude mixture was debenzoylated with sodium methoxide in methanol and the solution was neutralized with Amberlite IR-120 (H^+), filtered and evaporated. The residue was crystallized from ethanol-ethyl acetate to give 150 mg (32 %) of D-fucose, m.p. 134 – 141 °C. A mixed m.p. with authentic D-fucose gave no depression.

Microanalyses were carried out at Novo Microanalytical Laboratory.

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Structural Studies on a Water-soluble Arabinogalactan Isolated from Rapeseed (*Brassica napus*)

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A highly branched arabinogalactan isolated from rapeseed flour has been separated into a low molecular and a high molecular fraction by gel chromatography. The fractions had similar chemical compositions. For the structural investigation, methylation analysis of the original arabinogalactan and of products obtained after partial acid hydrolysis and after Smith degradation have provided the essential information in this study. A tentative structure for the arabinogalactan is presented.

RESULTS AND DISCUSSION

In a previous report,[1] the isolation of an arabinan and an arabinogalactan from rapeseed was described and structural studies on the arabinan were reported. Fractionation of the arabinogalactan on a Sepharose 2B column has now revealed that the arabinogalactan fraction contained at least two polysaccharides of different molecular weights. (Fig. 1). Similar observations on arabinogalactans from other sources have been reported elsewhere in the literature.[2] After repeated chromatographic separations on Sepharose 2B a high molecular weight fraction (A_H), a low molecular weight fraction (A_L) and a mixed fraction A_M were collected. (Fig. 1). All three fractions on hydrolysis yielded L-arabinose and D-galactose in the ratio 9:1 and these sugars accounted for more than 95 % of the materials. IR spectroscopy, paper chromatography and paper electrophoresis of the hydrolysates from A_H, A_M and A_L showed that no other sugar or uronic acid residues except for very small amounts of xylose were present. The optical rotations were similar and there was a close resemblance between the methylation analyses of A_H, A_L and A_M (Table 1). It therefore seems reasonable to assume that A_H, A_M and A_L differ only in their molecular weights and the following discussion is confined to structural studies of A_M. A sample of A_M, in 0.05 M aqueous sulfuric acid was kept at 82 °C and the change in optical rotation was followed. A rapid increase from $[\alpha]_{578}$ −111° to +20° over 5 h, followed by a slower increase to +65° in 29 h, was observed. This showed that the arabinose units in A_M are α-linked,[3] and have the L-configuration. Treatment of the hydrolysate with D-galactose oxidase, which should not oxidize L-galactose,[4] revealed that galactose had the D-configuration.

Methylation analysis [5] of A_M (Table 1, column 1) showed that A_M consisted of terminal L-arabinofuranosyl units and further of either L-arabinofuranosyl residues substituted at O−5 and O−2 or L-arabinopyranosyl units substituted at O−4 and O−2. The methylation analysis also demonstrated that A_M contained 3,6-di-*O*-substituted D-galactopyranosyl units and small amounts of either L-arabinofuranosyl units substituted at O−5 or L-arabinopyranosol units substituted at O−4.

A_M was subjected to partial acid hydrolyses under conditions designed to achieve partial and complete cleavage, respectively, of the furanosidic linkages. Methylation analysis of partially hydrolysed and reduced A_M (Table 1, column 4) revealed that furanosidic and small amounts of pyranosidic terminal L-arabinosyl units had been formed after the mild acid treatment. This indicates that A_M also contains, in addition to furanosidic L-arabinose units, small amounts of L-arabinopyranosyl units. The presence of both furanosidic and py-

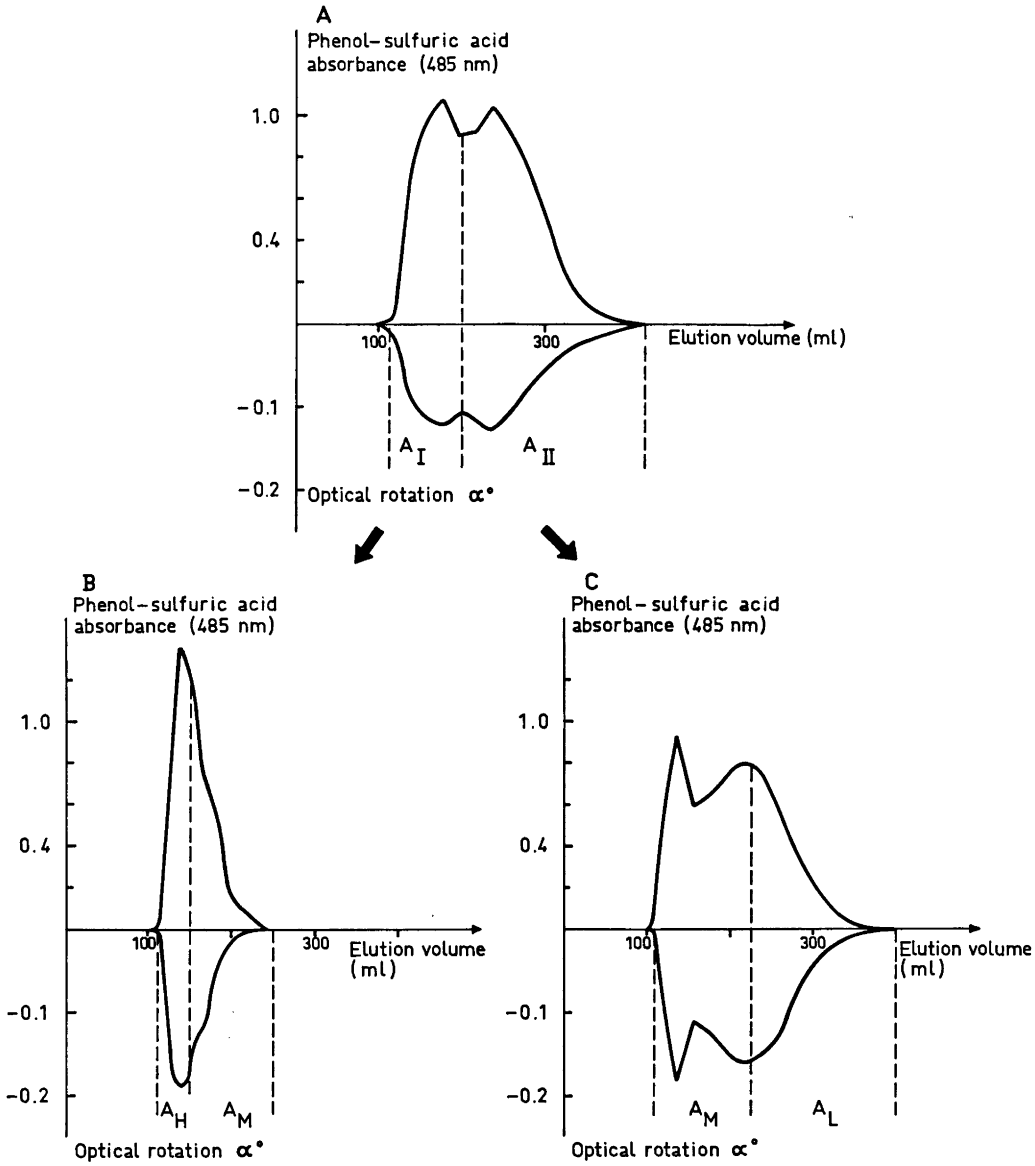

Fig. 1. Separation of a water-soluble arabinogalactan on Sepharose 2B into a high-molecular fraction (A_H) and a low molecular fraction (A_L).

ranosidic L-arabinose units has earlier been observed in arabinogalactans from other sources.[6] Methylation analyses of the more strongly degraded and reduced polymer (Table 1, columns 5 and 6) showed that a mixture of oligosaccharides containing D-galactose remained after the acid hydrolyses of A_M. Further, more 2,3,4-tri-*O*-methyl-D-galactose than 2,4,6-tri-*O*-methyl-D-galactose had been formed during the hydrolyses, indicating that the easily hydrolysed furanosidic L-arabinose units had been linked to O-3 of D-galactose. This indicates that A_M contains a backbone of (1→6) linked D-galactose units to which L-arabinose units are linked.

Table 1. Hydrolysis products (%) of (1) methylated A_H, (2) methylated A_M, (3) methylated A_L, (4) partially hydrolysed (2.5 h), reduced and methylated A_M, (5) partially hydrolysed (10 h), reduced and methylated A_M, (6) partially hydrolysed (21 h), reduced and methylated A_M, (7) periodate oxidized, reduced and methylated A_M, and (8) periodate oxidized, reduced, Smith degraded and methylated A_M.

Sugars	r^a	1	2	3	4	5	6	7	8
2,3,5-Tri-*O*-methyl-L-arabinose	0.40	48	46	46	55	12		9	35
2,3,4-Tri-*O*-methyl-L-arabinose	0.52	1	2	1	9	1			
3,5-Di-*O*-methyl-L-arabinose	0.75				10				16
2,3-Di-*O*-methyl-L-arabinose	1.03	3	3	3	6				16
3-Mono-*O*-methyl-L-arabinose	2.11	40	41	42	10			77	18
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.17				3	32	34		2
2,4,6-Tri-*O*-methyl-D-galactose	2.01					8	17		
2,3,4-Tri-*O*-methyl-D-galactose	2.90				4	29	33		2
2,4-Di-*O*-methyl-D-galactose	5.10	8	8	8	3	18	16	14	11

[a] Retention time of the corresponding alditol acetate on the OV-225 column, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-*D*-glucitol.

A_M was oxidized with periodate and part of the oxidized polymer was reduced and subjected to a methylation analysis.[7] (Table 1, column 7). The presence of 2,3,5-tri-*O*-methyl-L-arabinose in the methylation analysis of oxidised and reduced A_M showed that the oxidation had not gone to completion or that glycosidic linkages had been broken during the process, thereby generating new terminal units. The results of the methylation analysis of periodate oxidized, Smith degraded polysaccharide are summarized in Table 1, column 8. The presence of both 2,3-di-*O*-methyl-L-arabinose and 3,5-di-*O*-methyl-L-arabinose and small amounts of 2,3,4-tri-*O*-methyl-D-galactose in the analysis shows that oxidizable units have been linked to both O-2 and O-5 of the branching L-arabinose units and to some extent to O-3 of the D-galactose units. The latter result, further confirms the results from the partial acid hydrolysis experiment. The results from the analyses are presented in Fig. 2, which, however, does not necessarily represent the exact structure of the polysaccharide.

Arabinogalactans of plant origin have been studied extensively [7–15] and the function of arabinogalactans in the cell wall has been discussed.[16,17] One group of plant arabinogalactans are those related to the pectins.[14,15] They contain (1→4) linked β-D-galactopyranose residues with side chains of L-arabinofuranosyl residues. There are also arabinogalactans containing a backbone of branched β-D-galactopyranose units substituted at O-3 and O-6. The side chains are terminated with L-arabinofuranosyl residues or sometimes D-glucuronopyranosyl residues.[8–13] The acidic arabinogalactan from turnip rapeseed (*Brassica campestris*) [12] and an

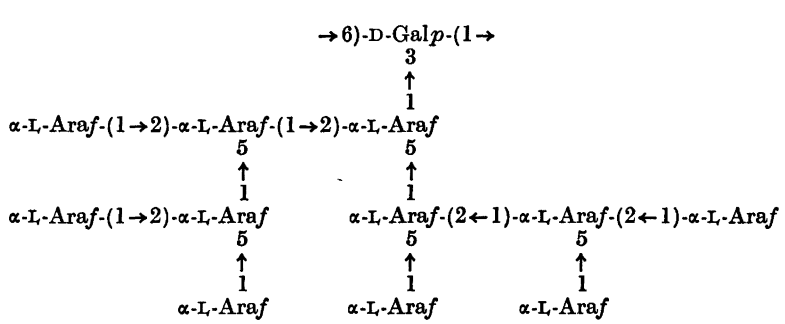

Fig. 2. A tentative structure of the arabinogalactan.

arabinogalactan from wheat flour[13] residues belong to this group. The arabinogalactan investigated in this work has structural similarities with the latter arabinogalactans, but it is more branched and has a much lower percentage of galactose.

EXPERIMENTAL

General methods, preparation of the arabinogalactan, sugar and methylation analysis and acid hydrolysis in the polarimeter are described in our investigation of a water-soluble arabinan isolated from rapeseed (*Brassica napus* var. *Sinus*).[1] Additional procedures were as follows.

Isolation of A_H, A_M and A_L. The arabinogalactan (200 mg) was fractionated on a Sepharose 2B column (1.5 × 75 cm) which was irrigated with water. The eluted carbohydrate material was detected by the phenol-sulfuric acid method [8] and by optical rotation. A high molecular fraction A_I and a low molecular fraction A_{II} were collected (Fig. 1). A_I and A_{II} were separated again and fractions were collected as shown in Fig. 1. The high molecular fraction A_H (10 mg), $[\alpha]_{578}^{20}$ −105° (*c* 0.1, water) contained arabinose: galactose in the ratio 89:11. The middle fraction A_M (85 mg), $[\alpha]_{578}^{20}$ −112° (*c* 0.1, water) contained arabinose: galactose in the ratio 91:9 and the low molecular fraction A_L (18 mg), $[\alpha]_{578}^{20}$ −115° (*c* 0.1, water), contained arabinose: galactose 91:9. In the hydrolysates from A_H, A_M and A_L small amounts of xylose could be detected.

Enzymatic oxidation of D-*galactose.* A_M (10 mg) with *myo*-inositol as internal standard was treated with 0.25 M sulfuric acid (4 ml) for 16 h at 100 °C. The hydrolysate was neutralized ($BaCO_3$), filtered, and treated with D-galactose oxidase (GALAX from KABI, Sweden). After 6 h the material was concentrated to dryness and silylated and the sugars were analysed by GLC. Only arabinose remained after the enzymatic treatment.

Analysis of partially hydrolysed A_M. A_M (64 mg) was hydrolysed in sulfuric acid (0.045 M) at 82 °C. Samples were withdrawn after 2.5, 10 and 21 h. The hydrolysates were neutralised ($BaCO_3$) and reduced ($NaBH_4$) and subjected to methylation analysis (Table 1, columns 4, 5 and 6). The residues from the hydrolyses were analysed for oligosaccharides. Paper chromatography and GC of the trimethylsilylated materials [9] revealed that no di- or trisaccharides were present in the hydrolysate from the material that was hydrolysed for 21 h. However, higher oligomers could be detected.

Analysis of periodate oxidized, reduced and Smith degraded A_M.[7] A_M (35 mg) was dissolved in 0.04 M sodium metaperiodate (12.0 ml), propanol (0.5 ml) was added and the mixture was kept in the dark at room temperature. The reaction was followed by optical rotation. When the oxidation was complete (28 h), ethylene glycol and then an excess of potassium borohydride were added and the solution was kept at room temperature for 24 h. The reaction mixture was neutralised (Dowex 50, H^+ form), filtered, dialysed against distilled water and concentrated. The residue was hydrolysed in sulfuric acid (0.5 M, 20 ml) at room temperature and samples were withdrawn after 0 and 20 h, neutralized ($BaCO_3$) and subjected to methylation analysis (Table 1, columns 7 and 8).

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Oxidation—Degradation of Methyl 2,3-Di-*O*-ethyl-4-*O*-propyl-α-D-glucopyranoside and Methyl 2,3-Di-*O*-ethyl-6-*O*-propyl-α-D-glucopyranoside

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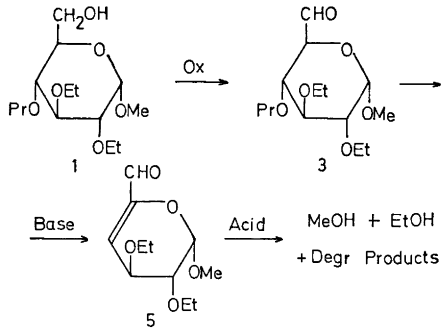
The degradation of methyl 2,3-di-*O*-ethyl-4-*O*-propyl-α-D-*gluco*-hexo-dialdo-1,5-pyranoside and methyl 2,3-di-*O*-ethyl-6-*O*-propyl-α-D-*xylo*-hexopyranosid-4-ulose by treatment with base followed by acid hydrolysis under mild conditions and with acid only have been investigated. These model experiments provide an understanding of the degradation of methylated hexodialdo-1,5-pyranosyl and hexopyranosyl-4-ulose residues in a polysaccharide on similar treatment.

A method for the specific degradation of polysaccharides starting from methylated polysaccharides with a limited number of free hydroxyl groups in defined positions has recently been described.[1,2] The hydroxyl groups are oxidized to carbonyl functions and subsequent treatment with base leads to β-elimination with the formation of unsaturated sugar residues. The latter are finally degraded by acid hydrolysis under mild conditions. Glycosyl groups (aglycones) and glycose residues (reducing) are released from the oxidized residue during these treatments. The course of the degradation may be followed by borohydride reduction, re-etherification using trideuteriomethyl or ethyl iodide, hydrolysis and characterization of the products. The results provide information on the sequence of sugar residues in the original polysaccharide. Other degradation methods, based upon β-elimination of sulfone derivatives of polysaccharides and of polysaccharides containing uronic acid residues, have also been described.[2]

The course of the degradation preceded by oxidation has been studied using partially etherified glycosides with free hydroxyl groups at C-2,[3] C-3[4,5] or C-4 and C-6[6] as model substances. We now report similar studies on glycosides having a free hydroxyl at C-4 or C-6.

RESULTS AND DISCUSSION

Methyl 2,3-di-*O*-ethyl-α-D-glucopyranoside was partially propylated using propyl iodide and silver oxide. The main reaction products, methyl 2,3-di-*O*-ethyl-4-*O*-propyl-α-D-glucopyranoside (*1*) and the corresponding 6-*O*-propyl derivative (*2*) were obtained in fair yields. They were identified by MS of the derived alditol acetates. The corresponding carbonyl derivatives, *3* and *4*, were prepared by oxidation with chlorine–dimethyl sulfoxide–triethylamine.[7] The identities of the carbonyl com-



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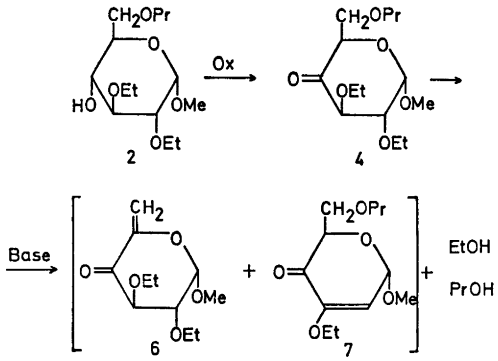
pounds were confirmed by reduction with sodium borodeuteride, whereby *1* gave a product monodeuterated at C-6, and *2* afforded a mixture of *2* and the corresponding D-galacto-pyranoside (1:7), deuterated at C-4.

Treatment of methyl 2,3-di-*O*-ethyl-4-*O*-propyl-α-D-*gluco*-hexodialdo-1,5-pyranoside (*3*) with 0.25 M sodium ethoxide in dichloro-methane-ethanol (2:1) yielded methyl 4-deoxy-2,3-di-*O*-ethyl-α-L-*threo*-hex-4-enodialdo-1,5-pyranoside (*5*), identified by its NMR spectrum. The formation of *5* is consistent with results of Beving and Theander,[8] who investigated the alkaline degradation of a related substance.

The unsaturated aldehyde (*5*) was treated with 50 % aqueous acetic acid at 100 °C, and the release of methanol and ethanol was followed by GLC. After 14 h, all the methanol (from C-1) and approximately 90 % of the ethanol (from C-2 and C-3) had been released. The other reaction products were not investigated. The related methyl 4-deoxy-β-L-*threo*-hex-4-enodi-aldo-1,5-pyranoside on treatment with acid yields 2,5-furandicarboxaldehyde and 4*H*-pyran-2-carboxaldehyde-4-one.[8] and these products were probably also formed in the present experiment. This type of degradation should be well suited for the specific degradation of polysaccharides, as is the chemically analogous uronic acid degradation.[8]

The degradation of *2* was expected to be more complicated as the β-elimination of its oxidized product, methyl 2,3-di-*O*-ethyl-6-*O*-propyl-α-D-*xylo*-hexopyranosid-4-ulose (*4*), could take two alternative courses, giving *6* or *7*. The carbonyl compound *4* was treated with 0.2 M sodium butoxide in butanol at 25 °C for 20 min, and the released alcohols were analysed by GLC. The molar proportions of



methanol, ethanol and propanol were 0.73, 1.45 and 0.35, respectively. We established that these alcohols were not formed by the action of heat on labile reaction products during GLC by removing the alcohols from the reaction mixture and injecting a sample into the gas chromatograph. On subsequent treatment with 50 % aqueous acetic acid at 100 °C for 12 h, the figures for the released alcohols increased to 0.95, 1.89 and 0.59. The amounts formed during the treatment with acid for 12 h as above were determined by removing the alcohols formed during the base treatment before treatment with acid. The figures, 0.24, 0.45 and 0.30, for methanol, ethanol and propanol, respectively, agree well with the other values.

These results demonstrate that on degradation of *4*, a high percentage of substituents in all positions is eliminated during the alkaline step and that the elimination of substituents at C-1, C-2 and C-3 is essentially complete after the mild acid hydrolysis. Elimination of the substituent at C-6, however, is incomplete. The elimination of substituents other than those at C-1 during the alkaline step may be a disadvantage when the method is applied to polysaccharides. If such a substituent is a sugar residue, it will be released as a reducing sugar residue, which will then be degraded further. This, and the incomplete elimination at C-6, make the results less clearcut than those obtained with the carbonyl group in other positions. This was apparent during studies of a bacterial polysaccharide.[9] Elimination of the substituent at C-1 during the alkaline step was also observed for a hexodialdo-1,5-pyranosid-4-ulose.[6]

Some glycosides containing carbonyl groups are considerably more sensitive to acid hydrolysis than their parent glycosides, for example, hexodialdopyranosides,[10] and the hexodialdo-1,5-pyranosid-4-ulose referred to above. When methyl 2,3-di-*O*-ethyl-4-*O*-propyl-α-D-*gluco*-hexodialdo-1,5-pyranoside (*3*) was treated with 50 % aqueous acetic acid at 100 °C for 12 h, the release of methanol, ethanol and propanol, per mol of starting material, were 0.38, 0.80 and 0.30 mol, respectively. Similar treatment of methyl 2,3-di-*O*-ethyl-6-*O*-propyl-α-D-*xylo*-hexo-pyranosid-4-ulose (*4*) released 0.75, 0.96 and 0.45 mol, respectively. These model ex-

periments indicate that the degradation of a methylated polysaccharide containing hexodialdo-1,5-pyranosyl or hexopyranosyl-4-ulose residues by treatment with acid under mild conditions may give useful structural information.

EXPERIMENTAL

Concentrations were performed at reduced pressure at bath temperatures not exceeding 40 °C. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. NMR spectra of all substances were recorded with a Varian A 60 A or a Varian XL-100 spectrometer, using tetramethylsilane as internal reference and were in agreement with the postulated structures. GLC separations were performed on a Perkin-Elmer F-30 instrument using a glass column containing 5 % UCON 50HB 2000 on Porapak Q (alcohols) and on a Hewlett-Packard 5830A instrument using a glass capillary column (25 m × 0.25 cm) wall-coated with SP-1000 (partially alkylated alditol acetates). For GLC-MS a Varian MAT 311-SS-100-gas chromatograph-mass spectrometer fitted with a glass column containing 3 % OV-225 on Gas Chrom Q was used. Hydrolyses were performed with 0.25 M sulfuric acid for 16 h at 100 °C.

*Methyl 2,3-di-*O*-ethyl-α-*D*-glucopyranoside.* Methyl 4,6-*O*-benzylidene-2,3-di-*O*-ethyl-α-D-glucoside[11] (4.7 g) in ethanol (20 ml) was hydrogenated at room temperature and atmospheric pressure using a catalyst of 10 % palladium on carbon (0.6 g) to give a chromatographically pure syrup (3.5 g), $[\alpha]_{578}^{25}$ +122° (*c* 1.4, chloroform).

*Methyl 2,3-di-*O*-ethyl-6-*O*-propyl-α-*D*-glucopyranoside (2).* Silver oxide (4.0 g) was added to methyl 2,3-di-*O*-ethyl-α-D-glucopyranoside (3.3 g) in propyl iodide (50 ml) and the mixture stirred for 4 h at 70 °C. The silver salts were filtered off, washed with chloroform and the combined filtrates concentrated to dryness. The reaction products were separated on a Silica Gel column (5 × 70 cm) irrigated with ethyl acetate – light petroleum (1:1). The separation was followed by TLC using the same solvent. The components eluted were identified by NMR and by MS of the derived alditol acetates.[12] The first fraction to be eluted, methyl 2,3-di-*O*-ethyl-4,6-di-*O*-propyl-α-D-glucoside, was obtained as a syrup (0.2 g), $[\alpha]_{578}^{25}$ +92° (*c* 1.3, chloroform). The next compound eluted (methyl 2,3-di-*O*-ethyl-6-*O*-propyl-α-D-glucopyranoside (*2*)) was also obtained as a syrup (1.3 g), $[\alpha]_{578}^{25}$ +100° (*c* 1.0, chloroform).

*Methyl 2,3-di-*O*-ethyl-4-*O*-propyl-α-*D*-glucoside (1).* Further elution of the column above yielded the title compound (*1*) as a chromatographically pure syrup (1.0 g), $[\alpha]_{578}^{25}$ +127° (*c* 1.1, chloroform). Unchanged starting material (0.9 g) was finally eluted.

*Methyl 2,3-di-*O*-ethyl-4-*O*-propyl-α-*D*-glucohexodialdo-1,5-pyranoside (3).* The oxidation reagent was prepared by adding dimethyl sulfoxide (3.5 ml) to a 1 M solution of chlorine in anhydrous dichloromethane (10 ml) in a sealed flask under vigorous stirring at −45 °C. A white precipitate appeared during the addition. The glucoside 1 (0.45 g) in dichloromethane (2 ml) was added with the aid of a syringe, and the reaction mixture was stirred at −45 °C for 3.5 h. Triethylamine (2.8 ml) was added and the mixture kept at the same temperature for further 10 min, and was then raised to room temperature. It was confirmed by TLC (chloroform–ethanol, 19:1) that all starting material had reacted. The product was purified by chromatography on a silica gel column (15 × 4 cm) irrigated with acetone–ethyl acetate, 1:1, and the last traces of dimethyl sulfone, formed during the oxidation, were removed on a column (30 × 2.5 cm) of Sephadex LH-20, irrigated with chloroform. The title compound (0.32 g) crystallized and was recrystallized from hexane, m.p. 86.5–88 °C $[\alpha]_{578}^{25}$ +129° (*c* 1.0, chloroform). Elemental analysis could not be performed because of the lability of the compound. The NMR spectrum showed, *inter alia*, a signal at δ 9.78, $J_{5,6} < 1$ Hz, assigned to the aldehyde proton. After addition of deuterated water (1 drop), the NMR showed signals at δ 0.93 (t, *J* 7 Hz, 3 H), 1.23 (t, *J* 7 Hz, 6 H), 1.54 (m, *J* 7 Hz, 2 H), 3.3–4.2 (complex signals, 13 H), 4.80 (d, $J_{5,6}$ 3.5 Hz, 1 H), and 4.85 (d, $J_{1,2}$ 3 Hz, 1 H). The substance crystallized as the *gem*-diol, as no carbonyl band but a strong hydroxyl band was present in the IR spectrum (KBr).

*Methyl 2,3-di-*O*-ethyl-6-*O*-propyl-α-*D*-xylohexopyranosid-4-ulose (4)* was prepared by oxidation of *2* (0.42 g), using the same procedure as above, except that ethyl acetate –light petroleum (1:1) was used for TLC and ethyl acetate–light petroleum (2:1) for column chromatography. The title compound was obtained as a chromatographically pure syrup (0.37 g), $[\alpha]_{578}^{25}$ +166° (*c* 1.9, chloroform). A strong carbonyl band was present in the IR spectrum at 1740 cm^{-1} and NMR showed signals at δ 0.92 (t, *J* 7 Hz, 3 H), 1.25 (t, *J* 7 Hz, 6 H), 1.54 (m, *J* 7 Hz, 2 H), 3.3–4.4 (complex signals 14 H), and 4.97 (d, $J_{1,2}$ 3.5 Hz).

Oxidation–reduction of 1 *and* 2. Oxidation of *1* (10 mg) in the presence of methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside (10 mg) was performed as described above. The product obtained on concentration was dissolved in ethanol (5 ml), and sodium borodeuteride (20 mg) was added. After 14 h at room temperature, the solution was neutralized with Dowex 50 (H^+) and the product analysed by GLC-MS of the derived alditol acetates. The yield of methyl 2,3-di-*O*-ethyl-4-*O*-propyl-α-D-glucopyranoside, monodeuterated at C-6, was estimated at 95 %. In an analogous experiment

2 was transformed to a mixture of D-glucose and D-galactose derivative, 1:7, deuterated at C-4, the total yield being 79 %.

Methyl 4-deoxy-2,3-di-O-ethyl-α-L-threo-hex-4-enodialdo-1,5-pyranoside (5). The aldehyde *3* (46 mg) was treated with 0.25 M sodium ethoxide in dichloromethane–ethanol (2:1, 4.5 ml) at room temperature for 50 min. Dowex 50 (H^+) was added to pH 7, filtered off, and the solution concentrated to dryness yielding *5* as a chromatographically pure syrup (31 mg), $[\alpha]_{578}^{25}$ +220° (*c* 1.0, chloroform). The NMR spectrum showed, *inter alia*, signals at: δ 1.24 (t, *J* 7 Hz, 6 H), 3.50 (s, 3 H), 4.27 (dd, $J_{3,4}$ 2.5 Hz, $J_{2,3}$ 8 Hz, 1 H), 5.06 (d, $J_{1,2}$ 2.5 Hz, 1 H), 5.86 (d, $J_{3,4}$ 2.5 Hz, 1 H) and 9.19 (s, 1 H).

Treatment of 5 with acid. A solution of *5* (10 mg) and 2-propanol (1 mg) in 50 % aqueous acetic acid (0.5 ml) was kept in a sealed tube at 100 °C. Analyses of alcohols by GLC showed that methanol (1 mol) and ethanol (1.8 mol) had been released after 14 h. In the analyses, molar responses of the alcohols and partial formation of acetate esters were accounted for.

Treatment of 4 with acid and base. A solution of *4* (10 mg) and 2-propanol (1 mg) in 0.2 M sodium butoxide in butanol (0.15 ml) was kept at room temperature for 20 min. The release of methanol (0.73 mol), ethanol (1.45 mol) and propanol (0.35 mol) was determined by GLC. On addition of 50 % aqueous acetic acid and heating for 12 h at 100 °C the values for the amounts of alcohols released increased to 0.95, 1.89 and 0.59 mol, respectively.

In a separate experiment, *4* (10 mg) was treated with base as described above, after which the reaction mixture was neutralized with acetic acid and concentrated to dryness. Treatment of the product with 50 % aqueous acetic acid containing 2-propanol as an internal standard at 100 °C for 12 h and analysis by GLC showed that methanol (0.24 mol), ethanol (0.45 mol) and propanol (0.30 mol) had been released.

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Intermolecular Hydride Transfer Reactions. VII. Isomerisation of Dihydropyran Derivatives Catalysed by Pyrylium Cations

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Isomerisation of dihydropyran derivatives in the presence of pyrylium cations has been studied. 4-Phenylflav-3-ene was quantitatively converted to 4-phenylflav-2-ene by means of 4-phenylflavylium perchlorate in boiling acetonitrile solution, suggesting greater stability of the 2-flavene isomer. Hydride transfer between the γ-positions of the 2-isomer and the flavylium cation was shown to be more favourable than hydride transfer between the α,α- or the α,γ-positions of the 3-isomer and the same cation. Treatment of 2,6-diphenyl- and 2,4,6-triphenyl-4*H*-pyrans with their corresponding pyrylium cations led to the formation of cinnamylideneacetophenone and 1,3,5-triphenylpent-2,4-dienone, respectively. Transformation of the 4*H*-pyran to the 2*H*-pyran catalysed by the pyrylium cation and subsequent valence isomerisation of the 2*H*-pyran leading to the most stable dienone isomer accounted for these products.

Formate reduction of 2,6-diphenyl- and 2,4,6-triphenylpyrylium cations led to the formation of 2,6-diphenyl-4*H*-pyran and 1,3,5-triphenylpent-2,4-dienone, respectively.

Recently we reported [1] that flavylium cations are quantitatively reduced into flavenes by means of formate anion. In this respect, 4-phenylflavylium perchlorate (*1a*) afforded a mixture (approximately 1:1) of 4-phenylflav-2-ene (*2a*) and 4-phenylflav-3-ene (*3a*) on treatment with sodium formate in boiling acetonitrile solution (Scheme 1A). The formation of carbon dioxide from the hydride donor is assumed to make the reaction irreversible, hence the ratio between the obtained flavenes should be kinetically controlled. However, the presence of unreacted flavylium salt (*1a*) alongside the produced flavenes *2a*, *3a* gives rise to the possibility of a concurrent intermolecular hydride transfer between the flavylium cation and the flavenes. Interconversion of the flavene isomers through a common transition state should be possible by this route. This would lead to an increased yield of the thermodynamically more stable flavene. Accordingly, the observed 1:1 ratio between the flavenes *2a* and *3a* obtained from the formate reduction of *1a* may not be related to the kinetically controlled reaction only.

The present work deals with the isomerisation of dihydropyran derivatives catalysed by pyrylium cations and furthermore a comparative study of the relative stabilities of 4*H*-pyrans *versus* 2*H*-pyrans and their open chain dienone valence isomers has been attempted.

An equimolar mixture of 4-phenylflav-2-ene (*2a*) and 4-phenylflavylium perchlorate (*1a*) in acetonitrile solution was recovered unchanged after keeping 3 days at 20 °C. Similar result was obtained from a mixture of the 4-phenylflav-3-ene (*3a*) and the flavylium salt *1a*. Thus, at room temperature, isomerisation of the flavenes *2a* and *3a* seems not to be important. When a mixture of 4-phenylflav-2-ene (*2a*) and 3-deuterio-4-phenylflavylium perchlorate (*1b*) was used, an equilibrium mixture consisting of 4-phenylflav-2-ene (*2a*), 3-deuterio-4-phenylflav-2-ene (*2b*), 4-phenylflavylium perchlorate (*1a*), and 3-deuterio-4-phenylflavylium perchlorate (*1b*) in equimolar ratio was obtained. The same equilibrium

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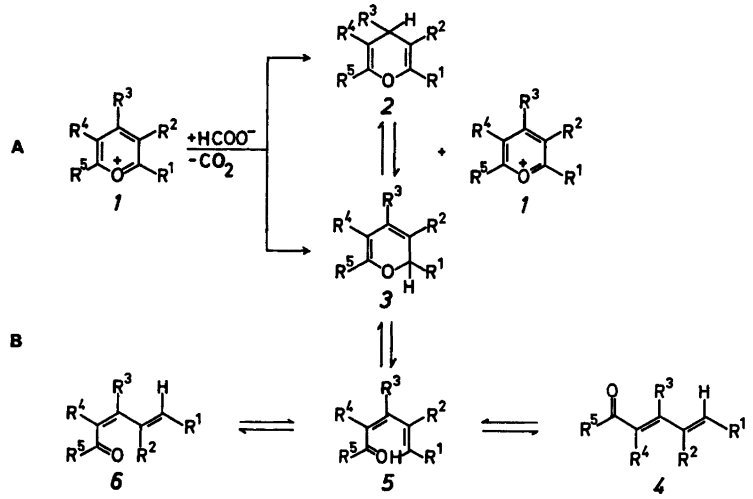

Scheme 1. Explanation of R^1-R^5, see Table 1

mixture was formed in a reaction starting with 3-deuterio-4-phenylflav-2-ene (*2b*) and 4-phenylflavylium perchlorate (*1a*). Hence a reversible hydride transfer between the γ-positions of 4-phenylflav-2-ene and 4-phenylflavylium cation takes place at room temperature. On the other hand, 4-phenylflav-3-ene (*3a*) was recovered unchanged on treatment with the 3-deuterated flavylium cation *1b*. These results suggest a more energetically favourable transition state for hydride transfer involving γ,γ positions rather than α,α or α,γ positions in the above-mentioned systems. The latter hydride transfer which could not be detected at room temperature took place at higher temperature. Thus, 4-phenylflav-2-ene (*2a*) alongside the 4-phenylflavylium cation (*1a*) were quantitatively obtained from 4-phenylflav-3-ene (*3a*) and the cation *1a* in boiling acetonitrile solution. When the 3-deuterated flavylium cation *1b* was used in the latter reaction, the expected 1:1 equilibrium mixture of deuterated and undeuterated flav-2-enes (*2b* and *2a*) besides the corresponding flavylium cations (*1b* and *1a*), was obtained. This indicates that 4-phenylflav-2-ene (*2a*) is thermodynamically more stable than 4-phenylflav-3-ene (*3a*) and that the isomerisation of *3a* into *2a* is possible under conditions similar to those used in the formate reduction of the cation *1a*. Since the above isomerisation is undetectable at room temperature, the formate reduction of *1a* was repeated at 20 °C. The ratio between the produced flavenes *2a* and *3a* in the latter case was found to be the same as that obtained in boiling acetonitrile. Since the yield of the relatively unstable isomer *3a* was not increased, it seems likely that isomerisation of *3a* into *2a*, can be neglected even when the formate reduction is carried out in boiling acetonitrile solution. Based on the roughly estimated ratio (^{1}H NMR) between *2a* and *3a*, the rate of the hydride transfer from the formate anion to the α- and the γ-positions of *1a* seems to be approximately the same.

Table 1. Substituents of compounds 1 – 6.

	R^1	R^2	R^3	R^4	R^5
a	C_6H_5	H	C_6H_5	$-(CH=CH)_2-$	
b	C_6H_5	D	C_6H_5		
c	C_6H_5	H	H	H	C_6H_5
d	C_6H_5	H	C_6H_5	H	C_6H_5
e	C_6H_5	H	CH_3	H	CH_3

While the equilibrium between 2*H*-pyrans and *cis*-dienones has been extensively studied [2–4] (Scheme 1B), little attention was given to the stabilities of these valence isomers *versus* the isomeric 4*H*-pyrans. In this respect it should be pointed out that for the flavenes *2a* and *3a* which are dihydropyran derivatives, the 4*H*-pyran *2a* being more stable than the

2*H*-pyran *3a* must also be more stable than the "dienones" which can arise through valence isomerisation of the latter. However, no change was observed in the ^{1}H NMR spectrum of *3a* (CD_3CN) over the temperature range 40 – 76 °C, suggesting the 2*H*-pyran structure *3a* to be the favoured valence isomer under the conditions used for the hydride transfer reactions.

A mixture of equimolar amounts of 2,6-diphenyl-4*H*-pyran (*2c*) and 2,6-diphenylpyrylium perchlorate (*1c*) in acetonitrile solution was recovered unchanged after keeping 3 d at 20 °C. However, boiling of the same mixture for 12 h yielded cinnamylidene acetophenone (*4c*)[5] besides the pyrylium salt *1c*. Hydride transfer from the γ-position of *2c* to the α-position of *1c* leading to the regeneration of the latter and formation of the 2*H*-pyran *3c* which subsequently undergoes isomerisation to the dienone *4c*, accounts for the observed products. In a similar reaction using 2,4,6-triphenyl-4*H*-pyran (*2d*) and 2,4,6-triphenylpyrylium perchlorate (*1d*) no isomerisation could be detected at 20 °C. However, from the reaction mixture of the 4*H*-pyran *2d* and the pyrylium salt *1d* in boiling acetonitrile solution, besides the recovery of *1d*, a crystalline compound of molecular formula $C_{23}H_{18}O$ was isolated. The dienone structure *6d* was assigned to the latter compound for reasons which will be discussed below. The formation of *6d* must be the outcome of valence isomerisation of the initially formed 2*H*-pyran *3d* through γ,α hydride transfer.

The quantitative transformation of the 4*H*-pyrans *2c* and *2d* to the dienones *4c* and *6d*, respectively, shows that the dienones are more stable than the 4*H*-pyrans as well as the 2*H*-pyrans *3c* and *3d*. While in the case of the flavenes *2a* and *3a*, the relative stabilities of the 4*H*-pyran, 2*H*-pyran and dienone structures could be assigned, such a relation could not be established for the 4*H*- and 2*H*-pyrans (*2c*, *d* and *3c*, *d*), since in the latter case the equilibrium between the pyrans is shifted to the more stable dienone forms.

Reduction of 2,6-diphenylpyrylium cation (*1c*) by means of sodium formate in boiling acetonitrile solution afforded, exclusively, the 4*H*-pyran *2c*. The formation of the latter must be the outcome of a kinetically controlled formate reduction of *1c*, since the 2*H*-pyran *3c* which may be concurrently formed, would be expected to isomerise to the dienone *4c* rather than to the 4*H*-pyran *2c*. Reaction of 2,4,6-triphenylpyrylium perchlorate (*1d*) with sodium formate in boiling acetonitrile afforded the dienone *6d* in almost quantitative yield. The dienone *6d* must be formed through valence isomerisation of the 2*H*-pyran *3d*. Under the conditions of the reaction, *3d* is expected to arise by two different routes, (i) hydride transfer from the formate anion to the α-position of the pyrylium cation *1d* or (ii) isomerisation of the 4*H*-pyran *2d* formed *via* an attack of the hydride to the γ-position of *1d*. In a trial to differentiate between these two possibilities, the reaction of the formate with *1d* was attempted at 20 °C, however, no reduction was observed.

The relation between the product distribution from the formate reduction of *1a*, *1c* and *1d* and the hydride acceptor ability of these cations will not be discussed in this work. However, it should be pointed out that the product distribution from hydride transfer to the flavylium cation *1a* was found to be greatly dependent on the nature of the hydride donor used. In the case of the formate anion as a hydride donor, the flavylium cation *1a* afforded a 1:1 mixture of the flavenes *2a* and *3a*.[1] From a mixture of *1a* and the 4*H*-pyran *2d* in acetonitrile at 20 °C, the pyrylium salt *1d*, and the flavenes *2a* and *3a* were obtained. In this reaction, in which the 4*H*-pyran *2d* acted as a hydride donor, the ratio between the flavenes *2a* and *3a* was found to be about 10:1 (^{1}H NMR). When the 4*H*-pyran *2c* was used as a hydride donor, the pyrylium salt *1c* and the flavene *2a* were isolated while the flavene *3a* could not be detected. These hydride transfer reactions are most likely kinetically controlled since they were carried out under conditions at which isomerisation of the flavene *3a* to *2a* catalysed by unreacted *1a* can be neglected.

The structural assignment of the dienone *6d* was based on its ^{1}H NMR spectrum which excludes the isomeric 2*H*-pyran form *3d*. The spectrum exhibited two doublets at δ 8.57 and 6.72 for two olefinic protons. The large vicinal coupling constant (J 16.0 Hz) is in complete agreement with the *trans* configuration of the γ,δ double bond ($R^2 = H$). The singlet at δ 6.95

was attributed to the proton in the α position (R^4=H). The s-*cis* conformation was assigned to the enone single bond system.[5] It seems likely that the low field chemical shift (δ 8.57) of one of the olefinic protons (γ or δ position) is related to the anisotropy effect exerted by the carbonyl group. Consequently, the *cis* configuration was assigned to the α,β double bond. The doublet at δ 8.57 may either be attributed to the proton in the γ position of *6d* or the proton in the δ position of *5d*. However, preference for conformation *6d* can be given on basis of the similarity of its ^{1}H NMR and UV with that of 4-methyl-6-phenyl-3,5-hexadien-2-one. The s-*cis* enone s-*trans* diene conformation *6e* has been assigned to the α,β-*cis* δ,γ-*trans* isomer of the latter dienone.[5]

The probable temperature dependent 2*H*-pyran/dienone equilibrium was examined by running the ^{1}H NMR spectrum of *6d* at −64 and 40 °C. However, no change in the spectrum was observed which indicates the stability of the dienone form *6d* over the above-mentioned temperature range. In this respect it should be pointed out that the 2,4,6-triphenyl-2*H*-pyran structure (*3d*) suggested by Dreux *et al.*[6] for the product of reaction of 2-hydroxy-2,4,6-triphenyl-2*H*-pyran and KBH_4 seems unlikely. While no ^{1}H NMR data are given, its m.p., IR and UV are very close to those of the dienone *6d*.

EXPERIMENTAL

The ^{1}H NMR spectra were recorded on a Varian A-60A instrument with TMS as internal standard. Infrared spectra were measured on a Perkin-Elmer 257 grating infrared spectrophotometer and ultraviolet spectra on a Coleman Hitachi 124 double beam spectrophotometer. Mass spectra were recorded on an AEI MS902 instrument.

The methods of preparation, melting points and ^{1}H NMR data of 4-phenylflavylium perchlorate (*1a*), 4-phenylflav-2-ene (*2a*), 4-phenylflav-3-ene (*3a*), 3-deuterio-4-phenylflavylium perchlorate (*1b*) and 3-deuterio-4-phenylflav-2-ene (*2b*) are reported in an earlier publication.[1] The pyrylium salts and 4*H*-pyrans used in this work are listed below with their most important physical data.

2,6-Diphenylpyrylium perchlorate (1c).[7] Yellow needles, m.p. 230 – 231 °C (acetic acid). ^{1}H NMR spectrum (TFA): δ 8.97 (J_{AB} 8.6 Hz, 1 H, AB_2-system), 8.48 (J_{AB} 8.6 Hz, 2 H, AB_2-system), 8.1 (10 H, m).

2,4,6-Triphenylpyrylium perchlorate (1d).[8] Yellow needles, m.p. 294 – 296 °C (acetic acid). ^{1}H NMR spectrum (TFA): δ 8.57 (2 H, s), 8.0 (15 H, m).

*2,6-Diphenyl-4*H*-pyran* (2c). 2,6-Diphenylpyrylium perchlorate (*1c*) (1.5 g; 0.0045 mol) was added in the course of 1 h to a stirred suspension of lithium aluminium hydride (0.40 g, 0.011 mol) in ether (75 ml). After decomposition of excess lithium aluminium hydride, the ethereal solution was filtered and dried ($MgSO_4$). 2,6-Diphenyl-4*H*-pyran (*2c*) (0.95 g, 95 % yield) was obtained after evaporation of the ethereal solution as needles, m.p. 100 – 101 °C. ^{1}H NMR spectrum ($CDCl_3$): δ 3.03 (*J* 3.5 Hz, 2 H, t), 5.37 (*J* 3.5 Hz, 2 H, t), 7.5 (10 H, m).

It should be noted that the reported method [9] for the synthesis of *2c* by reduction of *1c* with sodium borohydride in ether-water mixture gave a gummy product which required several recrystallisations.

*2,4,6-Triphenyl-4*H*-pyran* (2d). A much better yield of *2d* was obtained by a modification of the method reported by Dimroth *et al.*[10] as follows: 2,6-Diphenylpyrylium perchlorate (*1c*) (8.30 g, 0.025 mol) was gradually added (1 h) to a stirred Grignard solution (from bromobenzene (7.85 g, 0.050 mol) and magnesium (1.83 g, 0.75 mol)) in ether (175 ml). The reaction mixture was then poured into aqueous ammonium chloride solution and the ether layer was separated and dried ($MgSO_4$). The 4*H*-pyran *2d* (7.9 g, 90 % yield) was obtained after evaporation of the ethereal solution and crystallised in needles (ethanol), m.p. 111 °C. IR ($CHCl_3$): 1682, 1640 and 1600 cm^{-1}. ^{1}H NMR spectrum ($CDCl_3$): δ 4.33 (*J* 4.0 Hz, 1 H, t), 5.47 (*J* 4.0 Hz, 2 H, d), 7.4 (15 H, m).

General method for the isomerisation reactions (Table 2). An equimolar amount of the reac-

Table 2. Isomerisation experiments.

Reactants (1:1)	Temp. (°C)	Reaction period (h)	Result	
2a, 1a	20	72	Reactants [a]	
2a, 1a	80	15	Reactants [a]	
2a, 1b	20	2	*2a, 2b, 1a, 1b*	(1:1:1:1)
2b, 1a	20	2	*2a, 2b, 1a, 1b*	(1:1:1:1)
3a, 1a	20	72	Reactants [a]	
3a, 1a	80	15	*2a, 1a*	(1:1)
3a, 1b	20	72	Reactants [a]	
3a, 1b	80	15	*2a, 2b, 1a, 1b*	(1:1:1:1)
2c, 1c	20	72	Reactants [a]	
2c, 1c	80	12	*4c, 1c*	(1:1)
2d, 1d	20	72	Reactants [a]	
2d, 1d	80	12	*6d, 1d*	(1:1)

[a] Recovered unchanged.

tants in acetonitrile solution was stirred at room temperature or refluxed as indicated in the table. The reaction mixtures were then treated with ether and the precipitated salts were filtered and identified by ^{1}H NMR data and melting point determination. The filtrates were evaporated and the residues were identified by ^{1}H NMR data. Whenever possible, crystallisation of the residues was attempted and the products were further characterised by their melting points. Compounds *2a*, *3a*, *2c* and *2d* were recovered unchanged after refluxing their solutions in acetonitrile for 15 h.

*1,3,5-Triphenylpenta-2,4-dienone (*6d*) (Table 2).* This compound was obtained as pale yellow crystals (methanol), m.p. 125–126 °C. IR ($CHCl_3$): 1645, 1610 (weak), 1600 and 1580 cm^{-1}. UV (ethanol), λ_{max} (nm): 345 (ε 24 520) and 268 (ε 15 810). ^{1}H NMR spectrum (CD_3COCD_3): δ 6.72 (*J* 16.0 Hz, 1 H, d), 8.57 (*J* 16.0 Hz, 1 H, d), 6.95 (1 H, s), 7.7 (15 H, m). Molecular weight by MS: Found: 310.1353. Calc. for $C_{23}H_{18}O$: 310.1357.

*Reaction of 4-phenylflavylium perchlorate (*1a*) with sodium formate at room temperature.* A solution of 4-phenylflavylium perchlorate (*1a*) (0.50 g, 0.0013 mol) in acetonitrile (10 ml) was stirred with sodium formate (0.40 g, 0.006 mol) overnight at 20 °C. Ether (100 ml) was added and the precipitate was filtered. The residue (0.15 g, 40 % yield) obtained after evaporation of the filtrate was dissolved in deuteriochloroform. The ^{1}H NMR spectrum of this solution showed the characteristic signals of the Hβ, Hγ protons of 4-phenylflav-2-ene (*2a*) and the Hα, Hβ protons of 4-phenylflav-3-ene (*3a*), besides the aromatic protons signals. The integral ratio revealed a 1:1 ratio of the flavenes.

*Reaction of 2,6-diphenylpyrylium perchlorate (*1c*) with sodium formate.* A solution of 2,6-diphenylpyrylium perchlorate (*1c*) (0.40 g, 0.0012 mol) in acetonitrile (10 ml) was refluxed with sodium formate (0.40 g, 0.006 mol) for 5 h. Ether (100 ml) was added and the precipitate was filtered off. The filtrate after evaporation gave 2,6-diphenyl-4*H*-pyran (*2c*) (0.25 g, 90 % yield), identified by ^{1}H NMR.

*Reaction of 2,4,6-triphenylpyrylium perchlorate (*1d*) with sodium formate.* A solution of 2,4,6-triphenylpyrylium perchlorate (*1d*) (1 g, 0.0024 mol) in acetonitrile (15 ml) was refluxed with sodium formate (0.80 g, 0.012 mol) for 10 h. Ether (150 ml) was added and the precipitate was filtered. The oily residue (0.70 g) after evaporation of the filtrate afforded the dienone *6d* (0.45 g, 60 % yield), m.p. 125–126 °C on crystallisation from methanol.

When the above reaction was carried out at 20 °C for 48 h, the pyrylium salt *1d* was recovered unchanged.

*Reaction of 4-phenylflavylium perchlorate (*1a*) with 2,4,6-triphenyl-4*H*-pyran (*2d*).* A solution of 4-phenylflavylium perchlorate (*1a*) (0.25 g, 0.0006 mol) and 2,4,6-triphenyl-4*H*-pyran (*2d*) (0.20 g, 0.0006 mol) in acetonitrile (10 ml) was stirred at 20 °C for 48 h. Ether (150 ml) was added and the precipitated 2,4,6-triphenylpyrylium perchlorate (*1d*) (0.25 g, 95 % yield), m.p. 294–296 °C (acetic acid) was filtered. The crystalline residue after evaporation of the filtrate (0.17 g, 95 % yield) was identified by ^{1}H NMR to be a 10:1 mixture of 4-phenylflav-2-ene (*2a*) and 4-phenylflav-3-ene (*3a*).

*Reaction of 4-phenylflavylium perchlorate (*1a*) with 2,6-diphenyl-4H-pyran (*2c*).* A solution of 4-phenylflavylium perchlorate (*1a*) (0.30 g, 0.0008 mol) and 2,6-diphenyl-4*H*-pyran (*2c*) (0.19 g, 0.0008 mol) in acetonitrile (10 ml) was stirred at 20 °C for 48 h. Ether (150 ml) was added and the precipitated 2,6-diphenylpyrylium perchlorate (*1c*) (0.24 g, 90 % yield), m.p. 230–231 °C (acetic acid) was filtered. 4-Phenylflav-2-ene (*2a*) (0.21 g, 95 % yield), m.p. 109 °C (ethanol) was obtained after evaporation of the filtrate and was further characterised by ^{1}H NMR.

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Large-scale Laboratory Electrolysis in Organic Systems. III.[1] The Synthesis of α-Methoxyalkylamides. Cyclic Acylimmonium Precursors

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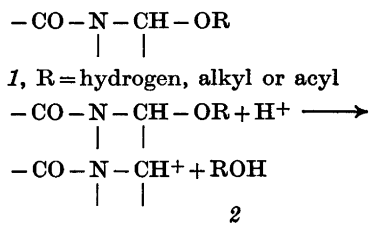
α-Methoxyalkylamides have been synthesized by anodic oxidation of *N*-formyl derivatives of amines, including pyrrolidine, piperidine, azacycloheptane, morpholine, piperazine and others. The methoxy compounds are of synthetic interest in electrophilic amidoalkylation as well as for the preparation of vinylamides.

α-Hydroxy-, alkoxy- and acyloxyalkylamides (*1*) are useful intermediates for synthetic purposes since they act as precursors for acylimmonium ions *2* by heterolytic cleavage of the carbon-oxygen bond under acidic conditions. They are important starting materials in electrophilic amidoalkylation reactions [2] and serve well for the synthesis of vinylamides [3–6] (Scheme 1). In a recent communication it was shown that *N*-acetoxymethyl-*N*-methylformamide reacts with arenes in the presence of trifluoroacetic acid yielding amidoalkylated products in high yield.[7]



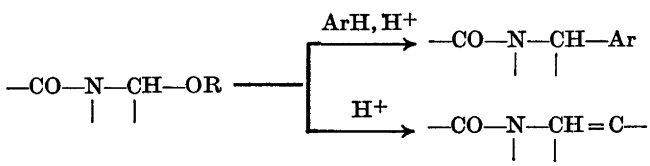
1, R = hydrogen, alkyl or acyl

2

Alkoxylated or acyloxylated *N*,*N*-dialkylamides have been prepared in a simple anodic oxidation process.[8–11] This reaction has now been extended to other methoxyamides (*1*, R = CH_3), in particular those where the nitrogen atom is part of a ring system. Amidoalkylation of arenes with such reagents gives rise to arenes substituted by a saturated heterocyclic ring.[6] Catalytic hydrogenation of such products should possibly cleave the heterocyclic ring yielding ω-aminoalkylarenes.

RESULTS

Anodic oxidation of *N*-formylated alicyclic amines in methanol containing tetrabutylammonium tetrafluoroborate as the supporting electrolyte was carried out in the concentric capillary gap cell previously described.[1] The results are summarized in Table 1. Besides the cyclic substrates some other amides were also studied. The high yields of products and the simplicity of operation make these reactions



Scheme 1.

* To whom inquiries should be addressed.

Table 1. Anodic methoxylation of amides (1 M) in 0.02 M Bu_4NBF_4/MeOH ($I = 50$ A, $U = 15-20$ V, $t = 30$ °C).

Substrate	MeOH/ dm³	Charge passed/ F mol⁻¹	Product	Yield g	Yield % [a]	B.p. °C/mmHg
N-Formylpyrrolidine	4	8.4	*N*-Formyl-2-methoxypyrrolidine	500	97	77–80/1.5
N-Formylpiperidine	4	8.4	*N*-Formyl-2-methoxypiperidine	545	95	69–75/1
N-Formylazacycloheptane	2	4.2	*N*-Formyl-2-methoxyazacycloheptane	300	96	102–105/4
N-Formylmorpholine	4	8.4	*N*-Formyl-3-methoxymorpholine	533	92	93–97/0.2
N,N'-Diformylpiperazine	3.5	7.7	*N,N'*-Diformyl-2-methoxypiperazine	548	91	170–174/0.8
N-Methylpyrrolidone	4	8.0	4-Methoxy-*N*-methylpyrrolidone [b]	492	88	66–70/1
N-Formyltetrahydroisoquinoline	0.5	1.05	*N*-Formyl-1-methoxytetrahydroisoquinoline	57	60	84 [c]
N,N-Diethylformamide	1.5	4.0	*N*-Ethyl-*N*-(1-methoxyethyl)-formamide	175	89	76–77/12
N,N-Diethylacetamide	1	2.5	*N*-Ethyl-*N*-(1-methoxyethyl)-acetamide	121	84	82–86/12
N,N-Diisopropylformamide	1	17.3	*N*-Isopropyl-*N*-(1-methoxy-1-methylethyl)formamide	38	24	67–69/0.8 [d]

[a] Material yield. [b] Contains 8 % of *N*-methoxymethylpyrrolidone. [c] M.p. [d] M.p. 66 °C.

synthetically useful. To our knowledge, no other convenient method is available for the synthesis of methoxylated amides. The methoxy compounds, with the exception of the methoxylated *N,N*-diethylamides,[11] have not been reported before.

We also attempted to prepare the corresponding acetoxylated amides but failed. This was probably due to the sensitivity of these compounds to the acidic conditions employed (the oxidations were run in acetic acid containing tetrabutylammonium tetrafluoroborate) resulting in elimination and further reactions of the enamides formed. A low yield of the product was obtained in the oxidation of *N,N*-diisopropylformamide in methanol. Since the product contains a tertiary α-methoxyalkyl group it is evident that this compound should be more sensitive to the reaction conditions (elimination) than the other methoxylated compounds. Therefore, the low yield of the product is not surprising.

EXPERIMENTAL

The starting materials were prepared by conventional formylation procedures (reactions between amines and formamide, ethylformate or acetic formic anhydride, respectively).[12,13] *N*-Methylpyrrolidone and *N,N*-diethylacetamide were of commercial quality. The anodic reactions were carried out as previously described.[1] Results and reaction conditions are given in Table 1. The reaction mixtures were worked up by evaporation of the solvent followed by distillation *in vacuo*. In the reaction with *N*-formyltetrahydroisoquinoline the product was isolated by filtering the precipitate from the cooled reaction mixture. A second crop was obtained by concentrating the filtrate, followed by cooling.

The purity of the products were checked by GLC (2 m × 0.3 cm 5 % NPGS on Chromosorb W column, Perkin-Elmer Model 880 Gas Chromatograph) and was found to be better than 95 % in all cases. The impurities consisted of starting material, enamide (elimination product) and bis-methoxylated material. The products were identified by their mass (LKB 9000 mass spectrometer at 70 eV) and ^1H NMR spectra (in $CDCl_3$; Jeol MH 100). These data were in complete agreement with the proposed structures.

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The Crystal Structures of γ-Aminobutyric Acid Choline Ester Diiodide and γ-Aminobutyric Acid Choline Ester (±)-Tartrate

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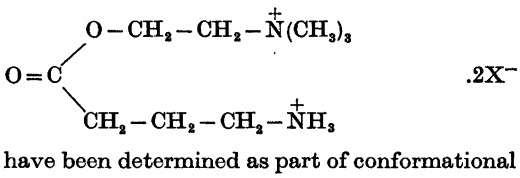
The crystal structures of γ-aminobutyric acid (GABA) choline ester diiodide, GAKOLI, and of GABA choline ester (±)-tartrate, GAKTRA, have been determined from diffractometer collected three-dimensional X-ray data and refined by full-matrix least-squares techniques to R values of 0.049 and 0.075 for GAKOLI and GAKTRA, respectively. The conformations of the GABA-moieties in the two crystal structures differ from one another, while the conformations of the choline moieties are almost the same. No hydrogen bonding involves the ester oxygen atoms.

The crystal structures of the diiodide and the (±)-tartrate of γ-aminobutyric acid choline ester, GABA choline ester



.2X⁻

have been determined as part of conformational studies of choline esters in the solid state. Of special interest to the author are compounds in which hydrogen bonding of the choline ester ion cannot *a priori* be excluded.

The biological activity of GABA choline ester has been studied by several groups, and it has been concluded,[1,2] that the compound has little acetylcholine-like activity and only weak GABA-like activity.

EXPERIMENTAL

GABA choline ester diiodide, GAKOLI. GABA choline ester dichloride was prepared from GABA and choline chloride according to directions of Daiichi Seizaku Co., Ltd.[3] GAKOLI was prepared by mixing saturated ethanolic solutions of GABA choline ester dichloride and potassium iodide. The instantaneously precipitated potassium chloride was filtered off, and upon standing colourless needleshaped crystals of GAKOLI were formed in the solution. Preliminary Weissenberg- and precession photographs showed the crystals to be monoclinic, space group $P2_1/c$. The unit cell parameters were refined by least-squares techniques from diffractometer measured 2θ-angles (Mo$K\alpha$ radiation, $\lambda = 0.7107$ Å) for 25 independent reflections. The density of the crystals was measured by flotation in a mixture of bromobenzene and methyliodide. The melting points given in this paper were determined on a Leitz hot stage microscope. Table 1 lists the unit cell parameters and other crystal data.

Three-dimensional diffraction data were measured at room temperature on a Nonius

Table 1. Crystal data.

	GAKOLI	GAKTRA
Stoichiometry	$C_9H_{22}N_2O_2I_2$	$C_{13}H_{26}N_2O_8$
Formula weight	444.10	338.36
Z	4	4
F(000)	848	728
Space group	$P2_1/c$	$Pna2_1$
a (Å)	11.660(1)	11.487(2)
b (Å)	14.384(1)	15.271(3)
c (Å)	9.499(1)	9.419(1)
β (°)	101.37(1)	
Cell volume (Å³)	1561.9	1652.2
D_X (g cm⁻³)	1.89	1.36
D_m (g cm⁻³)	1.90(1)	1.36(1)
μ(Mo$K\alpha$) (cm⁻¹)	41	1.2
μ(Cu$K\alpha$) (cm⁻¹)		9.8
$M.p.$ (°C)	188.5 – 191.5 (decomp.)	153.5 – 155.0 (decomp.)

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, and the scan angle was 1.0°. Background counts were taken for half the scanning time at each of the scan range limits. One standard reflection was measured after every 40 reflections. The intensity of this reflection diminished approximately 10 % during the measurement of the first *ca.* 3000 reflections, and a linear rescale factor was applied. During the measurement of the remaining reflections no systematic change in the intensity of the standard reflection was observed. All the data were measured from a single crystal with approximate dimensions $0.08 \times 0.08 \times 0.27$ mm. The crystal was sealed in a glass capillary and mounted with its elongated dimension, which was parallel to the crystallographic a-axis, along the ϕ-axis of the goniostat. Each of the 2865 independent reflections in the range $2.5 \leq \theta \leq 25.0°$ was measured twice. 3645 of the 5725 reflections ($hk \pm l$ and $h-k \pm l$) had $I_{net} \geq 2.5\sigma(I)$, 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. The reflections from both quadrants were used independently in the refinement. Lorentz and polarization corrections were applied, but no absorption corrections were made.

The trial structure was obtained by the heavy atom method.

GABA choline ester (±)-tartrate, GAKTRA. Freshly precipitated silver oxide (2 mmol) was suspended in an aqueous solution of (±)-tartaric acid (1 mmol) and an aqueous solution of GABA choline ester dichloride (1 mmol) was added. After stirring for 1 h the precipitate was filtered off and the solution evaporated *in vacuo*. Colourless flat elongated prismatic crystals were obtained from a solution of GAKTRA in dimethylformamide and a few drops of water, standing in a desiccator containing phosphorus pentaoxide. Preliminary Weissenberg- and precession photographs showed the crystals to be orthorhombic. The systematically absent reflections are $h0l$ when h is odd and $0kl$ when $k+l$ is odd, indicating that the space group is $Pnam$ or $Pna2_1$, but from considerations concerning the formula and the density of the compound, the centrosymmetric space group could be ruled out. The unit cell parameters were refined by least-squares techniques from diffractometer measured 2θ-angles (Mo$K\alpha$ radiation and/or Cu$K\alpha$-radiation, λ = 1.5418 Å) for 30 reflections. The density of the crystals was measured by flotation in a mixture of chlorobenzene and bromobenzene. The crystal data are listed in Table 1.

Two sets of intensity data have been measured, both at room temperature. Due to difficulties in growing and cutting appropriate crystals a tiny specimen was first examined and only a very low percentage of observed reflections was obtained. As better crystals were later obtained, another data set was collected.

The first set of data (I) was obtained from a single crystal with approximate dimensions $0.04 \times 0.08 \times 0.3$ mm using graphite monochromated Mo$K\alpha$-radiation and the second set (II) from a single crystal with dimensions $0.16 \times 0.26 \times 0.64$ mm using graphite monochromated Cu$K\alpha$-radiation. The crystals were sealed in glass capillaries and mounted with the elongated dimension, which was parallel to the crystallographic c-axis, along the ϕ-axis of the goniostat. The ω scan technique with a scan speed of 0.6° min^{-1} was employed, and the scan angles were 1.4 and 1.2° for measurement (I) and (II), respectively. One standard reflection was measured after every 25 reflections, showing no systematic decrease in intensity during the measurements. Using the first crystal, the reflections in the range $2.5 \leq \theta \leq 22.0°$ were measured once. From the second crystal the reflections in the range $2.5 \leq \theta \leq 50.0°$ were measured twice, and equivalent reflections were averaged. For data set I 477 of the 1089 independent reflections and for data set II 750 of the 918 independent reflections had $I_{net} \geq 2.5\sigma(I)$ and were regarded as observed. Lorentz and polarization corrections were applied, but no absorption corrections were made.

The trial structure was obtained from data set II by use of the 1974-version of MULTAN.[4] 15 of the highest peaks in the first calculated E-map were postulated to correspond to atomic positions. A refinement of this partial structure using the iterative tangent refinement procedure [5] gave a new E-map, from which the positions of 20 atoms were found. Out of these 20 atoms 12 were part of the mentioned partial structure. The positions of the remaining three atoms in the asymmetric unit [C(8), C(9) and C(10)] could be postulated from a subsequent Fourier synthesis.

REFINEMENT OF THE STRUCTURES

The trial structures were refined by full-matrix least-squares techniques. The quantity minimized was $\sum w(|F_o| - |F_c|)^2$ where weights were initially taken as unity. For GAKOLI the following weighting scheme was used during the last cycles of refinement: $w = 1/\{1 + [(F_o - B)/A]^2\}$ where $A = 20.0$ and $B = 45.0$. The X-ray atomic scattering factors used for hydrogen were those of Stewart, Davidson, and Simpson [6] and for all other atoms those of Cromer and Mann.[7] All nitrogen and oxygen atoms were treated as uncharged. All hydro-

Table 2. Final positional and thermal parameters for non-hydrogen atoms. The estimated standard deviations, referring to the last significant figure, are given in parentheses. Thermal parameters are $\times 10^2$. The temperature factor is defined by: exp $[-2\pi^2(U_{11}h^2a^{*2}+\cdots+2U_{12}hka^*b^*+\cdots)]$

Atom	x/a	y/b	z/c	U or U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
GAKOLI									
$I^-(1)$	.49856(4)	.15571(4)	.62418(5)	4.26(3)	5.08(3)	5.16(3)	0.59(2)	0.67(2)	−0.35(3)
$I^-(2)$	−.07120(4)	.15678(4)	−.26431(5)	4.20(3)	3.85(3)	6.26(3)	−0.34(2)	−0.15(2)	−0.13(3)
N(13)	.2893(6)	−.2257(5)	−.4145(7)	5.0(2)					
C(12)	.3123(7)	−.1368(5)	−.3344(8)	4.8(2)					
C(11)	.2039(7)	−.0904(6)	−.3078(8)	4.8(2)					
C(1)	.2332(7)	.0018(5)	−.2249(8)	4.6(2)					
C(2)	.2893(6)	−.0104(5)	−.0748(5)	4.0(2)					
O(3)	.3122(6)	−.0836(4)	−.0122(7)	6.7(2)					
O(4)	.3111(4)	.0710(3)	−.0047(5)	4.5(1)					
C(5)	.3716(7)	.0706(5)	.1422(8)	4.6(2)					
C(6)	.2946(6)	.0555(5)	.2494(7)	4.0(2)					
N(7)	.2006(5)	.1276(4)	.2552(6)	3.5(1)					
C(8)	.1046(8)	.1198(6)	.1280(9)	5.6(2)					
C(9)	.2514(7)	.2234(6)	.2639(9)	5.3(2)					
C(10)	.1535(8)	.1112(6)	.3880(9)	5.4(2)					
GAKTRA									
N(13)	−.0468(9)	.3521(7)	.3854(22)	3.4(7)	3.5(7)	3.9(8)	0.1(6)	−0.0(7)	−0.8(7)
C(12)	.0366(15)	.2798(10)	.3509(26)	6.9(12)	4.6(11)	4.5(11)	−0.2(10)	−1.0(11)	−1.6(9)
C(11)	.1556(16)	.2990(11)	.4110(22)	5.5(13)	6.7(11)	3.1(10)	0.8(9)	−0.1(10)	−2.1(9)
C(1)	.1590(15)	.2925(11)	.5735(−)	5.6(12)	6.3(12)	3.7(10)	−1.1(9)	−1.6(9)	−0.5(9)
C(2)	.2829(18)	.3184(11)	.6286(26)	7.5(16)	5.3(12)	2.3(12)	2.0(10)	−1.1(11)	−0.2(10)
O(3)	.3623(11)	.3408(9)	.5616(22)	5.0(8)	10.7(11)	4.7(8)	−1.0(8)	1.1(7)	−0.3(8)
O(4)	.2823(9)	.3058(7)	.7703(21)	6.7(9)	5.2(7)	4.7(9)	0.8(6)	−0.2(7)	−1.3(6)
C(5)	.3985(16)	.3185(12)	.8436(27)	6.1(13)	7.0(13)	6.2(12)	0.9(11)	−2.7(12)	−1.0(11)
C(6)	.4175(15)	.4130(11)	.8819(28)	5.6(12)	5.8(12)	6.6(14)	−0.7(10)	0.5(11)	−2.7(11)
N(7)	.3470(11)	.4456(8)	1.0047(21)	5.1(9)	5.2(8)	3.9(8)	0.3(6)	0.3(8)	−0.6(8)
C(8)	.2179(14)	.4403(13)	.9775(28)	3.4(10)	12.0(15)	9.0(18)	−0.5(10)	1.0(11)	−5.1(15)
C(9)	.3763(21)	.3982(12)	1.1452(28)	14.0(20)	5.2(12)	6.1(15)	0.1(13)	1.2(15)	1.1(11)
C(10)	.3810(16)	.5421(10)	1.0212(26)	9.9(14)	4.0(11)	5.6(13)	−0.9(10)	−0.6(13)	−1.5(10)
O(011)	.4689(10)	−.0059(8)	.7313(20)	8.1(9)	4.6(8)	4.0(8)	2.3(7)	1.6(7)	−0.0(6)
O(012)	.3916(10)	.1260(7)	.6833(20)	7.0(8)	4.5(7)	2.9(6)	1.2(6)	0.2(6)	0.9(6)
C(01)	.4207(14)	.0644(12)	.7643(24)	4.5(10)	4.4(11)	3.8(10)	−0.6(10)	1.0(10)	−1.0(11)
C(02)	.3920(15)	.0765(11)	.9284(25)	5.2(13)	6.4(12)	2.5(10)	0.6(10)	1.1(9)	−0.1(9)
O(02)	.4129(9)	−.0056(6)	.9981(19)	7.7(7)	4.3(6)	2.5(6)	1.9(6)	−0.7(6)	0.4(6)
C(03)	.2633(14)	.1039(10)	.9498(23)	5.4(12)	4.5(10)	2.1(10)	0.4(9)	1.1(8)	−0.7(8)
O(03)	.1881(9)	.0331(7)	.9058(21)	4.8(7)	6.3(8)	4.6(7)	−0.9(6)	−0.3(6)	−1.9(7)
C(04)	.2501(17)	.1254(12)	1.1099(23)	5.0(12)	5.5(12)	3.3(11)	1.1(11)	0.2(10)	0.5(11)
O(041)	.3064(9)	.1860(7)	1.1564(20)	6.1(8)	5.8(7)	2.8(7)	−0.3(7)	−0.8(6)	−1.4(6)
O(042)	.1781(11)	.0753(8)	1.1734(20)	10.1(11)	8.2(9)	4.8(8)	−1.5(8)	4.0(8)	0.8(8)

gen atoms in the GAKOLI structure were located in a difference Fourier-map calculated during the later stages of refinement, while a corresponding difference Fourier-map calculated for the GAKTRA structure only gave very weak indications of the positions of the hydrogen atoms. The final cycles of refinement of the GAKOLI structure included the positional parameters for all atoms and the thermal parameters for all non-hydrogen atoms (anisotropic for the iodide ions and isotropic for carbon, nitrogen, and oxygen atoms). The temperature parameters of the hydrogen atoms were fixed at arbitrary values. For the GAKTRA structure the final cycles of refinement included the positional and anisotropic thermal parameters for all non-hydrogen atoms. No hydrogen atoms were introduced in the calculations. Although anisotropic refinement hardly can be justified in

Table 3. GAKOLI. Final positional parameters for hydrogen atoms and the thermal parameters ($\times 10^2$) used. The estimated standard deviations (referring to the last significant figure) are given in parentheses.

Atom	x/a	y/b	z/c	U
H(131)	.247(6)	−.249(5)	−.370(7)	5.1
H(132)	.254(7)	−.198(5)	−.504(8)	5.1
H(133)	.352(7)	−.242(5)	−.419(7)	5.1
H(121)	.352(7)	−.158(5)	−.235(8)	5.1
H(122)	.344(6)	−.107(5)	−.385(8)	5.1
H(111)	.156(7)	−.072(5)	−.401(8)	5.1
H(112)	.162(7)	−.126(5)	−.258(8)	5.1
H(11)	.287(6)	−.033(5)	−.259(8)	5.1
H(12)	.161(6)	.028(5)	−.224(7)	5.1
H(51)	.418(6)	.019(5)	.169(7)	5.1
H(52)	.421(7)	.137(5)	.149(8)	5.1
H(61)	.254(7)	−.007(5)	.223(8)	5.1
H(62)	.329(6)	.052(5)	.339(7)	5.1
H(81)	.136(7)	.139(5)	.050(8)	5.1
H(82)	.070(6)	.052(5)	.129(8)	5.1
H(83)	.044(7)	.165(5)	.163(8)	5.1
H(91)	.291(6)	.225(5)	.344(8)	5.1
H(92)	.267(6)	.234(5)	.169(8)	5.1
H(93)	.192(6)	.267(5)	.266(7)	5.1
H(101)	.119(6)	.048(5)	.373(8)	5.1
H(102)	.201(7)	.126(5)	.469(8)	5.1
H(103)	.089(7)	.153(5)	.372(8)	5.1

view of the limited amount of data, this approach has been used. The reason for this is, that independent refinements using the two data sets give the same overall picture of the anisotropic thermal motions. The final R index ($\sum||F_o|-|F_c||/\sum|F_c|$) is 0.049 for GAKOLI and for GAKTRA 0.086 and 0.075 for data set I and II, respectively.

The calculations were performed on an IBM 1130 computer and an IBM 360/75 computer, using INDIFF,[8] a local version of *The N.R.C. 2A Picker Data Reduction Program*,[9] an updated version of *The X-Ray System*,[10], and *ORTEP*.[11] The observed and calculated structure factor data are available from the author on request.

RESULTS AND DISCUSSION

Tables 2 and 3 list final parameters and estimated standard deviations for non-hydrogen atoms and for hydrogen atoms, respectively. Unless otherwise stated the figures given for GAKTRA are based on data set II. The calculated bond lengths and angles involving only non-hydrogen atoms are given in Table 4. The atomic numbering used is shown in Fig. 1.

Packing arrangements

GAKOLI. The crystal packing is illustrated in Fig. 2. The iodide ions are situated in two layers, one rather puckered around $x=0$ and one nearly planar around $x=\frac{1}{2}$. Between these

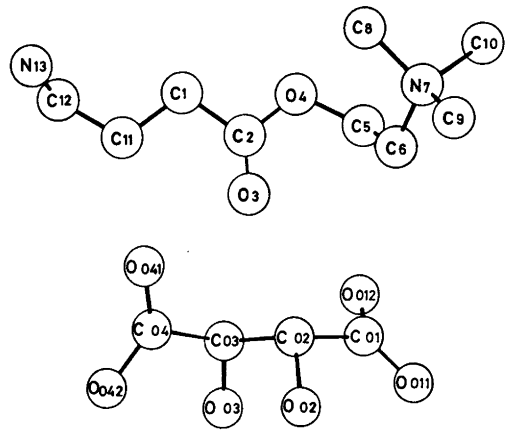


Fig. 1. The atomic numbering used for carbon, nitrogen, and oxygen atoms of GAKOLI and GAKTRA. The distances O(4)···C(8) and O(4)···N(7) are 3.01(1) and 3.11(1) Å for GAKOLI and 2.93(3) and 3.16(2) Å for GAKTRA.

Table 4. Bond lengths (Å) and angles (°) involving only non-hydrogen atoms and their estimated standard deviations, referring to the last significant figure, in parentheses.

	GAKOLI	GAKTRA Data set II	Data set I
N(13)–C(12)	1.49(1)	1.50(2)	1.49(3)
C(12)–C(11)	1.49(1)	1.51(3)	1.46(4)
C(11)–C(1)	1.55(1)	1.53(2)	1.57(3)
C(1)–C(2)	1.46(1)	1.57(3)	1.53(3)
C(2)–O(3)	1.21(1)	1.16(3)	1.17(4)
C(2)–O(4)	1.35(1)	1.35(3)	1.39(4)
O(4)–C(5)	1.43(1)	1.52(2)	1.59(3)
C(5)–C(6)	1.50(1)	1.50(3)	1.48(4)
C(6)–N(7)	1.52(1)	1.50(3)	1.47(4)
N(7)–C(8)	1.48(1)	1.51(2)	1.50(3)
N(7)–C(9)	1.50(1)	1.53(2)	1.59(4)
N(7)–C(10)	1.49(1)	1.55(3)	1.55(3)
∠N(13)–C(12)–C(11)	113.4(6)	111(1)	111(2)
C(12)–C(11)–C(1)	111.1(6)	113(2)	114(2)
C(11)–C(1)–C(2)	114.0(6)	110(1)	111(2)
C(1)–C(2)–O(3)	126.6(7)	127(2)	128(3)
C(1)–C(2)–O(4)	112.6(6)	107(1)	107(2)
O(3)–C(2)–O(4)	120.7(6)	126(2)	125(3)
C(2)–O(4)–C(5)	119.1(5)	115(2)	116(2)
O(4)–C(5)–C(6)	114.5(6)	111(1)	110(2)
C(5)–C(6)–N(7)	117.3(6)	115(2)	114(2)
C(6)–N(7)–C(8)	110.7(6)	113(2)	116(2)
C(6)–N(7)–C(9)	110.5(6)	113(1)	111(2)
C(6)–N(7)–C(10)	108.2(5)	109(1)	104(2)
C(8)–N(7)–C(9)	110.0(6)	110(2)	109(2)
C(8)–N(7)–C(10)	109.5(6)	109(1)	110(2)
C(9)–N(7)–C(10)	107.9(6)	108(2)	106(2)
O(011)–C(01)		1.25(2)	1.23(3)
O(012)–C(01)		1.26(2)	1.25(3)
C(01)–C(02)		1.59(3)	1.64(4)
C(02)–O(02)		1.44(2)	1.42(3)
C(02)–C(03)		1.55(2)	1.56(3)
C(03)–O(03)		1.44(2)	1.43(3)
C(03)–C(04)		1.55(3)	1.59(4)
C(04)–O(041)		1.21(2)	1.18(3)
C(04)–O(042)		1.28(2)	1.29(3)
∠O(011)–C(01)–O(012)		128(2)	129(3)
O(011)–C(01)–C(02)		116(2)	117(2)
O(012)–C(01)–C(02)		117(2)	114(2)
C(01)–C(02)–O(02)		108(1)	105(2)
C(01)–C(02)–C(03)		111(2)	108(2)
O(02)–C(02)–C(03)		110(1)	109(2)
C(02)–C(03)–O(03)		109(1)	113(2)
C(02)–C(03)–C(04)		106(2)	102(2)
O(03)–C(03)–C(04)		112(2)	112(2)
C(03)–C(04)–O(041)		117(2)	118(3)
C(03)–C(04)–O(042)		113(2)	111(2)
O(041)–C(04)–O(042)		129(2)	130(3)

layers the GABA choline ester ions are arranged. Only one close contact between two of these ions is observed. The distance $N(13)\cdots O(3)_{(x, -\frac{1}{2}-y, -\frac{1}{2}+z)}$ is 2.925(9) Å. This is, however, a polar rather than a hydrogen bonded contact. None of the three N–H···O contacts are even approximately linear as can be seen from Fig. 2 and Table 5, which lists some dimensions

Table 5. GAKOLI. Some dimensions describing the surroundings of N(13).

A	B	A···B Å	∠A−H···B°
N(13)−H(131)···I⁻(2)$_{(-x,-\frac{1}{2}+y,-\frac{1}{2}-z)}$		3.730(7)	173(6)
N(13)−H(132)···I⁻(2)$_{(-x,-y,-1-z)}$		3.701(6)	155(6)
N(13)−H(133)···I⁻(1)$_{(1-x,-y,-z)}$		3.612(7)	124(6)
N(13)−H(133)···I⁻(1)$_{(1-x,-\frac{1}{2}+y,\frac{1}{2}-z)}$		3.734(6)	115(6)
N(13)−H(131)···O(3)$_{(x,-\frac{1}{2}-y,-\frac{1}{2}+z)}$		2.925(9)	81(5)
N(13)−H(132)···O(3)$_{(x,-\frac{1}{2}-y,-\frac{1}{2}+z)}$		2.925(9)	63(4)
N(13)−H(133)···O(3)$_{(x,-\frac{1}{2}-,-\frac{1}{2}+z)}$		2.925(9)	101(6)

describing the surroundings of N(13). Four iodide ions form a rectangle. N(13) is situated 0.37 Å off the plane of these four iodide ions, and the four nitrogen-iodide distances are not very different. Two of these contacts, N(13)···I⁻(2)$_{(-x,-\frac{1}{2}+y,-\frac{1}{2}-z)}$ and N(13)···I⁻(2)$_{(-x,-y,-1-z)}$, may be described as rather weak hydrogen bonds. The iodide ions are also in close contact with the carbon atoms C(6) to C(10) of the quaternary ammonium group. This group is surrounded by six iodide ions, three of which are nested between the carbon atoms. The carbon-iodide distances are in the range 3.82 to 4.31 Å.

GAKTRA. The crystal packing is illustrated in Fig. 3. Only few and weak contacts between the GABA choline ester ions are observed. The tartrate ions are linked together in chains along the screw-axes. Although the positions of the hydrogen atoms could not be determined it seems reasonable to postulate the existence of a hydrogen bond O(02)−H···O(011)$_{(1-x,-y,\frac{1}{2}+z)}$. The O−O distance is 2.59(2) Å. No other close contacts between the tartrate ions are found. Most of the closer contacts in the crystal are found between the positively charged GABA choline ester ions and the negatively charged tartrate ions. Nine tartrate ions surround the GABA choline ester ion, and seven of these have van der Waals' or other contacts to the GABA choline ester ion. The existence of three hydrogen bonds N−H···O may be postulated, and these will be approximately linear, if the conformation around C(12)−N(13) is staggered in this crystal structure, as is approximately the case in the GAKOLI structure. The N···O distances are N(13)···O(12)$_{(x-\frac{1}{2},\frac{1}{2}-y,z)}$, 2.91(3) Å, N(13)···

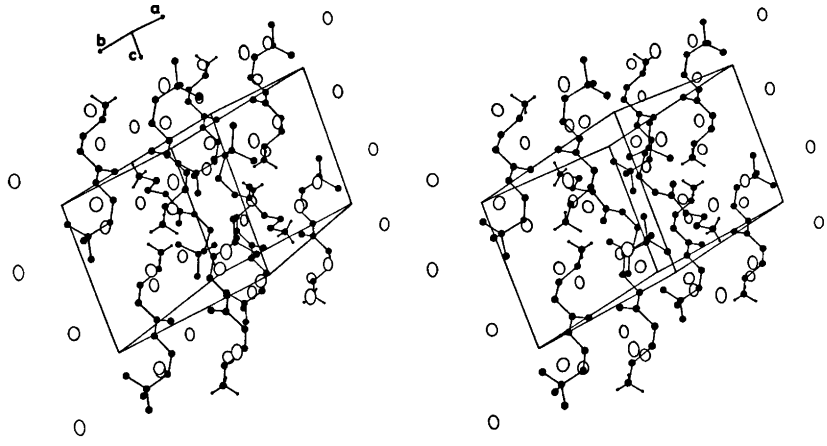


Fig. 2. Stereodiagram illustrating the packing of GAKOLI. All hydrogen atoms but the three bonded to N(13) have been omitted for clarity.

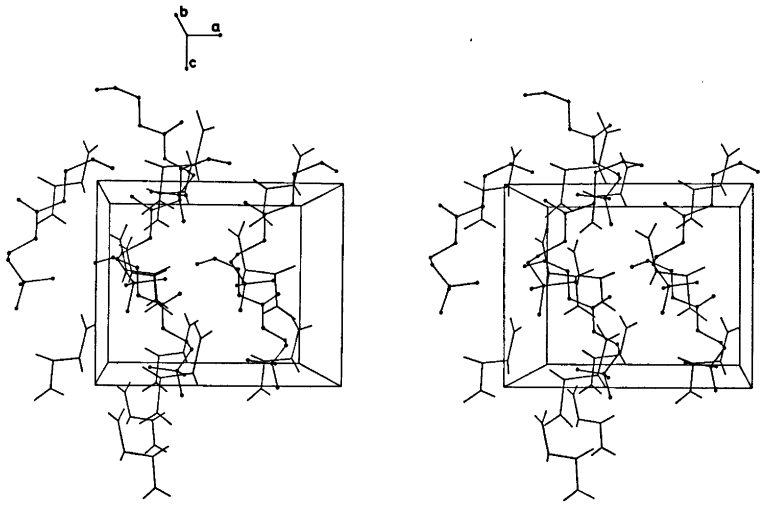


Fig. 3. Stereodiagram illustrating the packing of GAKTRA.

$O(041)_{(\frac{1}{2}-x,\frac{1}{2}-y,z-1)}$, 2.80(2) Å, and $N(13)\cdots O(011)_{(\frac{1}{2}-x,\frac{1}{2}+y,z-\frac{1}{2})}$, 2.76 Å. The distance $N(13)\cdots O(02)_{(\frac{1}{2}-x,\frac{1}{2}+y,z-\frac{1}{2})}$ is 2.87(2) Å. From model studies it seems most reasonable to assume this last contact not to be a hydrogen bond.

In summary the three possible $\overset{+}{N}-H\cdots O$ hydrogen bonds are found, and so is one of the two possible $O-H\cdots O$ hydrogen bonds. No *inter*-molecular hydrogen bond involving O(03) is observed. Three of the four carboxylate oxygen atoms act as hydrogen bond acceptors. O(011) accepts two hydrogen bonds, O(012) and O(041) accept one each, while no hydrogen bonding involves O(042). No close contacts to the ester oxygen atoms are observed.

The tartrate ion. As the estimated standard deviations on the dimensions of the tartrate ion are very high, importance can hardly be attached to the deviations from the dimensions determined by Ambady[12] and others. The torsion angle C(01)–C(02)–C(03)–C(04) is ±173(1)°. The distance from O(02) to the plane determined by O(011), O(012), C(01), and C(02) is 0.23 Å, while the deviation of O(03) from the plane through C(03), C(04), O(041), and O(042) is insignificant.

The GABA choline ester ion. The dimensions listed in Table 4 for GAKOLI are within the uncertainty in agreement with normally accepted values. The deviations between the corresponding figures for GAKOLI and GAKTRA are hardly significant.

The conformations of the GABA choline ester ion found in the two crystal structures are illustrated in Fig. 4, and torsion angles are listed in Table 6. The two conformations of the GABA-moiety are fundamentally different. In the crystal structures of GABA (zwitterion),[13] of GABA. HCl,[14] and of two

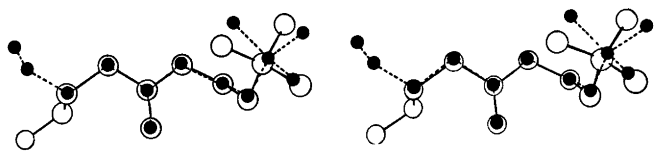


Fig. 4. A stereo view of the GABA choline ester ions of GAKOLI (○) and GAKTRA (●). The ions are viewed perpendicular to the plane of the ester group, and C(2) and the direction of C(1)–O(4) are defined to be common for both ions.

Table 6. Torsion angles of the GABA choline ester ion as found in GAKOLI and GAKTRA. The estimated standard deviations (referring to the last significant figure) are given in parentheses.

	GAKOLI	GAKTRA
N(13)–C(12)–C(11)–C(1)	±179.7(6)	±70(3)
C(12)–C(11)–C(1)–C(2)	∓71.8(9)	∓177(2)
C(11)–C(1)–C(2)–O(4)	∓178.5(7)	∓176(2)
C(1)–C(2)–O(4)–C(5)	∓176.8(7)	±174(2)
C(2)–O(4)–C(5)–C(6)	∓ 84.8(8)	± 86(2)
O(4)–C(5)–C(6)–N(7)	∓ 62.1(8)	± 74(2)
C(5)–C(6)–N(7)–C(8)	± 72.7(8)	∓ 62(2)

copper(II) complexes of GABA [15] a further variety of different conformations of GABA are found. This indicates that the conformation of GABA is highly dependent on the influence of the environment, and it falls in line with the results of NMR studies on a solution of GABA in D_2O, in which several different GABA conformers were found to coexist.[16] Theoretical studies especially on the effect of a solvent (water) on the relative stability of different conformers of GABA [17] also seem to indicate that a number of conformations with relatively small mutual energy differences are possible.

The conformation of the choline moiety is approximately the same in the two crystal structures. The torsion angles O(4)–C(5)–C(6)–N(7), ±62° in GAKOLI and ±74° in GAKTRA, are in the range found in most (but not all) crystal structures of compounds containing this atomic arrangement.[18–20] The torsion angle C(2)–O(4)–C(5)–C(6), for which much greater variations are found among the known crystal structures of choline esters,[18–20] is of the same size in GAKOLI and GAKTRA.

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Indirect Electrolytic Reduction of Derivatives of Hydroxylamine

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Several derivatives of hydroxylamine have been reduced by electrolytically generated Ti(III) or Fe(II) in the presence of suitable complexing agents. The preparative use of the indirect reduction was demonstrated and dependence of the rate of the reaction on the structure of the hydroxylamine derivative was investigated by polarography.

The reduction of hydroxylamine to ammonia *via* the radical NH_2. (or NH_3^+) by Ti(III), V(III), Cu(I), and Fe(II) has been the subject of many investigations.[1-9] Among other methods the enhancement of the polarographic waves of Ti(IV),[1] V(IV),[7] and Fe(III)[2] in suitable media has been used to measure the rate of the reaction between the metals in their lower oxidation state and hydroxylamine.

From these investigations, especially through the work of Tomat and Rigo,[5-9] it appears that only the unprotonated hydroxylamine reacts and that hydroxylamine, in certain cases at least, has to form a complex with the metal ion before electron transfer from metal to hydroxylamine takes place.

The formation of the amino radical as an intermediate was shown by a competition between aromatic substrate and metal ion for the radical;[9] aniline derivatives as well as ammonia were formed. It is not yet clear, whether the electron transfer to the amino radical occurs between solvent separated entities or within a complex.

The purpose of the present work is to investigate the reduction of derivatives of hydroxylamine by means of metal ions brought in a suitable oxidation state by electrolytic reduction. The rate of the reduction of different substrates was measured from the enhancement of the polarographic waves of Ti(IV) and Fe(III) by the substrates. As substrates, *N*-mono- and disubstituted hydroxylamines, aliphatic amine oxides, and heteroaromatic *N*-oxides were used.

RESULTS

Polarographic investigation

In alkaline solution hydroxylamine oxidizes the triethanolamine (TEA) complex of Fe(II); the catalytic increase of the polarographic wave of the Fe(III) complex in the presence of hydroxylamine has been used to calculate the rate of the reaction.[2]

The reaction between the Fe-(II)-triethanolamine complex and some substituted hydroxylamines has been investigated similarly. In Table 1 are given the rate constants for the reaction in a medium containing 2×10^{-4} M Fe(III), 4×10^{-2} triethanolamine and 0.1 M sodium hydroxide. The rate constants have been obtained by the method of Koutecky;[10] it is assumed that the reduction of a substituted hydroxylamine molecule requires the oxidation of two Fe(II) and that the amine radical does not abstract a hydrogen atom from the solvent or react in other ways.

In a similar way the reaction between Ti(III) and hydroxylamine derivatives has been investigated in oxalic acid (Table 2) and in EDTA (Table 3).

The reduction involves presumably the reduction of the unprotonated hydroxylamine derivative as it does for the unsubstituted hydroxylamine;[5] pH and the pK of the hydroxylamine derivative are thus important for the reaction. However, also the stability constants of the complexes of the complexing agents (oxalic acid, EDTA, triethanolamine) and of

Table 1. Pseudo first-order rate constants k (l mol^{-1} s^{-1}) of the reaction between electrolytically produced Fe(II) (from 2×10^{-4} M Fe(III)) and hydroxylamine derivatives (1.1×10^{-2} M) in 0.1 M NaOH containing 4×10^{-2} triethanolamine (TEA).

Added hydroxylamine	$-E_{\frac{1}{2}}$(V *vs.* SCE) of Fe-complex	k (l mol^{-1} s^{-1})
None	1.00_8	–
Hydroxylamine	1.01_5	780
N-Methylhydroxylamine	0.99_4	380
N-Ethylhydroxylamine	0.97_5	290
N-Isopropylhydroxylamine	0.97	190
N-*t*-Butylhydroxylamine	0.98_5	220
N-Benzylhydroxylamine	0.95_5	200
N,*N*-Diethylhydroxylamine	0.99	120
N-Hydroxypiperidine	0.98	120
N-Hydroxypyrrolidine	0.99	28
Triethylamine oxide	1.00	39
Pyridine *N*-oxide	1.00	23

Table 2. Pseudo first-order rate constants k (l mol^{-1} s^{-1}) of the reaction between electrolytically generated Ti(III) (from 10^{-3} M Ti(IV)) and derivatives of hydroxylamine (5×10^{-2} M) in 0.2 M oxalic acid; pH = 1.2.

Added hydroxylamine	$-E_{\frac{1}{2}}$(V *vs.* SCE) of Ti-complex	k (l mol^{-1} s^{-1})
None	0.30_5	–
Hydroxylamine	0.31_5	51
N-Methylhydroxylamine	0.32	32
N-Ethylhydroxylamine	0.32_5	13
N-Isopropylhydroxylamine	0.33_5	10
N-*t*-Butylhydroxylamine	0.31	28
N-Benzylhydroxylamine	0.33_5	28
N-Hydroxypyrrolidine	0.33	3.3×10^2
N-Hydroxypiperidine	0.31_5	1.4×10^2
N,*N*-Diethylhydroxylamine	0.32_5	3×10^2
A–OH [a]	0.31	1.6×10^5
Triethylamine *N*-oxide	0.32	123

[a] Concentration of Ti(IV) 5×10^{-5} M and of A–OH 2×10^{-3} M.

Table 3. Pseudo first-order rate constants k (l mol^{-1} s^{-1}) of the reaction between electrolytically generated Ti(III) (from 2×10^{-4} M Ti(IV)) and derivatives of hydroxylamine (10^{-2} M) in an acetate buffer pH 5.5 containing 10^{-3} M EDTA.

Added hydroxylamine	$-E_{\frac{1}{2}}$(*vs.* SCE) of Ti-complex	k (l mol^{-1} s^{-1})
None	0.46_5	–
Hydroxylamine	0.54	2.8×10^2
N-Methylhydroxylamine	0.51_5	6.3×10^2
N-Ethylhydroxylamine	0.49_5	47
N-Isopropylhydroxylamine	0.47_5	9.5
N-*t*-Butylhydroxylamine	0.46_5	1.8×10^2
N-Benzylhydroxylamine	0.48_5	1.3
N-Hydroxypiperidine	0.49_5	5.4×10^4
N-Diethylhydroxylamine	0.45	7.3×10^2
Pyridine *N*-oxide	0.46_5	1.3
Triethylamine *N*-oxide	0.46	78
Acetoxime	0.47_5	15

the hydroxylamines with Ti(III)/Ti(IV) resp. Fe(II)/Fe(III) are probably of importance, and these are not known at present. A discussion of the results in the tables will be deferred until more data are known; only the striking difference between primary and secondary hydroxylamines in the reduction by Fe(II) and Ti(III) should be pointed out.

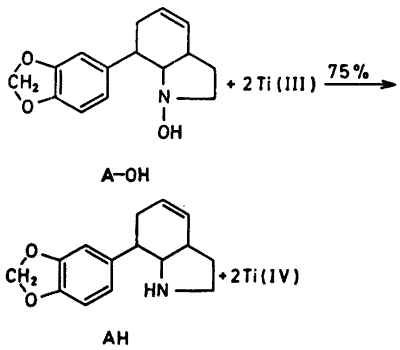
Preparative reduction

Hydroxylamine derivatives may be reduced chemically [11] by, a.o., zinc, iron or tin and acid, and presumably by Ti(III) under suitable conditions, *e.g.* in oxalic acid. Some aliphatic hydroxylamines are reducible electrolytically in slightly acid solution, but the reduction potential is quite near that of the reduction of hydrogen ions.

The reduction by electrolytircally egenerated Ti(III) or Fe(II) has certain advantages; it takes place at a less negative potential where hydrogen ions are not reduced and the low concentration of the metal salts facilitates the work-up compared to the reduction by chemical reagents where at least equivalent amounts of reagents must be used.

The conditions used in Tables 1, 2, and 3 have been chosen to show that reduction may be performed both in acid, nearly neutral, and alkaline solution. This may be of interest if stability or solubility of the substrate requires special considerations with respect to pH. Other conditions than those cited may work; generally, however, the rate decreases as pH decreases.

$$2Ti(IV) + 2e^- \rightarrow 2Ti(III)$$



Scheme 1.

The reduction in 0.2 M oxalic acid containing 10^{-2} M of 7-(1,3-benzodioxol-5-yl)-2,3,3a,6,7,7a-hexahydro-1-hydroxy-1*H*-indole (A – OH) may exemplify the reaction shown in Scheme 1. The indirect electrolytic reduction was in this case found to be preferable to chemical reduction.

Under similar conditions *N*-hydroxypiperidine yields 82 % piperidine determined polarographically through the *N*-nitrosamine.

EXPERIMENTAL

For the polarographic investigation a "multipurpose unit 563", AMEL, Milano, was used; a potentiostat from Tage Juul Electronics, Copenhagen, was employed for the preparative reductions.

The primary hydroxylamines were prepared by electrolytic reduction of the corresponding nitrocompounds in hydrochloric acid. The secondary hydroxylamines were gifts from Dr. P. E. Iversen and compound A – OH from Professor K. Torssell.[12]

Reduction of compound A – OH. A suspension of compound A – OH (1 g) in 0.2 M aqueous oxalic acid containing 20 % ethanol and 0.5 g of titanium tetrachloride was reduced at – 0.5 V (SCE), $n = 2$ F/mol. The reduction completed, the concentration of secondary amine (A) was determined by polarography after nitrosation,[13] yield 75 %. An excess of ammonia was added and the catholyte extracted twice with methylene chloride. A white precipitate, partly formed during the reduction, was filtered off, but was discarded, as an IR-spectrum did not indicate the presence of organic matter complexed with the mainly inorganic precipitate.

The methylene chloride was washed with water, dried and evaporated; the residue, 0.8 g, was dissolved in 10 ml of chloroform and dry hydrogenchloride added; on addition of ether the hydrochloride of A crystallized very slowly, m.p. 206 – 207 °C; after 4 days 0.48 g was obtained. (Found: C 64.27; H 6.74; N 4.70; Cl 12.56. Calc. for $C_{15}H_{18}ClNO_2$: C 64.40; H 6.51; N 5.01; Cl 12.67).

Reduction of N-hydroxypiperidine. *N*-Hydroxypiperidine (1 g) was reduced in 0.2 M aqueous oxalic acid containing 0.5 g of titanium tetrachloride, $n = 2$ F/mol. The reduction completed the content of the secondary amine, piperidine, was analyzed by polarography after nitrosation;[13] polarographically determined yield 82 %.

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The Orthoester Glycosylation Method. Variations in the Anomeric Composition of the Product with Aglycone Basicity in the Two-Step Procedure

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The steric outcome of the Kochetkov orthoester two-step glycosylation procedure, in which the 1,2-orthoester contains the same alcohol as that used for glycosidation, has been shown to depend on the basicity of the alcohol involved. Thus, whereas monochloroethanol gives mainly the β-D-glucopyranoside upon reaction with the appropriate 3,4,6-tri-*O*-acetyl-1,2-*O*-alkoxyethylidene-α-D-glucopyranose, dichloroethanol gives equal amounts of α-D- and β-D-glucopyranosides and trichloroethanol yields a preponderance of the α-D-glucopyranoside.

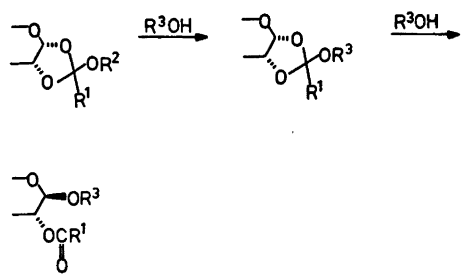


Fig. 1. R^1 = Me or Ph, R^2 = alkyl, R^3 = carbohydrate or other residue.

The potential use of sugar orthoesters for *trans*-1,2-glycoside synthesis was indicated in a study by Perlin who, in 1963, reported methyl 3,4,6-tri-*O*-acetyl-β-D-glucopyranoside as one of the major products on treatment of 3,4,6-tri-*O*-acetyl-1,2-*O*-(ethoxyethylidene)-α-D-glucopyranoside in methanol containing 1 % hydrogen chloride at room temperature for a few minutes.[1] Lemieux and Morgan [2,3] have used sugar orthoesters for *cis*-1,2-glycoside synthesis. It was, however, Kochetkov and co-workers who in a series of papers described the development of the orthoester glycosylation method into a versatile method for the synthesis of *trans*-1,2-glycosides.[4–7] The subject of *O*-glycoside synthesis has recently been reviewed.[8]

In the orthoester glycosylation method, the usual procedure is to first convert a simple alkyl orthoester into the orthoester of the alcohol to be used in the glycosylation step. The new orthoester is treated with more of the same alcohol in the presence of mercury(II) bromide or 2,6-lutidinium perchlorate. The usual solvents are nitromethane or chlorobenzene. Recent improvements include the use of molecular sieves in the synthesis of more complex orthoesters from simple ones.[9] The usual product is a 1,2-*trans*-glycoside, frequently produced in good yields. Exceptions to the expected steric outcome of the reaction have, however, been noted. Thus, in condensations of 3,4,6-tri-*O*-acetyl-1,2-*O*-(methoxyethylidene)-α-D-glucopyranose with benzyl 2-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside in nitromethane using mercury(II) bromide as promotor, the 3-*O*-α-D-glucopyranosyl-D-mannose derivative was formed in appreciable amounts, together with the expected corresponding β-linked disaccharide derivative.[10] Similar results were obtained by Kochetkov and co-workers who showed that treatment of 3,4,6-tri-*O*-acetyl-1,2-*O*-(methoxy-

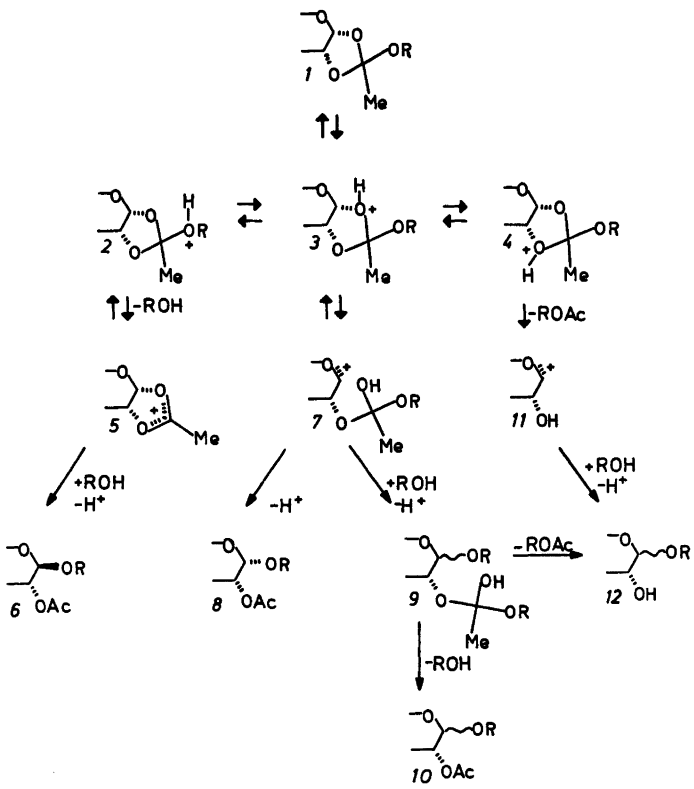


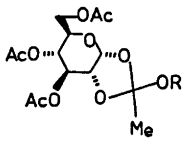
Fig. 2.

ethylidene)-α-D-glucopyranose or the corresponding orthoester of cyclohexanol with 2,6-lutidinium perchlorate in chlorobenzene containing the appropriate alcohol afforded 1,2-*cis*- as well as 1,2-*trans*-glycosides. The loss of acetyl in the 2-position was also observed.[11]

In the present paper, the variation in the proportion of *cis*-1,2- and *trans*-1,2-glycosides with the basicity of the alcohol used in the orthoester glycosylation procedure is discussed.

The various reactions which are possible in the orthoester glycosylation method are outlined in Fig. 2. For the sake of simplicity, the catalysis is represented as that of protonation. Depending on the site of the initial attack (by Lewis acid or proton) various products may be formed. The stable ion *5*, formed from *2*, with the positive charge on the alkoxyl oxygen, gives rise to the usual product, the 1,2-*trans*-glycoside. Attack at O-1 of the pyranose ring (*3*), however, may lead to the glycosyl cation (*7*) which reacts intramolecularly to give the *cis*-1,2-glycoside (*8*) or intermolecularly *via 9* to give an anomeric mixture of glycosides, retaining the acetyl as in 10 or with loss of acetyl at O-2 as in *12*. Attack at O-2 (4) would lead to the glycosyl cation *11* and, subsequently, to the anomeric mixture *12* with loss of acetyl at O-2.

The steric outcome of the orthoester glycosylation thus would seem to depend upon the initial site of Lewis acid attack or protonation. Varying the electron density at the alkoxyl oxygen in the orthoester starting material, *i.e.* the basicity of the alcohol ROH used in making the orthoester *1*, thus should influence the product distribution. In order to ascertain this, we have carried out glycosylations by the method described by Kochetkov and co-workers [4–7] using 2-chloro-, 2,2-dichloro- and 2,2,2-trichloroethanol as the alcohol component. The monochloro-, dichloro- and tri-



14, 15, 16

Fig. 3. 14: R = OCH_2CH_2Cl; *15*: R = OCH_2CHCl_2; *16:* R = OCH_2CCl_3.

chloro-orthoesters *14 – 16* (Fig. 3) were treated with 2 molar equivalents of the appropriate alcohol and 1/3 molar equivalent of mercury(II) bromide in refluxing nitromethane. The products were separated by chromatography and characterized. The composition of each reaction mixture was determined by GLC. Only traces of products other than the above pyranosides were observed. The following ratios of α:β glucopyranoside 2,3,4,6-tetraacetates were obtained: from the monochloroethyl orthoester *14* 16:84, from the dichloroethyl orthoester *15* 50:50 and from the trichloroethyl orthoester *16* 67:33. A clear correlation between the anomeric composition and the electron density at the oxygen atom of the alcohol to be glycosylated was thus established; for trichloroethanol, the α-D-glucoside predominates and for monochloroethanol, the β-D-glucoside. Ethanol yields the β-anomer exclusively.[5] The results indicate the need for careful consideration of the basicity of the free hydroxyl group in protected sugar moieties used in the synthesis of di- and oligosaccharides by the orthoester glycosylation procedure.

In the present work, products corresponding to glucosides deacetylated at O-2 (*13* in Fig. 2) were not observed. In studies where loss of acetyl from O-2 occurred, the reactions were proton catalyzed [1,11] and not Hg(II) catalyzed as in the present work.

EXPERIMENTAL

General methods. Melting points are corrected. Concentrations were performed at reduced pressure and a bath temperature below 40 °C. Optical rotations were recorded at room temperature (22 – 24 °C) using a Perkin-Elmer 141 instrument. NMR spectra were recorded on a JEOL JNM-PS-100 instrument. Analytical TLC was performed on precoated silica gel F_{254} plates (Merck). Sulfuric acid was used as spray reagent. Preparative separations were carried out on silica gel (particle size 0.040 – 0.063 mm) columns (Merck). GLC was performed on a Perkin-Elmer 900 instrument and an OV-225 column (silicone phase) at 250 °C. The relative molar response for anomeric pairs of glycosides were assumed to be 1:1. MS was performed on a Varian MAT-311-SS 111 MS-computer system. Spectra were recorded at 70 eV with an ionization current of 1000 mA and an ion source temperature of 135 °C. Mass spectra, recorded for all the compounds, gave the expected mass fragmentations.[12] Anomeric pairs gave identical fragmentation patterns, with the expected minor differences in peak intensities.

Preparation of orthoesters. 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (4.11 g, 10 mmol) in nitromethane (10 ml) was treated with the appropriate chloroethanol (20 mmol) in the presence of 2,6-lutidine (2.33 ml, 20 mmol) at 37 °C for 20 h. Aqueous silver nitrate (7.5 ml 2 M), water (12.5 ml) and acetone (25 ml) were added with stirring. The mixture was filtered and the filtrate extracted with chloroform. The combined chloroform extracts were washed with water, dried over magnesium sulfate, filtered and concentrated. The crystals obtained were recrystallized from ethanol. *3,4,6-Tri*-O-*acetyl-1,2*-O-*(2'chloroethoxyethylidene*-α-D-*glucopyranose (14)* was obtained in a 69 % yield, m.p. 121.5 – 122.5 °C, $[\alpha]_D$ + 25° (*c* 1.0, $CHCl_3$). NMR ($CDCl_3$): δ 5.76 (d, H-1), 4.42 (dd, H-2), 5.20 (t, H-3), 4.92 (dd, H-4), 3.94 (m, H-5), 4.20 and 4.24 (H-6 and H-6'), 2.12 (s, OAc), 1.73 (s, CCH_3), 3.55 – 3.85 (CH_2CH_2). First-order coupling constants (Hz): $J_{1,2}$ 5, $J_{2,3}$ 3, $J_{3,4}$ 3, $J_{4,5}$ 9. *3,4,6-Tri*-O-*acetyl-1,2*-O-*(2',2'-dichloroethoxyethylidene)*-α-D-*glucopyranose* (*15*) was obtained in a 67 % yield, m.p. 110.5 – 111.5 °C, $[\alpha]_D$ + 23° (*c* 1.1, $CHCl_3$). NMR ($CDCl_3$): δ 5.75 (d, H-1), 4.39 (dd, H-2), 5.16 (t, H-3), 4.90 (dd, H-4), 3.92 (m, H-5), 4.17 and 4.21 (H-6 and H-6'), 2.09 (s, OAc), 1.75 (s, CCH_3), 5.70 (d, $CHCl_2$), 3.89 (d, $CHCl_2$-CH_2O). First-order coupling constants (Hz): $J_{1,2}$ 5, $J_{2,3}$ 3, $J_{3,4}$ 3, $J_{4,5}$ 10, $J_{H-1',H-2'}$ of Cl_2CHCH_2O 6. *3,4,6-Tri*-O-*acetyl-1,2*-O-*(2',2',2'-trichloroethoxyethylidene)*-α-D-*glucopyranose (16)* was obtained in a 54 % yield, m.p. 79 – 80 °C, $[\alpha]_D$ + 22° (*c* 1.0, $CHCl_3$). NMR ($CDCl_3$): δ 5.82 (d, H-1), 4.49 (dd, H-2), 5.20 (t, H-3), 4.93 (dd, H-4), 3.97 (m, H-5), 4.19 and 4.23 (H-6 and H-6'), 2.11 (s, OAc), 1.80 (s, CCH_3), 4.14 (s, Cl_3CCH_2O). First-order coupling constants (Hz): $J_{1,2}$ 5, $J_{2,3}$ 3, $J_{3,4}$ 3, $J_{4,5}$ 9.

Preparation of glycosides. The appropriate orthoester (3 mmol), mono-, di- or trichloroethanol (6 mmol) and mercury(II) bromide (1 mmol) in dry nitromethane (12 ml) was refluxed for 15 min. The reaction mixtures were examined by GLC. The glycosides were separated by chromatography on dimethyl sulfoxide impregnated silica gel using diethyl

ether saturated with dimethyl sulfoxide containing 4 % water as solvent.[13] The individual substances were obtained by freeze-drying fractions containing the same substance. Monochloroethyl glucosides were recrystallized from ethanol, the others from diethyl ether–light petroleum. *2'-Chloroethyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside* was obtained in a 7 % yield, m.p. 77–78 °C, $[\alpha]_D$ +86° (*c* 1.2, $CHCl_3$). NMR ($CDCl_3$): δ 5.15 (d, H-1), 4.87 (dd, H-2), 5.49 (t, H-3), 5.05 (t, H-4), 3.5–3.9 (m, H-5), 4.2 (m, H-6 and H-6'), 2.02, 2.04, and 2.10 (s, OAc), 3.5–3.9 (m, $ClCH_2CH_2O$). First-order coupling constants (Hz): $J_{1,2}$ 4, $J_{2,3}$ 10, $J_{3,4}$ 10, $J_{4,5}$ 9.5. *2'-Chloroethyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside* was obtained in a 65 % yield, m.p. 118.5–119.5 °C, $[\alpha]_D$ −14° (*c* 1.1, $CHCl_3$) (Lit. values [14]m.p. 118.5–119.5 °C, $[\alpha]_D$ −13.7°). NMR ($CDCl_3$): δ 4.60 (d, H-1), 4.90–5.35 (m, H-2, H-3 and H-4), 4.0–4.4 (m, H-5, H-6 and H-6'), 2.02, 2.03, 2.06, 2.07 (s, OAc), 3.5–3.9 (m, $ClCH_2CH_2O$). First-order coupling constant (Hz): $J_{1,2}$ 8. MS: B_1 fragment – 60:[11] Found: m/e 248.0439 and 250.0420. Calc. for $C_{10}H_{13}{}^{35}ClO_5$: 248.0448, for $C_{10}H_{13}{}^{37}ClO_5$: 250.0418. In addition to the pure anomeric 2'-chloroethyl 2,3,4,6-tetra-*O*-acetyl-D-glucosides above, a 15 % yield of a mixed fraction was also obtained. *2',2'-Dichloroethyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside* was obtained in a 37 % yield, m.p. 86–87 °C, $[\alpha]_D$ +125° (*c* 1.2,$CHCl_3$). NMR ($CDCl_3$): δ 5.22 (d, H-1), 4.84 (dd, H-2), 5.47 (t, H-3), 5.04 (t, H-4), 3.9–4.3 (m, H-5, H-6 and H-6'), 2.05, 2.06, 2.10 (s, OAc), 3.9–4.3 (m, OCH_2CHCl_2), 5.84 (t, OCH_2CHCl_2). First-order coupling constants (Hz): $J_{1,2}$ 4, $J_{2,3}$ 10, $J_{3,4}$ 10, $J_{4,5}$ 9.5. MS: B_1 fragment – 60:[11] Found: *m/e* 282.0093, 284.0055. Calc. for $C_{10}H_{12}{}^{35}Cl_2O_5$: 282.0059, for $C_{10}H_{12}{}^{35}Cl^{37}ClO_5$: 284.0029. *2',2'-Dichloroethyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside* was obtained in a 38 % yield, m.p. 135.5–136.5 °C, $[\alpha]_D$ −12° (*c* 1.1, $CHCl_3$). NMR ($CDCl_3$): δ 4.66 (d, H-1), 4.8–5.3 (m, H-2, H-3 and H-4), 3.73 (m, H-5), 3.9–4.3 (m, H-6 and H6-'), 2.00, 2.03, 2.05 and 2.09 (s, OAc), 3.9–4.3 (m, OCH_2CHCl_2), 5.70 (dd, OCH_2CHCl_2). First-order coupling constant (Hz): $J_{1,2}$ 8. In addition to the pure anomeric 2',2'-dichloroethyl 2,3,4,6-tetra-*O*-acetyl-D-glucosides above, an 8 % yield of a mixed fraction was also obtained. *2',2',2'-Trichloroethyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside* was obtained in a 58 % yield, m.p. 99–100 °C, $[\alpha]_D$ +136° (*c* 1.0, $CHCl_3$). NMR ($CDCl_3$): δ 5.41 (d, H-1), 4.87 (dd, H-2), 5.53 (t, H-3), 5.07 (t, H-4), 4.0–4.4 (m, H-5, H-6, H-6' and OCH_2CCl_3), 2.03, 2.05 and 2.08 (s, OAc). First-order coupling constants (Hz): $J_{1,2}$ 4, $J_{2,3}$ 9.5, $J_{3,4}$ 9.5, $J_{4,5}$ 9.5. MS: B_1 fragment – 60:[11] Found: *m/e* 315.9716, 317.9703. Calc. for $C_{10}H_{11}{}^{35}Cl_3O_5$: 315.9670, for $C_{10}H_{11}{}^{35}Cl_2{}^{37}ClO_5$: 317.9640. *2',2',2'-Trichloroethyl 2,3,4,6-tri-O-acetyl-β-D-glucopyranoside* was obtained in a 17 % yield, m.p. 143–144 °C, $[\alpha]_D$ −24° (*c* 0.8, $CHCl_3$). NMR ($CDCl_3$): δ 4.84 (d, H-1), 4.9–5.3 (m, H-2, H-3 and H-4), 3.74 (m, H-5), 4.0–4.5 (m, H-6, H-6' and OCH_2CCl_3), 2.01, 2.04, 2.06 and 2.10 (s, OAc). $J_{1,2}$ 7 Hz. In addition to the pure anomeric 2',2',2'-trichloroethyl 2,3,4,6-tetra-*O*-acetyl-D-glucosides above, a 3 % yield of a mixed fraction was also obtained.

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Differences in Thiochrome Fluorescence Produced by Thiamine and Its Mono-, Di-, and Triphosphate Esters

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Equimolar amounts of thiamine and its mono-, di-, and triphosphate esters, when oxidized with potassium hexacyanoferrate(III) in an alkaline medium, were found to produce unequal intensities of thiochrome fluorescence. These differences were less pronounced if the oxidation medium contained ethanol. Assuming that the phosphate groups do not increase the fluorescence, then in 50 % ethanol (v/v); 74 % of thiamine and thiamine monophosphate, 85 % of thiamine diphosphate and 93 % of thiamine triphosphate are converted to thiochrome. The differences observed may be due to the formation of different amounts of nonfluorescent oxidation products, especially the disulfide derivative. This view is supported by the observation that thiamine and its phosphate esters differ in their stability to alkalinity.

In 1936 Jansen [1] found that oxidation of thiamine with potassium hexacyanoferrate(III) in an alkaline medium leads to the formation of the fluorescent thiochrome derivative. This reaction has frequently been used in the quantitative determination of thiamine. Methanol and ethanol, when present in the oxidation medium, favor the production of thiochrome from thiamine,[2,3] but there is no study on their possible effect on the formation of thiochrome from thiamine phosphate esters. In addition to thiamine, its mono-, di-, and triphosphate esters are found in biological materials [4–6] and can also be oxidized to fluorescent thiochrome derivatives. Lewin and Wei [7] claimed that thiamine and its mono- and diphosphate esters in equimolar amounts produce fluorescence of the same intensity. This view seems not to be generally accepted, because most investigators still treat thiamine phosphate esters with phosphatase [8] to liberate the thiamine before oxidation. The aim of the present study was to characterize the formation of thiochrome from thiamine phosphate esters, and to examine some factors influencing this reaction.

EXPERIMENTAL

Abbreviations. TMP = thiamine monophosphate, TDP = thiamine diphosphate and TTP = thiamine triphosphate.

Reagents. Thiamine and its mono- and diphosphate esters were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Thiamine triphosphate was prepared as described elsewhere.[9] All these preparations were crystallized three times and were free from inorganic phosphate. A standard solution of thiamine was made from the U.S.P. Reference Standard.[10] Thiochrome was obtained from Pfalz & Bauer, Inc., New York, U.S.A., and crystallized twice before use.

The quinine standard was 0.01 % (w/v quinine sulfate in 0.1 M H_2SO_4, diluted 1:10 and was taken to have a fluorescence of 100.

Formation of thiochrome. Thiamine and its phosphate esters were oxidized to thiochrome by the method of Lewin and Wei,[7] except that the oxidation medium contained ethanol. A sample (5 µl) of the compound was added to 3.0 ml of 50 % ethanol (v/v) and shaken, and after a few minutes 0.5 ml of alkaline hexacyanoferrate(III) reagent (15 ml of 15 % NaOH and 1 ml of 2 % potassium hexacyanoferrate(III)) was added. The mixture was agitated for 2 min, and 10 µl of 30 % H_2O_2 was added to destroy the yellow color of hexacyanoferrate(III). The fluorescence was then measured with a Zeiss PMQ II spectrophotometer fitted with a ZFM 4 fluorometer attachment (Carl Zeiss, Germany). The excitation wavelength was 365 nm and the emission maximum 430 nm.

Determination of phosphate. Total and hydrolyzable phosphate were determined according to Fiske and SubbaRow.[11] The hydrolyzable phosphate was determined after the specimen had been incubated in 1 M HCl for 10 min in a boiling water bath.

Infrared absorption analysis. Pellets containing 1 mg of thiamine compound in 120 mg of KBr were analyzed with a Beckman IR 10 spectrophotometer.

RESULTS

Effect of different alcohols on thiochrome formation and fluorescence. The effects of methanol, ethanol and propanol on thiochrome formation from a mixture of thiamine and its phosphate esters were compared (Fig. 1). In the light of this experiment, 50 % ethanol was chosen for the oxidation medium. When thiamine compounds were separately oxidized in media containing different proportions of ethanol, it was noted that the increase in thiochrome formation due to the presence of ethanol was greatest with thiamine, and smaller with TMP, TDP, and TTP, in this order. This conclusion can be drawn from the values of the index F/F_w in Table 1 as well.

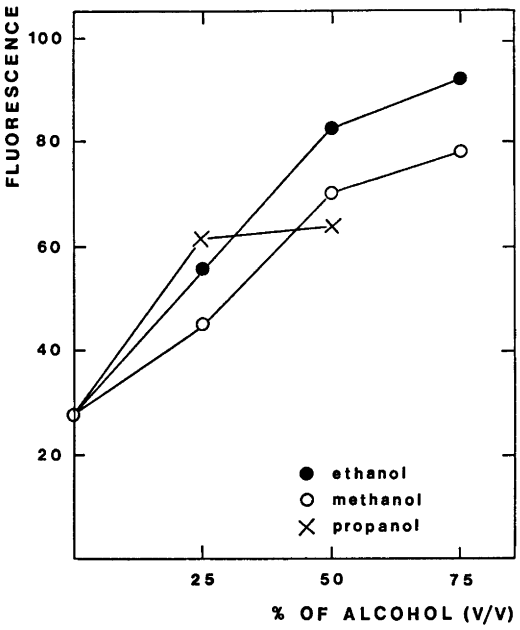

Fig. 1. The effect of different alcohols on formation of thiochrome from a mixture containing the same amounts of thiamine and its mono-, di-, and triphosphate esters.

When thiochrome was dissolved in 50 % ethanol its fluorescence was 2.5 times as intense as in water. Inclusion of potassium hexacyanoferrate(III) and NaOH in the assay medium was found to reduce the fluorescence of thiochrome to 70 % of the maximal. The presence of ethanol did not alter the pH of the assay medium, which was found to be 13.4 under all conditions used.

Differences in absorbance and in thiochrome fluorescence between thiamine and its phosphate esters. In Table 1 the absorbance in 0.1 M HCl is collated with the fluorescence of thiochrome derived from thiamine and its phosphate esters in water and in 50 % ethanol. When oxidized in 50 % ethanol, the thiamine compounds gave equal intensities of fluorescence, but after oxidation in an aqueous medium the intensities were unequal. The absorbances were also unequal. Because of these differences the concentrations were estimated in another way: by determining the total and hydrolyzable phosphate of the thiamine phosphate esters. As the data in Table 2 show, equimolar amounts of thiamine and its phosphate esters have the same absorbance, but do not produce the same

Table 1. Differences in absorbance and in thiochrome fluorescence between thiamine and its phosphate esters oxidized in 50 % ethanol and in water. The preparations were diluted to get equal thiochrome fluorescence in 50 % ethanol. The index F/F_w is the amount of fluorescence when the oxidation was performed in 50 % ethanol compared with that in water. $N = 6$.

	Absorbance $\times 10^{-3} \pm$ S.D. In 0.1 N HCl at 248 nm	Fluorescence $\pm$ S.D. In 50 % ethanol	F/F_w	In water
Thiamine	434 ± 6.34	27.60 ± 0.90	3.60	7.66 ± 0.25
TMP	422 ± 7.23	28.10 ± 0.57	3.34	8.41 ± 0.23
TDP	379 ± 6.47	28.00 ± 1.11	2.88	9.72 ± 0.15
TTP	370 ± 10.23	28.14 ± 0.16	2.84	9.91 ± 0.11

Table 2. The relationship between molarity, absorbance and fluorescence of thiochrome, thiamine and its phosphate esters. The preparations were diluted to get equal absorbance in 0.1 M HCl at 248 nm.

	Molarity ± S.D.[a] according to Weight	Total phosphate	Hydrolyzable phosphate	Relative absorbance ± S.D.[b]	Relative fluorescence ± S.D.[b] in 50 % ethanol Before hydrolysis	After hydrolysis	Correction index
Thiochrome	1.0	–	–	–	100 ± 0.5	–	–
Thiamine	1.0	–	–	100 ± 0.9	74 ± 0.3	74 ± 2.4	1.35
TMP	1.0	1.0 ± 0.01	–	100 ± 0.9	74 ± 0.4	74 ± 3.7	1.35
TDP	1.1	1.9 ± 0.02	1.0 ± 0.02	100 ± 0.6	85 ± 1.6	75 ± 2.5	1.18
TTP	1.0	3.2 ± 0.16	2.0 ± 0.03	100 ± 0.12	93 ± 1.3	79 ± 1.1	1.08

[a] $N = 3$. [b] $N = 4$.

intensity of thiochrome fluorescence. The molar absorption coefficient of thiamine and its mono-, di-, and triphosphate esters at 248 nm in 0.1 M HCl was 13 400.

When the oxidation medium contained 50 % ethanol, the fluorescence intensities produced by thiamine and thiamine monophosphate were equal, but the intensities from thiamine di- and triphosphate were higher (Table 2). To correct for the deficient thiochrome formation, the intensities produced by thiamine, TMP, TDP, and TTP must be multiplied by 1.35, 1.35, 1.18, and 1.08, respectively. On the other hand, if thiamine is taken as the standard, the fluorescence values of TMP need no correction but that of TDP and TTP must be multiplied by 0.87 and 0.80, respectively. The correction indices were found to be constant and independent of the concentration of thiamine compounds.

UV and IR absorption spectra of thiamine and its phosphate esters. The UV absorption spectra of the different thiamine phosphate esters were recorded in 0.1 M HCl, 0.1 M NaOH, and 0.05 M potassium phosphate buffer, pH 7.0. Under the same conditions, all thiamine compounds showed identical spectra. At 234 and 265 nm (the absorption maxima) molar absorption coefficients of thiamine and its phosphate esters in 0.05 M potassium phosphate buffer were found to be 10 900 and 8100 at pH 7.4 and 11 300 and 8250 at pH 7.0, respectively. These results are comparable with the corresponding values reported earlier for thiamine.[10,12–15]

The stabilities of the thiamine compounds were tested under conditions in which the UV spectra were measured. In 0.1 M HCl, absorbance at 248 nm was unchanged, ability to form fluorescent thiochrome was not reduced and no inorganic phosphate was liberated. In 0.1 M NaOH, in contrast, the ability to form fluorescent thiochrome disappeared within 1 min, but returned if the sample was acidified with HCl before oxidation. Neither a decrease in the absorbance at 230 nm nor liberation of inorganic phosphate occurred during the time needed for measurement of the UV spectra.

Characteristic differences, however, were found in the IR spectra of the different thiamine phosphate esters. Thiamine had a unique peak at 1050 cm^{-1}, whereas all three phosphate esters showed peaks at 1160 cm^{-1}. In the spectra of TMP, TDP, and TTP, additional peaks at 940, 1000, and 2560 – 2700 cm^{-1} became amplified as the number of phosphate groups increased. These results are in keeping with the structures of these esters presented in Fig. 2.

Inhibitory effect of alkalinity on formation of thiochrome from thiamine and its phosphate esters. Table 3 shows the effect of changing the pH values of the preincubation medium on formation of thiochrome from thiamine compounds. When the pH of the medium or preincubation time or both were increased, less thiochrome was formed, the effect being greater with thiamine than with its esters. The ester least susceptible to alkali was TTP, followed by TDP and TMP. At pH 9.0 the decrease in ability to form fluorescent thio-

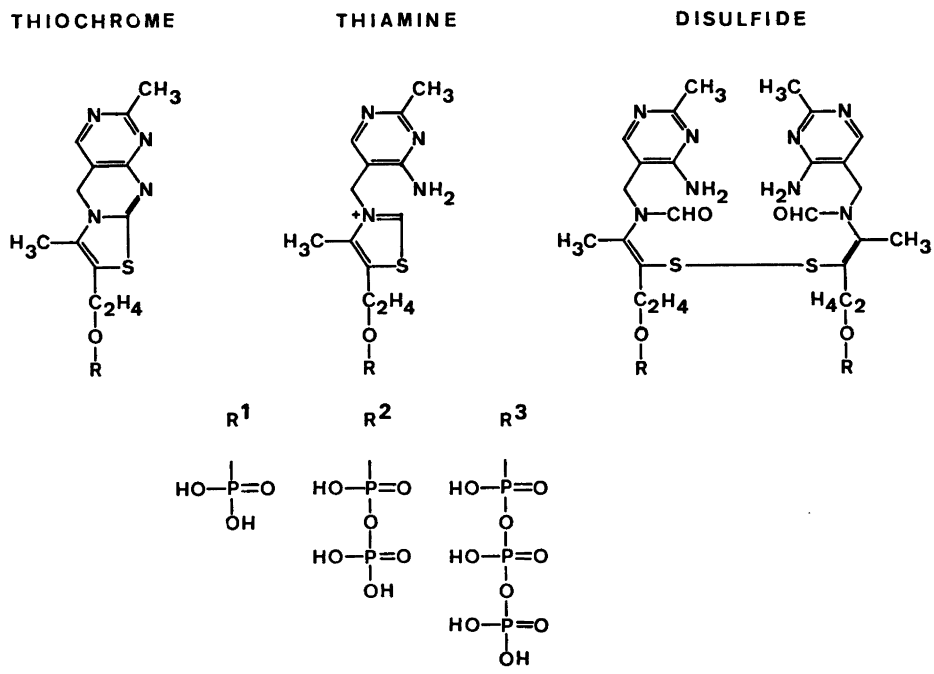

Fig. 2. Structures of thiamine, thiochrome and thiamine disulfide. R^1 = thiamine monophosphate (TMP), R^2 = thiamine diphosphate (TDP) and R^3 = thiamine triphosphate (TTP).

chrome after incubation for 48 h was irreversible, whereas at pH 8.0 it was reversible, provided the sample was acidified with HCl before oxidation.

DISCUSSION

It is not clear why thiamine esters give rise to different intensities of thiochrome fluorescence. They may produce different amounts of thiochrome, or the phosphate groups may intensify the fluorescence, or both mechanisms may be involved. Several observations support the first alternative. The other major oxidation product is the nonfluorescent disulfide (Fig. 2).[3] This is formed when thiamine is exposed to air in an alkaline medium, but can be reduced to thiamine with hydrochloric acid.[16] When incubated in an alkaline buffer, thiamine and its phosphate esters may well show the same tendency to form disulfide (Table 3). The experimental results suggest that on mild oxida-

Table 3. The stability of 1 mM thiamine and its phosphate esters in 0.5 M potassium phosphate buffer of different pH values at 24 °C.

Relative fluorescence										
pH of buffer	9.0				8.0			7.0		
Incubation time	5 min	1 h	24 h	48 h	2 h	24 h	48 h	2 h	24 h	48 h
Thiamine	73	37	36	8[a]	90	93	78[b]	100	97	72
TMP	94	89	79	66[a]	95	91	87[b]	94	91	94
TDP	100	91	60	45[a]	99	96	102	97	93	100
TTP	101	96	79	70[a]	99	97	101	99	99	101

[a] Irreversible change. [b] Reversible change.

tion in an aqueous medium, thiamine is most easily converted to the disulfide, whereas TMP, TDP, and TTP, in this order, show weaker tendencies to form corresponding disulfide derivatives. Thus possibly, the proportions of thiochrome and disulfide may differ. This notion is indirectly supported by observations on the effect of ethanol on thiochrome formation. In the oxidation of thiamine, Risinger and Pell [2] have suggested that solvents of low dielectricity will increase the formation of thiochrome, whereas those with a high dielectric constant will favor the production of disulfide. Comparison of the thiochrome fluorescence values derived from the oxidation of thiamine compounds in media with and without ethanol (Table 1, index F/F_w) showed that in the presence of ethanol, fluorescences of thiamine, TMP, TDP, and TTP were increased 3.6, 3.3, 2.9, and 2.8-fold, respectively, whereas that of thiochrome increased only 2.5-fold. The differences are small, but the tendency is clear: the more phosphorylated the thiamine compound, the smaller the effect of ethanol. Thus ethanol has the greatest effect on thiamine because thiamine has the greatest tendency to form disulfide. Furthermore, the weaker tendency of phosphorylated thiamines to form the disulfides may be due to the electrostatic repulsion between the phosphoric acid chains in the disulfide structure (Fig. 2).

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New Syntheses of the Bark Beetle Pheromones 2-Methyl-6-methylene-7-octen-4-ol (Ipsenol) and 2-Methyl-6-methylene-2,7-octadien-4-ol (Ipsdienol)

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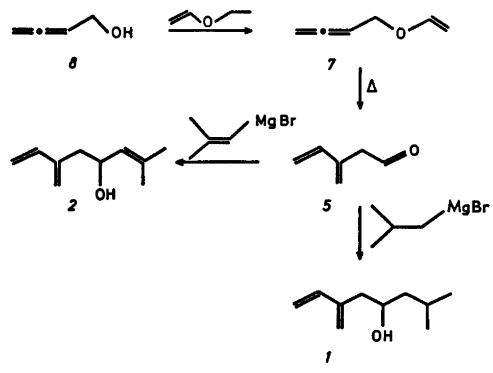
3-Methylene-4-pentenal (*5*) was prepared by a thermal [3,3] sigmatropic rearrangement of 2,3-butadienyl vinyl ether (*7*). Treatment of *5* with isobutylmagnesium bromide provided 2-methyl-6-methylene-7-octen-4-ol (ipsenol, *1*) while with isobutenylmagnesium bromide 2-methyl-6-methylene-2,7-octadien-4-ol (ipsdienol, *2*) was obtained. Furthermore, use of (+)-(2*S*,3*S*)-*N*,*N*,*N*′,*N*′-tetramethyl-2,3-dimethoxybutan-1,4-diamine as chelating agent in the latter reactions produced optically active *1* and *2* albeit in low optical yields. The synthesis of 2-methyl-6-methylene-(3*E*)-7-octadien-2-ol (*3*) is also described. Acid-catalyzed rearrangement of *3* gave only a small amount of *2*.

Various species of bark beetles are important pests to coniferous forests throughout the world; among these are beetles of the genus *Ips*. Silverstein *et al.*[1,2] isolated and characterized the following four compounds from the boring frass of male *Ips paraconfusus*: *trans*-verbenol, ipsenol (*1*), ipsdienol (*2*), and 2-methyl-6-methylene-(3*E*)-7-octadien-2-ol (*3*). The first three compounds were found to be main components of the aggregation pheromone complex of a number of *Ips* species[3] including *Ips typographus*, a serious pest to North European spruce forests.[4] As part of a group effort aiming at the control of *Ips typographus* several grams of compounds *1* and *2* were needed for use in field testing. The published syntheses at the time[5] were not suitable for such a scale and the present paper describes work which led to the successful preparation of both alcohols on a multigram scale. A synthesis of compound *3* is also described. However, the origin as well as the biological significance of this compound remain uncertain.[2] After this work was completed alternative syntheses of compounds *1* and *2* were reported;[6–8] two of them represent considerable improvement over the original syntheses and with respect to convenience may well be comparable to those described here. They involve a Reformatsky-type reaction of 2-bromomethyl-1,3-butadiene (*4*) with isovaleraldehyde[6] and 3-methyl-2-butenal,[7] respectively.

The route reported here involved the combination of a six-carbon fragment, 3-methylene-4-pentenal (*5*), with the four-carbon fragments derived from isobutyl bromide and isobutenyl bromide, respectively, as depicted in Scheme 1.

The aldehyde *5* was unknown, but the corresponding alcohol *6* had previously been reported[9] as a product formed in good yield by



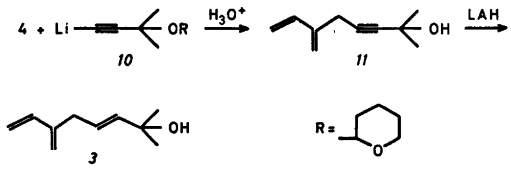
Scheme 1.

condensing isoprene with formaldehyde in the presence of stannic chloride. However, all attempts to reproduce this reaction were unsuccessful as only insignificant quantities of the alcohol were obtained.

We had previously shown [10] that 1,2,6-heptatriene rearranged thermally and quantitatively to 3-methylene-1,5-hexadiene. An analogous [3,3] sigmatropic rearrangement of 2,3-butadienyl vinyl ether (*7*) should lead to the aldehyde *5*. The allenic alcohol 2,3-butadienol (*8*) was a convenient starting material for the preparation of ether *7*. It was readily obtained from commercially available 2-butyne-1,4-diol by a simple two-step process.[11] Reaction of the allenic alcohol *8* with excess ethyl vinyl ether in the presence of stoichiometric amounts of mercury(II) acetate afforded the ether *7* in 60 % yield after 24 h. A by-product which was easily separated, proved to be 1-ethoxyethyl acetate probably formed by mercuric ion catalyzed addition of acetic acid to ethyl vinyl ether.[12] The use of catalytic amounts of mercury(II) acetate drastically reduced the yield of 7 and shorter reaction time had a similar effect. A better result was obtained employing mercury(II) trifluoroacetate [13] in the presence of 2,6-dimethylpyridine. Under these conditions the ether *7* was obtained in 69 % yield after one hour. The only by-product was a small amount (5 – 10 %) of acetaldehyde diethylacetal; ethanol is formed during the reaction. and its acid-catalyzed addition to ethyl vinyl ether must be responsible for the acetal formation.

The allenyl ether *7* was thermolysed by passing the compound under reduced pressure through a hot tube. The yields of aldehyde *5* varied depending on temperature and contact time. The latter was at first controlled by packing the tube with Pyrex wool. For complete conversion of the ether it was necessary to perform the reaction at tube temperature of 380 °C. However, at this temperature and in the presence of Pyrex wool the isomeric aldehyde 3-methyl-2,4-pentadienal (*9*) appeared as a by-product; it was invariably formed as a 5:1 mixture of (*E*)- and (*Z*)-isomers, respectively. The configuration of the isomers was established on the basis of the NMR spectrum. Due to the deshielding effect of the aldehyde group, the resonance for the methyl protons of the (*E*)-isomer appeared at δ 2.24, 0.19 ppm at lower field than that of the (*Z*)-isomer. At 395 °C compound *9* constituted the major part of the reaction product. It was possible to separate aldehyde *5* from the isomer *9* by fractional distillation, but not without significant loss of product. However, in the absence of Pyrex wool and with a tube temperature of 385 °C about 90 % conversion of *6* to aldehyde *5* occurred without formation of *9*. Apparently, the glass wool catalyzed the isomerization of *5*. Small amounts of isoprene were always detected in the thermolysis product showing that decarbonylation of *5* was a minor side reaction. The thermal conversion of *7* to *5*, an example of a [3,3] sigmatropic reaction, is the key reaction in the synthetic scheme. The reaction has recently also been studied by Cresson [14] who reports a quantitative conversion of *7* to aldehyde at 300 °C. The reaction conditions are not mentioned, and the aldehyde is only characterized by a UV spectrum. At least under our conditions at 300 °C a considerable amount of unreacted ether is present in the product.

Reaction of aldehyde *5* with isobutylmagnesium bromide in ether provided ipsenol in 62 % yield. A small amount of 3-methylene-4-penten-1-ol (*6*) was also formed; reduction of *5* would be expected with the above Grignard reagent, but the alcohol was easily separated from the main product. In a similar reaction between isobutenylmagnesium bromide and aldehyde *5* ipsdienol was obtained in 52 % yield. Replacing the Grignard reagents with the corresponding lithium derivatives in ether did not result in higher yields. The spectral properties of synthetic *1* and *2* were identical with those reported for the natural substances.[1,2] Furthermore, by reacting aldehyde *5* with the lithium compounds derived from each of the two bromides using equimolar amounts of (+)-(2*S*,3*S*)-*N*,*N*,*N*′,*N*′-tetramethyl-2,3-dimethoxybutan-1,4-diamine [15] as chelating agent, optically active ipsenol (*1*) and ipsdienol (*2*) were obtained. On the basis of the rotation values reported by Silverstein *et al.*[1,2] for the naturally occurring substances the optical yield for *1* was 6.3 % and for *2* 10.5 %. Optically active ipsenol has been recently prepared by Mori [16] from optically active starting material and the natural product has been shown,



Scheme 2.

to possess (*S*)-configuration. Work is in progress in our laboratory with the aim of establishing the absolute configuration of natural ipsdienol.

In another synthetic approach to the alcohol *2* the isomeric compound *3* was prepared by the sequence of reactions outlined in Scheme 2.

The rather difficultly accessible 3-bromomethyl-2,5-dihydrothiophene-1,1-dioxide was distilled under reduced pressure through a hot tube to give the bromide *4* in high yield.[17] Reaction of the bromide with the lithium derivative of 1,1-dimethyl-2-propynyl tetrahydropyranyl ether (*10*) in THF gave after hydrolysis the alcohol *11* in 39 % yield. Attempts to hydrogenate partially the triple bond using Lindlars catalyst, Pd/C or $Pd/CaCO_3$, were unsuccessful as more extensive reduction took place. The alcohol 2-methyl-6-hepten-3-yn-2-ol (*12*) was then prepared [18] as a model substance for further reduction studies. It was found that lithium aluminium hydride (LAH) in refluxing THF reduced *12* quite smoothly to 2-methyl-(3*E*)-6-heptadien-2-ol (*13*). When these conditions were applied to the alcohol *11* a 50 % yield of (*E*)-*3* was isolated. Its spectral properties were identical with those of the substance isolated from natural sources by Silverstein *et al.*[2] However, acid-catalyzed rearrangement of *3* gave a variety of products besides a minor amount of ipsdienol. Recently this rearrangement was achieved by Mori [8] *via* the acetate, but the reaction did not go to completion. Other synthetic routes to compound *3* have recently been reported.[20]

EXPERIMENTAL

NMR spectra were recorded on Varian A60A and HA100-15D spectrometers. Mass spectral data were obtained using an A.E.I. MS 902 mass spectrometer. A Perkin-Elmer model 457 spectrophotometer was used for IR spectra, and UV spectra were recorded on a Cary 14 spectrophotometer. Gas chromatographic analyses were performed with Varian Aerograph Models 90P and 711. Elemental analyses were carried out by Ilse Beetz Microanalytical Laboratory, 8640 Kronach, West Germany. All reactions were carried out under pure nitrogen.

2,3-Butadien-1-ol (8). The procedure was essentially that of Bailey and Pfeifer.[11] A solution of 200 g (1.91 mol) of 4-chloro-2-butyne-1-ol [21] in 300 ml of dry ether was slowly added to a stirred suspension of 100 g (2.63 mol) of lithium aluminium hydride in 2.5 l of dry ether. The reaction mixture was then heated under reflux overnight. Excess hydride was decomposed with water, and 20 % hydrochloric acid was added to bring the pH to about 2. The organic layer was separated and the water layer was extracted continuously for 48 h. The ether extracts were dried over anh. K_2CO_3 and the ether removed through a Vigreux column. Fractionation of the residue gave 82 g (62 %) of *8*, b.p. 60 °C/40 mmHg, n_D^{20} 1.4753 (lit.[10] 68–69°/45 mmHg, n_D^{20} 1.4754). NMR ($CDCl_3$): δ 2.62 (1 H, s), 4.1 (2 H, m), 4.8 (2 H, m), 5.28 (1 H, q). The IR spectrum was in accordance with that reported.[21] The compound is sensitive to oxygen, but can be stored for some time under N_2, preferably in the cold.

2,3-Butadienyl vinyl ether (7). In a typical run 1.8 g (4.2 mmol) of mercury(II) trifluoroacetate was added to 1 l of ethyl vinyl ether under vigorous stirring. 1.1 g (0.01 mol) of 2,6 dimethylpyridine was added and then 30 g (0.5 mol) of *8*. The reaction was monitored by GLC and was complete after 0.5–1 h at room temperature. Some K_2CO_3 was then added, and the volatile material was distilled through a Vigreux column. The residue was fractionated to yield 32 g (69 %) of *7*, b.p. 52 °C (120 mmHg), n_D^{23} 1.4645; ^1H NMR (CCl_4): δ 4.0 (2 H, m), 4.2 (2 H, m), 4.8 (2 H, m), 5.20 (1, H, q), 6.38 (1 H, dd). IR, ν_{max} (film): 1920 (s), 1640 (s) cm^{-1}.

GLC showed that a small amount of a second product was formed. This was separated by preparative GLC and identified as acetaldehyde diethylacetal by comparison with an authentic sample.

Method B. The reaction between *8* and ethyl vinyl ether was carried out using mercury(II) acetate in an analogous manner to that described in the literature.[22] Distillation gave *7* in 60 % yield. A second fraction, b.p. 58–65 °C (115 mmHg), which amounted to about half the weight of the ether *7*, was shown by GLC to consist of mainly one compound. This was separated by preparative GLC and identified as 1-ethoxyethyl acetate;[12] ^1H NMR ($CDCl_3$): δ 1.20 (3 H, t), 1.39 (2 H, d), 2.08 (3 H, broad s), 3.63 (2 H, m), 5.94 (1 H, q); the assignments are based on decoupling experiments.

3-Methylene-4-pentenal (5). Pyrolysis of 2,3-butadienyl vinyl ether (*7*) was carried out in a 2 cm wide and 60 cm long tube of pyrex glass kept at 385–390 °C in an electric oven. The ether was distilled into the tube at 0.2 mmHg, and the product was collected in a flask held at −78 °C.

As a rule only 20–30 g of 2,3-butadienyl vinyl ether was pyrolyzed each time, which required about 4–5 h. The product consisted of the aldehyde *5* contaminated with starting material and isoprene. Distillation provided pure *5* (80 %), b.p. 57–59 °C (60 mmHg), n_D^{24} 1.4743; IR (liq): 2710, 1710 (CHO), 1650, 1580, 980, 900 cm^{-1} (olefinic); ^1H NMR (CCl_4): δ 3.18 (2 H, d), 4.95–5.32 (4 H, m), 6.45 (1 H, dd), 9.55 (1 H, m).

The *2,4-dinitrophenylhydrazone* was prepared from *5* in the usual way, m.p. 123 °C (from methanol). Anal. $C_{12}H_{12}N_4O_4$: C, H, N.

With the tube at 395 °C and packed with 15 g of Pyrex wool *7* was converted to a product consisting of 10 % *5*, 75 % of a 5:1 mixture of (*E*)- and (*Z*)-3-methyl-2,4-pentadienal (*9*), and 15 % of unidentified products; ^1H NMR ($CDCl_3$) of (*E*)-isomer: δ 2.24 (3 H, s), 5.48 (1 H, d), 5.76 (1 H, d), 5.94 (1 H, d), 6.51 (1 H, d), 10.13 (1 H, d). The (*Z*)-isomer exhibited an identical spectrum except that the methyl resonance at 2.24 was shifted to 2.05.

2-Methyl-6-methylene-7-octen-4-ol (ipsenol), 1). A solution of 24 g (0.25 mol) of 3-methylene-4-pentenal (*5*) in 100 ml of dry ether was slowly added to a stirred solution of the Grignard reagent prepared from 38 g (0.28 mol) of 1-bromo-2-methyl-propane, 6.8 g (0.28 g.at.) Mg, in 200 ml dry ether. During the addition the reaction mixture was held at −20 °C. The cooling bath was removed and the mixture was stirred at room temperature overnight. The mixture was then cooled (ice) and saturated aqueous NH_4Cl was added. The aqueous layer was extracted with ether and the combined extracts were dried over $MgSO_4$. The solvent was removed on a rotatory evaporator and distillation of the residue through a short Vigreux column afforded 24.2 g (62 %) of *1*, b.p. 52 °C/1.3 mmHg, n_D^{24} 1.4692 (lit.[6] 109–110 °C/10 mmHg). ^1H NMR (CCl_4): δ 0.81 (3 H, d), 0.92 (3 H, d), 1.25 (2 H, m), ~1.70 (1 H, m), 2.02 (1 H, s), 2.22 (2 H, t), 3.7 (1 H, m), 4.9–5.4 (4 H), 6.37 (1 H, dd).

A compound present in the forerun was isolated by prep. GLC and shown to be 3-methylene-4-penten-1-ol (*6*); ^1H NMR ($CDCl_3$): δ 2.53 (2 H, t), 3.78 (2 H, t), 5.0–5.4 (4 H, m), 6.40 (1 H, dd).

2-Methyl-6-methylene-2,7-octadien-4-ol (ipsdienol, 2). To a cold (−30 °C) stirred solution of a Grignard reagent prepared from 40 g (0.3 mol) of 1-bromo-2-methyl-1-propene and 8 g (0.33 g.at.) Mg in 150 ml of THF and 700 ml of dry ether was added a solution of 24 g (0.25 mol) of the aldehyde *5* in 100 ml of dry ether. The cooling bath was then removed and the mixture stirred at room temperature overnight. The reaction mixture was decomposed with aqueous NH_4Cl and worked up as described under compound *1* above. Distillation gave 19.9 g (52 %) of *2*, b.p. 53 °C/0.05 mmHg (lit.[7] 54–59°/0.15 mmHg); ^1H NMR (CCl_4): δ 1.63 (3 H, d), 1.70 (3 H, d), 1.89 (1 H, s), 2.31 (2 H, d), 4.37 (1 H, m), 4.9–5.4 (4 H), 6.32 (1 H, dd).

2-Methyl-6-hepten-3-yn-2-ol (12). To a stirred solution of 50.3 g (0.3 mol) of 1,1-dimethyl-2-propynyl tetrahydropyranyl ether (*10*)[23] in 250 ml dry THF 135 ml of methyllithium (2.28 M in ether; 0.31 mol) was added at room temperature. After 2 h 36.3 g (0.3 mol) of allyl bromide was added with stirring. The reaction mixture was heated with reflux overnight and decomposed with water. The organic layer was separated and the solvents evaporated under reduced pressure. The residue was decomposed with aqueous methanol containing some *p*-toluenesulfonic acid and worked up in the usual way. Distillation gave 21.2 g (57 %) of *12*, b.p. 63–65 °C (9 mmHg), n_D^{20} 1.4620 (lit.[18] 68–69.5 °C (13 mmHg), n_D^{20} 1.4592); ^1H NMR ($CDCl_3$): δ 1.50 (3 H, s), 2.26 (1 H, s), 2.95 (2 H, m), 4.9–5.5) (4 H), 5.67 (1 H, dd).

2-Methyl-3E,6-heptadien-2-ol (13). The alcohol *12* (1.12 g; 9 mmol) in 25 ml dry THF was added dropwise to a cooled (ice) suspension of 0.33 g (7.8 mmol) lithium aluminium hydride in 35 ml dry THF. The reaction was followed by GLC, and all of the starting material had been consumed after 22 h. The product consisted essentially of *13* which was isolated by preparative GLC.

The IR spectrum was in accordance with published data.[19] ^1H NMR ($CDCl_3$): δ 1.30 (6 H, s), 1.67 (1 H, s), 2.8 (2 H, m), 5.0 (2 H, broad d, *J ca.* 14 Hz), 5.5–6.1 (3 H, m).

2-Methyl-6-methylen-7-octen-3-yn-2-ol (11). To a stirred solution of 35.3 g (0.2 mol) of the pyranyl ether *10*[23] in 150 ml of dry THF was added at room temperature 100 ml ethereal methyllithium (2.1 M; 0.21 mol). After 2 h 20.2 g (0.14 mol) of the bromide *4* in 50 ml THF was added dropwise. The reaction mixture was heated under reflux for 3 h and decomposed with water.

The organic layer was separated, and solvents evaporated under reduced pressure. The residue was treated with aqueous methanol containing some *p*-toluenesulfonic acid and worked up in the usual way. Distillation gave 7.7 g (36 %) of *11*, b.p. 61–63 °C (1.4 mmHg); ^1H NMR ($CDCl_3$): δ 2.53 (2 H, t), 3.78 (2 H, t), 5.0–5.4 (4 H, m), 6.40 (1 H, dd).

2-Methyl-6-methylene-(3E)-7-octadien-2-ol (3). A solution of 3.0 g (0.02 mol) of *11* in 50 ml dry THF was added dropwise to a stirred and ice-cooled suspension of 0.68 g (0.08 mol) lithium aluminium hydride in 90 ml dry THF. The reduction was complete after heating under reflux for 39 h as shown by GLC. When cold,

12 ml methanol and 40 ml saturated aqueous NH_4Cl was added successively.

The product was isolated with ether, and worked up in the usual way. Distillation gave 1.6 g (50 %) of *3*, b.p. 55–57 °C (1.4 mmHg); ^1H NMR ($CDCl_3$): δ 1.30 (6 H, s), 1.63 (1 H, s), 2.92 (2 H, m), 4.9–5.75 (6 H), 6.40 (1 H, dd).

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Mass Spectrometry of Onium Compounds. Part XXXI.* Methiodides of Cyclopentenyl- and Cyclopentadienylpyridines

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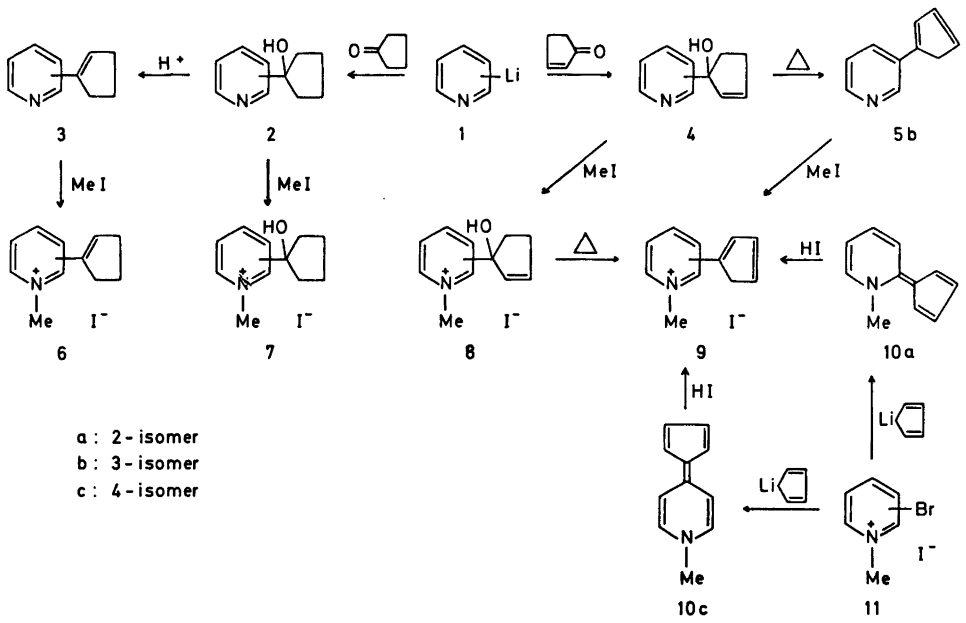
The 2-, 3-, and 4-isomers of 1′-cyclopentenyl-, 1′-hydroxy-2′-cyclopentenyl- and 1′,3′(4′)-cyclopentadienylpyridine methiodides have been synthesised and investigated by mass spectrometry. Pyrolytic demethylation before evaporation varied with the degree of unsaturation in the five-membered ring and with its position in the pyridine ring. Deprotonation of the 2- and 4-isomers yielded the volatile anhydro-bases which in the 1′-hydroxycyclopentenyl series also involves dehydration. The 3-isomers were partially reduced to dihydropyridines before evaporation. The structure analyses of the gaseous species are based on comparative fragmentation studies and on comparisons of ionisation and appearance potentials.

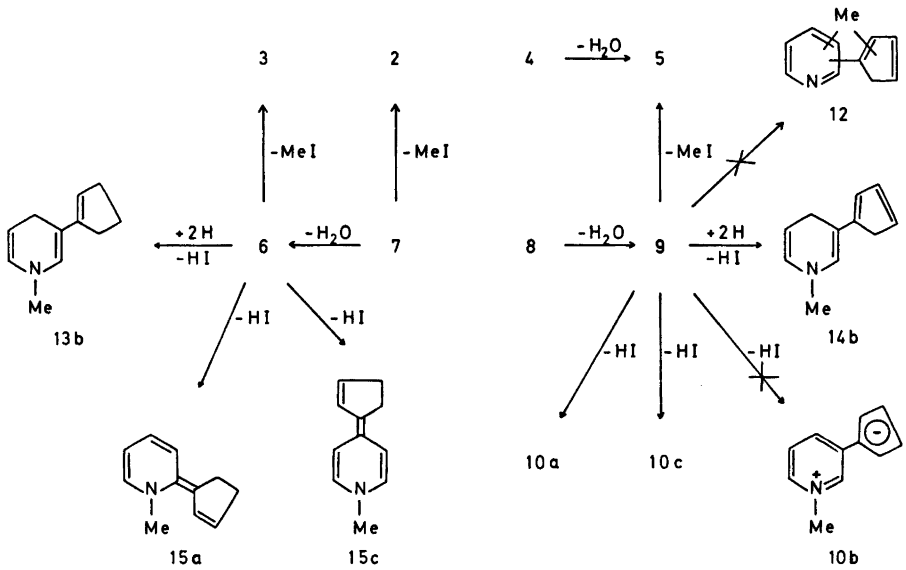
Elimination and dealkylation reactions are common pyrolytic reactions of quaternary ammonium salts in the mass spectrometer.[2] In recent investigations one and two electron reductions of aromatic and heteroaromatic onium systems have been demonstrated.[3] Valence isomerisation and skeletal rearrangements have been demonstrated in labile systems.[1] Stable betaines such as simple pyridinium-3-olates, however, are evaporated in the mass spectrometer without isomerisations to non-charged molecules.[4–6] Their conjugated acids, the 3-hydroxypyridinium salts, are largely dissociated into the pyridine betaine and the acid before evaporation.[6] By analogy to well-established behaviour in solution chemistry, anhydro-base formation may be an important pyrolytic process in heteroaromatic onium systems when the activated side-chain carries a hydrogen atom on the α-carbon atom.[3,7] Thus, the methiodides of the methyl 2- and 4-pyridylacetates furnished the respective volatile anhydro-bases in the mass spectrometer while the 3-isomer suffered a redox process to volatile dihydropyridines.[7] In the work reported herein we have investigated pyrolytic reactions in the mass spectrometer of pyridine methiodides with cyclopentenyl and cyclopentadienyl side-chains.

Syntheses. The desired methiodides or trideuteriomethiodides were prepared from the pyridines *2* – *4* and *5b* by reaction with methyl iodide or its trideuterio analogue. The cyclopentadienyl anhydro-bases *10a* and *10c* were prepared from the methiodide of the respective bromopyridine *11* and cyclopentadienyllithium;[8] protonation of *10a* and *10c* by HI yielded the corresponding conjugated acids *9*. Treatment of the unstable 3-cyclopentadienylpyridine *5b* with methyl iodide gave *9b*. The cyclopentenylpyridines *3* were prepared by acid dehydration of *2* [9,10] which were available from cyclopentanone and the respective pyridyllithium isomers. Similarly, the pyridyllithium isomers were reacted with 2-cyclopentenone to yield *4b* and *4c* as reported [8] for *4a*. The dehydration of *4b* was difficult to effect preparatively because the product (*5b*) was sensitive to polymerisation in the presence of acid or base, and *5b* was best obtained by pyrolysis of *4b* in purified sea sand at reduced pressure. The ^{1}H NMR spectrum ($CDCl_3$) showed that *5b* consisted mainly of the two cyclopentadienyl tautomers conjugated with the pyridine ring since the spectrum contained two methylene proton signals at δ 3.2 and 3.4 in the ratio 3:1 and no methine proton signal from the unconjugated isomer was seen. Similar tautomeric mixtures exist in the conjugated acids *9a* and

* Part XXX, see Ref. 1.



Scheme 1. Syntheses of pyridine methiodides.



Scheme 2. Thermal reactions in the mass spectrometer. Only one of the conjugated tautomers is shown for *12*, *13b*, *14b* and *15a*.

9c.[8] Thus, in the 2-isomer formed on protonation of the anhydro-base *10a* in trifluoroacetic acid (TFA) this is also apparent by the two different methyl proton signals at δ 4.3 and 4.4. Similarly the ^{1}H NMR spectrum in methanol-d_4 of the methiodide *9b*, which was prepared by methylation of *5b*, contained two methyl proton signals at δ 4.4 and 4.5.

Mass spectrometry. The methiodides were introduced directly into the ionisation chamber of the mass spectrometer. The spectra are sensitive to experimental conditions in accordance with pyrolytic transformations and consist of superimposed electron-impact induced fragmentation patterns of the pyrolytic gaseous species. The analyses are based on comparisons with the fragmentation patterns of the parent pyridines (Table 1) and on comparisons of appearance (AP) and ionisation (IP) potentials.

The spectrum of the cyclopentadienyl derivative *5b* has the molecular ion (*m/e* 143) as base peak. The hydroxy isomers *4* also have the base peak at *m/e* 143 [M − H_2O]. Pyrolytic dehydration of *4* to form *5* before ionisation was demonstrated by changes in the operating temperatures at 14 and 70 eV and was most pronounced when the indirect insertion system was used.

By analogy the involatile methiodides *8a* and *8c* were partially dehydrated and deprotonated before evaporation as the anhydro-bases *10a* and *10c* (*m/e* 157); in the trideuteriomethiodide analogues the molecular ion was at *m/e* 160. Pyrolysis is also supported by the fragmentation patterns which were compared with those of *10a* and *10c* (Table 1), and by the AP values (*m/e* 157) 6.6 and 6.7 eV which are in good agreement with the IP values 6.45 and 6.53 eV for the anhydro-bases *10a* and *10c*. The AP values further exclude transfer of the methyl group into the cyclopentadienyl system or into another pyridine position, since the resultant isomer (*12*, Scheme 2) would be expected to have AP value similar to the IP value (8.40 eV) of *5b*. The methiodides *9a* and *9c* were similarly converted to the anhydro-bases *10a* and *10c*, respectively, before evaporation.

The relative intensities of the *m/e* 143 species in the spectra from *8a* and *8c* were 100 and 15 %, respectively; the latter isomer has *m/e* 157 as base peak. The *m/e* 143 species corresponds to dehydration and demethylation to the cyclopentadienyl pyridines *5a* and *5c*; the spectra also contain the appropriate *m/e* 142 peak (high resolution) for methyl iodide. The value (8.30 eV) for the *m/e* 143 species from the 2-isomer (*8a*) is close to the IP value (8.40 eV) of the 3-cyclopentadienylpyridine *5b* which supports pyrolytic formation of *5a* since previous work has shown only small variations in the IP values between positional pyridine isomers.[7] The high AP value (9.9 eV) for the *m/e* 143 species from the 4-isomer, however, strongly indicates electron-impact fragmentations as the principal pathway.

The spectra of the hydroxycyclopentyl methiodides *7* were essentially those of the parent pyridines *2* together with methyl iodide in contrast to the above discussed hydroxycyclopentenyl derivatives *8*. Demethylation was also the major pyrolytic pathway in the cyclopentenyl series *6* which gave spectra very similar to those of the parent cyclopentenyl isomers *3* (Table 1) with high intensity of the molecular ion (*m/e* 145) and the base peak at [M − H]. The spectra from the 2- and 4-isomers of *6* also contain a peak at *m/e* 159 (relative intensity 3 and 20 %, respectively) corresponding to the anhydro-bases *15a* and *15c* (Scheme 2); the corresponding signal from the trideuteriomethiodide analogues of *6* were at *m/e* 162. A change of the anion from iodide to chloride in the 2-isomer increased the relative intensity to 10 %. The AP values for the *m/e* 159 species (*ca*. 6.4 and 6.35 eV, respectively) also show the expected close relationship to the IP values of the anhydro-bases *10a* and *10c*.[7] The cyclopentenyl anhydro-bases *15* have been drawn as the conjugated isomers although no evidence for this is provided; two conjugated isomers are possible for the 2-cyclopentenylidene derivative *15a*. The lower tendency for anhydro-base formation from *6* rather than from the cyclopentadienyl analogues *9* is probably due to greater stabilisation of the more conjugated anhydro-bases *10*.

The methiodides of the 3-isomers were largely demethylated before evaporation. Thus the base peak in the spectrum of the cyclopentadienyl derivative *9b* was at *m/e* 143 corresponding to *5b*, and its pyrolytic generation was confirmed by its AP value which was the same

as the IP value (8.40 eV) for the 3-cyclopentadienylpyridine *5b*.

The spectra of *9b* and *6b* also contain a signal corresponding to the reduced cation [M+H] at *m/e* 159 and *m/e* 161, respectively, together with a metastable peak for hydrogen expulsion to the cation or an isobaric ion. No evidence for deprotonation and evaporation of the resultant betaine *10b* was found. The relative intensity of [M+H] varied with the temperature and the length of time the sample was kept in the instrument and was increased from *ca.* 10 to 20 % on change of the iodide anion to chloride. The effect of the nature of the anion was most pronounced in the case of the hydroxycyclopentenyl methiodide *8b* whose very weak signal at *m/e* 159 was increased to *ca.* 20 % in the most favourable cases for the chloride; the genesis of the *m/e* 159 species involves dehydration and reduction. The AP values for this species from both *8b* and *9b* were *ca.* 7 eV which is similar to the IP values for the anhydro-bases *10a* and *10c* and are significantly different from the IP value (8.40 eV) of the 3-cyclopentadienylpyridine *5b*. A close relationship also exists between the AP value (6.30 eV) for the *m/e* 161 species from *6b* and the AP values (6.40 eV) for the suggested anhydro-bases *15a* and *15c*. In previous work we have shown that anhydro-bases and dihydropyridines derived from methiodides of pyridylacetates have similar IP values.[7] By analogy a thermally induced redox process with formation of a dihydropyridine before evaporation is postulated to explain the presence of the [M+H] species in the spectra.

The dihydropyridines *13b* and *14b* have been drawn as the generally more stable 1,4-dihydro-isomer, although no experimental evidence is available to differentiate between the 1,2- and 1,4-isomers; additional isomeric possibilities exist by different double bond locations in the five-membered ring. Differentiation between the possible isomers of *13b* and *14b* may not be possible from the spectra alone since the characteristic fragmentation is expected to be loss of a hydrogen atom or substituent from the pyridine sp^3-carbon with formation of the stable pyridinium ion;[11,12] by means of the trideuteriomethiodide analogues the initial hydrogen expulsion ([M+H]→[M]) was shown not to originate from the methyl group.

EXPERIMENTAL

^{1}H NMR spectra were recorded with a Varian A60-A instrument (60 MHz).

Mass spectra were recorded on an AEI MS-902 spectrometer attached to an AEI DS-30 data system. The compounds were introduced directly into the ion source kept at 220 °C. Low resolution (RP = 1000) spectra were recorded with 70 eV electron energy and 100 μA trap current. High resolution (RP = 10 000) spectra were recorded at 70 eV and 500 μA.

IP and AP values were obtained by semilog-plot interpretation of the ionisation efficiency curves as previously described.[13] The values are the average from three determinations, and the deviation was ±0.1 or ¹0.05 eV when the values are given with one or two decimal figures, respectively.

IP/AP values: *5a* as pyrolytic species from *8a*, 8.30 eV (*m/e* 143); *5b*, 8.40 eV (*m/e* 143); *10a* 6.45 eV (*m/e* 157); *10c*, 6.53 eV (*m/e* 157); *14b* or isomer(s) from *8b* and *9b*, *ca.* 7 eV (*m/e* 159); *15a* or isomer from *6a*, 6.4 eV (*m/e* 159); *15c* from *6c*, 6.35 eV (*m/e* 159); *13b* or isomer(s) from *6b*, 6.3 eV (*m/e* 161).

Table 1. Relative intensities of major ions in the mass spectra of pyridines *3* – *5* and *10*.

m/e	*3a*	*3b*	*3c*	*m/e*	*4a*	*4b*	*4c*	*m/e*	*5b*	*m/e*	*10a*	*10c*
145	67	80	78	161	10	37	80	143	*100*	*157*	*100*	*100*
144	*100*	*100*	*100*	160	2	16	20	142	50	156	92	10
143	10	10	15	146	2	84	14	141	8	155	8	2
130	32	30	30	144	14	50	17	117	20	154	22	3
117	20	20	22	143	*100*	*100*	*100*	116	14	142	16	5
115	8	8	10	142	24	32	30	115	27	130	48	5
106	23	2	2	132	2	49	60	89	10	115	5	15
79	28	7	6	117	73	77	18					
28	35	45	67	106	23	50	46					

(1-Hydroxycyclopentyl)pyridines (2) [9,10] were prepared from cyclopentanone and the respective pyridyllithium as described for the cyclopentenyl analogues (*4*) below. The yields were *ca.* 30 %.

(1-Cyclopentenyl)pyridines (3) were formed in 75 – 80 % yield by dehydration of the respective (1-hydroxycyclopentyl)pyridine in 3 parts of conc. H_2SO_4 at 60 °C.[9]

2-(1-Hydroxy-2-cyclopentenyl)pyridine (4) [8] was prepared as described for *4b* below.

*3-(1-Hydroxy-2-cyclopentenyl)pyridine (*4b*)*. A solution of 3-bromopyridine (15.8 g, 0.10 mol) in anhydrous ether (100 ml) was added dropwise with stirring at – 20 °C to a solution of butyllithium (0.11 mol) in ether (250 ml) in a nitrogen atmosphere. A reddish brown suspension of 3-pyridyllithium was formed.[14] The reaction mixture was stirred for 10 min before the temperature was lowered to – 50 °C and 2-cyclopentenone [15,16] (3.3 g, 0.04 mol) in anhydrous ether (50 ml) was added dropwise. The temperature of the stirred reaction mixture was allowed to reach 0 °C over 4 h and the mixture stirred for another hour at this temperature. The yellowish suspension was then poured into ice/water, the ether layer collected and the aqueous layer extracted with ether. The combined ethereal solutions were concentrated to *ca.* 20 ml and extracted with water (HCl) at pH 5.

Unreacted cyclopentenone remains in the ether solution. The aqueous solution was separated, brought to pH 8 – 9 and extracted with ether. Evaporation of the washed and dried ether solution left an oily product which slowly crystallised at 0 °C and was recrystallised from carbon tetrachloride; yield 2.0 g (37 %). The analytical specimen was sublimed at 40 °C/2.0 Pa; white solid with m.p. 110 – 112 °C. Anal. $C_{10}H_{11}NO$: C, H.

*4-(1-Hydroxy-2-cyclopentenyl)pyridine (*4c*)* was prepared as above from 4-pyridyllithium [14] except that the reaction was run at – 70 °C. The product was recrystallised from carbon tetrachloride: light petroleum (1:1); yield 1.8 g (33 %). The analytical specimen was sublimed at 30 °C/2.0 Pa, m.p. 124 – 125 °C. Anal. $C_{10}H_{11}NO$: C, H.

*3-(1,3(4)-cyclopentadienyl)pyridine (*5b*)*. 3-(1-Hydroxy-2-cyclopentenyl)pyridine (*4b*, 50 mg) and purified sea sand (*ca.* 2 g, Merck) were well-mixed and the mixture heated in a sublimation apparatus at 220 – 240 °C (oil bath) at 4.0 – 5.4 kPa. The yellowish volatile material was removed from the coldfinger by immersion in an ether solution. The combined products from 3 experiments were chromatographed on a neutral silica gel column wrapped in a dark coloured aluminium foil. Cooled ether was used as eluent and all operations were run under nitrogen as the product is very readily polymerised. The middle fractions (TLC) contained the *title compound* which was best stored in the ether solution under nitrogen in the cold; yield 40 mg (30 %). Elemental analysis was not carried out due to the instability of the compound, but the elemental composition was confirmed by high resolution MS. ^{1}H NMR ($CDCl_3$): δ 3.2 and 3.4 (CH_2 from different isomers, ratio 1:3), and 6.6 and 7.0 (3H, olefinic protons).

*Syntheses of methiodides (*6 – 8*)*. The substituted pyridine and excess methyl iodide (3 – 4 times by weight) were dissolved in ether or benzene and the solution left in the dark at room temperature. The methiodides were precipitated in 70 – 90 % yield. The reaction of 3- and 4-substituted pyridines was over in 24 h while 2-substituted pyridines required 1 – 2 weeks for the reaction to go to completion. The product was recrystallised once from ethanol to which was added a little ether.

The Me-protons in the NMR spectra (D_2O) of the 2-isomers appeared at δ 4.5 – 4.6 and of the 3- and 4-isomers at δ 4.3 – 4.5; in general the spectra were not well resolved.

N-*Methyl-(1-cyclopentenyl)pyridinium iodides (*6*)*.

6a: Decomp. 111 – 112 °C.[8]

6b: Decomp. 160 – 163 °C. Anal. $C_{11}H_{14}IN$: C, H.

6c: Decomp. 196 – 199 °C. Anal.: C, H.

N-*Methyl-(1-hydroxycyclopentyl)pyridinium iodides (*7*)*.

7a: Decomp. 115 – 120 °C. Anal.: $C_{11}H_{16}INO$: C, H.

7b: Decomp. 125 – 130 °C. Anal.: C, H.

7c: Decomp. 130 – 133 °C. Anal.: C, H.

N-*Methyl-(1-hydroxy-2-cyclopentenyl)pyridinium iodides (*8*)*.

8a: Decomp. 110 – 115 °C. Anal. $C_{11}H_{14}INO$: C, H.

8b: Decomp. 125 – 127 °C. Anal.: C, H.

8c: Decomp. 165 – 168 °C. Anal.: C, H.

N-*Methyl-3-(1,3- and 1,4-cyclopentadienyl)-pyridinium iodide (*9b*)* was prepared from the cyclopentadienylpyridine *5b* at 0 °C in ether solution. The product was sensitive to light and contact with air and was readily polymerised. Therefore no elemental analysis was carried out; NMR and MS data confirmed the structure assigned to the product.

*(*N-*Methyl-2- and 4-(1,3(4)-cyclopentadienyl)pyridinium iodides (*9a, 9c*)*. The anhydrobase (*10a* or *10c*) was dissolved in acetic acid and aq. HI added. Evaporation at reduced pressure left the solid title compounds identified by spectroscopy.[8]

Pyridinium chlorides for mass spectrometry studies were prepared by passing an aqueous solution of the iodide over a column of Amberlite IRA 400 in the chloride form.

Trideuteriomethiodides. The trideuteriomethiodide analogues *6, 8* and *9b* were prepared as above from *3, 4* and *5b* and equimolar amounts of trideuteriomethyl iodide.

N-*Methyl-2-cyclopentadienylidene-1,2-dihydropyridine (*10a*)* was prepared from cyclopentadienyllithium [17] and *N*-methyl-2-bromopyridinium iodide as described for the reaction

between cyclopentadienylsodium and *N*-methyl-2-iodopyridinium iodide;[8] m.p. 73–74 °C (pentane:ether).

N-Methyl-4-cyclopentadienylidene-1,4-dihydropyridine (10c) was synthesised in the same way from *N*-methyl-4-bromopyridinium iodide, m.p. 200 °C (slow decomp.).[8]

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Disubstituted 1,2,5-Selenadiazole *N*-Oxides. Preparation and Reactions*

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Treatment of 1,2-diketone dioximes with diselenium dichloride in dimethylformamide results in the formation of 1,2,5-selenadiazole *N*-oxides in moderate to good yields. Formation of the analogous di-*N*-oxides was not observed in any case. Thermolysis of some of the *N*-oxides gives the parent selenadiazoles as the major product. Photolysis of 2,1,3-benzoselenadiazole *N*-oxide gave a nearly quantitative yield of benzofurazan.

The 1,2,5-oxadiazole *N*-oxides (furoxans) have been known for almost a century and have since been intensely investigated,[2] but the corresponding 1,2,5-thiadiazole *N*-oxides were not recognized until recently [3] and have not yet been investigated in detail. The thiadiazole *N*-oxides were prepared in low yields from 1,2-diketone dioximes with excess of either sulfur dichloride in benzene or disulfur dichloride in dimethylformamide. The parent thiadiazoles were formed as well.

On the basis of these results, the parallel reaction with diselenium dichloride was examined to ascertain its usefulness for the preparation of the analogous selenium compounds.

RESULTS AND DISCUSSION

Preparation. A series of experiments were performed using the disubstituted glyoximes (*1a–c*) and the quinone dioximes (*1d,e*) (Chart 1) as substrates. The dioximes were allowed to react with a one molar excess of diselenium dichloride in dry dimethylformamide at ambient temperature. After hydrolysis and extraction with chloroform compounds *2a–e* crystallized on concentration. Preparative layer chromatography (PLC) of the mother liquors gave minor amounts of *2a–e* and the parent selenadiazoles *3a–c*. However, from *o*-benzoquinone dioxime (*1d*) benzofuroxan (*4*) was isolated as a by-product.

Methylphenylglyoxime (*1b*) could form two isomeric *N*-oxides, but only the isomer *2b* appears to be present (NMR, MS) in the crude product and in the product isolated from the reaction mixture by PLC. When *1b* is oxidized

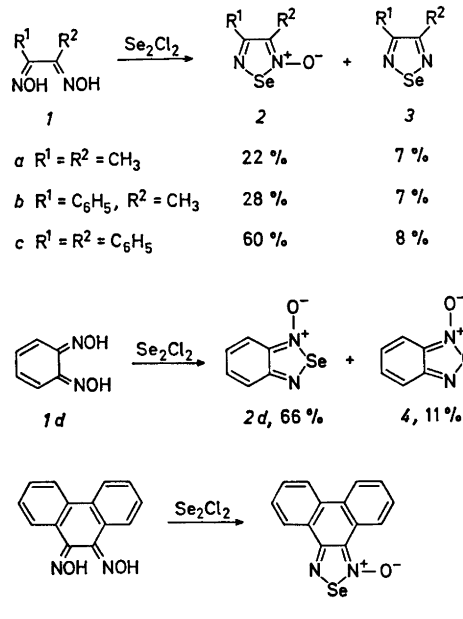
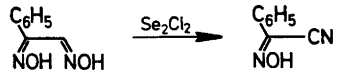


Chart 1.

* For a preliminary account of part of this work, see Ref. 1.

to methylphenylfuroxan, a mixture of the two isomers is formed.[4] Under equilibrium conditions, the more stable isomer is the analogue of *2b* with the exocyclic oxygen and the methyl substituent adjacent to each other.[4]

To obtain some insight into the limitations of this reaction, some experiments were carried out with phenylglyoxime (*1f*) (Chart 2) and the parent glyoxime. No products could be isolated from glyoxime. ^{1}H NMR analysis of the crude reaction mixture established the absence of NMR detectable amounts of 1,2,5-selenadiazole and its unknown *N*-oxide. Probably, degradation to cyanogen and water had taken place. Under the reaction conditions phenylglyoxime underwent only dehydration to give α-oximinobenzyl cyanide (*5*).



1f *5*, 90%

Chart 2.

No rationalization of the reactions leading to compounds *2* will be attempted. Mechanistic suggestions for formation of the analogous sulfur compounds are given by Pilgram.[3] Pilgram also suggests that the inability to detect any di-*N*-oxide might be due to their tendency to act as oxidizing agents, making them easily reduced by any S(I) or S(II) species present (in fact, pyridine and quinoline *N*-oxides are easily reduced using various sulfur compounds *e.g.* phenylsulfenyl chloride or disulfur dichloride[5]). Similar selenium species might also explain the formation of the deoxygenated compounds *3a–c*.

Reactions. Some thermolysis experiments were carried out on the *N*-oxides in high boiling solvents (decalin, xylene, see Experimental). In all cases the parent selenadiazoles were formed as major products (Chart 3). From the fused *N*-oxide *2d*, which decomposed more slowly than the disubstituted compounds, two products were isolated, benzoselenadiazole (*6*) and benzofurazan (*7*). No furazan formation was detected (TLC) from compounds *2b, c*.

When compound *2d* was photolyzed (wavelength >390 nm) in methylene chloride, a nearly quantitative yield of benzofurazan (*7*) was obtained.

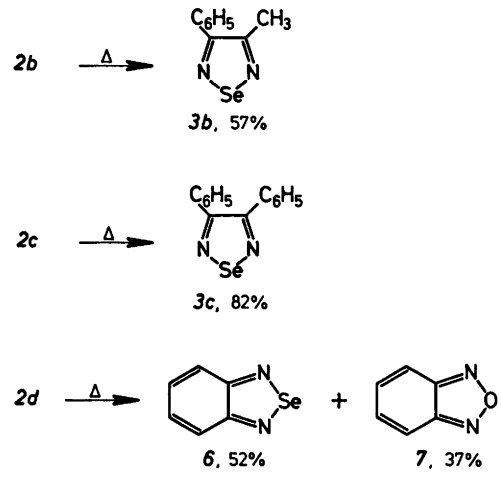


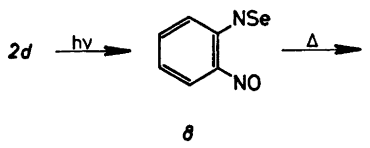
Chart 3.

The thermal results can be explained by assuming the operation of two pathways, *viz.* the reduction of *N*-oxide by solvent and a ring-opening-reclosure sequence involving the intermediacy of a nitroso-selenonitroso species which might either eliminate selenium to give a 1,2,5-oxadiazole or return to starting material. The analogous 1,2-dinitroso compounds are believed to be intermediates in the thermal interconversion of furoxans. The evidence is based mainly on spectroscopy.[6] However, 1,2-dinitrosobenzene has recently been claimed to be trapped by *p*-anisylazide.[7]

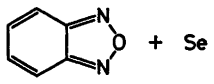
Provisional results suggest that an analogue of the latter pathway dominates in the photolysis of compound *2d*. When *2d* was photolyzed in a methanol-ethanol glass at 100 K a red-coloured intermediate (λ_{max} = 525 nm) was formed. It disappeared slowly upon heating the glass to 130 K and simultaneously formation of benzofurazan (*7*) occurred as observed in the low-temperature UV spectra. The intermediate can tentatively be identified as *8* (Chart 4).* The latter species can also be observed by flash photolysis at room temperature.[8]

The simplest synthesis of aromatic amine *N*-oxides often involves oxidation of the parent amine with a peracid.[5] However, when diphenylselenadiazole (*3c*) was oxidized with

* Benzothiadiazole *N*-oxide has been shown by low-temperature spectroscopy and flash photolysis to undergo reversible conversion to a compound believed to be *o*-nitroso-thionitrosobenzene.[8]



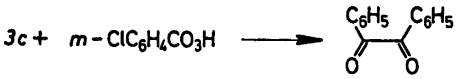
8



7

Chart 4.

m-chloroperbenzoic acid in chloroform only benzil (*9*) was isolated in low yield (Chart 5). No detectable amounts (TLC) of the *N*-oxide *2c* were present. The remaining starting material had probably been oxidized to benzoic acid. No material was extracted from the aqueous phase when benzoselenadiazole (*6*) was similarly oxidized (see Experimental).



9, 35%

Chart 5.

IDENTIFICATION OF PRODUCTS

The structural assignment of compounds *2* is based on spectroscopic evidence, on elemental analysis * and on the thermal reactions.

A characteristic feature of aromatic *N*-oxides is the presence of a strong absorption often found in the 1200 – 1300 cm^{-1} region due to the N – O stretching vibration.[5a] Compounds *2* all showed this absorption near 1350 cm^{-1} (Table 1). The values found are very close to those published for the analogous sulfur compounds [3] (*e.g.* benzothiadiazole *N*-oxide at 1365 cm^{-1} and diphenylthiadiazole *N*-oxide at 1360 cm^{-1}).

The NMR spectra (Table 1) of compounds *2a* and *2d* establish the non-equivalence of the 3,4-substituents (*2d*: the unsymmetrical pattern of the aromatic multiplet). This rules out a symmetrical structure, *e.g.* the isomeric and yet unknown Se-oxides. (The 1,2,5-thiadiazole *S*-oxide structure is known [9]).

Compound *2b*, for which two positional isomers *a priori* are possible, was identified by its mass spectrum * (Fig. 1, IP 70 eV, direct inlet at 90 °C). Provided that no essential thermal rearrangements occur prior to ionization (see below) the spectrum excludes one of the two possible isomers. The metastable defocussing technique showed that *m/e* 183 ($[C_6H_5CNSe]^+$) was formed from the molecular ion by loss of a fragment with mass 57 (CH_3CNO). The further fragmentation of *m/e* 183 is identical to that encountered in the spectrum of the parent selenadiazole (*3b*) and diphenyl-1,2,5-selenadiazole (*3c*).[10] The other positional isomer of *2b* would be expected to undergo

* All compounds gave elemental analyses within ±0.3 % units relative to the calculated values.

* Naturally abundant selenium is a mixture of 6 isotopes.

Table 1. Melting points and spectroscopic properties of *N*-oxides *2a-e* and methylphenylselenadiazole (*3b*).

Compound	M.p. °C [a]	IR (KBr) cm^{-1}	UV [b] (96 % ethanol) λ_{max} nm	log ε	λ_{max} nm	log ε	λ_{max} nm	log ε	^{1}H NMR [c] Aromatic, δ	Methyl, δ
2a	125(d) (A)	1350 (N – O)	245	3.39	285	3.87				2.58, 2.70
2b	175 – 180 (A)	1340 (N – O)	233	4.11	299	3.99			7.68 (5H)	2.69 (3H)
2c	131 – 132 (B)	1330 or 1345 (N – O)	243	4.30	316	4.00			7.0 – 7.5	
2d	180 – 182 (C)	1360 (N – O)	234	3.83	346	3.65	413	3.45	7.0 – 7.7	
2e	237 – 239 (D)	1380 (N – O)	238	4.50	302	4.15	377	4.05	–	
3b	70 – 72 (E)	–	223	3.85	305	4.00			7.0 – 7.5 (5H)	2.56 (3H)

[a] Recrystallization solvent given in parentheses; A, acetonitrile; B, ether; C, methanol; D, ethanol/benzene; E, light petroleum. [b] Vibrational fine structure is observed in the spectra of compounds *2d*, *e*. Only the major bands are given. [c] Spectra recorded at 60 MHz. Compounds *2a – c* were recorded in CF_3COOH, *2d* in DMSO-d_6 and *3b* in $CDCl_3$. *2e* was too insoluble to be recorded.

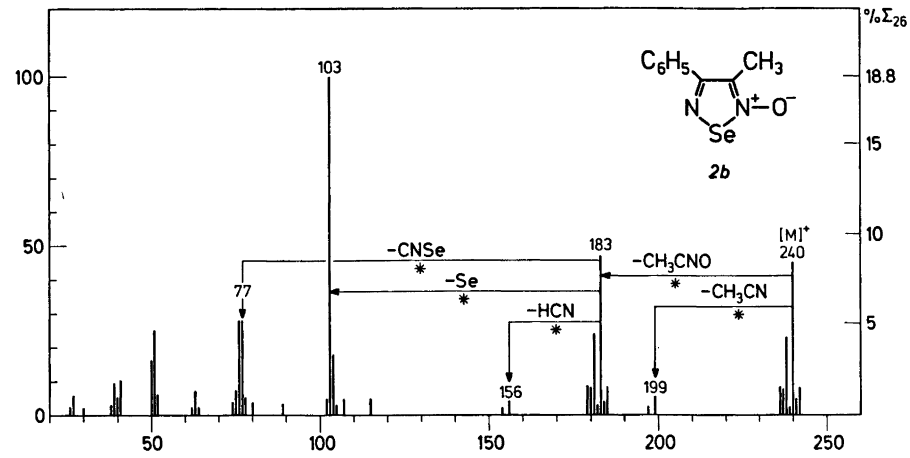


Fig. 1. Mass spectrum of compound *2b*.

cleavage to C_6H_5CNO and CH_3CNSe, either of which may be ionized. However, the absence of *m/e* 119 ($[C_6H_5CNO]^+$) and of *m/e* 121 ($[CH_3CNSe]^+$) in the spectrum of *2b* is important evidence for the assigned structure. The loss of CH_3CN from the molecular ion might be due to some thermal rearrangement prior to ionization, but *m/e* 199 is only of minor abundance (5.2 %).

The mass spectra of the remaining compounds were not investigated in detail. However, the masses of the ions found at the highest *m/e* values were identical with the calculated molecular weights. Compounds *3a,c*,[11] *4*,[12] *5*,[13] *6*,[14] and *7*[15] were identical (mixed melting point and IR) to authentic samples. *3b* was identical to a sample prepared analogous to compounds *3a,c*.

EXPERIMENTAL

Elemental analyses were carried out in the Microanalysis Department of this university. Spectroscopic analyses and PLC were performed as previously reported.[16]

*Dioximes.** These were prepared according to previously described methods: *1b* [4] (*anti*), *1c* [17] (*anti*), *1d* [15] (*amfi*? [18]), *1e*,[19] *1f* [20] (*amfi*) and glyoxime [21] (*anti*).

Reaction of 1,2-dioximes with diselenium dichloride. The reactions were performed analogously to the following procedure: *anti*-Diphenylglyoxime (*1c*, 1.50 g, 6.3 mmol) was dissolved in dry dimethylformamide (15 ml) and diselenium dichloride (2.90 g, 12.7 mmol) was slowly added with external cooling to keep the temperature at *ca.* 20 °C. After 18 h at room temperature the mixture was poured into water and extracted with chloroform. Compound *2c* (1.00 g) crystallized on concentration of the solvent. PLC (eluent: benzene – light petroleum – acetone, 7:7:1) of the oily residue gave diphenylselenadiazole (*3c*, 0.14 g ~ 8 %) and diphenylselenadiazole *N*-oxide (*2c*, 0.13 g, total yield 1.13 g ~ 60 %).

Thermolysis of 2,1,3-benzoselenadiazole N-oxide (2d). Compound *2d* (0.50 g) was suspended in decalin (10 ml) and the mixture was refluxed for 16 h. PLC (eluent: benzene, all operations were carried out at 5 °C due to the high volatility of benzofurazan) gave benzofurazan (*7*, 0.11 g ~ 37 %) and 2,1,3-benzoselenadiazole (*6*, 0.24 g ~ 52 %).

Thermolysis of 3-methyl-4-phenyl-1,2,5-selenadiazole N-oxide (2b). Compound *2b* (1.00 g) was suspended in decalin (20 ml) and the mixture was refluxed for 4 h. PLC (eluent: benzene – light petroleum – acetone, 7:7:1) gave 3-methyl-4-phenyl-1,2,5-selenadiazole (*3b*, 0.45 g ~ 57 % from reacted starting material) and starting material (0.15 g).

Thermolysis of diphenyl-1,2,5-selenadiazole N-oxide (2c). Compound *2c* (0.40 g) was suspended in xylene (10 ml) and the mixture was refluxed for 2 h. PLC (eluent: benzene – light petroleum – acetone, 7:7:1) gave diphenyl-1,2,5-selenadiazole (*3c*) (0.31 g ~ 82 %).

Photolysis of 2,1,3-benzoselenadiazole N-oxide (2d). Compound *2d* (0.50 g) was dissolved in methylene chloride (400 ml) and the solution was irradiated for 26 h with Thorn "Blue" lamps. A saturated solution of anthracene in acetone (absorbance > 2 below 390 nm) was used as cut-off filter. During the photolysis

* *1a* (commercially available) has the *anti* configuration.[22]

argon was bubbled through the solution. After partial evaporation of the solvent 0.12 g (~ 65 %) of selenium was isolated. PLC (eluent: benzene; all operations were carried out at 5 °C due to the high volatility of benzofurazan) gave benzofurazan (0.26 g ~94 % of reacted starting material) and starting material (0.04 g).

Peroxidation of diphenyl-1,2,5-selenadiazole (3c). Compound *3c* (0.50 g, 1.8 mmol) was dissolved in chloroform (5 ml). To this solution was added *ca.* 85 % *m*-chloroperbenzoic acid (0.54 g, *ca.* 2.7 mmol) in chloroform (10 ml). After 2 days at room temperature the reaction mixture was poured into 2 mol l^{-1} aqueous sodium hydroxide and extracted with chloroform. The chloroform solution was worked up by PLC (eluent: benzene – light petroleum, 1:1) into benzil (*9*) (0.10 g ~35 % of reacted starting material) and starting material (0.11 g).

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Preparation of 2-Deoxy-sugars by Hydrogenolysis of Benzoylated Glycopyranosyl Bromides. Part II*

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Hydrogenolysis of benzoylated glycopyranosyl bromides, having the substituents at C1 and C2 *cis*-oriented, gives 30 – 50 % yields of benzoylated 2-deoxy-pyranoses in addition to the expected 1,5-anhydro-alditol derivatives. The reaction has been used to prepare benzoylated 2-deoxy-D-*threo*-pentose, 2-deoxy-D-*lyxo*-hexose, 2,6-dideoxy-L-*arabino*-hexose and 2-deoxy-D-lactose.

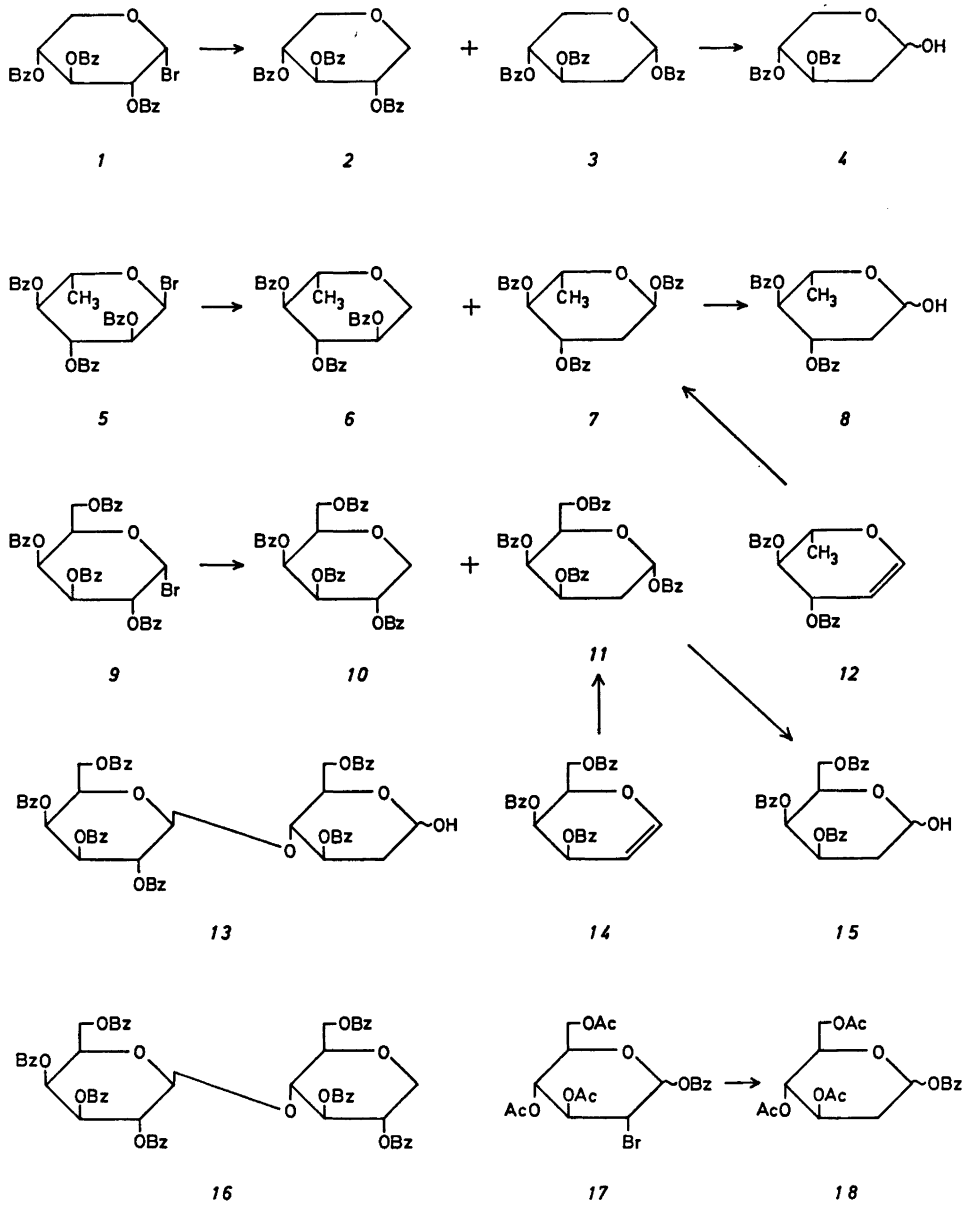
In a preceding paper it was shown that benzoylated 2-deoxy-pyranoses can be prepared by hydrogenolysis of benzoylated glycopyranosyl bromides, provided the latter have the benzoyloxy-group at C2 *cis* to the bromine atom at C1.[1] Thus hydrogenolysis of tri-*O*-benzoyl-β-D-arabinopyranosyl bromide gave tri-*O*-benzoyl-2-deoxy-β-D-*erythro*-pentopyranose in addition to the expected 1,5-anhydro-tri-*O*-benzoyl-D-arabinitol. Similarly, tetra-*O*-benzoyl-α-D-glucopyranosyl bromide gave substantial amounts of tetra-*O*-benzoyl-2-deoxy-α-D-*arabino*-hexopyranose. Benzoylated β-D-ribopyranosyl bromide or α-D-mannopyranosyl bromide, both of which have the substituents at C1 and C2 *trans* oriented, did not give 2-deoxy-sugars on hydrogenolysis.

The optimum yield of benzoylated 2-deoxy-D-ribose was obtained when the hydrogenolysis of tri-*O*-benzoyl-β-D-arabinopyranosyl bromide was carried out in ethyl acetate with palladium on carbon in the presence of 2 molar equivalents of triethylamine.[1] Other catalysts gave lower yields of benzoylated 2-deoxy-D-ribose. We have now studied the hydrogenolysis of tri-*O*-benzoyl-β-D-arabinopyranosyl bromide in the presence of a number of different amines, but otherwise under the conditions described previously.[1] With diisopropylamine or with diethylamine tri-*O*-benzoyl-2-deoxy-β-D-*erythro*-pentopyranose was obtained in 40 – 46 % yield, as determined from NMR spectra of the crude reaction products. Piperidine or propylamine gave *ca.* 25 % of the 2-deoxy-ribose derivative whereas tributylamine or pyridine only yielded traces. Thus hydrogenolysis under the conditions described previously with triethylamine as the base seems to give the best yield of 2-deoxy sugars. Under these conditions the hydrogenolyses of a number of benzoylated glycopyranosyl bromides have now been studied.

Hydrogenolysis of tri-*O*-benzoyl-α-D-xylopyranosyl bromide (*1*) under these conditions gave a crude product which, as seen from a ^{1}H NMR spectrum, contained *ca.* 40 % tri-*O*-benzoyl-2-deoxy-α-D-*threo*-pentopyranose (*3*) and 1,5-anhydro-tri-*O*-benzoyl-xylitol (*2*). Some *2* could be crystallized from the mixture, but pure *3* could not be obtained. In a separate experiment the crude reaction mixture was treated with hydrogen bromide and the resulting product was hydrolysed. This converted *3* into the corresponding 1-hydroxy-compound (*4*) whereas *2* was unchanged. Chromatography then gave 30 % crystalline *4* which on benzoylation yielded *3* and its β-anomer, both of which are known.[2]

Hydrogenolysis of 2,3,4-tri-*O*-benzoyl-α-L-quinovosyl bromide (*5*) under the same conditions gave a mixture which contained tri-*O*-benzoyl-2,6-dideoxy-α-L-*arabino*-hexopyranose (*7*) and the 1,5-anhydroalditol derivative (*6*). In this case *7* could be crystallized from the mixture in 40 % yield. Alternatively, treatment

* For Part I see Ref. 1.



of the reaction mixture with hydrogen bromide followed by hydrolysis gave the 1-hydroxy-compound (*8*), which on benzoylation yielded *7*. In order to confirm the structure of *7* it was prepared from 3,4-di-*O*-benzoyl-1,2,3-trideoxy-L-*arabino*-hex-1-enopyranose (*12*) which on treatment with hydrogen bromide yielded di-*O*-benzoyl-2,6-dideoxy-α-L-*arabino*-hexopyranosyl bromide. Treatment of the latter with silver benzoate gave *7* and its β-anomer.

Tetra-*O*-benzoyl-α-D-galactopyranosyl bromide (*9*) on hydrogenation gave a mixture which contained tetra-*O*-benzoyl-2-deoxy-α-D-*lyxo*-hexopyranose (*11*) (*ca.* 60 % as seen from an NMR spectrum) and 1,5-anhydro-tetra-*O*-benzoyl-D-galactitol (*10*). The products could

not be separated and the mixture was therefore treated with hydrogen bromide and hydrolysed as described above. Subsequent chromatography gave 39 % of the 1-hydroxy-compound (*15*). Benzoylation of the latter yielded (*11*) and its β-anomer. The structure of *11* was confirmed through its preparation from the unsaturated compound (*14*).

As a final example hepta-*O*-benzoyl-α-D-lactopyranosyl bromide was subjected to hydrogenolysis. The crude product contained *ca.* 40 % 2-deoxy-D-lactose heptabenzoate as seen from an NMR spectrum. Treatment with hydrogen bromide, hydrolysis, and chromatography gave the 1-hydroxy-2-deoxy-derivative (*13*) and the 1,5-anhydride (*16*).

Thus hydrogenolysis of benzoylated pyranosyl bromides with a 1,2-*cis* configuration seems to provide a convenient method for the preparation of benzoylated 2-deoxy-sugars. Benzoylated bromides with a 1,2-*trans* structure do not give 2-deoxy compounds.[1] Previous results have shown that acetylated pyranosyl bromides do not form 2-deoxy-sugars on hydrogenolysis.[1] We have now carried out a hydrogenolysis of tri-*O*-(*p*-methoxybenzoyl)-β-D-arabinopyranosyl bromide under the conditions described above and found that results similar to those of the benzoate were obtained.

The formation of benzoylated 2-deoxy-pyranoses must involve an acyl-migration from C2 to C1, probably *via* a cyclic intermediate. It was of interest to see whether acyl-migration from C1 to C2 could take place and to study this the two anomeric tri-*O*-acetyl-1-*O*-benzoyl-2-bromo-2-deoxy-D-glucopyranoses (α- and β-*17*) were hydrogenolysed. However, only the two 2-deoxy-D-glucose derivatives (α- and β-*18*) were obtained and no products resulting from acyl-migration were observed.

EXPERIMENTAL

Melting points are uncorrected. NMR spectra were measured on a Bruker HX-90E instrument using deuteriochloroform as solvent. 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.

*Hydrogenolysis of tri-*O*-benzoyl-α-D-xylopyranosyl bromide* (*1*). A solution of *1* (5.0 g) in ethyl acetate (35 ml) and triethylamine (2.65 ml) was hydrogenated at 1 atm. pressure for *ca.* 18 h in the presence of 1.0 g of 5 % palladium on carbon. The mixture was then filtered through carbon and the carbon was washed with dichloromethane. The filtrate was washed twice with water, dried and evaporated to a syrup (4.8 g). An NMR spectrum showed the presence of *ca.* 40 % of the 2-deoxy-compound (*3*). Crystallization from ether gave 1.05 g (25 %) of 2,3,4-tri-*O*-benzoyl-1,5-anhydro-xylitol (*2*), m.p. 145 – 148 °C (reported [3] m.p. 146 – 147 °C). An NMR spectrum further proved the structure. The products in the mother liquor could not be separated.

In a separate experiment the product from the hydrogenation of 5.0 g of *1* was dissolved in benzene (50 ml) saturated with hydrogen bromide and kept for 1.25 h. The benzene was then evaporated, tetrachloromethane was added twice and evaporated. The resulting syrup was dissolved in acetone (50 ml) and water (5 ml) and stirred overnight with silver carbonate (15 g). The silver salts were filtered off, the filtrate was diluted with dichloromethane and washed twice with water, dried and evaporated to a syrup (3.45 g). Crystallization from methanol (10 ml) gave 1.12 g (26 %) of *2*, m.p. 141 – 144 °C. Preparative TLC of the material in the mother liquor using ether-pentane (1:1) as eluent gave a further 560 mg (13 %) of *2*. A slower running fraction yielded 1.0 g (31 %) of 3,4-di-*O*-benzoyl-2-deoxy-D-*threo*-pentopyranose (*4*), m.p. 108 – 113 °C. Recrystallization from ether gave the pure product, m.p. 110 – 112 °C, $[\alpha]_D^{23}$ −76.8° (5 min) → −87.2° (42 h) (*c* 2.3, $CHCl_3$). Anal. $C_{19}H_{18}O_6$: C, H.

Benzoylation of 4. A solution of *4* (400 mg) in dichloromethane was added to a mixture of benzoyl chloride (0.5 ml) and pyridine (5 ml) at 0 °C. The mixture was kept over-night at room temperature and worked up in the usual way. The product was purified by preparative TLC (ether-pentane 1:2). The main fraction (361 mg) was crystallized from ether-pentane to give 62 mg of tri-*O*-benzoyl-2-deoxy-β-D-*threo*-pentopyranose, m.p. 162 – 166 °C. Recrystallization gave a product with m.p. 164.5 – 165.5 °C, $[\alpha]_D^{23}$ −104.8° (*c* 1.2, $CHCl_3$) (recorded [2] m.p. 160 – 162 °C, $[\alpha]_D$ −104°). Preparative TLC of the material in the mother liquor using benzene as eluent gave pure tri-*O*-benzoyl-2-deoxy-α-D-*threo*-pentopyranose (*3*) as a syrup, $[\alpha]_D^{23}$ +13.2° (*c* 0.9, $CHCl_3$) (reported [2] $[\alpha]_D$ +12.2). NMR spectra of both anomers were identical with those of previously described products.[2]

*Tri-*O*-benzoyl-α-L-quinovosyl bromide* (*5*). Benzoylation of L-quinovose [4] in the usual way with benzoyl chloride in pyridine gave a crude tetra-*O*-benzoyl-α-L-quinovopyranose, m.p. 120 – 121 °C. A ^1H NMR spectrum was in agreement with the structure. To the tetrabenzoate (5.0 g) in dichloromethane (5 ml) was added 30 % hydrogen bromide in glacial acetic acid (20 ml) and the mixture was kept for 2 h at room temperature. It was then diluted with

dichloromethane, washed with water and with aqueous sodium hydrogen carbonate, dried and evaporated. The residue was recrystallized from ether-pentane to give 3.53 g (76 %) of *5*, m.p. 158 – 160 °C. Recrystallization from ether gave a product with m.p. 158 – 160 °C, $[\alpha]_D^{20}$ −108° (*c* 3.3, $CHCl_3$). Anal. $C_{27}H_{23}BrO_7$: C, H.

^{1}H NMR: δ 7.00 (H1), 5.45 (H2), 6.37 (H3), 5.60 (H4), 4.65 (H5), 1.41 (H6); $J_{12}=4.0$ Hz, $J_{23}=J_{34}=J_{45}=9.8$, $J_{56}=6.2$.

*Hydrogenolysis of tri-*O*-benzoyl-α-*L*-quinovosyl bromide* (*5*). Hydrogenolysis of *5* (5.0 g) as described above gave 4.5 g of a crude product which was crystallized from methanol (15 ml) to give 1.8 g (41 %) of tri-*O*-benzoyl-2,6-dideoxy-α-L-*arabino*-hexopyranose (*7*), m.p. 108 – 118 °C. Recrystallization from methanol gave 1.4 g (30 %) with m.p. 122 – 124 °C and an additional recrystallization gave the pure product, m.p. 125 – 126 °C, $[\alpha]_D^{25}$ −38.5° (*c* 1.1, $CHCl_3$). Anal. $C_{27}H_{24}O_7$; C, H. ^{1}H NMR: δ 6.59 (H1), 2.47 (H2e), 2.27 (H2a), 5.82 (H3), 5.62 (H4), 4.30 (H5), 1.33 (H6); $J_{12e}=1.6$ Hz, $J_{12a}=3.4$, $J_{2e2a}=13.6$, $J_{2e3}=5.2$, $J_{2a3}=11.2$, $J_{34}=9.8$, $J_{45}=9.8$, $J_{56}=6.4$.

In a separate experiment the crude product (4.4 g) from the hydrogenolysis was treated with hydrogen bromide and then hydrolysed with aqueous acetone as described above. The product thus obtained (4.4 g) was chromatographed on a column of silica gel (400 g) eluting with ether-pentane (1:1). The first fraction to come off the column gave 1.77 g (42 %) of 1,5-anhydro-tri-*O*-benzoyl-6-deoxy-L-glucitol (*6*), m.p. 89 – 93 °C. Recrystallization from ethanol gave the pure product, m.p. 94 – 96 °C, $[\alpha]_D^{20}$ ~0° (*c* 9, $CHCl_3$). Anal. $C_{27}H_{24}O_7$: C, H. An NMR spectrum confirmed the structure.

The next fraction gave 1.02 g (31 %) of 3,4-di-*O*-benzoyl-2,6-dideoxy-L-*arabino*-hexopyranose (*8*), m.p. 94 – 97 °C, $[\alpha]_D^{25}$ +53.0° (*c* 4.2, $CHCl_3$); unchanged after 24 h. An NMR spectrum was in agreement with the structure and showed that a mixture of the two anomers was present.

Benzoylation of 8. To a mixture of pyridine (5 ml) and benzoyl chloride (0.2 ml) was added *8* (204 mg) in the course of 5 min at 0 °C. The mixture was kept for 1 h at 0 °C and for 2 h at room temperature. Work up in the usual way gave 265 mg (100 %) of almost pure *7*. Crystallization from ether-pentane gave 143 mg of a product with m.p. 124 – 125 °C, identical with that described above as seen from an NMR spectrum.

*Preparation of 7 from di-*O*-benzoyl-1,2,6-trideoxy-*L*-arabino-hex-1-enopyranose* (*12*). Hydrogen bromide was passed through a solution of *12* [5] (520 mg) in benzene (10 ml) for 15 min at 0 °C. The solvent was evaporated, carbon tetrachloride was added twice and again evaporated. An NMR spectrum of the syrupy residue (700 mg) showed that it was almost pure di-*O*-benzoyl-2,6-dideoxy-α-L-*arabino*-hexopyranosyl bromide. The product was stirred overnight with silver benzoate (2.0 g) in acetonitrile (10 ml). The silver salts were filtered off and the solvent evaporated. The residue (680 mg) was separated into two fractions by preparative TLC eluting 3 times with benzene. The slow-moving fraction (159 mg) was rechromatographed under the same conditions to give 101 mg (14 %) of *7*, m.p. 124 – 125 °C after crystallization from methanol. An NMR spectrum proved its identity with the product described above.

The fast-moving fraction gave 299 mg (42 %) of tri-*O*-benzoyl-2,6-dideoxy-β-L-*arabino*-hexopyranose as a syrup. ^{1}H NMR: δ 6.26 (H1), 2.78 (H2e), 2.29 (H2a), 5.68 (H3), 5.45 (H4), 3.98 (H5), 1.40 (H6); $J_{12e}=2.4$ Hz, $J_{12a}=9.6$, $J_{2e2a}=11.8$, $J_{2e3}=4.4$, $J_{2a3}=J_{34}=J_{45}=9.6$, $J_{56}=6.2$.

*Hydrogenolysis of tetra-*O*-benzoyl-α-*D*-galactopyranosyl bromide* (*9*). Penta-*O*-benzoyl-α-D-galactopyranose [6] (5 g) was dissolved in dichloromethane (10 ml) and 30 % hydrogen bromide in glacial acetic acid (20 ml) was added. After 2 h at room temperature more dichloromethane was added and the solution was washed with ice-water and aqueous sodium hydrogen carbonate, dried and evaporated. The residue (*ca.* 5 g) consisted of syrupy tetra-*O*-benzoyl-α-D-galactopyranosyl bromide (*9*) as seen from an NMR spectrum. The anomeric proton gave a doublet at 7.0 ppm; $J_{12}=4$ Hz. The crude bromide was hydrogenolysed as described above to give 3.9 g of a syrupy product which, as seen from an NMR spectrum, contained *ca.* 60 % of the 2-deoxy-galactose derivative (*11*) in addition to the anhydro-galactitol (*10*). These compounds could not be separated and the mixture was therefore treated with hydrogen bromide and subsequently hydrolysed as described above. The product thus obtained was chromatographed on a column of silica gel (400 g) eluting with ether-pentane (1:1).

The fastest moving fraction gave 1.1 g (27 %) of 1,5-anhydro-tetra-*O*-benzoyl-D-galactitol (*10*) as a syrup, $[\alpha]_D^{25}$ +89.6° (*c* 1.5, $CHCl_3$). (Found: C 70.90; H 4.85. Calc. for $C_{34}H_{28}O_9$: C 70.34; H 4.86). The structure was confirmed by NMR spectroscopy.

The next fraction gave 1.26 g (39 %) of syrupy 3,4,6-tri-*O*-benzoyl-2-deoxy-D-*lyxo*-hexopyranose (*15*), $[\alpha]_D^{23}$ +31.2° (*c* 1.5, $CHCl_3$). Anal. $C_{27}H_{24}O_8$: C, H.

Benzoylation of (*15*) (200 mg) with benzoyl chloride in pyridine as described above gave a syrup which was separated into two fractions by preparative TLC using benzene as eluent. The first fraction gave 180 mg (74 %) of tetra-*O*-benzoyl-2-deoxy-α-D-*lyxo*-hexopyranose (*11*) as a syrup, $[\alpha]_D^{20}$ +51.8° (*c* 3.9, $CHCl_3$). (Found: C 69.93; H 5.28. Calc. for $C_{34}H_{28}O_9$: C 70.34; H 4.86). ^{1}H NMR: δ 6.76 (H1), 2.65 (H2a), 2.38 (H2e), 5.82 (H3), 6.01 (H4), 4.3 – 4.9 (H5, H6); $J_{12e} \simeq 1$ Hz, $J_{12a}=3.8$, $J_{2e2a}=13$, $J_{2e3}=5.5$, $J_{2a3}=12$, $J_{34}=2.5$.

The next fraction consisted of tetra-*O*-benzoyl-2-deoxy-β-D-*lyxo*-hexopyranose, syrup, $[\alpha]_D^{25}$ +14.1° (*c* 6.3, $CHCl_3$), (reported[7] $[\alpha]_D$ +14.8°). An NMR spectrum confirmed the structure.

Preparation of 11 *from tri*-O-*benzoyl-1,2-dideoxy*-D-lyxo-*hex-1-enopyranose* (*14*). A solution of *14* (396 mg) in benzene was treated with hydrogen bromide, as described above for *12*, to give crude syrupy tri-*O*-benzoyl-2-deoxy-α-D-*lyxo*-hexopyranosyl bromide. An NMR spectrum was in agreement with the structure. The bromide was treated with silver benzoate in acetonitrile as described above to give 385 mg of a mixture of *11* and its β-anomer. Preparative TLC eluting 3 times with benzene gave 160 mg (32 %) of *11* and 128 mg (26 %) of tetra-*O*-benzoyl-2-deoxy-β-D-*lyxo*-hexopyranose. NMR spectra showed that they were identical with the products described above.

Hydrogenolysis of hepta-O-*benzoyl*-α-D-*lactopyranosyl bromide.* Octa-*O*-benzoyl-α,β-D-lactose[8] was treated with hydrogen bromide in glacial acetic acid for 2 h at room temperature. The mixture was then diluted with dichloromethane, washed with water and aqueous sodium hydrogen carbonate, dried and evaporated. This gave hepta-*O*-benzoyl-α-D-lactopyranosyl bromide as a syrup. An NMR spectrum showed H1 as a doublet at δ 6.8, J_{12} = 4.2 Hz.

The bromide (1.0 g) was hydrogenated as described above to give a crude product (900 mg) which contained *ca.* 40 % hepta-*O*-benzoyl-2-deoxy-α-D-lactopyranose as seen from an NMR spectrum. The product was treated with hydrogen bromide and then hydrolyzed as described above. The material thus obtained was separated into two fractions by preparative TLC using ether-pentane (1:1) as eluent.

The fast-moving fraction gave 298 mg (32 %) of the 1,5-anhydro-hepta-*O*-benzoyl-D-lactitol (*16*) as a syrup, $[\alpha]_D^{25}$ +59.5° (*c* 6.0, $CHCl_3$). Anal. $C_{61}H_{50}O_{17}$: C, H. Both ^1H and ^{13}C NMR spectra were in agreement with the structure.

The next fraction gave 245 mg (26 %) of hexa-*O*-benzoyl-2-deoxy-D-lactopyranose (*13*) as a syrup, $[\alpha]_D^{20}$ +64.6° (*c* 2.8, $CHCl_3$). Anal. $C_{54}H_{46}O_{15}$: C, H. An NMR spectrum confirmed the structure.

3,4,6-Tri-O-*acetyl-1*-O-*benzoyl-2-bromo-2-deoxy*-α-D-*glucopyranose* (α-*17*). To tri-*O*-acetyl-1-*O*-benzoyl-2-bromo-2-deoxy-β-D-glucopyranose[9,10] (β-*17*) (500 mg) in chloroform (5 ml) was added boron trifluoride etherate (1 ml) and the mixture was kept at room temperature for 24 h. It was then washed with aqueous sodium hydrogen carbonate, dried and evaporated. The residue (458 mg) was purified by preparative TLC using benzene-chloroform (1:1) as eluent. The first fraction gave 168 mg (34 %) of α-*17*, which was crystallized from ethanol, m.p. 107 −111 °C (reported[9] m.p. 112−113 °C). An NMR spectrum was identical with that described.[9] A second fraction gave 66 mg (13 %) of unchanged β-*17*.

Hydrogenolysis of β-*17* (500 mg) was performed as described above. The product (405 mg) was separated into two fractions by preparative TLC using 4 elutions with ethyl acetate-pentane (1:4). The fast-moving fraction gave 167 mg (40 %) of tri-*O*-acetyl-1-*O*-benzoyl-2-deoxy-β-D-*arabino*-hexopyranose (β-*18*), m.p. 85−87 °C. Recrystallization from ether-pentane gave a product with m.p. 86−87 °C, $[\alpha]_D^{20}$ −7.04° (*c* 3.4, $CHCl_3$). Anal. $C_{19}H_{22}O_9$: C, H. ^1H NMR: δ 6.52 (H1), 2.0−2.6 (H2), 5.0−5.4 (H3, H4), 3.89 (H5), 4.26 (H6), 4.16 (H6′); J_{12e} = 2.5 Hz, J_{12a} = 9.5, J_{56} = 4.8, $J_{56'}$ = 2.5.

The next fraction gave 92 mg (31 %) of tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-glucitol as seen from an NMR spectrum. It was not identified further.

Hydrogenolysis of α-*17* (190 mg) gave 130 mg of crude product. Preparative TLC (ethyl acetate-pentane, 2:1) yielded 63 mg (40 %) of tri-*O*-acetyl-1-*O*-benzoyl-2-deoxy-α-D-*arabino* hexopyranose (α-*18*), crystallized from ether, m.p. 115.5−117 °C, $[\alpha]_D^{20}$ +92.3° (*c* 1.2, $CHCl_3$). Anal. $C_{19}H_{22}O_9$: C, H. ^1H NMR: δ 6.52 (H1), 2.49 (H2e), 2.11 (H2a), 5.40 (H3), 5.16 (H4), 4.0−4.6 (H5, H6); J_{12e} = 1.4 Hz, J_{12a} = 3.4, J_{2e3} = 5.4, J_{2a3} = 11.4, J_{34} = J_{45} = 9.6.

Microanalyses were performed by Novo analytical laboratory.

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Short Communications

Biosynthetic Production of ^{14}C-Labeled Reference Glucuronides

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The conjugation of various aglycones with α-D-glucopyranosiduronic acid (α-D-glucuronic acid), a residue of UDPglucuronic acid, is catalyzed by microsomal UDPglucuronosyltransferase (EC 2.4.1.17).[1] The synthesized *R*-β-D-glucopyranosiduronic acids (glucuronides) are important detoxication products in mammalian tissues.[2] The glucuronides are excreted in the bile or urine. In studies of the glucuronide biosynthesis and excretion, labeled conjugates would be of great help. Because these compounds are not available or they are very expensive, we have developed a simple method for the production of ^{14}C-labeled reference glucuronides and used 4-methylumbelliferone, 4-nitrophenol, and ethylmorphine as model substrates. These aglycones are often used in studies of UDPglucuronosyltransferase.[1]

Materials and methods. Radioactive UDPglucuronic acid (^{14}C in glucuronic acid residue specific activity > 200 mCi/mmol) was purchased from NEN Chemicals GmbH (Frankfurt am Main, Federal Republic of Germany). 4-Nitrophenol, K_2EDTA and digitonin were obtained from E. Merck AG (Darmstadt, Federal Republic of Germany) and D-glucaro-1,4-lactone was purchased from Calbiochem (Los Angeles, USA). Ethylmorphine chloride (Ph.Nord.) was obtained from Lääketukku OY (Turku, Finland) and 4-methylumbelliferone, 3-methylcholanthrene, trypsin (type III), trypsin inhibitor (type II-o), α-D-glucuronic acid 1-phosphate, D-glucuronic acid sodium salt (grade II) and UDPglucuronic acid trisodium salt from Sigma Chemical Co. (St. Louis, Mo., USA). Olive oil was purchased from Fisher Scientific Company (Fairlawan, N.J., USA). 4-Methylumbelliferyl-β-D-glucuronide trihydrate and 4-nitrophenyl-β-D-glucuronide were obtained from Koch-Light Laboratories Ltd (Bucks, England).

Male rats (*Rattus norvegicus*) of Wistar Af/Han/Mol/(Han 67) strain aged 2–2.5 months, outbred by rotational mating system in the Laboratory Animal Centre of Kuopio University, were used as experimental animals. 3-Methylcholanthrene dissolved in olive oil was administered to rats intraperitoneally (20 mg/kg) as a single daily dose for five days to induce UDPglucuronosyltransferase. Microsomes and partly purified UDPglucuronosyltransferase were prepared as described earlier.[3]

In the preparation of ^{14}C-labeled glucuronides, ^{14}C-labeled UDPglucuronic acid (1 μCi) dissolved in 60 % ethanol was evaporated to dryness in an ice bath under a nitrogen stream. To the tube, 50 μl of 0.3 M potassium phosphate buffer (pH 7.0) containing 0.5 mM of aglycone,

Table 1. Biosynthesis of ^{14}C-labeled glucuronides and some other metabolites in 0.3 M potassium phosphate buffer pH 7.0 containing 0.5 mM aglycone, 80 mM K_2EDTA, 10 mM D-glucaro-1,4-lactone. The ratio of labeled intermediates was analyzed from the incubation mixture and the yield and purity from a lyophilized eluent after the chromatographic purification.

Aglycone	Ratio of labeled glucuronide: UDPGlcUA: GlcUA-1-P:GlcUA [a]	Yield/%	Purity/%
4-Methyl-umbelliferone	96:1:2:1	93	99
4-Nitrophenol	93:3:3:1	90	99
Ethylmorphine	50:12:22:16	45	95

[a] Abbreviations: UDPGlcUA = UDPglucuronic acid, GlcUA-1-P = α-D-glucuronic acid 1-phosphate and GlcUA = D-glucuronic acid.

80 mM of K_2EDTA and 10 mM of D-glucaro-1,4-lactone was added. The reaction was started by adding 10 µl of partly purified UDPglucuronosyltransferase and immersing the tube into a 38 °C water bath. After a 2 h incubation period the reaction was stopped by adding 100 µl of absolute ethanol and denatured protein was spun down. The supernatant was applied as a band on Whatman No. 1 paper and the paper was developed for 20 h with ethyl acetate:acetic acid:water (6:3:4) as solvent using a descending chromatographic technique. Glucuronides and other metabolites of UDP-glucuronic acid were localized on the paper with aid of reference samples as described earlier.[4] The strips containing conjugates were eluted by 5 ml of distilled water and dried by lyophilisation.

From every step small aliquots were taken and analyzed by the same chromatographic method. The amounts of different intermediates of UDPglucuronic acid metabolism in samples were analyzed by a Wallac LSC 8100 liquid scintillation counter as earlier described.[4]

During development of the method, 0.3 M Tris-HCl and 0.3 M potassium citrate buffers as incubation medium, and native rat liver microsomes as enzyme source were also used. In addition 2-aminophenol was tested as aglycone.

Results and discussion. After an incubation of radioactive UDPglucuronic acid, aglycone and partly purified UDPglucuronosyltransferase in potassium phosphate buffer pH 7.0 containing K_2EDTA and D-glucaro-1,4-lactone, labeled 4-nitrophenyl-β-D-glucuronide and 4-methylumbelliferone-β-D-glucuronide contained more than 90 % of the radioactivity of the reaction mixture. If ethylmorphine was used as aglycone the yield was decreased to the half. By the method described we failed to produce 2-aminophenyl-β-D-glucuronide. No labeled spot was found, but the radioactivity was spread over a large area. The tailing may be due to the lability of *N*-glucuronides of aromatic amines.[5] If 4-nitrophenol or 4-methylumbelliferone were used as aglycone, the glucuronide yield after the chromatographic separation and elution contained more than 99 % of the radioactivity of the elution mixture. The purity of ethylmorphine glucuronide by the same procedure was found to be about 95 % (Table 1). When native microsomes were used as UDPglucuronosyltransferase source a lower glucuronide yield was obtained. This was probably due to the lower specific activity of UDPglucuronosyltransferase in native microsomes and partly due to the sedimentation of radioactive material with denatured microsomes.[4]

EDTA and D-glucaro-1,4-lactone were added to the reaction mixture to optimize the production of glucuronides. EDTA is a powerful inhibitor of UDPglucuronic acid pyrophosphatase [6] which shares the substrate UDPglucuronic acid with UDPglucuronosyltransferase. D-Glucaro-1,4-lactone is a specific inhibitor of β-glucuronidase.[7] This enzyme hydrolyses β-glucuronides to free aglycone and D-glucuronic acid. The use of these inhibitors in the reaction mixture containing native microsomes as enzyme source greatly improves the yield of glucuronide synthesis. If partly purified UDP-glucuronosyltransferase is used as an enzyme source, the effect of these inhibitors on the yield is small, because the preparation contains only traces of β-glucuronidase and UDP-glucuronic acid pyrophosphatase.

If the incubation was carried out in 0.3 M potassium citrate buffer pH 7.0 containing 10 mM of D-glucaro-1,4-lactone, the radioactive metabolites, 4-nitrophenyl-β-D-glucuronide, UDPglucuronic acid, α-D-glucuronic acid 1-phosphate and free D-glucuronic acid were found in the ratio of 60:1:12:17 in the reaction mixture. K_2EDTA could not be used in this mixture, because it caused a sediment to form, but citrate itself inhibits UDPglucuronic acid pyrophosphatase. When 0.3 M Tris-HCl buffer pH 7.0 containing 80 mM of K_2EDTA and 10 mM of D-glucaro-1,4-lactone was used, labeled metabolites could be found in the ratio of 92:4:2:2, respectively. In every buffer the final purity of 4-nitrophenyl-β-D-glucuronide after chromatographic purification was better than 99 %, but if citrate or Tris-HCl buffers were used the yield was poorer than in phosphate buffer.

The method described is reproducible, easy to perform and can also be applied for preparation of labeled glucuronides from unlabeled UDPglucuronic acid and a labeled aglycone.

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Redetermination of the Crystal Structure of Lactoylcholine Iodide

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The crystal structure of lactoylcholine iodide, $CH_3CHOHCOOCH_2CH_2N^+(CH_3)_3,I^-$, has been determined by Chotia and Pauling,[1] but unfortunately neither coordinates nor any description of the crystal packing was published. The lactoylcholine ion is, in contrast to most other choline ester ions, a potential hydrogen bond donor. The redetermination of the crystal structure was undertaken as part of solid state studies of choline ester salts, in which hydrogen bonding of the choline ester ion cannot *a priori* be excluded.

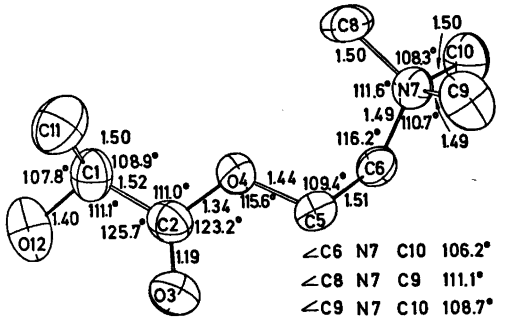

Fig. 1. (*S*)-Form of the lactoylcholine ion. The torsion angles are: C11 – C1 – C2 – O4 – 86.0°; C1 – C2 – O4 – C5 177.4°; C2 – O4 – C5 – C6 158.8°; O4 – C5 – C6 – N7 82.6°; C5 – C6 – N7 – C8 – 61.2°. The estimated standard deviations on bond lengths and angles are about 0.01 Å and 0.7°, respectively. The drawings were produced by ORTEP.[8]

Bond lengths and angles calculated from the final parameters (Table 1) are shown in Fig. 1 and are in general agreement with accepted values. A stereo view of the crystal packing is given in Fig. 2. O12 seems to be hydrogen bonded to $I^-_{(1-x,y-\frac{1}{2},\frac{1}{2}-z)}$. The distance O12..I$^-$ is 3.45(1) Å, and the angle O12 – H12..I$^-$ is 146(7)°. As in the crystal structures of acetylcholine β-resorcylate[2] and of two salts of GABA-choline ester[3] no hydrogen bonding involves the ester oxygen atoms O3 and O4.

Experimental. (*R,S*)-Lactoylcholine iodide was prepared according to published directions.[4] Yellow elongated prisms were obtained by recrystallization from ethanol and preliminary X-ray diffraction photographs showed these crystals to be identical with those investigated earlier.[1]

Crystal data. (*R,S*)-Lactoylcholine iodide, $C_8H_{18}NO_3I$, M = 303.14, m.p. 120.0 – 120.5 °C. Space group $P2_1/c$, $a = 9.878(4)$, $b = 12.254(5)$, $c = 10.134(5)$ Å, $\beta = 90.50(4)°$, $V = 1227$ Å^3. $D_m = 1.64$ g cm^{-3}, $Z = 4$, $D_c = 1.64$ g cm^{-3}. Linear absorption coefficient for X-rays [λ (Mo$K\alpha$) = 0.7107 Å], $\mu = 26.2$ cm^{-1}. $F(000) = 600$. The unit-cell parameters were refined by least-squares techniques from the θ angles measured for 22 reflections on a NONIUS three-circle automatic diffractometer. The density was measured by flotation in a mixture of bromobenzene and ethyl iodide. The melting point was determined on a Leitz hot stage microscope.

Intensity data were collected on the diffractometer from a crystal with dimensions 0.12 × 0.18 × 0.36 mm using Mo$K\alpha$ radiation and omega scan. 2163 reflections in the range $2.5 \leq \theta \leq 25.0°$ were measured twice and symmetry-related reflections were averaged. Out of these 1543 had $I_{net} \geq 3.0\sigma(I)$, where $\sigma(I)$ is the standard deviation from counting statistics. No absorption corrections have been made.

The structure was solved by the heavy atom method and refined by full matrix least-

Table 1. Final positional and thermal parameters. The estimated standard deviations, referring to the last figure, are given in parentheses. Thermal parameters are × 10^2. The temperature factor is defined by:

$\exp[-2\pi^2(U_{11}h^2a^{*2}\cdots + 2U_{12}hka^*b^* + \cdots)]$.

ATOM	X/A	Y/B	Z/C	U11	U22	U33	U12	U13	U23
I-	.27466(6)	.45031(5)	.31466(6)	6.49(4)	7.65(4)	5.45(4)	-1.36(3)	0.44(2)	0.62(3)
O12	.6416 (9)	.0887 (6)	.4701 (7)	12.0(6)	6.4(4)	6.3(4)	-1.7(4)	1.8(4)	0.3(3)
C11	.5507(10)	.2537(10)	.5460(11)	7.3(6)	9.2(7)	8.1(7)	-0.3(6)	1.6(5)	-0.9(6)
C 1	.6297 (9)	.1998 (8)	.4386 (8)	7.0(5)	6.6(6)	4.5(4)	-1.5(4)	0.0(4)	0.4(4)
C 2	.7676 (8)	.2535 (7)	.4301 (8)	5.8(5)	5.7(5)	4.7(4)	1.0(4)	0.4(4)	0.6(4)
O 3	.8677 (7)	.2239 (6)	.4857 (8)	6.3(4)	9.7(5)	10.4(5)	0.9(4)	-0.7(4)	5.0(5)
O 4	.7630 (5)	.3420 (5)	.3526 (5)	4.8(3)	5.2(3)	6.1(3)	-0.3(3)	-0.3(2)	1.5(3)
C 5	.8881 (8)	.4023 (7)	.3422 (9)	4.1(4)	5.4(5)	7.1(6)	-0.1(4)	-0.3(4)	0.8(4)
C 6	.8850 (7)	.4703 (6)	.2182 (8)	4.3(4)	5.2(5)	5.2(4)	0.2(3)	1.1(3)	0.1(3)
N 7	.8132 (6)	.5774 (5)	.2270 (6)	4.7(3)	4.8(4)	4.0(3)	-0.2(3)	0.2(3)	0.1(3)
C 8	.6663 (8)	.5627 (8)	.2599(10)	4.8(4)	7.4(6)	6.9(5)	2.1(4)	0.9(4)	0.5(5)
C 9	.8818(10)	.6506 (8)	.3243 (8)	9.8(7)	5.8(5)	4.8(5)	-0.2(5)	-0.7(5)	-0.7(4)
C10	.8219(10)	.6282 (8)	.0924 (8)	8.1(6)	7.6(6)	4.2(4)	-1.0(5)	-0.2(4)	1.4(4)
H12	.694 (9)	.076 (7)	.385 (10)	6.3					

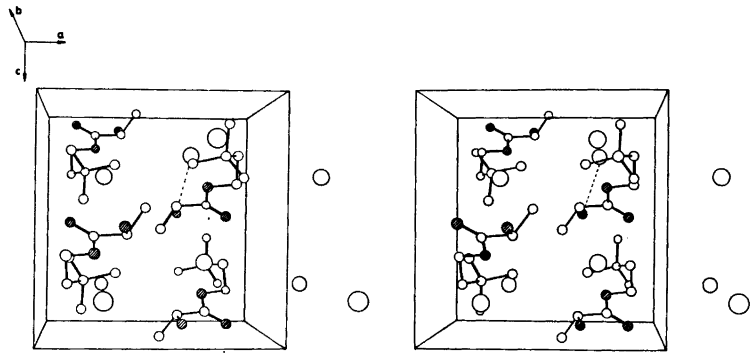

Fig. 2. A stereo view of the packing of lactoylcholine iodide. The possible hydrogen bond O12..$I^-_{(1-x,y-\frac{1}{2},\frac{1}{2}-z)}$ is indicated by a broken line. Oxygen atoms are shaded.

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Propellicene or Bi-2,13-pentahelicenylene

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Aromatic compounds having structures with interesting topologies, *e.g.* the helicenes,[1,2] cyclophanes,[3–5] and circulenes [6–9] have long fascinated the synthetic chemists. We now wish to report the synthesis of a new hydrocarbon, $C_{44}H_{24}$, which has the geometry of a two-bladed propeller. The compound, which we would like to call propellicene or bi-2,13-pentahelicenylene [12] is built from two pentahelicene units, with the terminal rings linked together at C_2 and C_{13} by single bonds (Fig. 1).

We recently described a procedure, using the Wittig reaction, whereby various [2.2.2.2]-cyclophanes and related compounds can be prepared.[10,11] Such compounds may serve as precursors for topologically interesting compounds like those mentioned above.

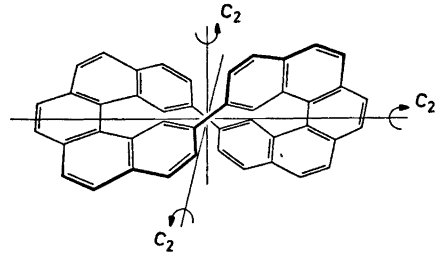
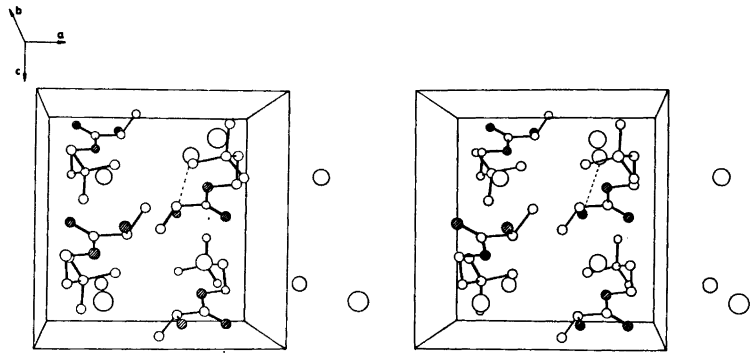


Fig. 1. Proposed structure of propellicene or bi-2,13-pentahelicenylene with the three symmetry axes.



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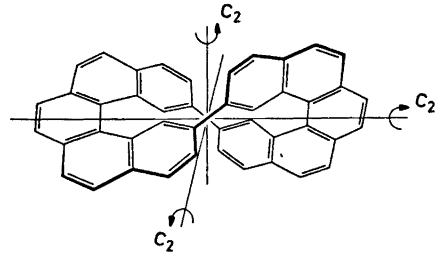

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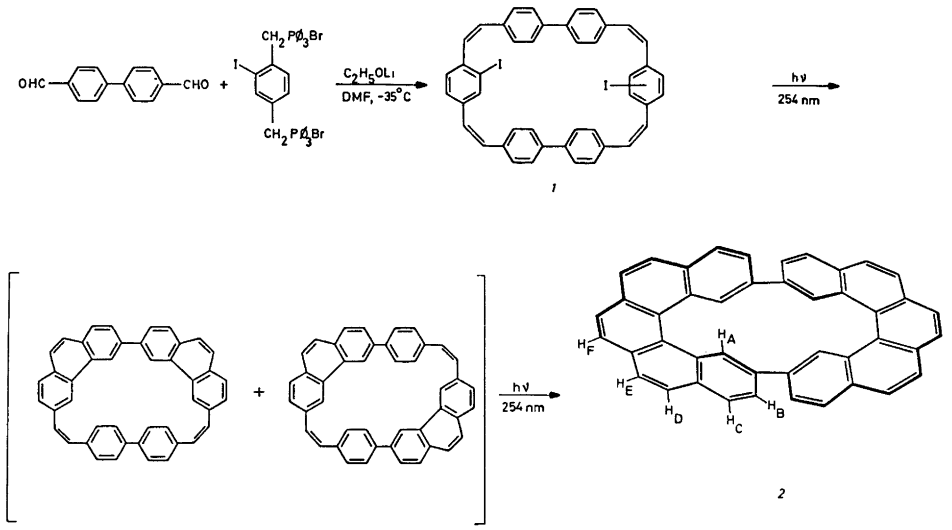

Scheme 1.

One example of the versatility of this procedure is the two-step synthesis of the title compound from relatively simple starting materials. Thus di-iodo[2.2.0.2.2.0]paracyclophanetetraene [13] was obtained as a mixture of two isomers in 4.5 % yield from 4,4′-biphenyldicarbaldehyde and the bistriphenylphosphonium salt from 1,4-bis-(bromomethyl)-2-iodobenzene. When irradiated with light from a low pressure mercury lamp, the isomeric compound mixture, *1*, gave 70 % yield of propellicene, *2* (Scheme 1).

Its structure follows from NMR, MS and UV data. The ^{1}H NMR spectrum in deuteriochloroform is of first order at 270 MHz showing an AMX-pattern and singlets; H_A at δ 8.56 (1), H_B 7.12 (1), H_C 7.80 (1), $J_{AB}=1.5$ Hz, $J_{AC}<0.5$ Hz, $J_{BC}=8$ Hz, H_D and H_E 7.91 (2) broad singlet and H_F 7.96 (1). The corresponding chemical shifts for the protons in pentahelicene are: H_A 8.47, H_B 7.47, H_C 7.89, H_D and H_E 7.83 and H_F 7.79.[14] The shift difference for H_B is clearly caused by the proximity of the other pentahelicene unit in propellicene.

The mass spectrum of propellicene is very simple (Fig. 2). The molecular ion is the base peak. Fragmentations are due to the loss of hydrogens and splitting of the molecule in half. Doubly charged and triply charged ions are frequent. The doubly charged molecular

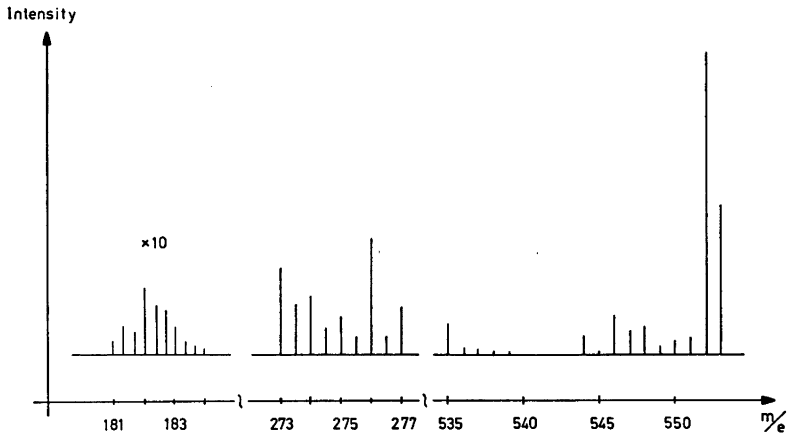

Fig. 2. Mass spectrum of propellicene or bi-2,13-pentahelicenylene.

ion and the singly charged ion with the same mass to charge ratio can be distinguished by their isotopic pattern.

The UV spectrum of propellicene in cyclohexane shows two distinct maxima at 241 nm (log $\varepsilon = 5.11$) and 284 nm (4.85) and shoulders at 225, 233, 305, 315, and 340 nm. Pentahelicene absorbs at somewhat shorter wavelengths [15] which indicates that there is some change in molecular geometry between the two compounds or possibly that there is some interaction between the helicene units in propellicene.

Propellicene has three perpendicular C_2-axes of symmetry and thus belongs to the same symmetry point group, D_2, as a two-bladed propeller (Fig. 1). The molecule is chiral and the two enantiomers should be stable and separable. The barrier for interconversion must be higher than that of pentahelicene.[1] Further work on the stereochemistry of propellicene and on the interaction between the molecular halves is under way.

Experimental. NMR spectra were recorded on a Bruker WH 270, mass spectra on an AEI MS 902, UV spectra on a Beckman DK 2 and IR spectra on a Beckman IR 9 instrument. Photochemical experiments were run in a Rayonet reactor model RPR 100.

Wittig reaction. 4,4'-Biphenyldicarbaldehyde [11] (0.01 mol) and the bistriphenylphosphonium salt from 1,4-bis-(bromomethyl)-2-iodo benzene [16] (0.01 mol) were suspended in dry dimethylformamide (250 ml) in a three-necked flask, equipped with a mechanical stirrer and a dropping funnel. The flask was kept at −35 °C in a thermostated cooling bath and flushed with nitrogen. A solution of lithium ethoxide in ethanol was added dropwise to allow for consumption of the red ylid between successive additions. The addition was stopped when no formation of an ylid could be detected, usually after 24 h. The yellow reaction mixture was diluted with an equal amount of water and extracted with diethyl ether three times. The ethereal solution was washed with water several times, dried over sodium sulfate and the solvent evaporated. The residue was chromatographed on silica gel with carbon tetrachloride as eluent. The first yellowish fraction contained di-iodo[2.2.0.2.2.0]paracyclophanetetraene, *1*, as a mixture of isomers which was recrystallised from carbon tetrachloride to give 0.18 g, 4.5 % of a yellow solid melting at 180–85 °C. ^{1}H NMR: δ 7.84 (1, broad singlet, protons adjacent to iodine), 7.46–7.07 (10, complex multiplet, aromatic protons) and 6.65–48 (4, complex multiplet, olefinic protons). MS: *m/e* 812 (100 %), 686 (20), 685 (20), 684 (48), 555 (20), 554 (40), 553 (28), 552 (44), 407 (12), 406.5 (8), 406 (12), 277 (20), 276 (20), 275 (12), 274 (12). Abs. mass; found 812.044 ± 0.003; calc for $C_{14}H_{30}I_2$ 812.046.

Propellicene. A water-cooled solution of cyclophane *1* (100 mg) in benzene (150 ml) was irradiated in a quartz cell with light from a low pressure mercury lamp (254 nm) for 3 h. The solvent was distilled off, and the residue was chromatographed on silica gel with carbon tetrachloride as eluent. A white crystalline compound identified as propellicene *2* (48 mg, 70 %) was isolated. Abs. mass; found 552.190 ± 0.003; calc. for $C_{44}H_{24}$ is 552.188.

Acknowledgements. We thank the Swedish Natural Science Research Council for financial support.

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Degradation of Cellobiose in Hydroxide and Hydrogen Carbonate Solution

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The formation of 3-deoxy-2-*C*-(hydroxymethyl)pentonic acids from cellobiose is less important in sodium hydrogen carbonate solution than in sodium hydroxide, while the relative amounts of 2-deoxytetronic, 3-deoxypentonic, and 3,4-dideoxypentonic acids were much larger. 3,4-Dideoxypentonic acid is formed *via* 3-deoxypentulose present in large amount after the treatment with sodium hydrogen carbonate solution. The formation of cyclic non-electrolytes from the same intermediate explains the low yield of acids derived from the reducing moiety in cellobiose.

Efforts to develop sulfur-free processes for the production of wood pulp have focussed attention on cooking with sodium hydrogen carbonate in the presence and absence of oxygen.[1] Significantly, the degradation products from carbohydrates differ markedly from those produced by conventional treatments at high hydroxide ion concentration. In the present work an attempt is made to elucidate the carbohydrate reactions by experiments with cellobiose in the absence of oxygen.

RESULTS AND DISCUSSION

The alkali treatment in sodium hydroxide was carried out under conditions such that the added cellobiose and the glucose formed as an intermediate [2] were consumed completely. The yield of organic acids was 90.3 % by weight (Table 1) demonstrating that the amount of non-electrolytes (including a minor amount of sugars) was comparatively small. Most of the non-electrolytes were held irreversibly by the ion exchangers under the applied conditions.

In the experiment in sodium hydrogen carbonate solution, 15.5 % of the added cellobiose remained as disaccharides. Cellobiose and its isomers (C-2-epimer + cellobiulose) were present in about equal amounts. Calculated as a percentage of degraded disaccharide the yield of organic acids was only 17.6 %. The yield of hexoses amounted to 36.6 % indicating that the liberation of glucose by β-elimination occurred at an appreciable rate even in this medium, while the consecutive degradation of the hexoses was rather slow. In addition a large amount of 3-deoxypentulose was present after the hydrogen carbonate treatment. The total yield of isolated acids and sugars was only 61.4 %. The results show that large quantities of other compounds held irreversibly by the ion exchangers were formed from the reducing glucose moiety. Similar observations were made in a recent study of hot alkali treatment of hydrocellulose in hydrogen carbonate solution.[3] It was found that a large proportion of the degraded glucose moieties was converted to a complex mixture of cyclic compounds. These reactions are less important in sodium hydroxide solution. According to Koetz and Neukom [4] an important reaction path is *via* 3-deoxypentulose.

These results, together with the observation that the relative amounts of those monocarboxylic acids derived from the reducing glucose moiety were quite different in these media, show that some reaction paths of particular importance in hydroxide solution are less important in hydrogen carbonate solution and *vice versa*. The results given in Table 1 confirm

Table 1. Carboxylic acids and monosaccharides formed during hot alkali treatment of cellobiose at 97 °C. Weights refer to 1 g of degraded disaccharides.

	0.3 M NaOH 2 h mg	0.3 M $NaHCO_3$ 5 h mg
Acids		
3-Deoxy-2-*C*-hydroxymethyl-*threo*-pentonic	215 [a]	18
3-Deoxy-2-C-hydroxymethyl-*erythro*-pentonic	57 [a]	4
2-Hydroxypropanoic	171 [a]	1
3-Deoxy-*arabino*-hexonic	94 [a]	13
3-Deoxy-*ribo*-hexonic	25 [a]	4
3-Deoxytetronic (2,4-Dihydroxybutanoic)	71 [a]	7
1,4-Anhydro-3-deoxypentitol-2-carboxylic	48	7
3,4-Dideoxypentonic (2,5-Dihydroxypentanoic)	44	9
Glycolic	19 [a]	6
3-Deoxy-*threo*-pentonic	9	12
3-Deoxy-*erythro*-pentonic	4	4
2-Deoxytetronic (3,4-Dihydroxybutanoic)	9 [a]	25
Glyceric	4 [a]	5
2-*C*-Methylglyceric	4	–
Formic	75	53
Acetic	24	8
Dicarboxylic	30 [b]	–
Monosaccharides		
Glucose		198
Mannose		38
Fructose		127
3-Deoxypentulose		75
Total	903	614

[a] Observed in earlier investigations.[11,12] [b] Identified: 3,4-dideoxyhexaric (5 mg), 2,3,4-trideoxyhexaric (4 mg), deoxytetraric (2 mg), 2,3-dideoxypentaric (2 mg), oxalic (2 mg) and tartronic (1 mg).

that the well-known benzilic acid rearrangement of liberated 4-deoxy-2,3-hexodiulose to 3-deoxy-2-*C*-(hydroxymethyl)pentonic acids [5] is a predominant reaction of the reducing glucose moiety in sodium hydroxide. In hydrogen carbonate solution this reaction is less important.

Since 3-deoxypentulose is also formed after bicarbonate treatment of hydrocellulose [3] this intermediate (II in Fig. 1) was probably derived from the reducing glucose moiety. A reaction route *via* 4-deoxy-2,3-hexodiulose (I) with elimination of C-1 as formic acid was previously postulated by Koetz and Neukom.[4] The results discussed above suggest that the relative importance of fragmentation reactions compared to benzilic acid rearrangements is much greater in mildly alkaline solutions than in sodium hydroxide. Accordingly, formic acid was the most abundant acid produced in hydrogen carbonate solution.

3,4-Dideoxypentonic acid has previously been isolated in small amounts after treatment of hydrocellulose with sodium hydroxide.[6] Table 1 shows that the amount of this acid relative to the 3-deoxy-2-*C*-(hydroxymethyl)-pentonic acids was larger in the experiments with hydrogen carbonate solution. This observation together with the fact that 3-deoxypentulose is formed in large amounts strongly suggests that 3,4-dideoxypentonic acid (III) is formed from 3-deoxypentulose (II) by β-hydroxyelimination at C-4 followed by isomerization and subsequent benzilic acid rearrangement. This reaction path was confirmed in experiments with 3-deoxy-*erythro*-pentose (27 mg) in 0.14 M sodium hydroxide. After 90 min at 75 °C the yield of 3,4-dideoxypentonic acid was 28 %. Except for 3-deoxytetronic and 2-hydroxypropanoic acids (total yield 9 %) no non-volatile carboxylic acids were observed.

The absence of 4-hydroxybutanoic acid indicates that the hydrolytic cleavage of the dideoxypentosulose intermediate was negligible. The isolated non-electrolyte fraction (13 %) contained only a trace amount of unreacted 3-deoxypentoses. Evidently, cyclic compounds strongly held by the resins were formed in large amounts.

The finding that 2-deoxytetronic acid (IV), a minor product in sodium hydroxide, was the second most abundant carboxylic acid in hydrogen carbonate supports the conclusion that fragmentation is favoured compared to benzilic acid rearrangement. Most likely this compound is formed together with glycolaldehyde from the same dicarbonyl precursor by the reaction route illustrated in Fig. 1.

As seen in Table 1 the formation of 3-deoxypentonic acids is also favoured at low alkalinity. The isomerization of the reducing glucose moiety to a 3-hexulose moiety has been demonstrated recently.[3] A loss of C-1 as formaldehyde by a reverse aldol reaction will then give rise to glucopyranosylarabinose and glucopyranosylribose which will be rapidly decomposed by β-elimination of glucose. The 3-deoxypentosulose formed as intermediate will after benzilic acid rearrangement give rise to the two diastereomeric 3-deoxypentonic acids. Except for the initial reaction steps, this reaction path parallels the one which gives rise to 3-deoxy-2-*C*-(hydroxymethyl)pentonic acids. In agreement with previous results with hydrocellulose [6] the *threo* form was more abundant than the *erythro* form. The same is true for the formation of 3-deoxypentonic acids during alkali treatment of xylan.[7]

It is noteworthy that the formation of 1,4-anhydro-3-deoxypentitol-2-carboxylic (anhydroisosaccharinic) acid [8] was depressed less by the decrease in alkalinity than was the formation of the related 3-deoxy-2-*C*-(hydroxymethyl)-pentonic acids. The other monocarboxylic acids were formed mainly from the liberated glucose [9] (*e.g.* 2-hydroxypropanoic, 3-deoxytetronic, and 3-deoxyhexonic acids) or were present in small amounts only.

Small amounts of dicarboxylic acids were produced during the sodium hydroxide treatment of cellobiose. The relative composition was similar to that observed after hot alkali treatment of hydrocellulose.[10] No detectable amounts of dicarboxylic acids were obtained after the treatment in hydrogen carbonate solution.

EXPERIMENTAL

Paraffin oil was layered on a boiled solution (65 ml) of $NaHCO_3$. Cellobiose (1.5 g) dissolved in 10 ml of boiled water was injected into the aqueous solution. The final concentration was 0.3 M in $NaHCO_3$. The flask was kept at 97 °C for 5 h and then cooled in ice-water. The excess $NaHCO_3$ was removed by stirring with a cation exchange resin (H^+). Sodium hydroxide was added and the solution kept at pH 8.5 for 2 h.

Larger amounts of carboxylic acids were formed in parallel experiments with 0.3 M NaOH. In the experiment reported in Table 1 the amount of cellobiose was therefore decreased to 0.2 g in order to maintain constant

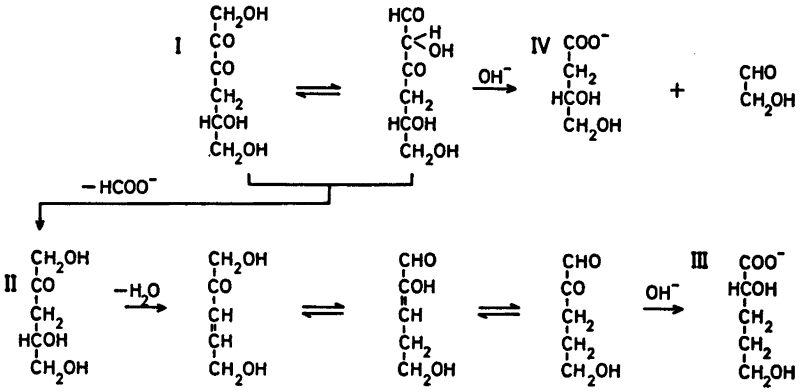

Fig. 1. Degradation of 4-deoxy-2,3-hexodiulose (I) to 3-deoxypentulose (II), 3,4-dideoxypentonic acid (III) and 2-deoxytetronic acid (IV).

pH in the reaction solution. The duration of the treatment was 2 h. Excess sodium hydroxide was removed by adding a cation exchange resin (H^+) until pH 8.5 was obtained.

The analyses were made after a group separation of non-electrolytes, non-volatile monocarboxylic acids and dicarboxylic acids on an anion exchange column. The acids were separated on a preparative scale by anion exchange chromatography and the fractions analyzed on anion exchange columns coupled to a three-channel analyzer.[13,14] GLC-MS was used for final identification.[15] Formic and acetic acids were determined separately.[16]

The sugars were analyzed by partition chromatography on an anion exchanger in the sulfate form,[17] by anion exchange chromatography in 0.075 M potassium tetraborate [18] (with the columns coupled to a two-channel analyzer) and by GLC and GLC-MS.[15]

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Rotational Barriers of the Carbon-Nitrogen Bond in Hydroxy and Methoxy Substituted Aromatic Thioamides. Influence of Hydrogen Bonding

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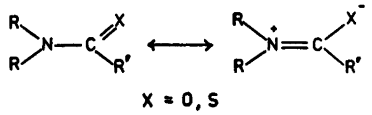
The barrier to rotation around the C—N bond in *o*- and *p*-hydroxy and methoxy substituted *N*,*N*-dimethylthiobenzamides and thionaphthamides has been studied by the NMR technique. It was found that *o*-hydroxy substitution decreases the barrier ($\Delta G^{\ddagger}$) by 10—20 kJ/mol and *o*-methoxy substitution increases the barrier by 10—20 kJ/mol. NMR, IR and UV data were used to elucidate the strength of hydrogen bonding and the conformational situation. Comparatively weak intramolecular hydrogen bonds were found in the *o*-hydroxy derivatives due to a sterically induced twist around the Ar—C(S) bond. In the transition state for the rotation the molecule is free from steric strain and a stronger hydrogen bond in this state is suggested as an important contribution to the barrier-lowering effect of *o*-hydroxy substitution. No significant effect of intermolecular hydrogen bonding on the barrier in the *p*-hydroxy derivatives was observed. Comparison is made with the analogous dithioesters.

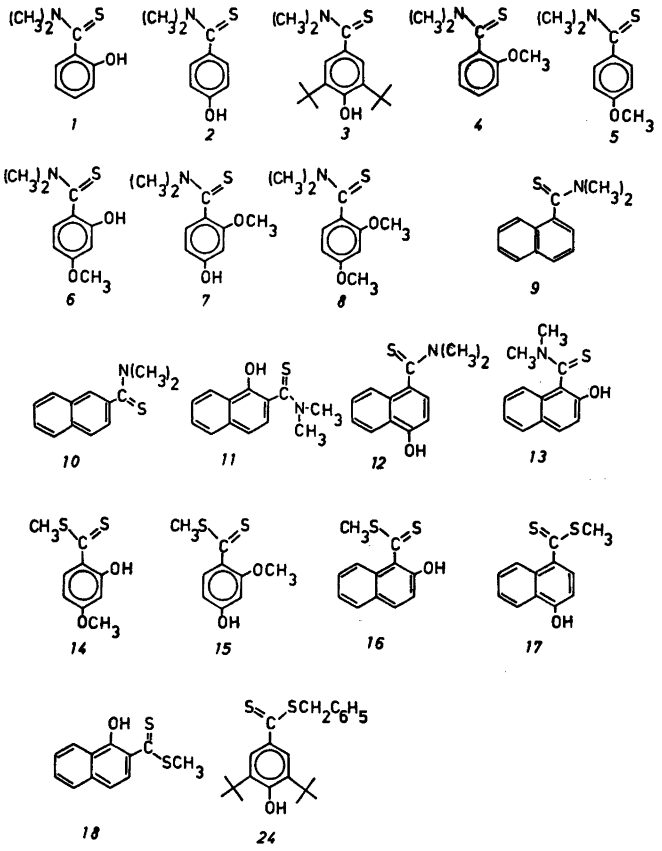
Amides and thioamides have been extensively studied in order to obtain information about the barrier to internal rotation around the carbon-nitrogen bond.[1] In all likelyhood this barrier is associated with the partial double bond character of the C—N bond. The barrier to thioamide rotation in *N*,*N*-dimethylthiobenzamide is 77.0 kJ/mol [2] which is about 25 kJ/mol lower than the barrier in dimethylthioformamide.[3] The observed lowering of the barrier has been attributed to electronic effects; competitive conjugation is believed to decrease the transition state energy more than the ground state energy. The same trend is observed in the analogous amide systems. The effect of *meta* and *para* substitution in *N*,*N*-dimethylbenzamides correlates well with Hammett's σ or, better, σ^+ constants with ϱ values of +1.6 and +1.1, respectively.[4]



Scheme 1.

Introduction of an *ortho* substituent in benzamides usually produces a noticeable effect on the barrier. The substituents Cl, NO_2, Me, and MeO all cause an increase in barrier height in *N*,*N*-dibenzylbenzamide.[5] Thus, the barrier seems to be determined primarily by the steric demands of an *ortho* substituent, presumably through inhibition of resonance between the ring and the carbonyl group in the transition state.

The barrier in *o*-hydroxy- and *o*-amino-*N*,*N*-dialkylbenzamides is dramatically lower than those of normal *ortho*-substituted *N*,*N*-dialkylbenzamides.[4—6] This reduction has been attributed to enhanced conjugative stabilization of the transition state due to intramolecular hydrogen bonding [4] and to the existence of a "quasiaromatic chelated ring" created by the hydrogen bond leading to a decrease in C—N bond order.[6] In view of the steric strain in the planar molecule, it seems doubtful whether an optimal O···H—O arrangement for hydrogen bonding, required by the latter proposal, is possible. Furthermore, the barrier increases in solvents which may enter into intermolecular hydrogen bonding to the amide.



Scheme 2.

In our opinion further experimental studies are necessary before the observed barrier-lowering effect of *o*-hydroxy and *o*-amino substituents can be understood.

This paper reports a study of the effects of hydroxy and methoxy substitution in the *ortho* and *para* positions in *N*,*N*-dimethylthiobenzamides and *N*,*N*-dimethylthionaphthamides on the torsional barrier around the C–N bond. Special attention has been devoted to the effects of inter- and intramolecular hydrogen bonding and of steric interference. The compounds studied are presented in Scheme 2.

RESULTS

Barrier measurements. The free energy of activation at the coalescence for the thioamides and the thionaphthamides are summarized in Table 1. As the coalescence temperatures range from −16 to +186 °C and an attempt has been made to use the same solvent for all compounds, the number of available solvents is highly reduced. With two exceptions, all spectra were recorded in *o*-dichlorobenzene (ODC). The low solubility of *2* in ODC made it necessary to use dimethyl sulfoxide (DMSO) instead. Some of the compounds were studied in several solvents. No significant difference in $\Delta G^{\ddagger}$ was obtained for the *para*-hydroxy derivatives in ODC and DMSO, despite the capacity of the latter solvent to form strong hydrogen bonds with phenols.[7] On the other hand, a drastic increase of the barrier was observed for *N*,*N*-dimethyl-2-hydroxy-4-methoxythiobenzamide in pyridine-d_5, indicating that this solvent is able to form strong hydrogen bonds to the solute.

Table 1. Coalescence data for *N,N*-dimethylthiobenzamides and *N,N*-dimethylthionaphthamides.

Compound	Solvent	$\delta\nu$/Hz[a]	T_c/K	$\Delta G^{\ddagger}_c$/kJ mol^{-1}
1	ODC	25.5	302.5	64.0
2	$(CD_3)_2SO$	19.4	343.6	73.6
3	ODC	24.4	331.1	70.3
4	ODC	32.3	440.0	93.7
5	ODC	26.2	335.3	71.1
6	$CDCl_3$	13.9	258.7	55.6
	pyridine-d_5	19.0	298.1	63.6
7	ODC	31.3	431.2	91.6
	$(CD_3)_2SO$	27.4	428.9	91.6
8	ODC	30.6	419.5	89.1
9	ODC	51.5	458.9	95.8
10	ODC	34.2	358.7	75.3
11	ODC + C_6H_5F	26.8	258.1	54.0
	$CDCl_3$	21.3	256.7	54.4
12	ODC	48.4	441.0	92.5
	$(CD_3)_2SO$	41.2	438.7	92.5
13	ODC	48.5	405.6	84.5

[a] At 60 MHz.

Table 2. Thermodynamic parameters for *6* and *11* from the complete lineshape analyses.

Compound	Solvent	$\Delta G^{\ddagger}_{298}$/kJ mol^{-1}	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J mol^{-1} K^{-1}
6	ODC + C_6H_5F (3:1)	55.6	57 ± 2[a]	4 ± 8
11	$CDCl_3$	54.4	51 ± 1	-10 ± 2

[a] The errors are standard deviations from a least-squares plot.

Table 3. IR and NMR data on hydrogen bonding.

Compound	IR[a]			NMR[b]
	ν_{OH}(free)/cm^{-1}	ν_{OH}(complexed)/cm^{-1}	$\Delta\nu_{OH}$/cm^{-1}	δ
1	3590	3250	340	7.20
3	3630	–	–	5.25
6	3590	3140	450	9.20
7	3590	3200	390	8.30
11	3580	3130	450	9.42
13	3585	3310	275	7.20
14	–	2880	(700)[c]	12.83
15	3580[d]	3360[d]	220	6.50
16	3585	3455	130	6.62
17	–	–	–	5.85
18	–	2650	(930)[c]	14.20
24	–	–	–	5.83

[a] 5 % in $CDCl_3$. Cell thickness = 0.1 mm. [b] *Ca.* 10 % in $CDCl_3$. [c] With a value of 3580 cm^{-1} for ν_{OH}(free). [d] Saturated solution (*ca.* 3 %).

A complete lineshape analysis for the *N*-methyl signals was performed for two compounds, *6* and *11*. These two compounds show barriers much lower than those of ordinary aromatic *N*,*N*-dimethylthioamides. The methoxy signal in *6* and TMS for *11* were used as reference signals for determination of T_2. The results from the complete lineshape analyses are summarized in Table 2.

IR and NMR data on hydrogen bonding. An important factor in the understanding of the low barriers of the *o*-hydroxy-*N*,*N*-dimethylthiobenzamides is the strength of hydrogen bonds in these compounds. Likewise, hydrogen bonding in the *p*-hydroxy-*N*,*N*-dimethylthiobenzamides can affect their barriers.

IR data on hydrogen bonding for *o*- and *p*-hydroxythiobenzamides and -thionaphthamides are shown in Table 3. In addition, the table contains data for four analogous dithioesters.

The concentration dependence of the intensity ratio between the bands of free and associated forms was also studied. The *p*-hydroxy substituted derivatives, except *3*, showed a concentration dependence typical for intermolecularly hydrogen bonded systems. Upon dilution, the relative intensity of the sharp band assigned to unassociated OH-stretching band increased at the expense of the intensity of the broad band at lower frequency assigned to the associated form. On the other hand, the *o*-hydroxy substituted derivatives exhibited no concentration dependence. One exception was *13*, which showed a concentration dependence of the intensity ratio.

Although there is a large number of contributions to the ^{1}H NMR chemical shift change accompanying interactions such as hydrogen bonding, and although it is unlikely that the hydrogen bonding contribution can be separated from the other terms, the position of the hydroxy proton chemical shifts has been found to be linearly related to $-\Delta H$, the enthalpy of the hydrogen-bonding interaction, in a series of 30 phenol-base systems.[7] The chemical shifts for the hydroxy protons of the thioamides and the corresponding dithioesters in about 10 % deuteriochloroform solution are summarized in Table 3. Fig. 1 shows a plot of $\Delta\nu_{OH}$ against δ_{OH}. A good correlation is obtained including intra- as well as intermolecularly hydrogen-bonded compounds.

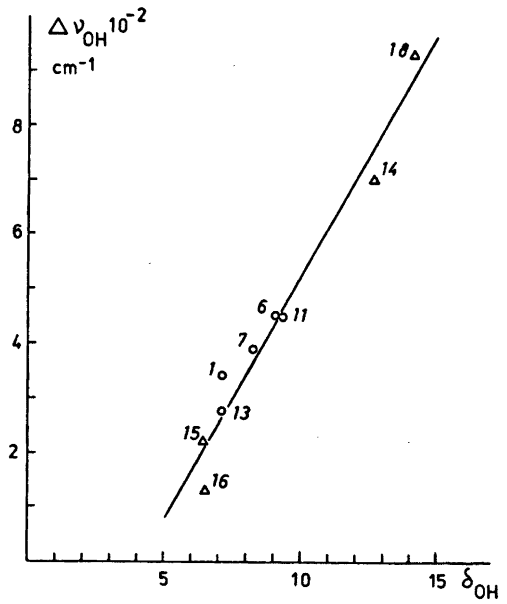


Fig. 1. Infrared $\Delta\nu_{OH}$ *versus* NMR chemical shifts for the phenolic protons in $CDCl_3$ solutions. (△ = dithioesters, ○ = thioamides).

The IR and NMR results make it possible to estimate the strength of the hydrogen bond and to determine whether the compounds in question are intra- or intermolecularly hydrogen bonded. Thus, the two dithioesters *14* and *18* are obviously intramolecularly hydrogen bonded by strong bonds. The analogous thioamides *6* and *11* and compound *1* are likewise intramolecularly hydrogen bonded *but with much weaker bonds.*

Both the dithioesters and the thioamides with a hydroxy group in the *para* position are intermolecularly hydrogen bonded, *but the bonds are stronger in the thioamide series than in the dithioester series*, in contrast to the *o*-hydroxy derivatives.

UV measurements. The UV spectra of the thioamides and of some of the corresponding dithioesters are listed in Table 4. The spectra were recorded in heptane containing up to 10 % of dichloromethane for solubility reasons.

DISCUSSION

Rotational barriers. Certain generalizations emerge from the results of the barrier measurements:

Table 4. Ultraviolet spectra of thioamides and dithioesters in heptane containing 0.5 % CH_2Cl_2 if not otherwise stated.

Compound	λ_{max}/nm	log ε	λ_{max}/nm	log ε	λ_{max}/nm	log ε
N,N-Dimethylthiobenzamide [a]	395	2.50	282	3.93	250	3.95
N-Methyl-1,2,3,4-tetrahydro-isoquinoline-1-thione [b]	425	2.09	323	3.81	299	3.71
1	377[c]	2.90	290	4.01	242	3.91
4	379[c]	2.48	281	4.03	245	3.93
5	389[c]	2.73	276	4.16	253	4.18
2	383[c]	2.75	276	4.13	259	4.06
6	370[c]	3.16	288	4.04	279	4.04
8	378[c]	2.75	283	4.11	255	4.09
9	379[c]	2.58	304[d]	3.89	275	4.13
10	384[c]	2.84	295[d]	4.03	285.5	4.13
					275	4.11
13	382[c]	3.07	334	3.56	285	4.06
11	373[c]	3.46	346	3.49	289[d]	4.13
					277.5	4.21
14	450[c,d]	2.53	381	4.26	324	4.21
15	486[c]	2.30			325	4.12
18	439	4.04	422	4.06	330	4.37
16	478[c,d]	2.46	401	3.08	328	3.85

[a] Ref. 9, in heptane. [b] Ref. 35, in heptane. [c] In heptane + 10 % CH_2Cl_2. [d] Shoulder.

(1) Introduction of a methoxy group in the *ortho* position of a *N,N*-dimethylthiobenzamide raises the barrier by 10 – 20 kJ.

(2) Introduction of a hydroxy group in the *ortho* position lowers the barrier by 10 – 20 kJ.

(3) Introduction of a hydroxy or a methoxy group in the *para* position lowers the barrier by 2 – 9 kJ.

(4) The barrier in *N,N*-dimethyl-1-thionaphthamide is *ca.* 20 kJ higher than in the 2-substituted isomer.

(5) The influence of the solvent is small, except for the *ortho*-hydroxy substituted derivatives in basic solvents such as pyridine, in which the intramolecular hydrogen bond is broken.

It is known that the angle between the thioamide plane and the aromatic ring in *N,N*-dimethylthiobenzamide is *ca.* 40°,[8–11] indicating that steric interaction in the planar form is considerable. Steric interference between the *E*-methyl group and the *ortho* hydrogen prevents a coplanar conformation around the Ph – C(S) bond. In the transition state for the rotation, the interfering methyl group is twisted out of the region in which steric interaction can be expected. The rotation can thus be coupled with a decrease of the angle around the Ph – C(S) bond, resulting in more effective conjugation between the thiocarbonyl group and the benzene ring with accompanying gain in delocalization energy. Moreover, this energy gain is larger in the transition state than in the initial state, since in the transition state the cross conjugation with the dimethylamino group is inhibited. The π-barrier accompanying rotation around the benzene-thiocarbonyl bond is also affected by substitution in the ring. This effect will change the energy of the transition state relative to that of the initial state. In the *ortho*-methoxy derivatives and in the α-thionaphthamides, the thiocarbonyl group is kept out of the plane in the transition state as well, which increases its energy, with a higher barrier as a consequence.

It has been clearly demonstrated that amides and thioamides coordinate with the oxygen and sulfur atoms on hydrogen bonding to protic species. The torsional barriers of such compounds are expected to be higher in protic solvents than in other solutions. Hydrogen bonding may stabilize the polar structure represented by the charge-separated resonance

notation and thus increase the double bond character of the C–N bond. This has been found to be the case for amides dissolved in protic solvents (*e.g.*, water [12] and formamide[13]).

The effect of added phenol on the rate of rotation around the C–N bond in *N*-benzyl-*N*-methyl-2-chlorobenzamide and its thio analogue in ODC has been studied by Siddall *et al.*[14] They found that $\Delta G^{\ddagger}$ increased with phenol concentration, whereas $\Delta S^{\ddagger}$ never deviated significantly from zero. The effect of phenol on the thioamide barrier was, however, much less marked than on the amide barrier and close to the experimental error. This lesser tendency of the thioamide barriers to be affected by protic solvents is consistent with the weaker association of thioamides with such species.[15]

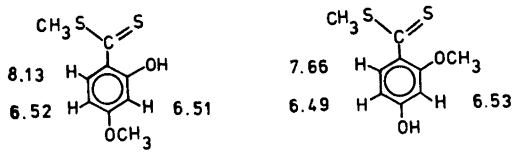
Instead of raising the barrier, introduction of an *ortho*-hydroxy group has the opposite effect, and it is obvious that we must seek another model for the rotational process in these systems.

In the *ortho*-hydroxythiobenzamide derivatives, a moderately strong intramolecular hydrogen bond was experimentally verified. The strength of this bond is dependent on the dihedral angle between the amino group and the thiocarbonyl group (θ) as well as the dihedral angle between the phenyl ring and the thiocarbonyl group (ϕ). According to the simple electrostatic model of the hydrogen bond, maximum strength of the bond is obtained with a planar thioamide since then the negative charge on the sulfur atom is largest.

The energy of the hydrogen bond depends also on the distance between the sulfur and oxygen atoms, and thus on the angle ϕ, the ideal distance for hydrogen bonding being the situation where this angle is zero or near zero.

Due to the steric interference discussed above, both the angles θ and ϕ cannot be zero simultaneously. If the sulfur atom is forced nearer the plane of the ring owing to hydrogen bonding, the dimethylamino group has to be twisted or pyramidalized. However, it seems unlikely that the sulfur atom lies in the phenyl plane in the *ortho*-hydroxy derivatives for the following reasons.

Firstly, it has been observed that in secondary *ortho*-hydroxythiobenzamides, where the single substituent on nitrogen is in the *Z* position and consequently the sulfur atom may lie in the plane, the NMR signal of the *ortho* hydrogen is shifted 0.5–1 ppm to lower field compared to the signals of the other aromatic protons.[16] Such a downfield shift is not observed in *N*,*N*-dimethylthiobenzamide nor in the *ortho*-hydroxy derivatives. A comparison with the dithioesters *14* and *15* is interesting. A strongly deshielding region in the plane of the dithioester function makes the *ortho* hydrogen shift to lower field if the sulfur atom lies in the plane of the ring. The hydrogen atom moves out of the deshielding region if the thiocarbonyl group is forced out of the plane as in *15*.



14 *15*

Scheme 3.

The fact that the ring hydrogen atom in the *ortho* position in *ortho*-hydroxy substituted *N*,*N*-dimethylthiobenzamides is not shifted downfield compared to the methoxy analogue supports the suggestion that the sulfur atom does not lie in the benzene plane.

Secondly, the distance between the sulfur and oxygen atoms becomes greater when the thiocarbonyl group is twisted, which is consistent with the observed weaker hydrogen bonds in the *ortho*-hydroxy compounds compared to those in the corresponding dithioesters.

Thirdly, the UV spectra (*vide infra*) of these compounds are perfectly consistent with the hypothesis that the *ortho*-hydroxy derivatives are twisted around both the Ph–C(S) and the C(S)–N bonds.

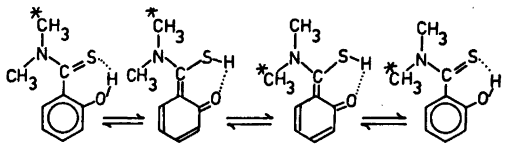
Thus, in the initial state for the rotation both the angles θ and ϕ are larger than zero. The magnitudes of these angles are determined by the maximum energy gain obtained by resonance and hydrogen bonding, balanced by the steric interaction between the *E*-methyl and the *ortho* hydrogen. The overall result must be a lower energy compared to a situation with no hydrogen bond.

In the transition state, the sulfur atom can move still nearer the ideal distance for hydro-

gen bonding, that is in the plane of the ring. Though the electronic conditions for hydrogen bonding are more favourable in the planar thioamide this may be more than balanced by the better steric conditions in the transition state. It is believed that an important contribution to the barrier lowering effect of an *ortho*-hydroxy group is a stronger hydrogen bond in the transition state than in the initial state.

An examination of the effect of *para*-methoxy substitution in unsubstituted [2] and *ortho*-hydroxy substituted *N,N*-dimethylthiobenzamide reveals that an enhanced conjugative stabilization of the transition state also plays an important role in the barrier lowering effect of an *ortho*-hydroxy group.

Another mechanism for the rotational process that cannot be ruled out involves a rapid tautomeric equilibrium prior to the rotation.

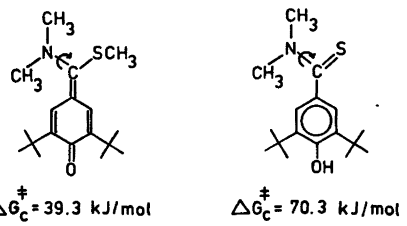


Scheme 4.

If the first step is fast and reversible, the observed rate constant for the rotation is a weighted mean value between those for rotation of the thioamide and of the quinone methide forms:

$$k_{obs} = pk_{thioamide} + (1-p)k_{quinone\ methide}$$

where p = mol fraction of thioamide. The tautomeric equilibrium must be in favour of the thioamide, since no evidence (UV, IR and NMR) for the existence of the quinone methide has been obtained. However, this does not preclude the possibility of the existence of a



Scheme 5.

kinetically significant concentration of the quinoid form. We found a much lower barrier to C–N rotation in a quinone methide compared to that of the corresponding thioamide in an analogous *para*-substituted system.

Entropy of activation. In the preceding discussion the free energies of activation of many compounds have been compared despite the fact that they are measured at very different temperatures. This is of course only meaningful if the entropy of activation ($\Delta S^{\ddagger}$) is close to zero. Fortunately, numerous studies on *tert* amides and thioamides appear to confirm this assumption.[17–27]

In their study on the effect on the activation parameters of added phenol, Siddall *et al.* found that $\Delta S^{\ddagger}$ is never significantly different from zero although $\Delta G^{\ddagger}$ increased with phenol concentration.[14]

The model for the rotational process suggested above involves intramolecular hydrogen bonds in the initial state as well as in the transition state for the *ortho*-hydroxy substituted thiobenzamides. Consequently, no marked change in the intermolecular interactions is to be expected during the course of the rotation.

The entropies of activation for *6* and *11* were measured to $+4 \pm 8$ and -10 ± 2 J/mol K, respectively. These results are in harmony with the expected small change in order in the solution during the rotation.

Ultraviolet spectra. The ultraviolet spectra of thiones, such as thioamides and dithioesters, contain a characteristic long-wavelength, low-intensity band and more intense bands at shorter wavelengths. It has been shown that the former band is due to the excitation of a nonbonding electron from the sulfur atom to an antibonding π orbital, an $n \rightarrow \pi^*$ transition.[28–31].

Hydrogen bonding usually causes significant perturbations of the electronic transitions of the system, and the most commonly observed effect is the blue shift of the $n \rightarrow \pi^*$ transition[32–34] whereas the shift in the $\pi \rightarrow \pi^*$ transition can be either towards the blue or towards the red, depending on the change in polarity on excitation.

Comparing the two hydrogen bonded, *ortho*-hydroxy substituted derivatives *1* and *6* with the two *ortho*-methoxy analogs *4* and *8*, one finds that the $n \rightarrow \pi^*$ band appears at shorter

wavelength in the hydrogen bonded species but that the shifts are small, 2 and 8 nm.

It has been shown that successive *N*-methylation of thiobenzamide causes hypsochromic shifts of the $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ bands due to a departure from coplanarity caused by interference between the methyl group and the ring hydrogen atoms.[6] According to this observation, the $\pi\rightarrow\pi^*$ bands of *1* and *6* would be expected to appear at longer wavelength than in *4* and *8*, respectively, due to the decrease of the angle around the Ar–C(S) bond upon hydrogen bonding. This is what was observed, but the shifts are again small, 9 and 5 nm. This observation is in line with the hypothesis that the *ortho*-hydroxy derivatives are twisted around the Ar–C(S) bond. A coplanar arrangement around this bond would make the red shift much more pronounced.

It is interesting to make a comparison with the two dithioesters *14* and *15*. In *14* there is no steric interaction between the *S*-methyl group and the ring hydrogen atoms so that the molecule can adopt a planar, strongly hydrogen bonded form. Thus, $\pi\rightarrow\pi^*$ band of *14* is split into a doublet, the long wavelength part of which is shifted 57 nm towards longer wavelength compared to the short wavelength part, while the other remains close to the position of the single band in *15*. A similar splitting of this band was also found in *N*-methyl-1,2,3,4-tetrahydroisoquinoline-1-thione, where coplanarity is enforced by ringclosure.[35]

Furthermore, the $n\rightarrow\pi^*$ band of *14* is shifted 36 nm towards shorter wavelengths compared to that in *15*, in agreement with the normal behaviour of $n\rightarrow\pi^*$ bands upon hydrogen bonding.

Sandström observed an increase in oscillator strength of the $n\rightarrow\pi^*$ band on going from thiobenzamide to *N*,*N*-dimethylthiobenzamide,[9] and ascribed this to the increasing departure from coplanarity between the thioamide group and the benzene ring, which causes an increased mixing of the $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ transitions. Large oscillator strengths were found for the thioamides studied in this investigation as well, but the effect is much more pronounced for the *ortho*-hydroxy derivatives (Table 5). This enhanced effect can be understood in view of a departure from coplanarity in the thioamide group. The lone pair orbitals on the sulfur atom can now mix with the *p* orbital on the nitrogen atom since these orbitals are no longer orthogonal.

Table 5. Oscillator strengths for the $n\rightarrow\pi^*$ transition in some thiobenzamides.

Compound	Oscillator strength
Thiobenzamide	0.0035
N,*N*-Dimethylthiobenzamide	0.0046
1	0.011
6	0.021

The oscillator strengths have been calculated with the aid of the formula

$$f = 4.31 \times 10^{-9} \times \varepsilon_{max} \times 2(\nu_{max} - \nu_{0.5})$$

where $\nu_{0.5}$ is the wave number on the low frequency side of the band at which the extinction has decreased to half of the maximum value.

EXPERIMENTAL

The NMR spectra were recorded on a Varian A-60 or Varian A-60 A spectrometer equipped with Varian V-6031 variable temperature probe and V-6040 temperature controllers. Chemical shifts were measured by the side-band technique using an HP 200 CD Audio Oscillator. The frequencies were measured with an HP 3734 Electronic Counter. The shifts are given downfield from TMS. The evaluation of rate constants and the measurement of temperature and T_2 were performed as previously described.[36] IR spectra were recorded on a Perkin-Elmer Model 221 prism-grating instrument and UV spectra on a Unicam SP 800 B Ultraviolet Spectrometer.

Preparations. I. Gompper *et al.* have synthesized a number of dithiocarbonic acids and esters from phenols and carbon disulfide in a manner analogous to the Kolbe salicylic acid synthesis.[37] The hydroxyaryl dithioesters were synthesized by this procedure (I*a* corresponds to "Verfahren" A or B, I*b* to "Verfahren" C or D in Ref. 37).

The *N*,*N*-dimethyl-hydroxyaryl thioamides were prepared by either of four different methods:

II. The corresponding ethoxycarbonylmethyl hydroxyaryl dithioester was allowed to react with 2 equiv. of dimethylamine in boiling benzene solution for 2–3 h. The solution was evaporated and the residue recrystallized,

when necessary after prior column chromatography.

III. This method is a variant of the Willgerodt-Kindler reaction.[38] An aromatic hydroxyaldehyde (*e.g.* salicylic aldehyde) was mixed with 1.5 equiv. of sulfur and heated to 120 °C in an oil bath. Dimethylamine was slowly bubbled through the mixture for 1 – 3 h. The still warm, thick oil was poured onto ice-water. The solution was neutralized with hydrochloric acid and extracted with chloroform. The organic phase was dried over $MgSO_4$, followed by evaporation. The residue was chromatographed on an aluminia or silica column and recrystallized. (From salicylic aldehyde 2-hydroxy-*N,N*-dimethylthiobenzamide was formed).

IV. Viola *et al.* have found that *N,N*-dimethylcarbamyl chloride reacts with activated aromatic compounds in the presence of Friedel-Crafts catalysts to give *N,N*-dimethyl thioamides.[39]

V. Aromatic thioamides, not containing hydroxy groups, were prepared by thionation of the corresponding amide with phosphorus pentasulfide.

The following compounds were described previously: *8*,[39] *14*,[37] *16*.[37]

Methyl 4-hydroxy-2-methoxydithiobenzoate (*15*) was prepared from resorcinol monomethylether by method I*b*. Recryst. from benzene-ligroin (3:2); yield 38 %, m.p. 126.5 – 128 °C. Anal. found: C 49.7; H 4.58; S 29.9. Calc. for $C_9H_{10}O_2S_2$: C 50.4; H 4.71; S 29.9.

Methyl 4-hydroxy-1-dithionaphthoate (*17*) and *methyl 1-hydroxy-2-dithionaphthoate* (*18*) were prepared from 1-naphthol by method I*b*. The two isomers were separated by chromatography on a silica column (benzene-ether). *17* was recrystallized from benzene-ligroin (1:2). Yield 19 %, m.p. 113 – 114 °C. *18* was recrystallized from benzene-ligroin (1:3). Yield 12 %, m.p. 75 – 77 % °C. Anal. $C_{12}H_{10}OS_2$: C, H, S.

Ethoxycarbonylmethyl 4-hydroxy-2-methoxydithiobenzoate (*19*) was prepared from resorcinol monomethylether by method I*b*. Recryst. from benzene-ligroin (1:1); yield 18 %, m.p. 106 – 107 °C. Anal. found: C 50.8; H 4.97; S 22.3. Calc. for $C_{12}H_{14}O_4S_2$: C 50.3; H 4.92; S 22.4.

Ethoxycarbonylmethyl 2-hydroxy-1-dithionaphthoate (*20*) was prepared from 2-naphthol by method I*a*. Recryst. from 90 % methanol; yield 25 %, m.p. 117.5 – 118.5 °C. Anal. $C_{15}H_{14}S_2O_3$: C, H, S.

Ethoxycarbonylmethyl 4-hydroxy-1-dithionaphthoate (*21*) and *ethoxycarbonylmethyl 1-hydroxy-2-dithionaphthoate* (*22*) were prepared from 1-naphthol by method I*b*. The isomers were separated on an alumina column (benzene-ether). *21* was recrystallized from ethanol; yield 27 %, m.p. 118.5 – 119.5 °C. *22* was recrystallized from ligroin; yield 4 %, m.p. 69 – 70.5 °C. Anal. $C_{15}H_{14}O_3S_2$: C, H. S.

Ethoxycarbonylmethyl 3,5-di-tert-*butyl-4-hydroxydithiobenzoate* (*23*) was prepared from 2,6-di-*tert*-butylphenol by method I*a*. Recryst. from 90 % ethanol; yield 61 %, m.p. 101 – 102 °C. Anal. $C_{19}H_{28}O_3S_2$: C, H, S.

Benzyl 3,5-di-tert-*butyl-4-hydroxydithiobenzoate* (*24*) was prepared from 2,6-di-*tert*-butylphenol by method I*a*. Recryst. from ethanol; yield 57 %, m.p. 120 – 121.5 °C. Anal. found: C 70.3; H 7.44; S 17.4. Calc. for $C_{22}H_{28}OS_2$: C 70.9; H 7.57; S 17.2.

2-Hydroxy-N,N-*dimethylthiobenzamide* (*1*) was prepared from salicylic aldehyde by method III. Recryst. from benzene-ligroin (2:5); yield 53 %, m.p. 65 – 67 °C. Anal. $C_9H_{11}NOS$: C, H, N, S.

4-Hydroxy-N,N-*dimethylthiobenzamide* (*2*) was prepared from *p*-hydroxybenzaldehyde by method III. Recryst. from 80 % ethanol; yield 22 %, m.p. 166 – 168 °C. Anal. $C_9H_{11}NOS$: C, H, N, S.

3,5-Di-tert-*butyl-4-hydroxy*-N,N-*dimethylthiobenzamide* (*3*) was prepared from *23* by method II. Recryst. from ethanol; yield 86 %, m.p. 163 – 164 °C, lit.value:[40] 163.5 – 164.5 °C.

4-Hydroxy-2-methoxy-N,N-*dimethylthiobenzamide* (*7*) was prepared by two methods. A. From *19* by method II. Recryst. from 70 % ethanol; yield 54 %, m.p. 143 – 144 °C. B. From resorcinol monomethylether by method IV. Recryst. from 70 % ethanol; yield 45 %, m.p. 144 – 145 °C. Anal. $C_{10}H_{13}NO_3S$: C, H, N, S.

2-Hydroxy-4-methoxy-N,N-*dimethylthiobenzamide* (*6*) was prepared from 2-hydroxy-4-methoxybenzaldehyde by method III. Recryst. from benzene-ligroin (2:3); yield 34 %, m.p. 79 – 80 °C. Anal. $C_{10}H_{13}NO_2S$: C, H, N, S.

2-Methoxy-N,N-*dimethylthiobenzamide* (*4*) was prepared from 2-methoxy-*N,N*-dimethylbenzamide by method V. The product was distilled in *vacuo*, b.p. 145 – 155 °C/0.07 kPa; yield 57 %. Anal. found: C 60.9; H 6.58; N 7.05; S 16.6. Calc. for $C_{10}H_{13}NOS$: C 61.5; H 6.71; N 7.17; S 16.4.

2-Hydroxy-N,N-*dimethyl-1-thionaphthamide* (*13*) was prepared from *20* by method II. Recryst. from 90 % ethanol or benzene-ligroin (1:2); yield 58 %, m.p. 146 – 147 °C. Anal. $C_{13}H_{13}NOS$: C, H, N, S.

1-Hydroxy-N,N-*dimethyl-2-thionaphthamide* (*11*) was prepared from *22* by method II. Recryst. from ethanol; yield 52 %, m.p. 84 – 85 °C. Anal. found: C 68.0; H 5.84; N 5.98; S 14.1. Calc. for $C_{13}H_{13}NOS$: C 67.5; H 5.66; N 6.05; S 14.1.

4-Hydroxy-N,N-*dimethyl-1-thionaphthamide* (*12*) was prepared from *21* by method II. Recryst. from benzene, yield 66 %, m.p. 168 – 170 °C. Anal. $C_{13}H_{13}NOS$: C, H, N, S.

N,N-*Dimethyl-1-thionaphthamide* (*9*) was prepared from *N,N*-dimethyl-1-naphthamide by method V. Recryst. from 90 % ethanol; yield 40 %, m.p. 68.5 – 70 °C. Anal. $C_{13}H_{13}NS$: C, H, N, S.

N,N-*Dimethyl-2-thionaphthamide* (*10*) was prepared from *N,N*-dimethyl-1-naphthamide by method V. Recryst. from 90 % ethanol;

yield 21 %, m.p. 97–98 °C. Anal. $C_{13}H_{13}NS$: C, H, N, S.

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Formation of Aromatic Compounds from Carbohydrates. IV.* Chromones from Reaction of Hexuronic Acids in Slightly Acidic, Aqueous Solution

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Two new chromones, 3.5.6-trihydroxy-2-methylchromone (*1*) and 3,5,8-trihydroxy-2-methylchromone (*2*) have been isolated and identified from treatment of D-glucuronic or D-galacturonic acid in aqueous solutions of pH 3.5 and 4.5, respectively, at 96 °C. Compound *2* was also isolated from similar treatment of D-xylose. The MS spectra of compounds *1*, *2* and 3,8-dihydroxy-2-methylchromone (*6*) [1,2] are discussed. The former two (*1*, *2*) seem to be new compounds.

In previous papers [1,2] the isolation and identification of a series of phenolic compounds, furanes and reductic acid from treatment of hexuronic acids or pentoses in slightly acidic, aqueous solution, were reported. Also isolated were 3-acetyl-2,3,6-trihydroxycyclohexanone (*3*), 5,6,7,8-tetrahydro-3,5-dihydroxy-2-methyl-8-oxo-benzopyrone (*5*) and two unidentified chromones.[2] The former two compounds (*3* and *5*) were shown to be precursors of, respectively, 2,3-dihydroxyacetophenone (*4*) and 3,8-dihydroxy-2-methylchromone (*6*) (the predominant phenolic reaction product). The yields of *5* and *6* from D-glucuronic acid, were 1.7 and 3.4 %, respectively. Based upon these findings, tentative mechanisms were later presented [3] for the formation of *4* and *6* (see DISCUSSION).

This paper describes the identification of the prevously [1,2] isolated chromones *1* and *2*, isolated from D-glucuronic acid in 0.7 and 0.2 % yields, respectively, in smaller amounts from D-galacturonic acid and *2* also from D-xylose.

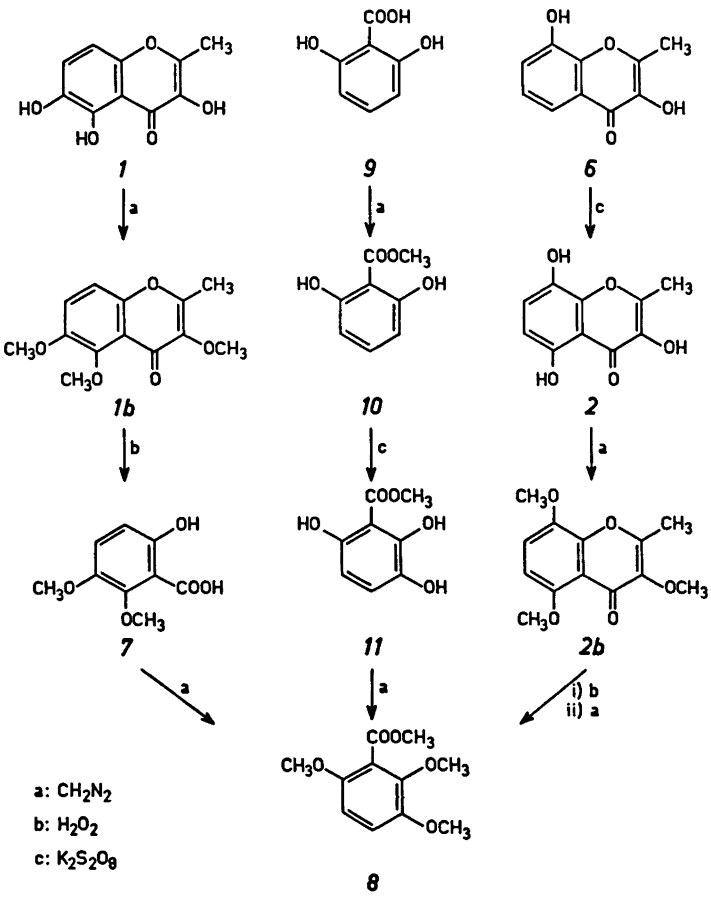
* Part III. See Ref. 14.

RESULTS

Compound *1* gave green and compound *2* reddish-violet colour with acidic ferric chloride. Both compounds corresponded to the formula $C_{10}H_8O_5$ as shown by elemental analysis and they resembled the chromone *6* closely in their MS fragmentation pattern as well as in their UV and IR spectra, indicating 3-hydroxy-2-methylchromones. NMR spectroscopy and MS of their acetates (*1a* and *2a*) indicated three acetyl groups. NMR spectra (Table 1) of *1* and *2* showed that each had two adjacent aromatic protons. Both substances gave a positive reaction to Wilson's boric acid test,[4] indicating a hydroxyl group at position 5.

Compound *1* was methylated with diazomethane, and the tri-*O*-methyl ether (*1b*) oxidized according to Aso [5] with hydrogen peroxide to acid *7*. This was further converted to methyl-2,3,6-trimethoxybenzoate (*8*) with diazomethane. The latter compound was shown to be identical (IR, NMR and MS) with an authentic sample prepared from 2,6-dihydroxybenzoic acid (*9*) *via* oxidation of the methyl ester (*10*) to *11* with potassium persulfate [6] followed by methylation.

Authentic *2* was synthesized from compound *6* by oxidation with potassium persulfate in a yield of 26 % calculated on reacted *6* and the identity with the isolated sample shown by IR, NMR, MS, melting points and chromatographic properties. It was further shown that compound *2* could be converted to the ester *8 via* methylation, oxidation and treatment with diazomethane. Treatment of *1* and *2*



with diazomethane gave, in addition to *1b* and *2b*, 3,6-dimethoxy-5-hydroxy-2-methylchromone (*1c*) and 3,8-dimethoxy-5-hydroxy-2-methylchromone (*2c*), respectively, which were chromatographically separated.

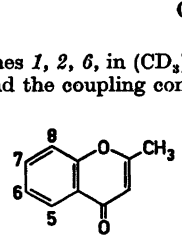
The structures of compounds *1* and *2* are fully established by the data and reactions presented above. Some results from the NMR spectral and MS studies of compounds *1*, *2*, *6* and their derivatives, which are of general interest to the chemistry of chromones and related compounds are discussed below.

DISCUSSION

The ^1H NMR spectra of 2-methylchromones have been discussed previously.[7] The ^1H NMR spectral data for compounds *1*, *2*, *6*, their methyl ethers and acetates are collected in Table 1. The proton H-7 was localized in the spectra of the dimethyl ethers *1c*, *2c* and *6b* by its long range coupling to the adjacent methoxyl group.[8a] The mutual assignment of H-7 and H-8 in compound *1a* was confirmed by the addition of the shift reagent "Eu(fod)$_3$", which shifted the signal at δ 7.48 (H-7) most (*cf.* Ref. 8b). In compound *6* and its acetate *6a* the aromatic protons form an ABC-system, which was analyzed by computer simulation.

MS spectra of 2-methylchromones have been previously [9–11] reported, and give fragmentation patterns similar to those of flavones.[12,13] Thus, the most prominent peaks correspond to the molecular ions, to losses of 28 and 29 mass units and to retro-Diels-Alder reactions (exemplified by fragment A and A+1 in Fig. 1). The fragmentation of compounds *6* and *6b*, as well as of *1* and *2* and their methoxy derivatives, was supported by the appearence of metastable peaks. We found

Table 1. ^{1}H NMR spectral data for chromones *1*, *2*, *6*, in $(CD_3)_2SO$ and for their derivatives in $CDCl_3$. The chemical shifts (δ) are given in the table and the coupling constants (J; without sign) as footnotes.



Compounds	5-H	6-H	7-H	8-H	CH_3	OCH_3	OAc
1 [a]			7.26	6.89	2.41		
2 [b]		6.55	7.09		2.40		
6 [c]	7.50	7.22	7.17		2.44		
1a [a]			7.48	7.34	2.51		2.31; 2.33; 2.34
2a [b]		6.98	7.40		2.40		2.32; 2.35; 2.39
6a [d]	8.08	7.37	7.41		2.41		2.41; 2.37
1b [a]			7.25	7.04	2.34	3.84; 3.87; 3.91	
2b [b]		6.61	7.02		2.41	3.85; 3.87; 3.88	
6b [e]	7.80	7.28	7.12		2.50	3.91; 3.98	
1c [a]			7.21	6.83	2.43	3.88; 3.92	
2c [b]		6.68	7.11		2.50	3.88; 3.90	

[a] $J_{7,8}$ 9.0 Hz. [b] $J_{6,7}$ 8.5 Hz. [c] $J_{5,6}$ 6.5, $J_{5,7}$ 3.3 and $J_{6,7}$ 7.5 Hz. [d] $J_{5,6}$ 8.3, $J_{5,7}$ 1.1 and $J_{6,7}$ 8.0 Hz. [e] $J_{5,6}$ 8.0, $J_{5,7}$ 2.0 and $J_{6,7}$ 8.0 Hz.

that peak A+1 is more intense than peak A in the spectra of the 3-hydroxychromones, while peak A predominates when the hydroxyl group at C-3 is etherified. This is in agreement with previous results on flavones.[13] The methoxychromones also gave fragmentation patterns similar to methyoxyflavones,[13] exhibiting strong peaks for M−15, M−43, A−15 and A−43. The strong peak B was found for all 3-hydroxychromones, in agreement with previous results[12] for flavones. In methoxychromones, however, A−15 and B are isobaric, but no metastable peak could be found confirming the formation of the latter. Peak M−46 seems to be characteristic for all 3-hydroxy- and 3-methoxychromones and A−29 for chromones having a hydroxyl or methoxyl group at C−8.

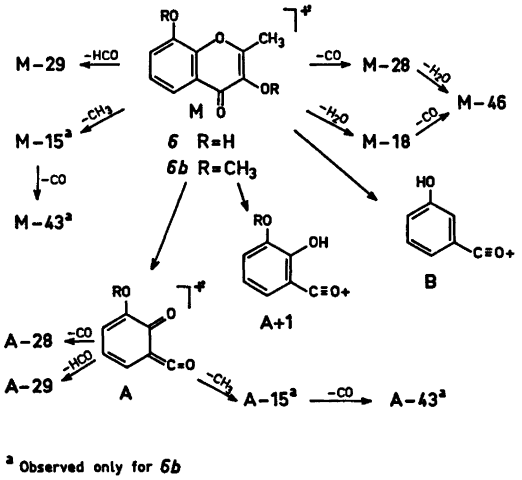


Fig. 1. Fragmentation on electron impact of compounds *6* and *6b*.

In the present and the previous studies[1,2] on the formation of aromatic compounds from reaction of hexuronic acids or pentoses under slightly acidic conditions, the only precursors isolated so far are compounds *3* and *5*. The following tentative mechanisms are suggested[3] for the formation of the corresponding phenolic compounds *4* and *6* from these precursors (Fig. 2). We are fully aware that the acyclic intermediates may be different and that other alternatives are possible for the subsequent reactions. Both *5* and *6* are formed from pentoses or hexuronic acids, but not from hexoses according to our recent investigations,[14] which strongly suggest that C_5-fragments are involved in the formation of the acyclic C_{10}-intermediates. The formation of monocyclic γ-pyrones from D-fructose or D-glucose *via*

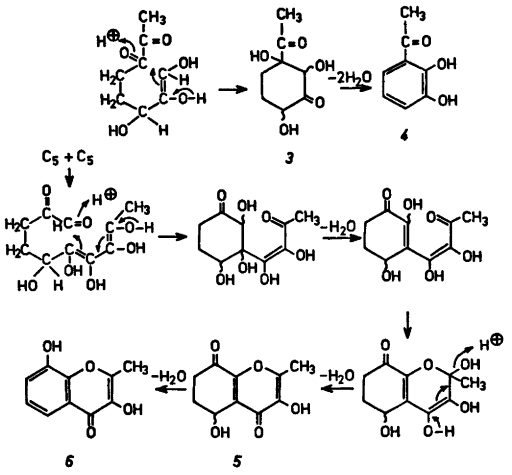


Fig. 2. Tentative mechanisms for the formation of compounds *4* and *6*.

closure of dicarbonyl sugars has been discussed previously.[15]

Anderegg and Neukom have studied the formation of *6* from ascorbic acid and pentoses,[16,17] and a mechanism for its formation from D-ribose was suggested,[17] based on our findings of *5* as a precursor. The suggested acyclic intermediate is somewhat different from ours, but many equally probable mechanisms may be constructed. Aso, who first isolated the chromone *6* (named alginetin) from treatment of alginic acid in aqueous solution at 160 °C,[5] suggested a mechanism for its formation,[18] which in the light of our isolation of *5* may be discarded or be considered to be a less likely alternative.

The formation of the cyclic intermediates *3* and *5* under weakly acidic conditions and their rapid transformation into the phenolic compounds *4* and *6* in high yields resembles the biosynthesis of phenolic compounds.[19,20] There are also many biogenetically modelled syntheses related to our findings. These studies have mainly been concerned with cyclization of poly-β-carbonyl compounds (see Ref. 21 for a summary), but the synthesis of a 2,6-diketodeoxyheptonic acid and its ready cyclization to 3-dehydroquinic acid provide an example from the shikimate route.[22]

EXPERIMENTAL

Melting points are corrected. TLC was performed on Silica Gel HF_{254} (Merck) with 9:1 dichloromethane-acetonitrile as solvent. Silicic acid (100 mesh Mallinckrodt) was used for column chromatography. The plates were studied in UV-light before treatment with (a) diazotized sulfanilic acid or (b) ferric chloride as spray reagents. Sublimations (or distillations) were performed at 0.5 mmHg in an electrically heated tube. NMR spectra were recorded at 100 MHz.

3,5,6-Trihydroxy-2-methylchromone (1) [1,2] was recrystallized from ethanol, m.p. 223 – 224 °C. Found: C 57.2; H 3.9. Calc. for $C_{10}H_8O_5$: C 57.7; H 3.9. MS [IP, 70 eV; *m/e* (% rel. int.)]: 209(11), 208(100, M), 180(14, [M – 28]), 179(15, [M – 29]), 162(6,[M – 46]), 153(33,[A + 1]), 137 (27,[B]), 124(6,[A – 28]), 57(12), 55(14), 43(20). IR (KBr): 1640 (s), 1620 (s), 1600(s), 1560 (s), 1470 (broad), 1370 (m), 1310 (broad), 1265 (s,) 1215 (broad), 1155 (w), 1105 (s), 1050 (s), 995 (s), 830 (s), 790 (s) cm^{-1}. UV [abs. ethanol (log ε)]: 238(4.17), 255(4.24), 295(sh, 363), 367 (3.62). (NaOH/ethanol): 235(4.07), 500(3.25) nm.

Acetylation of *1* (Ac_2O/pyr.) yielded the triacetate (*1a*) recrystallized from benzene-light petroleum (b.p. 40 – 60 °C). m.p. 165 – 166 °C. Found: C 58.1; H 4.3. Calc. for $C_{16}H_{14}O_8$: C 57.5; H 4.2. MS [IP 70 eV; *m/e* (% rel. int.)]: 334(1, M), 292(14), 250(32), 209(13), 208(100), 179(14), 163(7), 137(9), 136(5),121(8), 107(6), 63(6), 55(6), 51(7), 43(61). Methylation (CH_2N_2) of compound *1* yielded a mixture of the di- and tri-methyl ethers in ratio 1:5, which were separated on a silicic acid column with 9:1 dichloromethane-acetontrile as solvent.

3,5,6-Trimethoxy-2-methylchromone (1b) was recrystallized from carbon tetrachloride, m.p. 130.5 – 132 °C. Found: C 62.9; H 5.6. Calc. for $C_{13}H_{14}O_5$: C 62.4; H 5.6. MS [IP 70 eV; *m/e* (% rel. int.)]: 250(63, M), 236(16), 235(100, [M – 15]), 233(10), 221(12,[M – 29]), 220(11), 217(16), 207(18,[M – 43]), 204(5,[M – 46]), 203(12), 192(32), 180(18,[A]), 165(19,[A – 15]), 137(21,[A – 43]), 78(17), 77(15), 55(12), 53(11), 50(12), 43(56).

The dimethyl ether was positive towards Wilson's boric acid test [4] and towards spray b, indicating a free hydroxyl at C-5. Since it is previously known [23] that 5-hydroxyflavones are difficult to methylate with diazomethane, the compound was assigned *5-hydroxy-3,6-dimethoxy-2-methylchromone (1c)*. It was recrystallized from aqueous ethanol, m.p. 80.5 – 82.5 °C. Anal. $C_{12}H_{12}O_5$: C, H. MS [IP 70 eV; *m/e* (% rel. int.)]: 237(20), 236(100, M), 235(16), 221(52,[M – 15]), 218(13, [M – 18]), 208(13, [M – 28]), 207(12,[M – 29]), 203(10), 194(12), 193(90,[M – 43]), 190(12,[M – 46]), 178(22), 166(19,[A]), 151(15,[A – 15]), 150(18), 123(18, [A – 43]), 79(22), 67(10), 55(10), 51(11), 43(26).

3,5,8-Trihydroxy-2-methylchromone (2) was synthesized in 28 % yield from *6* by oxidation

with $K_2S_2O_8$ in the same way previously reported [23] for 5-hydroxy-3,7-dimethoxyflavone, and recrystallized from ethanol.* M.p. 254–256 °C (dec.). Found: C 57.2; H 3.7. Calc. for $C_{10}H_8O_5$: C 57.7; H 3.9.[MS IP 70 eV; *m/e* (% rel. int.)]: 209(12), 208(100, M), 180(10, [M–28]), 179(13, [M–29]), 162(6,[M–46]), 153(60,[A+1]), 137(22, [B]), 124(4,[A–28]), 123(4,[A–29]), 57(18), 55(15), 43(20). IR (KBr): 1645 (w), 1620 (s), 1560 (s), 1470 (broad), 1420 (w), 1360 (w), 1270 (s), 1215 (s), 1165 (w), 1095 (m), 1035 (m), 1005 (m), 960 (m), 800 (m), 745 (m) cm^{-1}. UV[abs. ethanol (log ε)]: 248(4.35), 370 (3.65) and (NaOH/ ethanol): 235(sh, 3.23), 365(3.79) nm.

Acetylation (Ac_2O/pyr.) yielded the *triacetate* (*2a*), recrystallized from benzene, m.p. 175.5–179.5 °C (dec.). Anal. $C_{16}H_{14}O_8$: C, H. MS[IP 70 eV; *m/e* (% rel. int.)]: 334 (1, M), 292(20), 250(25), 209(14), 208(100), 207(13), 179(7), 153(6), 43(90).

Methylation (CH_2N_2) of compound *2* yielded a mixture of the di- and tri-methyl ether in ratio 3:1. Separation was effected in the same way as for compounds *1b* and *1c*.

3,5,8-Trimethoxy-2-methylchromone (*2b*) crystallized on storage, m.p. 106–110 °C. Found: C 62.9; H 5.8. Calc. for $C_{13}H_{14}O_5$: C 62.4; H 5.6. MS [IP 70 eV; *m/e* (% rel. int.)]: 251(15), 250(100, M), 249(14), 235(84,[M–15]), 233(17), 232(13,[M–18]), 221(19,[M–29]), 220(16), 217(30), 207(24,[M–43]), 206(25), 205(14), 204(15,[M–46]), 192(17), 191(12), 180(26,[A]), 177(12), 165(12,[A–15]), 151(20, [A–29]), 137(28,[A–43]), 123(14), 122(18), 78(13), 77(15), 55(10), 53(14), 51(12), 43(59).

For the reasons given for *1c* the dimethyl ether was assigned *5-hydroxy-3,8-dimethoxy-2-methylchromone* (*2c*), recrystallized from aqueous ethanol, m.p. 100–102.5 °C. Anal. $C_{12}H_{12}O_5$: C, H. MS [IP 70 eV; *m/e* (% rel. int.)]: 237(22), 236(83, M), 222(39), 221(85,[M–15]), 207(8, [M–29]), 206(27), 203(17), 193(13), 190(4, [M–46]), 178(24), 166(13,[A]), 151(21,[A–15]), 138(6,[A–28]), 137(10,[A–29]), 135(12), 133(12), 123(23,[A–43]), 79(19), 77(12), 69(18), 67(18), 55(35), 54(17), 53(31), 51(25), 43(100).

3,8-Dihydroxy-2-methylchromone (*6*).[1,2] MS [IP 70 eV; *m/e* (% rel.int.)]: 193(13), 192(100, M), 174(3,[M–18]), 164(16,[M–28]), 163(21, [M–29]), 146(15,[M–46]), 137(56,[A+1]), 136(21,[A]), 121(50,[B]), 118(16), 108(11,[A–28]), 107(15,[A–29]), 81(14), 80(13), 79(16), 77(12), 65(19), 63(24), 55(28), 53(30), 52(32), 51(27), 50(15), 43(46). IR(KBr): 1640 (m), 1620 (m), 1600 (s), 1550 (broad), 1480 (s), 1435 (m), 1390 (m), 1325 (w), 1290(s), 1245 (s), 1220 (broad), 1150 (w), 1095 (m), 1025 (s), 980 (s), 890 (w), 830 (w), 750 (s) cm^{-1}. UV [abs. ethanol (log ε)]: 238(sh, 441), 242(4.46), 331(3.85) and (NaOH/ ethanol). 255(4.37) nm.

Acetylation (Ac_2O/pyr.) yielded the *diacetate* (*6a*), recrystallized from benzene–light petroleum (b.p. 60–80 °C), m.p. 126.5–127 °C (lit. value [5,18] 125 °C). Anal $C_{14}H_{12}O_6$: C,H. MS [IP 70 eV; *m/e* (% rel. int.)]: 276(3, M), 235(4), 234(26), 193(12), 192(100), 163(7), 137(9), 136(5), 121(8), 107(6), 63(6), 55(6), 51(7), 43(88).

Methylation (CH_2N_2) of compound *6* yielded the *dimethyl ether* (*6b*) recrystallized from hexane, m.p. 105–106 °C (lit. value [5,18] 105 °C). Anal. $C_{12}H_{12}O_4$: C, H. MS [IP 70 eV; *m/e* (% rel. int.)]: 220(100, M), 219(25), 205(40, [M–15]), 202(37,[M–18]), 191(12,[M–29]), 190(20), 177(24), 174(10,[M–46]), 151(20, [A+1]), 150(49,[A]), 135(4,[A–15]), 122(64, [A–28]), 121(21,[A–29]), 120(14), 119(11), 107(23,[A–43]), 91(15), 77(15), 76(17), 67(12), 65(15), 53(11), 51(20), 50(14), 43(34).

6-Hydroxy-2,3-dimethoxybenzoic acid (*7*). Compound *1b* was oxidized with H_2O_2 in the same way as previously reported [5] for *6b*. To *1b* (250 mg) were added aqueous Na_2CO_3 (1 %, 175 ml) and aqueous H_2O_2 (3 %, 50 ml), and the mixture left at room temperature overnight. After acidification, extraction with ethyl acetate (3 × 50 ml), drying (Na_2SO_4) and evaporation, the compound (80 mg) was purified by silicic acid column chromatography (9:1 chloroform-acetic acid), yielding 60 mg *7* (30 %). After sublimation the crystals melted at 79–80.5 °C. (lit. value [24] 77–79 °C). Found: C 55.2; H 5.1. Calc. for $C_9H_{10}O_5$: C 54.6; H 5.1.

Methyl-2,6-dihydroxybenzoate (*10*) [25] was oxidized to *methyl-2,3,6-trihydroxybenzoate* (*11*) in analogy with a previous related synthesis;[6] recrystallized from benzene, m.p. 136–139 °C (lit. value [26] 139–140.5 °C).

Methyl-2,3,6-trimethoxybenzoate (*8*) was obtained in an amorphous state from compound *11* by methylation (CH_2N_2). It was identical (NMR, MS) with methylated (CH_2N_2) compound *7*, and with oxidized and methylated *2b*. (Lit. value [27] m.p. 57.0–57.5 °C). MS [IP 70 eV; *m/e* (% rel. int.)]: 227(19), 226(100, M), 211(55), 195(55), 183(38), 180(18), 179(20), 164(14), 137(40), 45(18). ^1H NMR, δ ($CDCl_3$): 3.73 (3 H, s), 3.78 (3 H, s), 3.85 (3 H, s), 3.87 (3 H, s) 6.59 (1 H, d, *J* 9.3 Hz), 6.88 (1 H, d, *J* 9.3 Hz).

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The Conformation of 4,4,8,8-Tetramethylcyclodecanone

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The slow-exchange ^1H NMR spectrum below $-100\,°C$ shows that 4,4,8,8-tetramethylcyclodecanone has a single unsymmetric conformation. The appearance of one α-proton at very low field and one β-proton at very high field is best explained on the basis of a conformation of boat-chair-chair type. A single exchange process is observed by dynamic NMR spectroscopy ($\Delta G^{\ddagger} = 9.5 - 10$ kcal/mol),* although two processes would be expected; both must therefore have the same barrier height.

Dieckmann cyclization of the diethyl ester of 4,4,8,8-tetramethylundecanedioic acid gives, in addition to a good yield of the 20-membered cyclic diketone, a difficultly reproducible and small yield of the 10-membered cyclic monoketone.[1] This compound, 4,4,8,8-tetramethylcyclodecanone, is of particular conformational interest inasmuch as the 1,5-relationship of the two *gem*-dimethyl groups prevents this ring from adopting the generally favoured quadrangular [2323] conformation ** of cyclodecane derivatives.[3] Fig. 1 demonstrates that one methyl group would then have to point into the ring, which is sterically impossible. Unsubstituted cyclodecanone has been shown [4] to take this type of conformation with the carbonyl group in the position shown.

Four alternative quadrangular conformations, whose calculated energies [5] are reasonably low for cyclodecane itself, all have corner atoms in 1,5-relationship and should thus be able to accommodate the two *gem*-dimethyl groups. The resulting possible conformations for 4,4,8,8-tetramethylcyclodecanone are shown in Fig. 2; the ring skeleton [2233] gives conformation A, [1333] gives B, [1324] gives C and D, and [1414] gives E.

In the case of a similarly substituted cyclodecane, 4,4,8,8-tetramethylcyclodecanecarboxylic acid, the crystal was found [3] to contain a 4:1 mixture of conformations of type A and B. The spectral data for our ketone, however, fit neither of these,* nor a mixture of the two, but are in agreement with a single unsymmetric conformation such as C or D.

PROOF OF CONFORMATIONAL HOMOGENEITY

The infrared spectra of 4,4,8,8-tetramethylcyclodecanone in the crystalline state and in CS_2-solution show negligible differences. This, together with the relatively high melting point of 105 °C, would in itself indicate conformational homogeneity. Since, however, the spectrum in either phase shows broad and numerous absorptions, the possibility that both phases contain the same conformer mixture had to be ruled out. Only an expanded crystal lattice can accept a conformer mixture, and the demonstration by differential scanning calorimetry of a perfectly normal [6] entropy of melting ($\Delta H_m = 3.9$ kcal/mol; $\Delta S_m = 10.4$ e.u.) and no solid phase transition point down to $-80\,°C$,

* Before having obtained a slow-exchange NMR spectrum, we suggested [6] conformation A as the most likely candidate.

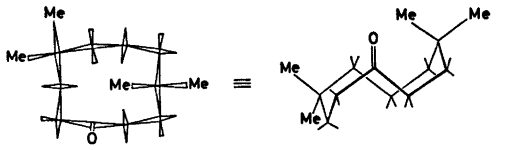

Fig. 1. The sterically impossible [2323] conformation of 4,4,8,8-tetramethylcyclodecanone.

* 1 kcal = 4.184 kJ.

** This notation indicates the number of bonds in each "side" separating the "corner" atoms.[3]

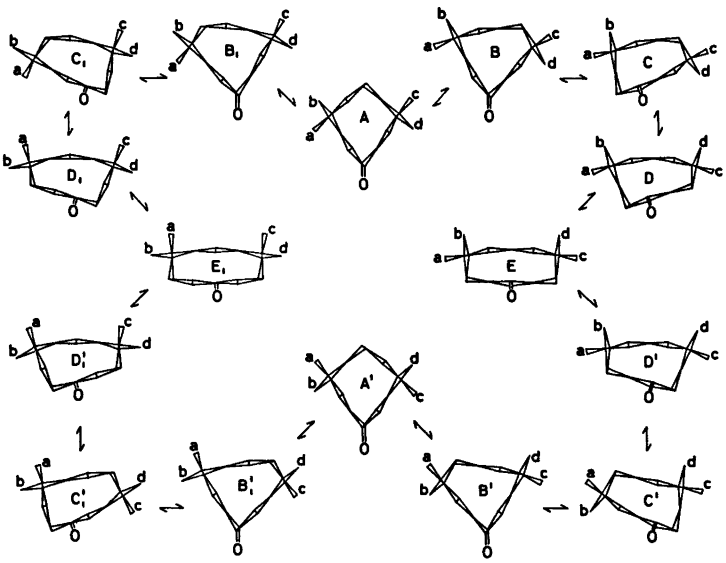

Fig. 2. Conformational interconversion scheme for five possible conformations of 4,4,8,8-tetra methylcyclodecanone.

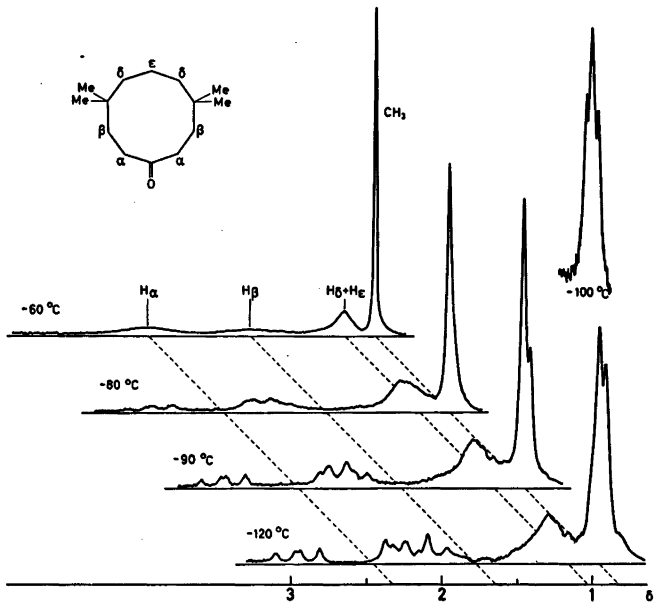

Fig. 3. 100 MHz ^{1}H NMR-spectra of 4,4,8,8-tetramethylcyclodecanone in CS_2-solution at −60, −80, −90, and −120 °C. The inset in the upper right-hand corner shows the methyl region in CCl_2F_2-solution at −100 °C.

excluded a conformer mixture. The numerous and broad IR-absorptions then already suggest that the single conformer might be unsymmetric and perhaps not even very rigid.

THE LOW-TEMPERATURE NMR SPECTRUM

The 100 MHz ^{1}H NMR-spectrum was of the expected fast-exchange type down to −20 °C.

On further cooling of CS_2-solutions (Fig. 3) broadening of all methylene signals occurred at about $-60\,°C$, the final slow-exchange spectrum being reached at about $-100\,°C$ (coalescence temp. $\sim -70\,°C$). Broadening of the methyl line started at $-80\,°C$, the final slow exchange spectrum being reached at about $-120\,°C$ (coalescence temp. $\sim 85\,°C$). One methyl line is at higher field than the remaining massif of presumably three lines, which remain unresolved in this solvent. In CCl_2F_2-solution at $-100\,°C$ a methyl line is seen resolved both on the high- and the low-field side of a central massif presumably representing the remaining two methyl groups (Fig. 3). On further cooling this broadens and fuses with the low-field line.

The complexity of this low-temperature spectrum allowed only an indirect interpretation. This was based on integrated intensities of the four main spectral areas and correlation with the chemical shifts of the α,β,δ and ε CH_2- and the CH_3-protons in the fast-exchange spectrum. It gave as a result that at all temperatures from $-80\,°C$ and below the intensities in the four spectral regions $\delta = 3.1-2.5$, $2.5-1.5$, $1.5-0.93$, and $0.93-0.5$, represent protons in a ratio close to 1:4.8:13, assuming that a single conformation (26 protons) is present. Thus, the lowest region must represent a single α-proton, the next-lowest region the remaining three α-protons and a single β-proton, the next-highest region the remaining three β-protons and five of the δ- and ε-protons, and the highest region twelve methyl protons and the remaining δ- (or ε-) proton. The latter absorption is actually discernible on the high-field side of the methyl lines.

This unsymmetric intensity distribution, as well as the presence of more than two types of methyl groups, immediately excludes the symmetric conformations A and E. It also excludes conformation B since the local symmetry around the corner position of the carbonyl group would require that not only one, but two, of the α-protons appear in the lowest region, namely those that are eclipsing the carbonyl group on either side. It is not possible, however, from the NMR-spectra to make a choice between the unsymmetric conformations C and D. These are both based on equivalent mirror image [1324] ring skeletons which are not genuinely quadrangular of type, but also classifiable [7] as extremes of a biangular family [28] commonly represented (Fig. 4) in the intermediate form, sometimes referred to as boat-chair-chair.* This intermediate [28] conformation is for cyclodecane calculated [5] to be a low barrier (1 kcal/mol) between two mirror image [1324] minima, but it may instead very well be a shallow energy minimum in our substituted ketone, since the *gem*-dimethyl groups require maximal staggering in the adjoining ring bonds; this can be achieved by moving the inherent partial eclipsing in these bonds of the [1324] forms C and D to the next ring bonds between unsubstituted carbon atoms by passing over to the [28] form (Fig. 4). Also the broad infrared absorptions can be well understood by the existence of a shallow minimum of this type.

All conformations of the [28] family satisfy the symmetry requirements and can explain the unusual chemical shifts observed. The [28] conformation of Fig. 4, chosen for the present discussion but actually representing also the possible conformations C and D, has just one α-proton (marked H_α) eclipsing the carbonyl group and thus expected at unusually low field. In cyclodecanone, where the carbonyl group by symmetry arguments must be similarly situated next to a corner position, one of the four α-protons has been reported [4] as low as $\delta = 3.41$. Among the β-protons it seems most likely that the one marked $H\beta$ is the one at unusually low field, since this is also in the plane of the carbonyl group; this, by the way, might constitute an argument that the [28] form shown in Fig. 4 represents the actual conformation rather than C or D, where this proton gets somewhat out of the carbonyl plane. One of the δ-protons (marked $H\delta$) sits across the ring just above the carbonyl plane

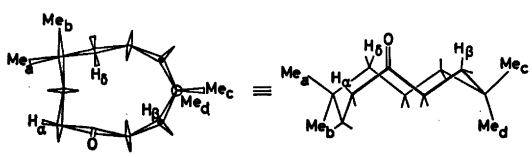

Fig. 4. The [28] conformation of 4,4,8,8-tetramethylcyclodecanone.

* The boat-chair of cyclooctane [8] is closely related and is in our notation given the symbol [26] of a biangular conformation.[7]

and so is expected to come at unusually high field. This is exactly what is observed for the [26] conformation of cyclooctane [8] where one δ-proton in the same transannular relationship to the carbonyl group comes as high as at $\delta = 0.63$. Similarly, one of the methyl groups of our cyclodecanone (marked Me_a in Fig. 4) is closer to the carbonyl group than the others, and is probably the one which appears at slightly higher field than the other three.

It may be wondered why 4,4,8,8-tetramethylcyclodecanone adopts none of the two conformers A and B, found [3] for the very closely related 1-carboxylic acid, or the conformer E, largely preferred by cyclodecane-1,6-dione in solution.[9,10] One reason may be the well-established tendency [4,9,10] for the carbonyl group of large-ring ketones to avoid corner positions and to prefer to sit next to corners. Another reason may be that the absence of one of the transannular H−H 1,5-interactions, exactly as in the [2323] conformation of cyclodecanone itself and other medium-ring ketones,[4,8,9,10] favours the [28] family.

THE CONFORMATIONAL EXCHANGE PROCESS

The multi-step interconversion scheme shown in Fig. 2 has been constructed by employing the type of corner-moving elementary step discussed earlier,[7,11] which consists in eclipsing the bond between the old and the new corner. The choice of paths becomes restricted by the requirement that *gem*-dimethyl substituted corners cannot be "moved", since this would lead to intermediate conformations with methyl groups pointing into the ring. The only exception is of course the conversion within the [28] family from one extreme C to the other extreme D, whereby the *gem*-dimethyl group moves directly from one non-genuine corner to the next in an outward motion.[7] It is interesting that no other conformations than the actual low-energy candidates for the stable equilibrium conformation are needed as intermediates. Rapid passage by a certain number of steps along this closed loop will lead to full site exchange for any type of proton on any of the five conformational types, and to a mixing of the NMR-signals from the individual components of any conformer mixture. Whether one or more processes can be observed by dynamic NMR-spectroscopy will depend [7] on the symmetry of the conformers and, in the case of a mixture, on the relationship between its constituents.

The situation observed in the present case (Fig. 3) is not a very clear one inasmuch as the spectral changes take place at somewhat different temperatures in the various regions of the spectrum. However, due to the very different magnitudes of the chemical shift splittings, the estimated activation free-energies come out not significantly different in the range 9.5 − 10 kcal/mol. Also, the clear direct development in the low-temperature NMR spectrum of an α-CH_2 signal corresponding to one proton and a methyl signal corresponding to one methyl group, and not *via* intermediate spectra showing signals corresponding to two of each, is only in accord with a single observed site-exchange process.

Nevertheless, the interconversion scheme in Fig. 2 shows that only if the conformation has a symmetry axis (A) or a symmetry plane (E) will full exchange of all constitutionally equivalent protons be possible in a single process. Thus, A can be converted to its mirror image A′ *via* the path $A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D \rightleftharpoons E \rightleftharpoons D' \rightleftharpoons C' \rightleftharpoons B' \rightleftharpoons A'$ and E can be converted to the identical conformation E_1 *via* the path $E \rightleftharpoons D \rightleftharpoons C \rightleftharpoons B \rightleftharpoons A \rightleftharpoons B_1 \rightleftharpoons C_1 \rightleftharpoons D_1 \rightleftharpoons E_1$, whereby only the critical barrier on each path is observable. In the case of an unsymmetric conformation two processes must in principle be observable.[7] Thus C may interconvert with its mirror image C′ *via* the path $C \rightleftharpoons D \rightleftharpoons E \rightleftharpoons D' \rightleftharpoons C'$ and acquire by averaging an apparent symmetry plane (as in E), whereby *cis*-related methyl groups and methylene hydrogens become equivalent (a = c, b = d), or it may interconvert with the identical conformation C_1 *via* the path $C \rightleftharpoons B \rightleftharpoons A \rightleftharpoons B_1 \rightleftharpoons C_1$ and acquire by averaging an apparent twofold symmetry axis (as in A), whereby *trans*-related methyl groups and methylene hydrogens become equivalent (a = d, b = c).

The only possible conclusion is that the critical barrier on both interconversion paths have closely similar values. It seems unrealistic to attempt strain energy calculations on these compounds, but for the parent unsubstituted cyclodecane the corresponding barriers have

been realiably calculated.[5] Fixing the energy of the [1324] conformation (C and D) as zero, the highest barrier on the first path (~6 kcal/mol) comes between [1333] and [2233] (B⇌A), and the highest barrier on the second path (~15 kcal/mol) between [1324] and [1414] (D⇌E). It is likely that the former barrier should increase somewhat on *gem*-dimethyl substitution. It is even more likely that the latter barrier should decrease considerably on carbonyl substitution, since the severe transannular H−H 1,5-interaction in the transition state has been relieved by conversion of one CH_2-group into a carbonyl group, the interaction being now between a δ-hydrogen and the oxygen.

EXPERIMENTAL

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

Calorimetric measurements were performed with a Perkin-Elmer Differential Scanning Calorimeter IB.

^{1}H NMR spectra were recorded on a Varian HA 100 15D instrument.

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The Crystal and Molecular Structure of 6-Methylmercaptopurine Riboside Monohydrate

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The crystal structure of 6-methylmercaptopurine riboside monohydrate has been determined by X-ray diffraction methods using 3988 observed reflections collected on a counter diffractometer. The crystals are monoclinic, space group $P2_1$, with cell dimensions $a = 7.912(2)$ Å; $b = 18.120(1)$ Å; $c = 4.848(1)$ Å; $\beta = 105.70(2)°$. The structure was refined to a conventional R-factor of 0.040, the standard deviations in bond lengths and angles involving non-hydrogen atoms are 0.002 Å and 0.1°, respectively.

The molecular and crystal structure is discussed and the results compared to those obtained from magnetic resonance experiments.

Several sulfur containing nucleic acid analogues are effective metabolic inhibitors used in cancer chemotherapy. The adenosine analogue 6-methylmercaptopurine riboside (9-β-D-ribofuranosyl-6-methylthiopurine) is a strong inhibitor of several mouse tumors and is also effective against some human leukemias as well as other tumors which have become resistant to 6-mercaptopurine therapy.[1] It has been shown that the riboside is mistaken for adenosine at the enzymatic level and is incorporated into the nucleic acids.[2] The action of this drug, either administered alone or in combination with other agents, is to influence the purine metabolic pathways.

Previous investigators have found similarities between enzymatic reactions and reactions in the solid state due to ionizing radiation.[3] In an attempt to study reactions induced by ionizing radiation in chemotherapeutic agents, magnetic resonance experiments (ESR and ENDOR) are being carried out on irradiated mercaptopurine crystals.[4,5] Although the relative orientations of the purine rings in the unit cell of the crystals can be predicted from the ESR/ENDOR data, a detailed stereochemical comparison of molecular directions cannot be made without a complete structural analysis of the crystals.

Using one of the crystals grown for the magnetic resonance experiments, an analysis of the crystal and molecular structure of 6-methylmercaptopurine riboside (6MeMPR) was undertaken.

EXPERIMENTAL

Powdered samples of 6MeMPR obtained from Nutritional Biochemical Company were recrystallized from water at a constant temperature of 30 °C. A crystal fragment of approximate dimensions $0.3 \times 0.5 \times 0.5$ mm was cut from a larger crystal and used for the X-ray experiments. Data were collected on a SYNTEX $P\bar{1}$ four-circle diffractometer (graphite crystal monochromated Mo$K\alpha$ radiation, $\lambda = 0.71069$).

The crystals are monoclinic; systematic absences are k odd for $(0k0)$ indicating $P2_1$ or $P2_1/m$ as possible space groups of which the latter could be ruled out for this optically active compound. Cell parameters were determined by a least-squares fit to the diffractometer settings for 15 general reflections.

Intensity data were recorded using the $\theta/2\theta$ scanning mode using a scan speed of 2° min^{-1} for reflections with sin θ/λ below 0.65 Å^{-1} and scan speeds varying from 1 to 2° min^{-1} depending on the intensity for reflections with sin θ/λ between 0.65 and 0.95 Å^{-1}. Reflections in the latter interval were measured only if a quick scan gave an intensity larger than a preset value. 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 limits.

From the 4348 unique reflections recorded, 3988 with $I > 2.50\sigma(I)$ were retained for the structure analysis. The standard deviations for the intensities were calculated by $\sigma(I) = [C_T + (0.02C_N)^2]^{\frac{1}{2}}$ where C_T is the total number of counts and C_N is the scan count minus background count. The usual corrections were made for Lorentz and polarization effects, but no correction was applied for absorption or extinction.

Scattering factors used were those of Doyle and Turner for S, O, N, and C,[6] and of Stewart, Davidson and Simpson for H.[7] Description of the computer programs used are given in Refs. 8 and 9. The quantity minimized in the least-squares calculations was $\sum w\Delta F^2$ where w is the inverse of the variance of the observed structure factors.

CRYSTAL DATA

6-Methylmercaptopurine riboside monohydrate, $C_{11}H_{14}O_4N_4S.H_2O$, monoclinic, $a = 7.912(2)$ Å; $b = 18.120(1)$ Å; $c = 4.848(1)$ Å; $\beta = 105.70(2)°$; ($t = 18 \pm 1$ °C); $V = 669.1$ Å^3; $M = 316.34$; $Z = 2$; $F(000) = 332$; $D_{calc} = 1.570$ g cm^{-3}. Absent reflections: $(0k0)$ for k odd. Space group $P2_1$ (No. 4).

STRUCTURE DETERMINATION

The structure was solved by application of noncentrosymmetric direct methods using the program assembly MULTAN.[9] Phases for 175 reflections with $|E| > 1.5$ were determined with the best starting set of phases yielding an absolute figure of merit of 1.31. An E-map based on these phases revealed 26 peaks, of which 18 could be related to a molecule of the anticipated geometry. The remaining three non-hydrogen atoms were localized by the use of successive Fourier refinements; the positions of the hydrogen atoms were calculated from stereochemical considerations after a preliminary refinement. Using the data with $\sin\theta/\lambda < 0.65$ the positional parameters, anisotropic parameters for non-hydrogen atoms and isotropic parameters for hydrogen atoms were refined by least-squares methods to a conventional R-factor of 0.04. The goodness of fit, $S = [\sum w\Delta F^2/(m-n)]^{\frac{1}{2}}$, was 3.53, however, and a weight analysis indicated systematic errors present for the low-angle data. The least-squares procedure was then repeated with all the data; the weights for the 1231 reflections with $\sin\theta/\lambda < 0.6$ were multiplied with a factor varying from 0 to 1 for reflections with $\sin\theta/\lambda$ varying from 0 to 0.60. The remaining 2757 reflections were given normal weights. The refinement converged to a conventional R-factor of 0.040, $R_w = 0.043$ and $S = 1.77$.

Table 1. Atomic parameters and their estimated standard deviations. Atoms marked with asterisks belong to the ribose moiety. The anisotropic temperature factor has the form $\exp -2\pi^2[(U11a^{*2}h^2 + U22b^{*2}k^2 + U33c^{*2}l^2 + 2U12a^*b^*hk + 2U13a^*c^*hl + 2U23b^*c^*kl)]$.

ATOM	x	y	z	U11	U22	U33	U12	U13	U23
S	.3299(1)	.5362(0)	.7324(1)	.0285(2)	.0257(1)	.0352(2)	-.0006(1)	.0093(1)	-.0103(1)
N1	.5317(2)	.4532(1)	.4850(3)	.0218(4)	.0276(4)	.0401(5)	-.0029(3)	.0091(4)	-.0095(3)
N3	.4288(2)	.3595(1)	.1253(4)	.0264(7)	.0283(14)	.0449(9)	.0004(8)	.0129(6)	-.0100(9)
N7	.0477(2)	.4339(1)	.2854(3)	.0228(7)	.0215(4)	.0383(6)	-.0014(4)	.0117(5)	-.0067(4)
N9	.1103(2)	.3481(1)	-.0074(3)	.0219(6)	.0180(4)	.0316(6)	-.0014(4)	.0074(5)	-.0047(4)
C2	.5519(2)	.3997(1)	.3037(3)	.0219(5)	.0319(5)	.0471(6)	-.0010(5)	.0120(4)	-.0129(5)
C4	.2676(2)	.3773(1)	.1435(4)	.0210(5)	.0175(5)	.0295(7)	-.0010(4)	.0081(4)	-.0034(5)
C5	.2274(2)	.4305(1)	.3243(4)	.0215(5)	.0168(5)	.0297(7)	-.0007(4)	.0092(5)	-.0022(5)
C6	.3687(2)	.4675(1)	.5000(3)	.0233(5)	.0182(4)	.0272(6)	-.0016(4)	.0079(4)	-.0027(4)
C8	-.0163(2)	.3845(1)	.0850(3)	.0207(4)	.0225(4)	.0384(5)	-.0006(3)	.0089(4)	-.0057(4)
C1*	.5448(2)	.5736(1)	.6812(4)	.0388(5)	.0468(7)	.0505(9)	-.0106(5)	.0081(6)	-.0239(6)
OW	.1324(2)	.3352(1)	.4237(3)	.0313(5)	.0279(4)	.0443(6)	.0048(3)	.0155(4)	.0097(4)
O1*	-.0946(2)	.2899(1)	-.3786(3)	.0280(5)	.0191(4)	.0311(5)	-.0017(4)	-.0022(4)	.0041(4)
O2*	.1985(2)	.1819(1)	-.3559(3)	.0377(5)	.0234(4)	.0405(5)	.0007(4)	.0201(4)	-.0059(4)
O3*	-.0292(2)	.1056(1)	-.1067(4)	.0469(5)	.0182(5)	.0362(7)	-.0003(4)	.0095(5)	.0064(5)
O5*	-.3367(2)	.2578(1)	-.0392(3)	.0358(5)	.0761(4)	.0488(5)	.0170(4)	.0102(4)	-.0243(4)
C1*	.0848(2)	.2935(1)	-.2406(3)	.0260(5)	.0172(4)	.0247(5)	-.0012(4)	.0054(4)	-.0010(4)
C2*	.1466(2)	.2163(1)	-.1323(3)	.0246(5)	.0164(4)	.0246(5)	.0023(4)	.0048(4)	-.0006(4)
C3*	-.0224(2)	.1832(1)	-.0874(3)	.0276(5)	.0193(4)	.0228(5)	.0010(4)	.0075(4)	.0014(4)
C4*	-.1611(2)	.2163(1)	-.3398(4)	.0256(6)	.0189(7)	.0226(7)	-.0016(5)	.0055(5)	-.0012(6)
C5*	-.3434(3)	.2223(2)	-.3017(6)	.0259(9)	.0358(11)	.0343(12)	-.0015(8)	.0068(8)	-.0049(10)

ATOM	x	y	z	B	ATOM	x	y	z	B
H2	.663(5)	.387(2)	.313(8)	3.3	HO3*	.015(5)	.088(2)	.038(9)	3.6
H8	-.126(5)	.371(2)	.018(8)	3.3	HO5*	-.415(5)	.276(2)	-.023(8)	3.6
H1*1	.542(5)	.607(2)	1.043(8)	4.9	H1*	.156(5)	.311(2)	-.358(7)	2.5
H1*2	.585(5)	.595(2)	.717(8)	4.9	H2*	.227(4)	.217(1)	.033(6)	2.5
H1*3	.618(4)	.546(1)	.958(7)	4.9	H3*	-.043(4)	.201(1)	.088(6)	2.5
HW1	.217(4)	.014(2)	.431(6)	3.7	H4*	-.174(4)	.191(1)	-.506(6)	2.5
HW2	.075(4)	.011(2)	.521(6)	3.7	H5*1	-.423(6)	.245(3)	-.462(10)	2.5
HO2*	.150(6)	.145(3)	-.377(11)	3.6	H5*2	-.480(6)	.178(3)	-.318(10)	2.5

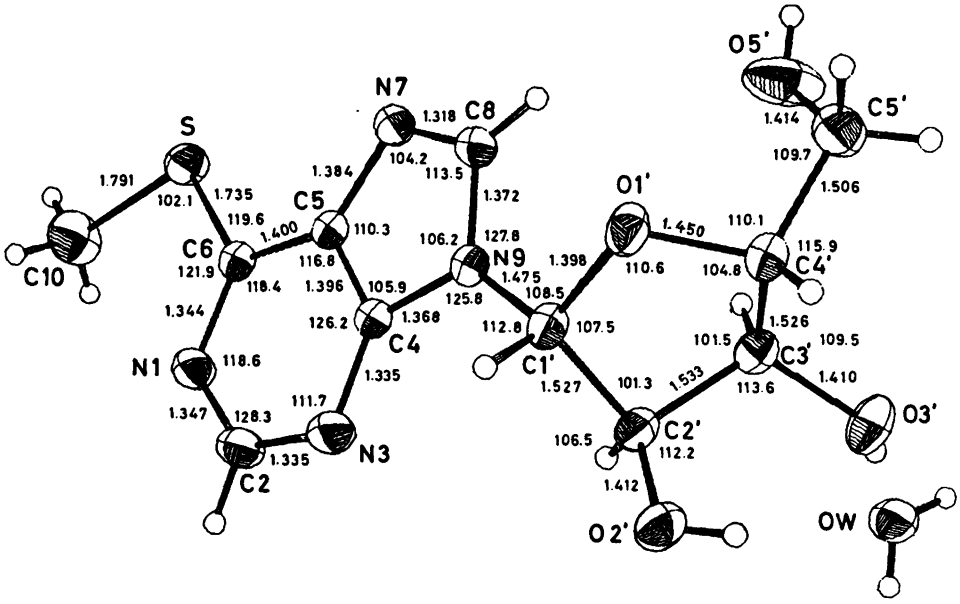

Fig. 1. Perspective view of the molecule as seen along c* showing bond lengths (Å) and angles (°). Non-hydrogen atoms are represented by thermal ellipsoids defined by the principal axes of thermal vibration and scaled to include 50 % probability.

Table 1 lists the final parameters and their estimated standard deviations. Tables of observed and calculated structure factors are available from the authors.

DISCUSSION

Fig. 1 shows the conformation, thermal ellipsoids, and bond lengths and angles involving non-hydrogen atoms. Estimated standard deviations are 0.002 Å and 0.1°, respectively. C–H bond lengths were found in the range 0.78–1.01 Å, mean value 0.92 Å; for O–H the range is 0.72–0.86 Å, mean value 0.77 Å.

The 6-methylmercaptopurine moiety. The geometry of this part of the molecule is virtually identical to that found for 6-methylmercaptopurine trihydrate.[10] The purine part is planar with no atom situated more than 0.012 Å out of the least-squares plane through the 9 atoms. The sulfur atom is also close to the plane (0.015 Å) whereas the distance from the plane to C10 is 0.143 Å; the torsional angle N1–C6–S–C10 is 5.2°. The normal to the purine plane forms an angle of 46.5° with the two-fold screw axis (*b*).

The glycosidic bond N9–C1′ is of normal length, 1.475 Å; the C1′ atom is displaced 0.112 Å from the purine plane. The dihedral angle C4–N9–C1′–O1′ is −167.9°.

The ribose ring has the C3′ *endo* conformation with the C3′ atom situated 0.58 Å from

Table 2. Hydrogen bond distances (Å) and angles (°).

D	H	A	D–A	H–A	>D–H···A
OW	HW1	N1 $(1-x, -\frac{1}{2}+y, 1-z)$	2.971	2.21	172
OW	HW2	N7 $(-x, -\frac{1}{2}+y, 1-z)$	2.912	2.06	168
O2′	HO2′	OW $(x, y, -1+z)$	2.844	2.20	145
O3′	HO3′	OW (x, y, z)	2.845	2.08	177
O5′	HO5′	N3 $(1-x, y, z)$	2.881	2.21	157

the plane through C1′, C2′, C4′, and O1′. The deviations of the atoms defining the plane are as follows: C1′ (−0.041 Å), C2′ (0.024 Å), C4′ (−0.026 Å), O1′ (0.032 Å). The configuration of the sugar ring is described by the following torsional angles about the ring bonds: 7.1° for (O1′−C1′), −28.2° for (C1′−C2′), 36.9° for (C2′−C3′), −34.0° for (C3′−C4′), and 17.3° for (C4′−O1′). The corresponding values for the phase angle of pseudorotation P and the amplitude of pucker τ_m[11] are 7.9 and 37.3°, respectively. The conformational angles of the side chain are (O1′−C4′−C5′−O5′) = −67.7° and (C3′−C4′−C5′−O5′) = 51.1°. The configuration of the ribose moiety is thus the same as the one found for cytidine.[12] Bond lengths and angles are found to be quite normal; the C1′−O1′ bond is shorter than O1′−C4′ by 0.05 Å, the three ring C−C distances are equal to 1.529 Å within twice their standard deviation and the three hydroxy C−O bonds 1.412 Å. The C−C−C, C−C−O and C−O−C angles are 101.4, 106.2, and 110.6°, respectively, as compared to 101.7, 105.8, and 109.3° given as average values for five-membered sugar rings.[13]

Molecular packing. All hydroxyl hydrogen atoms are engaged in hydrogen bonding; dimensions are given in Table 2. 6-Methylmercaptopurine riboside molecules are linked through hydrogen bonds from O5′ in one molecule to N3 in another; chains along the *a*-axis are thus formed. The other hydrogen bonds all involve water molecules which are bonded approximately tetrahedrally to nitrogen and oxygen atoms of four different 6MeMPR molecules. The hydrogen bonds involving N1, N3 and N7 are situated approximately in the purine plane. No particularly short contacts are found to the sulfur atom, and apart from the hydrogen bonds mentioned the intermolecular contacts are of the van der Waals type.

It is of interest to compare crystallographic directions with the corresponding directions obtained from magnetic resonance (ESR/ENDOR) experiments. Pugh and Alexander report the angle between the normal to the purine plane and the *b*-axis from several independent measurements of magnetic interactions to be in the range 44−48°,[4] close to the value 46.5° given above. The same authors also show that the direction cosines for either the C2−H or the C8−H bond in the *a*bc* coordinate system are (0.97, 0.23, 0.05). In the present work the corresponding numbers for C8−H are found to be (0.96, 0.27, 0.09), given in the same reference frame. The C8−H direction is thus within the standard deviation (3°) the same as that determined from the ENDOR experiments, and the combined data demonstrate that the radical formation on irradiation is due to hydrogen addition to the C2 position.

Sagstuen and Alexander report that the C6−S bond direction in a radical formed by hydrogen abstraction from the methyl group is less than 0.8° from that in the undamaged molecule.[5] Furthermore, one of the methyl carbon-proton directions is approximately 12° from the direction of the in-plane methyl carbon-proton bond in the undamaged molecule. This angle corresponds to a rotation about the S−C10 bond to increase the conjugation of the unpaired electron with the π-molecular orbital of the purine moiety.

The above results indicate that even if large amounts of energy are deposited on crystals of this highly conjugated purine molecule, it is not reasonable to expect any large changes in molecular geometry to occur, even in areas in close proximity to the ultimate center of radiation damage. This is in agreement with other magnetic resonance experiments on irradiated purine and pyrimidine compounds (Ref. 14 and references cited therein).

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Pseudomonas Cytochrome *c* Peroxidase. XII. Product Inhibition Studies

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Kinetic studies of the reaction mechanism of *Pseudomonas* cytochrome *c* peroxidase (PaCCP) were made by the method of product inhibition using oxidized cytochrome *c* (551 *P. aeruginosa*) and oxidized *Pseudomonas* azurin as products. Inhibition by the two oxidized substrates was linearly non-competitive towards the respective reduced electron donor and towards hydrogen peroxide. Although a full kinetic analysis is experimentally impossible in a peroxidase-type reaction, the results do provide some evidence in favour of an ordered reaction mechanism in which hydrogen peroxide is the first to add to PaCCP and electron donor the second.

Pseudomonas cytochrome *c* peroxidase (PaCCP, EC 1.11.1.5) isolated from *P. aeruginosa* catalyzes the peroxidatic oxidation of the *c*-type cytochromes and azurin from the same organism.[1] The reaction mechanism of PaCCP has been studied by initial velocity techniques and a sequential mechanism with ordered addition of substrates has been proposed for the enzyme.[2,3]

Alberty [4] and Cleland [5] have shown product inhibition studies to be useful in determining the mechanistic features of two-substrate reactions, particularly when both the end-products can be used. Because of the irreversible nature of the peroxidase-type reaction, however, initial velocity as well as product inhibition studies are limited to the oxidation of the electron donor only, and thus neither technique allows a full kinetic analysis of the peroxidase reaction. Nevertheless, product inhibition studies using oxidized electron donors, *i.e.* oxidized cytochrome *c* (551 *P. aeruginosa*) and oxidized azurin, as product inhibitors were undertaken to obtain additional data to confirm the proposed mechanism of the PaCCP-reaction.

The occurrence of a ternary complex in the reaction mechanism was shown by comparing maximal reaction velocities in the presence and absence of a constant concentration of oxidized cytochrome *c*.

As in the earlier studies,[2,3] all initial velocity measurements were performed in such a way that the phase of initial delay [1] was avoided, *i.e.* PaCCP was incubated with the reduced substrates before the reaction was started by addition of hydrogen peroxide.

MATERIALS AND METHODS

Pseudomonas *cytochrome c peroxidase (PaCCP)* was prepared from acetone-dried cells of *P. aeruginosa*.[6] The ratio A_{407}/A_{280} of the preparation was 4.5 and the specific activity was 100 – 105 U/mg, measured as reported earlier.[7] The concentration of PaCCP was determined spectrophotometrically using A (1 %, 1 cm) equal to 12.1 at 280 nm.[8] The molar concentration of the enzyme was calculated using a molecular weight of 43 200.[8]

Yeast cytochrome c *peroxidase (YCCP)* was prepared as described previously.[9] The ratio A_{407}/A_{280} of the preparation was 1.3. The concentration of the enzyme was determined on the basis of the total hematin content, measured as pyridine ferrohemochrome according to Paul *et al.*[10]

Horse heart cytochrome c was a commercial preparation from Sigma Chemical Co., Type III, 98 %, used without further purification. The extinction coefficient $\Delta\varepsilon_{550}$ (red.-ox.) = 19.6 mM^{-1} cm^{-1} was used for the spectrophotometric determination of the cytochrome concentration.[11]

Pseudomonas *cytochrome c-551 (Pa-cyt-551) and* Pseudomonas *azurin* were prepared from acetone-dried cells of *P. aeruginosa* by the method of Ambler [12] and Ambler and Brown.[13] The purity of the preparation [A_{551}(red.) − A_{570}(red.)/A_{280}] was 1.18 and that of azurin [A_{625}(ox.)/A_{280}] 0.49. Both azurin and Pa-cyt-551 were homogeneous on disc electrophoresis. The concentration of Pa-cyt-551 was determined spectrophotometrically applying the millimolar absorptivity $\Delta\varepsilon_{551}$(red.-ox.) = 19.0 mM^{-1} cm^{-1}, and the concentration of azurin by using ε_{625}(ox.) = 5.1 mM^{-1} cm^{-1}.[1] Cytochrome was reduced according to Yonetani and Ray [14] using anaerobic gel filtration on Sephadex G-25. Azurin was reduced with solid sodium dithionite, and excess reductant was removed by exhaustive dialysis against sodium phosphate buffer, pH 6.0, $\mu = 0.01$. Oxidized cytochrome and azurin were prepared by oxidizing with potassium ferricyanide, and excess oxidant was removed by dialysis against phosphate buffer, pH 6.0, $\mu = 0.01$.

Hydrogen peroxide solutions were prepared from Merck Perhydrol (30 % H_2O_2). Peroxide concentration was determined enzymatically with YCCP using horse heart cytochrome *c* as substrate according to Yonetani.[11]

Measurements of reaction rates. The activity of PaCCP was assayed spectrophotometrically by measuring the rate of peroxidatic oxidation of fully reduced substrates by the enzyme in sodium phosphate buffer, pH 6.0, $\mu = 0.01$. The reaction was initiated by mixing 10 µl of hydrogen peroxide solution with the reaction mixture (2.0 ml) containing varying amounts of reduced cytochrome or azurin and, in inhibition studies, oxidized cytochrome or azurin as well. The reaction was followed by recording the disappearance of the electron donor or the appearance of the product. All velocities were measured on a Cary 15 recording spectrophotometer with the cell compartment thermostated at 25 °C. Reaction mixtures were incubated at 25 °C before the reaction was initiated. Initial velocities were determined from the slopes of the recorded lines and expressed in terms of mol reduced substrate oxidized per mol of enzyme and second.

Disc electrophoresis was carried out in polyacrylamide gel according to the procedure of Maurer using the basic gel system No. 1a (pH 8.9, 7 % gel).[15] The protein bands were stained with Coomassie brilliant blue G-250 (Serva, Heidelberg) by the method of Diezel *et al.*[16]

Instruments. Spectrophotometric measurements were performed with a Beckman DU-2 spectrophotometer. Enzymatic activities were measured with a Cary 15 recording spectrophotometer. pH was measured with a Radiometer pH meter 27 fitted with a combination glass-calomel electrode. Beckman pH 7 buffer No. 3501 was used for standardization.

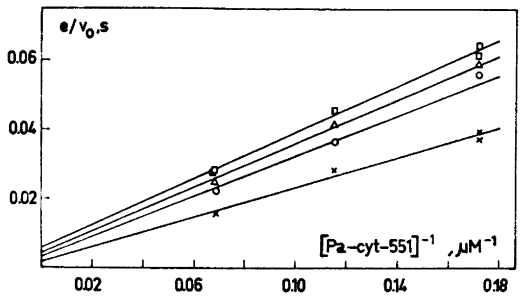

Fig. 1. Product inhibition of *Pseudomonas* cytochrome *c* peroxidase by oxidized cytochrome *c*-551 with reduced cytochrome as the variable substrate. Inhibitor concentrations were (µM): ×, 0; ○, 8.4; △, 14.6; □, 20.8. Enzyme concentration was 1.6 nM and hydrogen peroxide 81.6 µM.

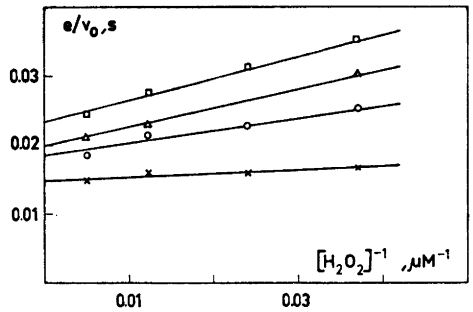

Fig. 2. Product inhibition of PaCCP by oxidized cytochrome *c*-551 with H_2O_2 as the variable substrate. Inhibitor concentrations were (µM): ×, 0; ○, 8.4; △, 14.6; □, 20.8. Enzyme concentration was 1.6 nM and reduced cytochrome 14.5 µM.

Reagents were of analytical grade if not otherwise stated.

RESULTS

The primary Lineweaver-Burk plots appeared to be linear within experimental error in all cases. Inhibition by oxidized Pa-cyt-551 towards hydrogen peroxide was non-competitive in the sense defined by Cleland,[5] which requires alteration of the slopes and intercepts of the Lineweaver-Burk plots. The inhibition towards reduced Pa-cyt-551 was non-competitive too, although it was difficult to interpret the primary plot of Fig. 1 unambiguously. However,

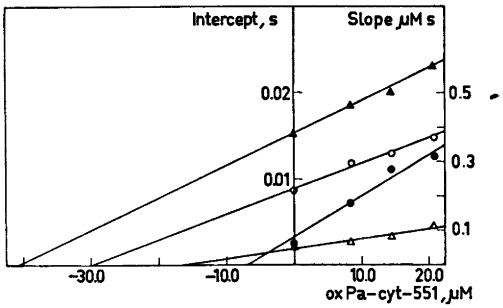

Fig. 3. Replots of slopes (○, ●) and intercepts (△, ▲) of the primary Lineweaver-Burk plots of Figs. 1 and 2 against inhibitor concentration. Open symbols represent data from Fig. 1 and solid symbols slopes and intercepts from Fig. 2. The apparent $K_{i\ intercept}$ and $K_{i\ slope}$-values calculated from the horizontal intercepts of the plot are given in Table 1.

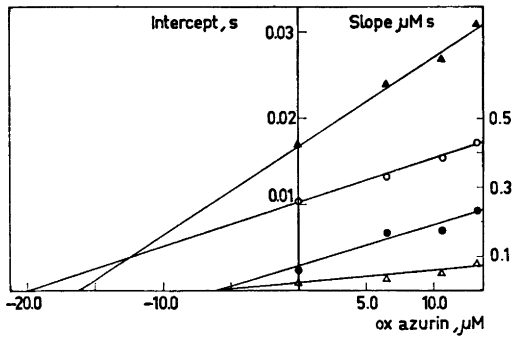

Fig. 6. Replots of slopes (○, ●) and intercepts (△, ▲) of the primary Lineweaver-Burk plots of Figs. 4 and 5 against inhibitor concentration. Open symbols represent slopes and intercepts from Fig. 4 and solid symbols those from Fig. 5. The apparent K_{ii} and K_{is}-constants calculated from the horizontal intercepts of the plot are given in Table 1.

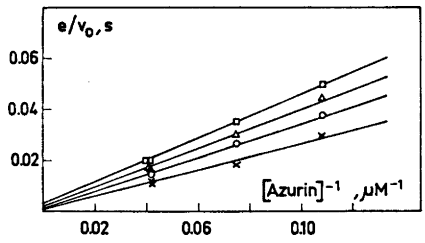

Fig. 4. Product inhibition of PaCCP by oxidized azurin with reduced azurin as the variable substrate. Inhibitor concentrations were (μM): ×, 0; ○, 6.6; △, 10.6; □, 13.2. Enzyme concentration was 1.6 nM and hydrogen peroxide 81.6 μM.

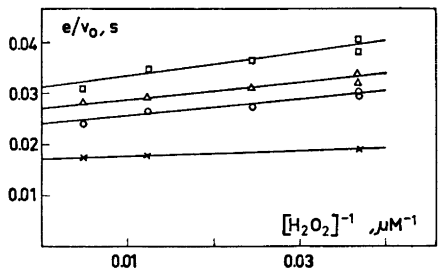

Fig. 5. Product inhibition of PaCCP by oxidized azurin with hydrogen peroxide as the variable substrate. Inhibition concentrations were (μM): ×, 0; ○, 6.6; △, 10.6; □, 13.2. Enzyme concentration was 1.6 nM and reduced azurin 13.3 μM.

the Dixon plot,[17] *i.e.* the plot of reciprocal initial velocity *versus* inhibitor concentration, derived from the data of Fig. 1 showed non-competitive inhibition towards reduced Pa-cyt-551. The Dixon plot from the data of Fig. 2 gave similar results, thus confirming the non-competitive inhibition by oxidized Pa-cyt-551 towards hydrogen peroxide. The replots of slopes and intercepts of the primary Lineweaver-Burk plots against inhibitor concentration were linear, and from the horizontal intercepts of these plots apparent inhibition constants ($K_{i\ slope}$ and $K_{i\ intercept}$) could be calculated.[5]

When oxidized azurin was used as product inhibitor, linear non-competitive inhibition was

Table 1. Inhibition constants for *Pseudomonas* cytochrome *c* peroxidase. Type of inhibition: Linear noncompetitive.

Inhibitor	Variable substrate	Fixed substrate	K_{is} μM	K_{ii} μM
ox Pa-cyt-551	Pa-cyt-551	H_2O_2	30.0	16.5
ox Pa-cyt-551	H_2O_2	Pa-cyt-551	7.0	40.8
ox azurin	azurin	H_2O_2	20.0	6.9
ox azurin	H_2O_2	azurin	6.4	16.4

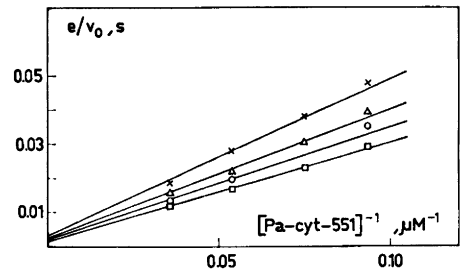

Fig. 7. A double reciprocal plot of initial rate data taken in the presence of 18.4 μM oxidized cytochrome *c*-551 as product inhibitor. Reduced Pa-cyt-551 was the variable substrate. Concentrations of the fixed substrate (H_2O_2) were (μM): ×, 7.2; △, 14.4; ○, 28.8; □, 144.0. Data were taken at 25 °C and pH 6.0 in sodium phosphate buffer $\mu = 0.01$. The PaCCP-concentration was 1.6 nM.

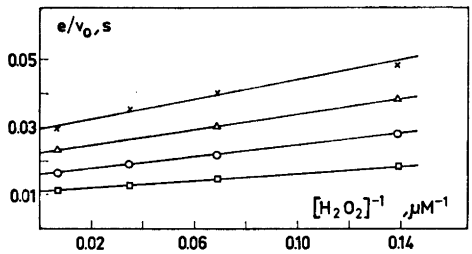

Fig. 8. A double reciprocal plot of initial rate data taken in the presence of 18.4 μM oxidized Pa-cyt-551 as product inhibitor. Hydrogen peroxide was the variable substrate. Concentrations of the fixed substrate (red Pa-cyt-551) were (μM): ×, 10.7; △, 13.3; ○, 18.4; □, 27.8. Other experimental details were as given in the legend to Fig. 7.

observed towards reduced azurin as well as towards hydrogen peroxide. The non-competitive inhibition towards reduced azurin was not immediately apparent, but Dixon plots confirmed non-competitive inhibition for both substrates. Replots of slopes and intercepts of the primary plots *versus* the concentration of oxidized azurin were linear, and the apparent inhibition constants (K_{is} and K_{ii}) calculated from the plot are given in Table 1.

Initial velocity measurements in the presence of a constant concentration of oxidized Pa-cyt-551 (18.4 μM) gave linear Lineweaver-Burk plots. The secondary plots of intercepts and

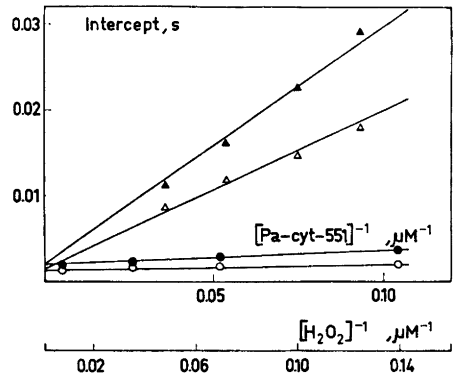

Fig. 9. Secondary plots of primary plot intercepts (see Figs. 7 and 8) *versus* the reciprocal of fixed substrate concentration. Circles, whether open or solid, represent data in which H_2O_2 was present as the fixed substrate. Triangles, whether open or solid, represent data taken with Pa-cyt-551 as the fixed substrate. Open circles or triangles represent data taken in the absence of product inhibitor. Solid circles or triangles represent data taken in the presence of oxidized Pa-cyt-551 (18.4 μM) as product inhibitor. Experimental details are given in the legend to Fig. 7.

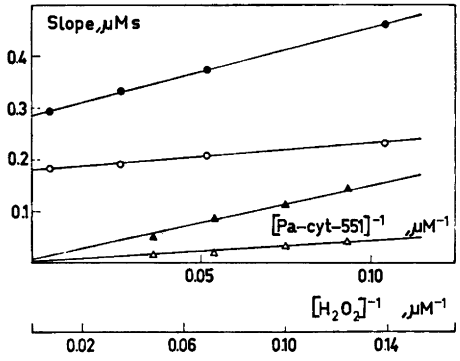

Fig. 10. Secondary plots of primary plot slopes (see Figs. 7 and 8) *versus* the reciprocal of the fixed substrate concentration. Circles, whether open or solid, represent data taken with H_2O_2 as the fixed substrate. Triangles, whether open or solid, represent data taken with Pa-cyt-551 as the fixed substrate. Open circles or triangles represent data taken in the absence of product inhibitor. Solid circles or triangles represent data taken in the presence of oxidized Pa-cyt-551 (18.4 μM) as product inhibitor. Experimental details are given in the legend to Fig. 7.

slopes are shown in Figs. 9 and 10, respectively, together with data obtained in similar experiments in the absence of the oxidized cytochrome. The results were analyzed using the initial-rate equation of Dalziel [18]

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (3)$$

The kinetic constants (ϕ_0, ϕ_1, ϕ_2, ϕ_{12}) were calculated from the secondary plots of intercepts and slopes. The kinetic constants obtained in the presence of the product inhibitor were increased compared to those of the uninhibited reaction. The reciprocal of the maximal reaction velocity, ϕ_0 calculated from Fig. 9, was 1.5×10^{-3} s, while that of the inhibited reaction ϕ_0' was 2.2×10^{-3} s. In contrast, the Michaelis constants of hydrogen peroxide and reduced cytochrome *c*-551 were essentially independent of the presence of the inhibitor.

DISCUSSION

The product inhibition of the *Pseudomonas* cytochrome *c* peroxidase reaction by oxidized cytochrome *c*-551 towards reduced cytochrome has earlier been studied in a simple experiment using one inhibitor concentration only.[2] The Lineweaver-Burk plot of this preliminary experiment was interpreted as showing competitive inhibition. The type of inhibition could not be checked in other plots because of the single inhibitor concentration used.

The present study, however, shows that inhibition by oxidized cytochrome and azurin towards their respective reduced substrates as well as towards hydrogen peroxide is non-competitive. The type of inhibition against reduced cytochrome and azurin cannot be unambiguously derived from the Lineweaver-Burk plots (Figs. 1, 4), but the occurrence of non-competitive inhibition in both cases has been verified using Dixon plots.[17]

Some qualitative conclusions can be drawn from the results even though only one product is available for inhibition studies and the kinetic analysis is thus incomplete.[5] On the basis of the intersecting initial velocity patterns, it has been suggested that the PaCCP-reaction follows a sequential reaction mechanism.[2,3] The present product inhibition study helps further to rule out a ping-pong mechanism, since such a mechanism would require competitive inhibition by oxidized cytochrome towards hydrogen peroxide, which is not the case.

The mechanisms to be more thoroughly considered are Ordered Bi Bi,[19] Rapid Equilibrium Random Bi Bi,[19] and the Theorell-Chance mechanism.[20] In a random mechanism, inhibition by one end-product against either substrate should be competitive,[5] and so this mechanism is not proposed for PaCCP. On the other hand, the non-competitive inhibition patterns against both reduced cytochrome (or azurin) and hydrogen peroxide are in accordance with an Ordered Bi Bi mechanism [5] in which cytochrome is the second substrate to add to the enzyme and oxidized cytochrome the first product to leave. If reduced cytochrome were the first substrate, and thus oxidized cytochrome the second product, inhibition should be competitive against reduced cytochrome and non-competitive against hydrogen peroxide. The data obtained do not support this sequence, and furthermore, such an order of substrate addition would make PaCCP unique among peroxidases.

According to Alberty,[4] the product inhibition method is capable of detecting ternary complexes of even very short life-times. Consequently, a distinction between an Ordered Bi Bi and Theorell-Chance mechanism can be made in experiments in which the product of the second substrate is added initially. In either mechanism adding the product of the first substrate initially has no effect on the maximal velocity. In an ordered ternary complex mechanism the effect of the product inhibitor on the maximum velocity of the reaction reveals that it is the product of the second substrate.

Non-competitive inhibition in itself implies a reduced maximal velocity in the presence of the inhibitor. Moreover, a full two-substrate kinetic study in the presence of a constant concentration of oxidized cytochrome *c*-551 showed a decreased maximal velocity compared to that of the uninhibited reaction (Fig. 9), which confirms the occurrence of a ternary complex in the PaCCP-reaction. A similar experimental arrangement has earlier been used to verify a ternary complex in the reaction of pig heart malic dehydrogenase.[21]

The results of the product inhibition studies seem to support an ordered reaction mechanism in which hydrogen peroxide adds to the peroxidase as the first substrate and electron donor as the second.

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Reaction of Sugar Esters with Hydrogen Fluoride. XIV. Rearrangement of D-Xylose and D-Lyxose Derivatives

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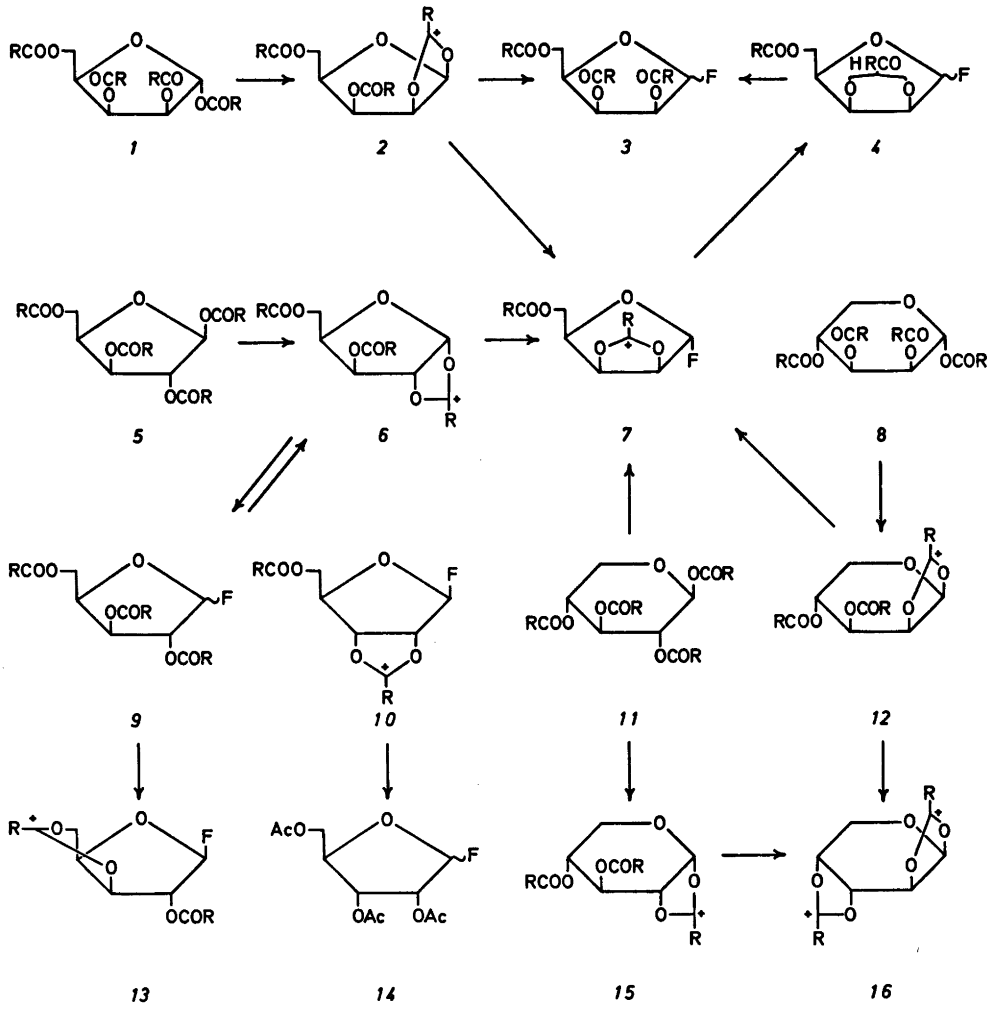
Prolonged treatment of tetra-*O*-benzoyl-α-D-xylofuranose with anhydrous hydrogen fluoride gave tri-*O*-benzoyl-α-D-lyxofuranosyl fluoride. Reaction of the tetraacetates of D-xylofuranose, D-lyxofuranose, D-xylopyranose, and D-lyxopyranose with hydrogen fluoride yielded complicated mixtures of products, which were analyzed by ^{19}F NMR spectroscopy. ^{13}C NMR spectra of some dioxolanylium ions have been measured in hydrogen fluoride solution.

In previous papers the reaction of benzoylated xylo- and lyxo-pyranoses with anhydrous hydrogen fluoride (HF) was described.[1,2] Tetra-O-benzoyl-β-D-xylopyranose (*11b*) was completely converted into a dibenzoxonium ion (*16b*) derived from D-arabinopyranose.[1] Tetra-*O*-benzoyl-α-D-lyxopyranose (*8b*) gave the same ion; but in addition it underwent ring-contraction to a lyxofuranose derivative.[2] Benzoylated and acetylated ribo- and arabino-pyranoses were converted to ribofuranose derivatives on prolonged treatment with HF.[3] In order to get a complete picture of the behaviour of pentose esters towards HF the reactions of some additional derivatives of xylose and lyxose have now been investigated.

A solution of tetra-*O*-benzoyl-α-D-lyxofuranose (*1b*) in anhydrous HF was kept at +5 °C and ^1H NMR spectra were measured at intervals. The tetrabenzoate (*1b*) reacted immediately in HF to form a product the ^1H NMR spectrum of which could not be analyzed. A ^{13}C NMR spectrum (Table 3), however, showed that it must be the 1,2-benzoxonium ion (*2b*).[4] Hydrolysis of the HF solution after 15 min gave a good yield of tri-*O*-benzoyl-α-D-lyxofuranosyl fluoride (α-*3b*). When (*1b*) was kept in HF for 4 days at +5 °C, or for *ca.* 24 h at room temperature, the initially formed ion (*2b*) disappeared and the 2,3-benzoxonium ion (*7b*) was formed as the sole product as seen from NMR spectra (Tables 1 and 3). When this solution was hydrolysed it gave a mixture of dibenzoylated lyxofuranosyl fluorides (*4b*). Benzoylation yielded (α-*3b*) and a small amount of the corresponding β-anomer (β-*3b*).

When tetra-*O*-benzoyl-β-D-xylofuranose (*5b*) was dissolved in HF at 0 °C it reacted at once and gave the 1,2-benzoxonium ion (*6b*). The ^1H NMR spectrum of this ion could not be analyzed, but ^{13}C NMR data are presented in Table 3. Hydrolysis gave a good yield of tri-*O*-benzoyl-β-D-xylofuranosyl fluoride (β-*9b*). When (*5b*) was kept in HF it underwent further reaction and after *ca.* 24 h at room temperature it was completely converted into a product which, as seen from ^1H NMR spectra of the solution, contained the 2,3-benzoxonium ion (*7b*), identical with the ion described above. However, hydrolysis and subsequent benzoylation gave a mixture of tri-*O*-benzoyl-α-D-lyxofuranosyl fluoride (α-*3b*) and tri-*O*-benzoyl-β-D-xylofuranosyl fluoride (β-*9b*) in a ratio of 2.3:1. The two products could not be separated but were identified through their ^1H and ^{19}F NMR spectra. The xylofuranosyl fluoride is not formed from unreacted (*6b*). Probably some 3,5-benzoxonium ion (*13b*) is formed together with the 2,3-ion (*7b*). Hydrolysis and benzoylation of (*13b*) will then give the tribenzoylated fluoride (β-*9b*).

The reaction of tetrabenzoyl-α-D-lyxopyranose (*8b*) with HF, which has been investigated previously,[2] has now been followed by NMR spectroscopy. A solution of (*8b*) in HF immedi-



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a, R= CH_3

b, R= C_6H_5

ately formed a compound which was different from the known tri-*O*-benzoyl-α-D-lyxopyranosyl fluoride.[2] Its spectrum could not be analyzed, but it is assumed to be the 1,2-benzoxonium ion (*12b*). On further reaction with HF for *ca.* 200 h at +5 °C (*12b*) was converted into a mixture of the 2,3-benzoxonium ion (*7b*) and the dibenzoxonium ion (*16b*). Hydrolysis at this stage has been shown to give products that may be derived from these ions.[2]

Thus the reaction of benzoylated xylo- and lyxo-furanoses, with HF is similar to the reaction of benzoylated or acetylated arabino- and ribofuranoses, which give the 2,3-dioxolanylium ion (*10*).[5] This ribofuranose ion is also formed as the only detectable product when acetylated or benzoylated arabino- and ribo-pyranoses are subjected to prolonged treatment with HF.[3] The benzoylated xylo- and lyxo-pyranoses react differently, the former giving no furanose derivative at all.[1]

Table 1. ^{1}H and ^{19}F chemical shifts and observed first order coupling constants of some pentofuranose derivatives. The pentosyl fluorides were measured in deuteriochloroform solution, the ions (*6* and *7*) in anhydrous HF.

Compound	H1	H2	H3	H4	H5, H5′	Φ_F	J_{1F}	J_{2F}	J_{4F}	J_{12}	J_{23}	J_{34}	J_{45}	$J_{45'}$	
α-3a						−122	63.0	10.0	0						
β-3a						−128.7	67.0	22.5	6.5						
α-3b	6.03	5.83	6.09	5.06	4.6−4.8	−121.0	61.0	8.5	2.2	0.8	5.4	6.0	6.0	6.0	$J_{13}=0.6$
β-3b	6.10	5.48	6.21	5.00	4.6−4.8	−133.8	67.0	21.5	6.0	3.5	5.8	5.8	6.0	6.0	
α-9a	5.95	5.13	5.56	4.74	4.31,4.13	−134.8	61.8	17.2	1.4	3.6	6.5	6.5	4.8	4.1	$J_{55'}=-12.4$
β-9b	5.96	5.67	5.95	5.09	4.5−4.8	−118.5	61.5	4.5	6.0			5.8	6.0	6.0	
α-14a						−133.1	67.7	20.7							
β-14a						−116.6	61.8	4.2	6.9						$J_{3F}=2.3$
6a	7.42	6.08	5.90							4.8	≃0	3.6			$H_3C-C^+=2.90$
7a	6.27	6.1−6.6		5	4.5−4.8		55			≃0					$H_3C-C^+=2.90$
7b	6.58	6.3−6.8		5.6	5.1−5.3		55			≃0					

In view of this it was of interest also to study the reaction of acetylated xyloses and lyxoses with HF.

Treatment of these acetates with HF for 48 h followed by acetylation gave complex mixtures of products which were not separated. ^{1}H NMR spectra of these mixtures were very complex; but since most of the products were glycosyl fluorides they could be conveniently analysed by ^{19}F NMR spectroscopy. Glycosyl fluorides give simple ^{19}F spectra with a wide range of chemical shifts,[6,7] and signals do therefore usually not overlap. Furthermore, the ^{19}F spectra were measured by pulsed Fourier technique allowing the detection of small amounts of products. In Fig. 1 is shown the ^{19}F NMR spectrum of the mixture of acetylated glycosyl fluorides which was obtained when tetra-*O*-acetyl-α-D-lyxopyranose was treated with anhydrous HF for 48 h.

When tri-*O*-acetyl-α-D-xylofuranosyl fluoride (*9a*) was dissolved in HF at +5 °C it gave at once the 1,2-acetoxonium ion (*6a*) as seen from an NMR spectrum of the solution (Table 1). When the HF solution was kept at +5 °C or at room temperature further reactions took place. When the reaction was finished after *ca.* 24 h a rather complex ^{1}H NMR spectrum was obtained. It indicated, however, that the 2,3-acetonium ions (*7a* and *10a*) were present; their signals are given in Table 1. Hydrolysis of the HF solution after 48 h reaction and acetylation of the product gave a mixture of tri-*O*-acetyl-pentosyl fluorides, which were analyzed by ^{19}F NMR spectroscopy.

It was found (Table 2) that almost equal amounts of lyxo-(*3a*) and ribo-furanosyl fluorides (*14a*) were obtained. The formation of lyxofuranosyl fluorides is analogous to the behaviour of tetra-*O*-benzoyl-D-xylofuranose (see above) and may be explained through a mechanism similar to that proposed for the rearrangement of arabinofuranose into ribofuranose derivatives.[5] The rearrangement of *9a* into a ribofuranosyl fluoride, involving an inversion at C3 and formation of the ion *10a*, was unexpected and is not in agreement with the behaviour of other sugar esters towards HF.

Treatment of tetra-*O*-acetyl-α-D-lyxofuranose (*1a*) with HF for 48 h gave a similar result, however, smaller amounts of ribofuranosyl fluorides were found (Table 2). The acetylated

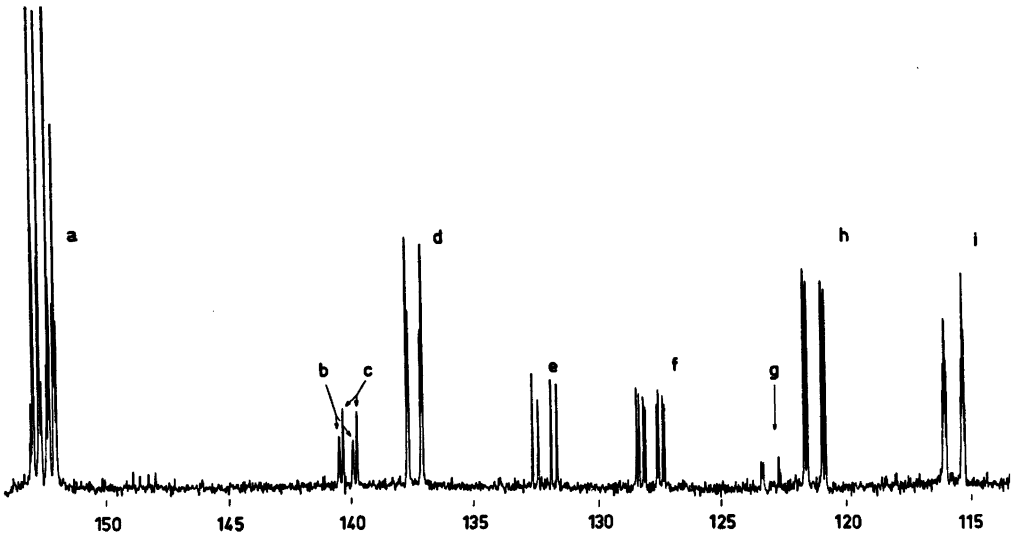

Fig. 1. ^{19}F NMR spectrum of a mixture of acetylated pentosyl fluorides. *a*, β-Ara*p*; b, β-Rib*p*; c, α-Lyx*p*; d, α-Ara*p*; e, α-Rib*f*; f, β-Lyx*f*; g, β-Ara*f*; h, α-Lyx*f*; i, β-Rib*f*.

xylo- and lyxo-pyranoses (*8a* and *11a*) were treated with HF for 48 h. This gave a very complex mixture of glycosyl fluorides (Table 2). In both cases considerable amounts of tri-*O*-acetyl-ribofuranosyl fluorides were formed, showing that the two acetates (*8a* and *11a*) react to some extent differently from the corresponding benzoates.

In view of the unexpected formation of ribofuranosyl fluorides from the acetates of xylose and lyxose it was decided to reinvestigate the reaction of acetylated arabinose and ribose with HF, using the rather sensitive ^{19}F NMR spectroscopy to analyse the products. The results (Table 2) are in agreement with those found previously [3,5] and show that both the acetylated pyranoses and furanoses give ribofuranosyl fluorides on prolonged treatment with HF. Small amounts of lyxofuranosyl fluorides were, however, also detected.

It should be pointed out that since the reaction mixtures resulting from the treatment of acetylated pentoses with HF, were analysed by ^{19}F NMR spectroscopy (Table 2) only fluorine containing products were found. Compounds which do not contain fluorine are also present.

Table 2. Relative yields (%) of acetylated pentosyl fluorides obtained by prolonged reaction of acetylated pentoses with anhydrous HF. Products were analyzed by ^{19}F NMR spectroscopy.

Starting compound (acetylated D-pentoses)	Products Pyranosyl fluorides				Furanosyl fluorides			
	β-Ara	α-Ara	α-Lyx	β-Rib	α-Rib α-*14a*	β-Rib β-*14a*	α-Lyx α-*3a*	β-Lyx β-*3a*
α-Lyxofuranose (*1a*)					8	10	72	10
Xylofuranosyl fluoride (α,β-*9a*)				4	20	31	30	15
α-Lyxopyranose (*8a*)	20	14	2	1	12	24	15	12
β-Xylopyranose (*11a*)	4	3	2		15	41	26	9
β-Ribofuranose				2	21	71	4	trace
α-Arabinofuranose	3	2		1	22	71	1	trace
β-Ribopyranose				3	21	76	trace	
β-Arabinopyranose	57	20			10	13		

The reaction shown in Table 2 were carried out with acetylated D-pentoses and the products probably belong to the D-series. However, since they were identified by NMR spectroscopy only, this has not been proved.

The ^{19}F NMR spectra of some of the glycosyl fluorides shown in Table 2 are known.[6,7] Previously unreported spectral data are presented in Table 1. Tri-*O*-acetyl-α- and -β-D-xylofuranosyl fluoride (*9a*) were obtained by brief treatment of 3,5-di-*O*-acetyl-1,2-*O*-isopropylidene-α-D-xylofuranose with HF at −70 °C followed by acetylation. Brief treatment of tetra-*O*-acetyl-β-D-ribofuranose with HF gave a mixture of the anomeric tri-*O*-acetyl-D-ribofuranosyl fluorides (α- and *β-14a*). The two anomers could not be separated, but their ^{19}F NMR data could be obtained from the mixture and are very similar to those of the corresponding benzoates.[7] Analogous treatment of tetra-*O*-acetyl-D-lyxofuranose (*1a*) gave a mixture of α- and *β-3a* from which ^{19}F NMR data were obtained.

Proton NMR spectra of a number of dioxolanylium ions in hydrogen fluoride have been measured in the course of the present and previous work.[3,4,5] ^{13}C NMR spectra have now been measured on a series of dioxolanylium ions in hydrogen fluoride solution and spectral data of ions derived from pentoses are shown in Table 3. The cationic carbon of benzoxonium ions is found at *ca.* 187 ppm, in agreement with results found for other phenylsubstituted onium ions.[8,9] The corresponding carbon in acetoxonium ions resonates at *ca.* 198 ppm (*17a* and *10a*, Table 3). The other two carbon atoms of the dioxolanylium ring are in most cases shifted downfield *ca.* 20 ppm relative to the carbon atoms of acylated sugars. A few $^{13}C-^{19}F$ coupling constants were also measured (Tables 3); they are in agreement with values found previously for glycosyl fluorides.[10]

EXPERIMENTAL

^{1}H NMR spectra and thin layer chromatography was performed as described previously.[4] ^{19}F NMR spectra were obtained at 84.68 MHz on a Bruker HX-90E instrument using pulsed Fourier technique. Position of signals (*Φ*F) are in ppm relative to internal trichlorofluoromethane. ^{13}C NMR spectra were measured at 0 °C on a Bruker WH-90 instrument. Hydrogen fluoride containing 10 % deuterium fluoride (for the deuterium lock) was used as solvent, which was contained in a Teflon sample tube. Position of signals are in ppm relative to internal $(CH_3)_3SiCH_2CH_2CH_2SO_3Na$.

*Reaction of tetra-*O*-benzoyl-α-D-lyxofuranose (1b) with HF. For 15 min.* A solution of *1b*[11] (1.16 g) in anhydrous HF (4 ml) was kept at 0 °C for 15 min. Dichloromethane was then added and the mixture was poured on ice. The organic phase was washed with aqueous $NaHCO_3$, dried and evaporated. The product (1.1 g) was purified by preparative TLC with ether-pentane (1:1) as eluent. The main fraction gave 771 mg (81 %) of pure tri-*O*-benzoyl-α-D-lyxofuranosyl fluoride (*α-3b*) as a syrup,

Table 3. ^{13}C NMR spectral data of dioxolanylium ions in anhydrous hydrogen fluoride containing 10 % deuterium fluoride.

Dioxolanylium ions	Chemical shifts (ppm) and $J^{19}F-^{13}C$) values (Hz) C1	C2	C3	C4	C5	R−C(=O)+	H_3C-C^+
2b	124.0	90.27	75.13	87.28	68.18	186.98	
7b	114.19	97.10	95.47	84.29	66.68	187.13	
	229.4	47.1					
6b	121.60	95.08	79.35	85.27	67.66	187.56	
17a[5]	122.31	89.75	74.48	83.12	67.14	198.37	18.13
17b[5]	121.08	89.82	75.91	83.12	67.72	187.50	
10a[5]	115.46	98.07	97.03	88.52	69.60	197.77	17.74
	230.9	47.10		2.9			
15b[3]	116.01	82.73	71.55	70.90	67.14	186.76	
16[3]	112.50	86.83	80.72	77.47	64.46	187.37	
						186.85	

$[\alpha]_D^{20}$ −15.5° (*c* 0.46, $CHCl_3$). Anal. $C_{26}H_{21}FO_7$: C, H. NMR data are shown in Table 1.

For 4 days. Treatment of *1b* (1.05 g) with HF for 4 d at +5 °C as described above gave 875 mg of a product which was benzoylated in the usual manner with benzoyl chloride (0.5 ml) in pyridine (5 ml). The product thus obtained (875 mg) was separated into two fractions by preparative TLC (ether-pentane 1:1). The fast moving fraction gave 483 mg (58 %) of *α-3b*, $[\alpha]_D^{20}$ −17.0° (*c* 7, $CHCl_3$). ^1H and ^{19}F NMR spectra proved its identity with the product described above. The next fraction gave 296 mg of a mixture of tri-*O*-benzoyl-*β*-D-lyxofuranosyl fluoride (*β-3b*) and the tetrabenzoate (*1b*). Crystallization from ether and recrystallization gave the pure *β*-fluoride (*β-3b*), m.p. 62–63 °C, $[\alpha]_D^{20}$ −14.13° (*c* 0.72, $CHCl_3$). Anal. $C_{26}H_{21}FO_7$: C, H. NMR data are in Table 1.

Reaction of tetra-O-benzoyl-β-D-xylofuranose (5b) with HF. For 10 min. Treatment of *5b* [12] (1.63 g) with HF (5 ml) for 10 min. at 0 °C as described above gave 1.47 g of crude product. Purification by preparative TLC (ether-pentane 1:1) gave 1.007 g (75 %) of pure tri-*O*-benzoyl-*β*-D-xylofuranosyl fluoride (*β-9b*) as a syrup, $[\alpha]_D^{22}$ +61.5° (*c* 11, $CHCl_3$). Anal. $C_{26}H_{21}FO_7$: C, H. NMR data are given in Table 1.

For 4 days. Reaction of *5b* (2.0 g) with HF (5 ml) for 4 d at +5 °C gave a crude product (1.7 g) which was benzoylated immediately. The product (1.82 g) was purified by preparative TLC (ether-pentane 1:1). The main fraction (1.113 g, 68 %) was a mixture of tri-*O*-benzoyl-α-D-lyxofuranosyl fluoride (*α-3b*) and tri-*O*-benzoyl-*β*-D-xylofuranosyl fluoride (*β-9b*) in a ratio 2.3:1 as seen from a ^{19}F NMR spectrum. ^1H and ^{13}C NMR spectra further confirmed that these two products were present. All attempts to separate them were unsuccessful.

Treatment of acetylated pentoses with anhydrous HF. The acetylated pentose (500 mg) was dissolved in HF (1 ml) and the solution was kept at room temperature for 48 h. It was then diluted with chloroform and poured on ice. The organic phase was dried ($MgSO_4$) and the solvent was evaporated. The rather unstable product was immediately acetylated with acetic anhydride in pyridine. Work up in the usual way gave a syrupy product which was analyzed by ^{19}F NMR spectroscopy. The results are shown in Table 2. ^1H NMR spectra were also measured but were in most cases too complicated to be analyzed.

Tri-O-acetyl-α- and β-D-xylofuranosyl fluoride (α- and β-9a). 3,5-Di-O-acetyl-1,2-*O*-isopropylidene-α-D-xylofuranose [13] (810 mg) was dissolved in anhydrous HF (2 ml) at −78 °C and the solution was kept for 30 min. Work up as described above followed by acetylation with acetic anhydride in pyridine gave 690 mg (84 %) of a product which was a mixture of α- and *β-9a* in a 1.4:1.0 ratio as seen from ^1H and ^{19}F NMR spectra.

The two anomers were separated by preparative TLC using ether-pentane (3:1) as eluent. The fast-moving fraction gave 240 mg (30 %) of *α-9a* as a syrup, $[\alpha]_D^{22}$ +52.5° (*c* 7.4, $CHCl_3$). Anal. $C_{11}H_{15}FO_7$: C, H. Spectral data are shown in Table 1.

The next fraction gave 180 mg (22 %) of syrupy *β-9a*, $[\alpha]_D^{22}$ +8.4° (*c* 3.9, $CHCl_3$). Anal. $C_{11}H_{15}FO_7$: C, H. NMR data were in agreement with those reported previously.[7]

Tri-O-acetyl-α- and -β-D-ribofuranosyl fluoride (α- and β-14) were obtained as a 1:3 mixture by treatment of tetra-*O*-acetyl-*β*-D-ribofuranose with HF for 10 min at room temperature as described above. ^{19}F NMR data were measured on the mixture (Table 1).

Tri-O-acetyl-α- and -β-D-lyxofuranosyl fluoride (α- and β-3a) were obtained in the same way as a mixture by brief treatment of *1a* [14] with HF.

Microanalyses were performed by NOVO analytical laboratory. The Bruker WH-90 instrument was provided by The Danish National Science Research Council.

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The Asymmetric Nature of the Conformation of 4,4,7,7-Tetramethylcyclononanone

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Dynamic ^{13}C NMR spectroscopy reveals two conformational exchange processes for this nine-membered ring. It must therefore have an asymmetric [234] conformation with the carbonyl group in a non-corner position. This explains earlier difficulties in understanding details of the ^1H spectrum on the basis of a symmetric [333] conformation with the carbonyl group in a corner position.

We have previously reported[1] that a conformational process with very high activation energy ($\Delta G^{\ddagger} = 65$ kJ/mol) is observed for 4,4,7,7-tetramethylcyclononanone by dynamic ^1H NMR spectroscopy, and that the splitting of all chemical shifts into two in the slow-exchange spectrum suggests a triangular [333] conformation * with a two-fold symmetry axis and the carbonyl group in a corner position (A, Fig. 1).

The observation of a different triangular ring skeleton [225] for cyclononanone itself in its crystalline $HgCl_2$ adduct,[3] as well as the unsymmetric non-corner position of the carbonyl group both in this adduct and for the free molecule in solution,[4] would by itself be insufficient as an argument against our assignment. The 1,4-relationship of the gem-dimethyl substituents excludes the [225] skeleton and might be expected to favour the [333] skeleton (A, Fig. 1).

There exists, however, a third type of triangular ring-conformation [234] which is unsymmetric and can also accommodate the methyl groups in corner positions,[2,5] but will place the carbonyl group next to a corner (B, Figs. 1 and 2). Such a conformation would be in better accord with recently accumulated X-ray, NMR and dipole moment data not only for other medium-ring cycloalkanones, where relief of transannular H—H 1,5-interactions is an energetic advantage, but also for large-ring cycloalkanones and "diametric" cycloalkanediones. In these latter cases only H—H 1,4-interactions (*gauche* interactions) can be invoked to explain that the carbonyl group is never at a corner but usually next to it. Thus, in addition to the data for cyclononanone already mentioned, cyclooctanone[6,7] and cyclooctane-1,5-dione[7] have both been shown to take the boat-chair or [26] conformation with the carbonyl group(s) next to a corner. Cyclodecanone has the usual [2323] conformation[4] and 4,4,8,8-tetramethylcyclodecanone the unusual [28] conformation,[8] both with the carbonyl group next to a corner. Cyclodecane-1,6-dione adopts in the crystal a [2323] conformation with both carbonyl groups next to corners,[9] and in solution a [1414] conformation with no genuine corners.[7,10] Cycloundecanone has a crystal conformation of [335] type with the carbonyl group two bonds away from a corner,[11] and such a single conformation would be in agreement with data for the solution.[7] Cyclododecanone,[4] *cis*-2,12-dibromocyclododecanone,[12] and cyclododecane-1,7-dione[10] all take the familiar [3333] conformation with the carbonyl group(s) next to a corner. Cyclotetradecanone has both in solution[4] and in the solid[13] a mixture of conformers, and these are in the solid all of the usual [3434] type with the carbonyl group in any but the corner positions. A similar situation is indicated for

* This notation indicates the number of bonds in each "side" separating the "corner" atoms.[2]

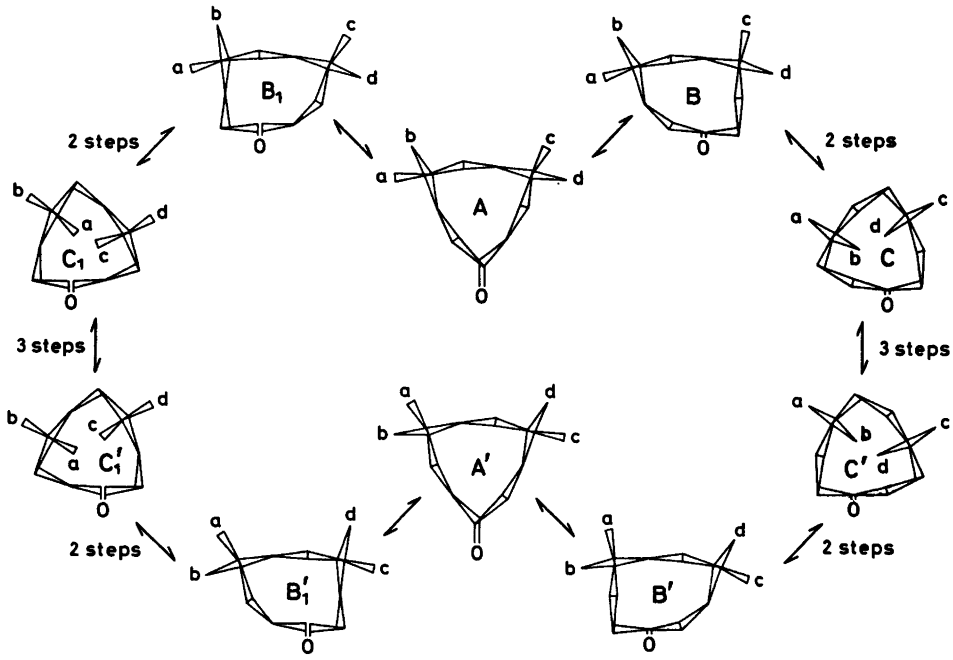

Fig. 1. Conformational interconversion scheme for 4,4,7,7-tetramethylcyclononanone.

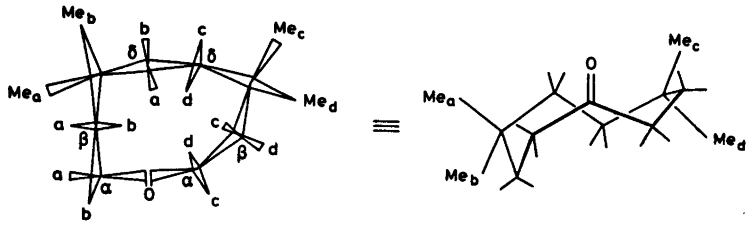

Fig. 2. The [234] conformation of 4,4,7,7-tetramethylcyclononanone.

cyclohexadecanone [4] and cyclohexadecane-1,9-dione.[10]

Some anomalies in the ^{1}H NMR-spectrum of 4,4,7,7-tetramethylcyclononanone which we have already noted [14] would become explicable on the basis of the unsymmetric conformation B exchanging rapidly even at low temperature with B_1 *via* A so as to obtain the apparent symmetry of A. Thus, the observed [14] strong geminal splitting of the chemical shifts of the β- and δ-hydrogens and the methyl groups seems unlikely in conformation A where the anisotropic carbonyl group points outwards from the corner position, but can be readily understood on the basis of an averaging between B and B_1 where in each (Fig. 2) the carbonyl group comes close to one inner β-hydrogen (c), one inner δ-hydrogen (a), and one methyl group (a). Furthermore, the very small geminal splitting of the α-hydrogens which is observed [14] in the presence of large quantities of europium shift reagent is hardly possible for conformation A, outer and inner hydrogens being very different, but may well result from an averaging between B and B_1. Finally, in view of the very low-field position observed [4,6,8] for α-hydrogens "eclipsing" the carbonyl group in other medium-ring ketones, the chemical shift is here not at sufficiently low field to correspond to the two carbonyl-

eclipsing α-hydrogens in A, but may well correspond to the averaging of only one low-field and one normal as expected for B and B_1 (a and d in Fig. 2).

NMR-SPECTROSCOPY

Attempts to observe a second process of lower energy in the 100 MHz ^1H NMR-spectrum were inconclusive; signal broadening took place in $CHCl_2F$ from −100 to −130 °C, but no resolved slow-exchange spectrum could be obtained. On the other hand, the 15 MHz ^{13}C NMR-spectrum (Fig. 3) has now in fact revealed two processes. In addition to a splitting of the methyl carbon line at 29.2 ppm, identified by its coupling, ($T_c = 37$ °C, $\Delta G^{\ddagger} = 67$ kJ/mol) corresponding to the process observed earlier in the ^1H spectrum,[1] a further broadening of most lines, especially the methyl carbon lines, was seen below −100 °C. Since the carbonyl carbon line remains sharp, as does the quaternary γ-carbon line (presumably because both γ-carbons have similar chemical shifts) and of course also signals from the solvent and TMS, the broadening is not ascribed to increased viscosity, but to the freezing of an exchange process. Since this ketone has been shown earlier[15] to be conformationally homogeneous, the only conceivable further spectral change is the splitting of all carbon lines, except that for carbonyl carbon, into just two lines. General viscosity broadening did take place on further cooling in several solvents and prevented the observation of a resolved slow-exchange spectrum. Nevertheless, the free energy barrier for this process can be estimated to be in the range 22−27 kJ/mol, assuming coalescence temperatures from −150 to −160 °C and $\Delta\nu$ from 15 to 60 Hz.*

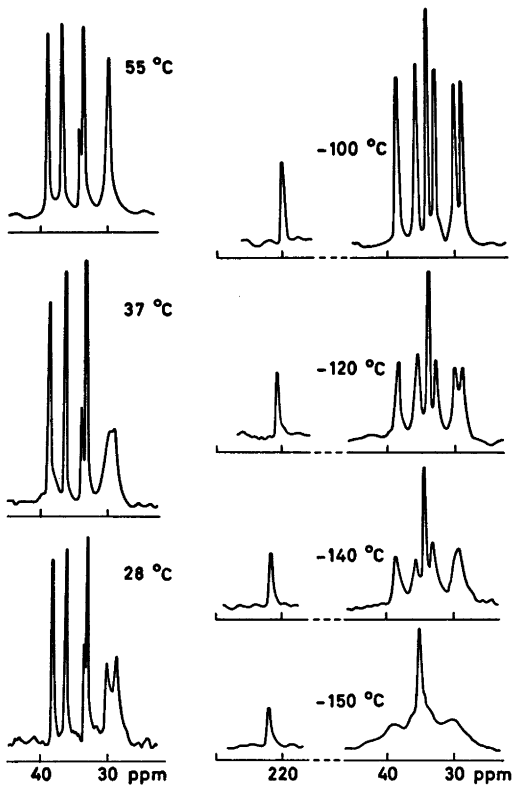

Fig. 3. 15 MHz ^{13}C NMR spectra of 4,4,7,7-tetramethylcyclononanone in $CDCl_3$ solution from 28 to 55 °C (left row) and in $CHClF_2$/$CHCl_2F$ solution from −100 to −150 °C (right row).

Thus, the real conformation lacks symmetry, in accord with conformation B, so that also this ketone shows a normal medium-ring behaviour in the sense that the carbonyl group is in a non-corner position.

THE CONFORMATIONAL EXCHANGE MECHANISM

The complete interconversion scheme in Fig. 1 consists of six repetitions of the three-step corner-moving cycle proposed earlier for full site-exchange in the [333] conformation of cyclononane.[16] In each cycle the [234] conformation occurs twice as intermediate; only the low-energy versions B are shown in Fig. 1, the other [234] intermediates with *gem*-dimethyl groups in non-corner positions are of high

* One referee is not convinced that the observed line broadening is due to an exchange process, since quaternary carbons are less influenced by viscosity effects than those carrying hydrogens. We observe, however, identical broadening in vinyl chloride and various mixtures with $CHClF_2$ and the ^{13}C spectra of similar cyclic ketones[4,7] in the same solvents show sharp lines for CH_2-carbons down to −175 °C. In the meantime the crystal structure of 4,4,7,7,-tetramethylcyclononanone has been solved by Per Groth; it reveals the proposed [234] conformation. A low-energy process *must* therefore exist, and it seems unlikely that its activation energy can be lower than the value estimated here.

energy and not populated. All [333] intermediates C, on the other hand, are shown, even though these are also of high energy.

If the stable conformation had been the symmetric one A as concluded earlier, the only possible interconversion would have been with the mirror image A′ to exchange geminal methyls and hydrogens *via* a nine-step path $A \rightleftharpoons C \rightleftharpoons C' \rightleftharpoons A'$ (or $A \rightleftharpoons C_1 \rightleftharpoons C_1' \rightleftharpoons A'$). The critical barrier should occur in one of the steps between C and C′ when the 5 – 6 bond passes *syn*-eclipsing, since then the inner methyl groups b and d interact strongly. This interaction between two methyl groups in 1,4-relationship can never be bypassed in any scheme for geminal exchange in such ring systems, and explains why the observed barrier (67 kJ/mol) is so much higher than for 4,4,8,8-tetramethylcyclodecanone (~42 kJ/mol) where the methyl groups are in 1,5-relationship and the most severe 1,4-interaction occurs between one methyl group and one hydrogen.[8] The possible importance of an additional strain due to ring size cannot be excluded, however, although nine- and ten-membered rings are not very different with respect to ring strain. The observed barrier for geminal exchange in cyclodecanone (30.6 kJ/mol) is in fact even higher than in cyclononanone (27.2 kJ/mol).[4]

If B is taken as the stable conformation, as now shown, a partial exchange with the identical conformation B_1 over two identical barriers *via* the already mentioned two-step path $B \rightleftharpoons A \rightleftharpoons B_1$ becomes possible; all *trans*-related substituents (Fig. 2) thereby become pairwise identical (a = d; b = c). One would expect these barriers to be less strongly increased by the presence of the *gem*-dimethyl groups as compared to the barrier observed [4] by ^{13}C spectroscopy in cyclononanone (21 kJ/mol) for the same type of partial exchange, as indeed now found to be the case (22 – 27 kJ/mol). Exchange of B with its mirror image B′ *via* the seven-step path $B \rightleftharpoons C \rightleftharpoons C' \rightleftharpoons B'$ over the very high critical barrier already discussed makes all *cis*-related substituents pairwise identical (a = c; b = d). Full exchange requires that both processes occur.

EXPERIMENTAL

The ^{13}C NMR spectra were recorded in a Jeol Fourier transform instrument (JNM-FX 60) operating at 15 MHz using external deuterium lock. For the higher temperatures the solvent was $CDCl_3$, for the lower temperatures a mixture of $CHClF_2$ and $CHCl_2F$. The number of pulses varied from 400 to 900, pulse intervals were 4 s. The lines were identified by their coupling pattern in the off-resonance decoupled spectrum. Ppm is given relative to tetramethylsilane.

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Structure and Hydrogen Bonding in the Enol Form of 5,6-Diacetyl-2,9-dimethyl-4,7-decanedione in the Solid State

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Low temperature (−165 °C) diffractometer data for 5,6-diacetyl-2,9-dimethyl-4,7-decanedione, $C_{16}H_{26}O_4$, were collected to $2\theta = 70°$ using graphite-monochromated Mo$K\alpha$ radiation. There are 2 molecules in a $P\bar{1}$ cell with dimensions $a = 8.938(1)$, $b = 9.406(1)$, $c = 11.030(2)$ Å, $\alpha = 67.09(1)$, $\beta = 70.26(1)$, $\lambda = 74.57(1)°$. Full matrix least-squares refinements have yielded a conventional R value of 0.040. The compound crystallizes in the dienol form with two short, asymmetric, intramolecular hydrogen bonds of lengths 2.457(1) and 2.438(1) Å. A slight double- and single- bond localization exists in the enol rings.

Interest in the structural chemistry of enolized β-diketones stems from the fact that these compounds possess a short intramolecular hydrogen bond. Structure determinations of these compounds differ on the issue of the symmetry of the hydrogen bond. From gas phase electron diffraction studies it has been deduced that the enol ring of acetylacetone,[1,2] and its trifluoro [1] and hexafluoro derivatives [3] possess C_{2v} symmetry, implying symmetrical hydrogen bonds. X-Ray and neutron diffraction investigations seem to indicate that this is the exception rather than the rule in the solid state. Evidence of C_{2v} symmetry has hereto only been obtained in the crystal structure of bis(*m*-bromobenzoyl)methane [4] whereas small, though significant, departures from such symmetry have been found in the crystal structures of dibenzoylmethane,[5,6] benzoylacetone,[7] and tetraacetylethane.[8] In all enolized β-diketones so far studied in the solid state, there are intramolecular non-bonded repulsions which may account for the relative broad range in hydrogen bond lengths observed (2.45 − 2.50 Å). A crystal structure determination of 5,6-diacetyl-2,9-dimethyl-4,7-decanedione was carried out to study the influence from such interactions on the enol ring geometry. A low temperature study was chosen in order to increase the amount of data and to minimize the thermal motions.

EXPERIMENTAL

Synthesis. The synthesis of the title compound was carried out by a procedure slightly modified from that used for tetraacetylethane.[9] Commercially obtained 6-methyl-2,4-heptanedione (*1*) was first converted into its sodium salt by addition of a stoichiometric amount of sodium hydride in ether. To the resulting suspension a slight excess of finely granulated I_2 was slowly added. When the reaction had ceased, ether was added to dissolve the crude reaction product, and sodium iodide was filtered off. By slow evaporation large transparent colourless crystals of 5,6-diacetyl-2,9-dimethyl-4,7-decanedione (*2*) were formed, m.p. 96 °C. The yield was 60 − 70 %. (Synthesis of several other β-diketone dimers have also successfully been carried out by this procedure. The yield of tetraacetylethane was found to be higher by this modified procedure than by that previously reported.[9]) IR and NMR spectroscopy on *2* as well as *1* confirmed the reaction product to be 5,6-diacetyl-2,9-dimethyl-4,7-decanedione. The NMR spectra of *1* and *2* were virtually identical, except for the CH_2 (keto form) and vinyl-CH resonances in the spectrum of *1*, which were absent in the spectrum of *2*, and that the broad enol-OH signal at δ 15.4 in the spectrum of *1* reappeared as a sharp signal at δ 17.0 in the spectrum of *2*.

X-Ray crystallography. The compound was recrystallized from ether and well developed

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crystals were obtained. Preliminary film investigations revealed triclinic symmetry. A nearly spherical crystal with approximate diameter 0.35 mm was arbitrarily mounted on the SYNTEX $P\bar{1}$ diffractometer equipped with an Enraf-Nonius gas-flow cooling device, modified by H. Hope. The temperature in the stationary N_2 stream was -165 °C at the crystal site. Cell dimensions at -165 °C were determined by a least-squares adjustment of the setting angles of 30 high-angle reflections. Intensity data were collected with graphite-monochromated Mo$K\alpha$ radiation ($\lambda = 0.71069$ Å) and the $\omega - 2\theta$ scanning mode. The scan speed varied from 2 to 4°/min (in 2θ), dependent on intensity calculated from a fast prescan. A symmetrical scan range of 2° corrected for spectral dispersion was applied and the ratio of the total background time to the time of integration was 0.7. Data were collected in one hemisphere of the reciprocal lattice in two steps. In the second step ($40° \leqslant 2\theta \leqslant 70°$) a rejection level was applied so as to avoid measurements of reflections with intensities less than $\sim 5\sigma(I)$. The intensities of three standard reflections were measured at regular intervals showing no significant decline in intensity. A total of 4840 reflections were measured of which 4663 had intensities greater than $2\sigma(I)$, estimated from counting statistics, and were considered to be observed. The remaining 177 were rejected from the structure refinement. Corrections for Lorentz and polarization effects were applied to the intensities and their e.s.d. and a 2 % uncertainty due to instrument instability was included in the latter.

Structure solution and refinements. Statistical tests strongly indicated the space group $P\bar{1}$. The structure was solved by direct methods [10]* and refined by full-matrix least-squares techniques. The function minimized was $M = \sum w(F_o - F_c)^2$, $w = \sigma(F_o)^{-2}$. The coordinates of the hydrogen atoms were calculated and included in the refinement with isotropic thermal parameters. Refinements terminated with $R_w = 0.051$, $R = 0.041$, and $S = 2.55$.** A refinement involving only the heavy atom parameters and the 2700 structure factors for which $\sin\theta/\lambda > 0.55$ Å^{-1}, was finally carried out. The values arrived at were $R_w = 0.039$, $R = 0.038$, and $S = 1.38$. However, only small parameter shifts ($< 2.0\sigma$) were obtained by exclusion of low angle data. The following calculations, as well as the discussion, are based upon parameters from the latter refinement except for results involving hydrogen atoms. These are derived from the refinement of the full data set. Atomic scattering factors were those of Doyle and Turner [12] for carbon and oxygen atoms; for the hydrogen atoms those of Stewart, Davidson, and Simpson.[13] Positional and thermal parameters are given in Table 1, and atom numbering

* Unless otherwise stated programs used are described in Ref. 11.

** Standard deviations of unit weight $S = [\sum W(F_o^2 - F_c^2)^2/(m-n)]^{\frac{1}{2}}$

Table 1. Fractional coordinates and thermal parameters with estimated standard deviations. Expression for anisotropic vibration is: $\exp[-2\pi^2(h^2a^{*2}U11 + \cdots + 2klb^*c^*U23)]$. Hlmn is bonded to Clm.

ATOM	X	Y	Z	U11	U22	U33	U12	U13	U23
O1A	1.86778(18)	.67196(11)	-.03827(8)	.0147(2)	.0235(3)	.0169(2)	-.0042(2)	.0016(2)	-.0007(2)
O2A	1.18392(9)	.66725(13)	.17453(9)	.0185(2)	.0348(4)	.0212(3)	-.0052(2)	-.0005(2)	-.0140(3)
O1B	.44269(11)	.95967(11)	.28476(11)	.0163(3)	.0198(3)	.0268(3)	.0023(2)	-.0058(2)	-.0075(2)
O2B	.41818(9)	.68439(18)	.32672(9)	.0186(2)	.0218(3)	.0222(3)	-.0028(2)	-.0015(2)	-.0064(2)
C1A	.81143(13)	.71141(13)	-.07677(10)	.0215(3)	.0288(3)	.0139(3)	-.0024(3)	-.0058(2)	-.0055(2)
C2A	.91489(11)	.78161(10)	.09928(9)	.0165(3)	.0143(3)	.0121(2)	-.0037(2)	-.0016(2)	-.0038(2)
C3A	.84546(10)	.72237(10)	.13892(8)	.0122(2)	.0149(3)	.0117(2)	-.0036(2)	-.0014(2)	-.0042(2)
C4A	.94939(10)	.78112(11)	.21835(9)	.0113(3)	.0199(3)	.0141(3)	-.0048(2)	-.0018(2)	-.0065(2)
C5A	.88591(12)	.71015(15)	.36088(10)	.0138(3)	.0321(4)	.0154(3)	-.0088(3)	-.0009(2)	-.0105(3)
C6A	1.88422(11)	.74959(13)	.41134(10)	.0145(3)	.0225(3)	.0159(3)	-.0049(2)	-.0043(2)	-.0071(2)
C7A	.93533(16)	.73002(18)	.56313(12)	.0253(4)	.0352(5)	.0161(3)	-.0068(3)	-.0053(3)	-.0102(3)
C8A	1.84883(17)	.91514(16)	.33889(14)	.0267(4)	.0246(4)	.0259(4)	-.0118(3)	-.0002(3)	-.0096(3)
C1B	.68888(16)	1.85237(12)	.86674(12)	.0256(4)	.0142(3)	.0225(3)	-.0058(3)	-.0038(3)	-.0039(2)
C2B	.59426(12)	.92156(11)	.15765(10)	.0174(3)	.0147(3)	.0161(3)	-.0013(2)	-.0046(2)	-.0052(2)
C3B	.66985(10)	.76262(10)	.19068(9)	.0115(2)	.0134(2)	.0131(2)	-.0019(2)	-.0026(2)	-.0048(2)
C4B	.57139(10)	.64728(11)	.27656(9)	.0112(2)	.0156(3)	.0143(3)	-.0029(2)	-.0027(2)	-.0044(2)
C5B	.63598(11)	.47598(11)	.31777(10)	.0145(3)	.0145(3)	.0177(3)	-.0043(2)	-.0049(2)	-.0024(2)
C6B	.52833(12)	.37378(12)	.32892(11)	.0176(3)	.0191(3)	.0185(3)	-.0084(2)	-.0012(2)	-.0059(2)
C7B	.59164(22)	.28199(15)	.38884(15)	.0421(6)	.0166(3)	.0288(4)	-.0186(4)	-.0109(4)	-.0023(3)
C8B	.48558(16)	.41458(16)	.19164(14)	.0244(4)	.0277(4)	.0265(4)	-.0048(3)	-.0184(3)	-.0121(3)

ATOM	X	Y	Z	B	ATOM	X	Y	Z	B
H1A1	.8764(19)	.6754(18)	-.1523(17)	4.4(2)	H1A2	.7556(19)	.8145(19)	-.1863(16)	4.4(4)
H1A3	.7293(19)	.6468(18)	-.0241(16)	4.4(4)	H5A1	.8562(17)	.6845(17)	.4210(14)	3.3(2)
H5A2	.7855(18)	.7865(16)	.3667(14)	3.3(4)	H6A	1.1913(15)	.6777(14)	.4617(12)	2.5(2)
H7A1	1.0115(19)	.7589(18)	.5976(16)	3.5(2)	H7A2	.9118(19)	.6247(28)	.6172(17)	3.5(4)
H7A3	.8370(20)	.8079(19)	.5782(16)	3.5(4)	H8A1	1.1171(20)	.9398(18)	.3639(16)	3.3(2)
H8A2	.9441(20)	.9928(19)	.3408(17)	3.3(4)	H8A3	1.0833(19)	.9285(18)	.2318(17)	3.3(4)
H1B1	.6288(20)	1.1465(19)	.0499(16)	6.3(2)	H1B2	.7356(19)	1.0410(19)	-.0187(17)	6.3(4)
H1B3	.7662(20)	1.0567(18)	.0992(17)	6.3(4)	H5B1	.6548(17)	.4451(16)	.4135(15)	2.9(2)
H5B2	.7421(17)	.4586(15)	.2522(14)	2.9(4)	H6B	.4181(15)	.3940(14)	.3955(13)	2.3(2)
H7B1	.5251(19)	.1332(19)	.3879(16)	4.2(2)	H7B2	.6125(19)	.1773(18)	.4676(17)	4.2(4)
H7B3	.6972(21)	.1883(18)	.3142(17)	4.2(4)	H8B1	.4160(20)	.3427(19)	.1998(16)	3.3(2)
H8B2	.5898(20)	.3993(18)	.1191(17)	3.3(4)	H8B3	.4387(19)	.5263(19)	.1595(16)	3.3(4)
H0A	1.1138(22)	.6618(21)	.8659(19)	6.4(4)	H0B	.3976(22)	.8873(23)	.2878(18)	6.5(4)

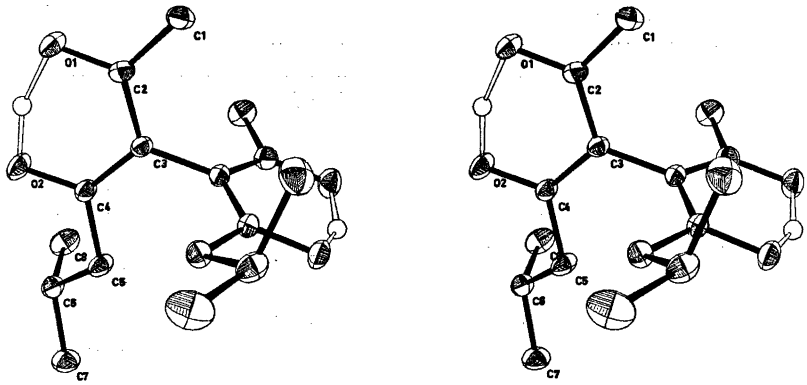


Fig. 1. Stereoscopic illustration of the molecule, showing atom numbering scheme. Thermal ellipsoids are scaled to include 50 % probability [Johnson, C. K. *ORTEP* ORNL-3794, Oak Ridge National Laboratory, Oak Ridge 1965].

is shown in Fig. 1. Bond lengths and angles are given in Table 2. Estimated standard deviations were derived from the final correlation matrix of least-squares refinements.

CRYSTAL DATA

5,6-Diacetyl-2,9-dimethyl-4,7-decanedione $C_{16}H_{26}O_4$, triclinic; $P\bar{1}$, $a = 8.937(1)$, $b = 9.406(1)$, $c = 11.030(2)$ Å, $\alpha = 67.09(1)$, $\beta = 70.26(1)$, $\gamma = 74.57(1)$, $V = 794.2$ Å^3. (Dirichlets reduced cell), $D_{obs} = 1.14$ g cm^{-3}, $D_{calc} = 1.18$ g cm^{-3}, $Z = 2$, $M = 282.4$ g mol^{-1}, $F(000) = 308$.

RESULTS AND DISCUSSION

The molecule crystallizes in the *cis*-dienolic form and the structure therefore comprises two independent determinations of the enol system (A and B). The π-electrons in the enol rings appear to be almost entirely delocalized, although a slight, but significant, single and double bond fixation is apparent. Except for the C1 – O1 bonds, equivalent bonds between heavy atoms in the enol rings agree to within twice their combined estimated standard deviations. These bond lengths correspond closely to those given in a recent neutron diffraction study of tetraacetylethane.[8] The somewhat shorter C – O bonds of the neutron study may be explained by the higher thermal motions of the oxygen atoms at room temperature. The short intramolecular hydrogen bonds of the enol rings [O1A – O2A; 2.457(1) Å, O1B – O2B; 2.438(1) Å], agree reasonably well with that of tetraacetylethane [2.450(3) Å]. In agreement with the findings in the neutron study of tetraacetylethane, the protons HA and HB appear to be located asymmetrically in these bonds. The observed deviations from C_{2v} symmetry in the heavy atom positions are consistent with the asymmetries in the proton positions. The hydrogen atoms in both enol rings thus seem to be more closely attached to the oxygen atoms tied to the carbon atom carrying the more branched substituent. This is to be expected since a hydrogen atom at this position would tend to be somewhat less acidic. The difference in the asymmetry of the proton positions in the two enol rings is not significant.

As may be seen from Table 2, the $sp^3 - sp^3$ and $sp^2 - sp^2$ carbon-carbon bond lengths are normal, with mean values of 1.528 and 1.499 Å, respectively. The average angle at sp^3-carbon atoms is 111.4°. C – H bond lengths range from 0.90 to 1.04 Å with estimated standard deviations of 0.03 Å. C – C – H and H – C – H angles are found between 104 and 119° (e.s.d. ~2°).

Least-squares planes through the enol rings and surrounding atoms are given in Table 3. Deviations from planarity probably arise from non-bonded repulsions (see below). The angle between the normals to the enol ring planes was calculated to be 85.7°. A rigid-body motion analysis [14] for each of the enol rings was carried out including the atoms in plane 2A and B. The good agreement between observed and calculated vibration tensor elements (A; $\Delta U_{ij} = 0.0008$ Å^2, B; $\Delta U_{ij} = 0.0007$ Å^2) suggests that

Table 2. Bond distances and bond angles. Upper values from the refinement with an inner cut off at sin $\theta/\lambda = 0.55$, lower values from the refinement of all data.

Bond distances (Å)	A	B	Bond angles (°)	A	B
C1 – C2	1.492(1) 1.490(2)	1.498(1) 1.495(2)	C1 – C2 – C3	121.5(1) 121.7(1)	121.8(1) 121.9(1)
C2 – C3	1.422(1) 1.420(1)	1.422(1) 1.417(1)	C2 – C3 – C4	118.2(1) 118.5(1)	118.0(1) 118.3(1)
C3 – C4	1.409(1) 1.406(1)	1.407(1) 1.405(1)	C2 – C3 – C3′	121.0(1) 121.0(1)	120.2(1) 120.2(1)
C4 – C5	1.508(1) 1.508(2)	1.499(1) 1.499(2)	C3 – C4 – C5	121.7(1) 121.9(1)	122.9(1) 123.6(1)
C5 – C6	1.528(1) 1.527(2)	1.535(1) 1.534(2)	C4 – C5 – C6	115.0(1) 115.3(1)	113.6(1) 113.8(1)
C6 – C7	1.530(2) 1.525(2)	1.523(2) 1.524(2)	C4 – C3 – C3′	120.7(1) 120.5(1)	121.8(1) 121.5(1)
C6 – C8	1.523(2) 1.522(2)	1.529(2) 1.525(2)	C5 – C6 – C7	109.6(1) 109.8(1)	109.6(1) 109.6(1)
C3A – C3B	1.482(1) 1.487(1)		C5 – C6 – C8	111.3(1) 111.3(1)	111.0(1) 111.0(1)
O1 – C2	1.288(1) 1.289(1)	1.282(1) 1.281(1)	C7 – C6 – C8	110.2(1) 110.3(1)	110.6(1) 110.5(1)
O2 – C4	1.295(1) 1.295(1)	1.298(1) 1.299(1)	O1 – C2 – C3	121.4(1) 121.1(1)	121.4(1) 121.4(1)
O1···O2	2.457(1) 2.454(1)	2.439(1) 2.439(1)	O2 – C4 – C3	121.6(1) 121.5(1)	121.3(1) 121.0(1)
O2 – H	1.20 1.20(2)	1.08 1.08(2)	O1 – C2 – C1	117.2(1) 117.2(1)	116.7(1) 116.7(1)
O1···H	1.30 1.30(2)	1.42 1.42(2)	O2 – C4 – C5	116.7(1) 116.5(1)	115.8(1) 115.7(1)
			C2 – O1···H	99.4	100.1
			C4 – O2 – H	99.9	103.3
			O1···H – O2	160. 160.(1)	156. 156.(1)

the enol rings may be regarded oscillating rigid bodies. Corrections in bond lengths range from 0.002 to 0.003 Å and have not been applied. These do not, however, alter the observed asymmetries in the enol ring bond lengths. The principal axes of the thermal vibration ellipsoids for the individual carbon and oxygen atoms were also calculated. Maximum root mean squares amplitudes range from 0.139 to 0.229 Å for carbon atoms and 0.174 to 0.208 Å for oxygen atoms.

The shortness of the intramolecular hydrogen bond in bis-3-(6-methyl-2,4-heptanedione) is essentially due to various short non-bonded repulsive contacts between the bulky substituents at the α position and the alkyl substituents

Table 3. Deviations (Å) from least-squares planes. For atom numbering see Fig. 1. Deviations of atoms not defining the planes in parentheses.

	1 A	2 A	1 B	2 B
O1	0.011	0.028	−0.004	0.000
O2	−0.006	0.000	0.005	0.021
C1	(−0.103)	−0.073	(0.013)	−0.011
C2	−0.020	0.001	0.004	−0.006
C3	0.012	0.029	0.003	−0.010
C4	0.002	0.011	−0.009	−0.007
C5	(−0.065)	−0.060	(−0.022)	−0.023
C3*[a]	(0.033)	0.054	(0.061)	0.030

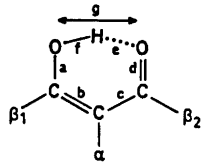
[a] The C3 atom of the adjacent enol ring.

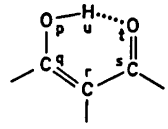
at the β positions. In particular the contacts from C3A to C2B (2.51 Å), to C4B (2.52 Å), and to H5B2 (2.56 Å)*, and contacts from C3B to C2A (2.53 Å), to C4A (2.51 Å), and to H5A2 (2.59 Å) lead to decrease in the internal angles at C3A and C3B from the expected value of 120 to 118.2 and 118.0°, respectively. On the other hand, the internal angles at C2 A,B and C4 A,B are opened as a result of non-bonded repulsions between the oxygen atoms, and repulsions between the enolic proton and C2 and C4. The enol ring geometry represents a compromise between these forces and as a result the hydrogen bond is shorter than the equilibrium distance for this type of hydrogen bond, and there is an increase of conjugation in the alternating bond system. The enolic proton is "pinched" between the two oxygen atoms – its presence reduces the oxygen-oxygen repulsive forces.

The effect of non-bonded repulsions on the enol ring geometry becomes evident when systems carrying large substituents in the α- and β-positions are compared. Table 4 gives a summary of bond distances and angles from some of the more accurate studies so far reported. In benzoylacetone [9] and dibenzoylmethane [5,6] the phenyl substituents are almost coplanar with the enol ring, the steric repulsions from these substituents resist the opening of the enol ring angle adjacent to this group (q and s). Repulsions are therefore relieved by opening the central angle (r). In benzoylacetone there is only one phenyl substituent and the

* H5B2 is attached to C5B.

Table 4. Bond distances and angles in some *cis*-enolized β-diketones.





Compound	a	b	c	d	e	f	g	Ref.
Tetraacetylethane	1.289(3)	1.402(3)	1.423(3)	1.277(3)	1.319(9)	1.194(9)	2.450(4)	8
5,6-Diacetyl-2,9--dimethyl-4,7-decane-dione	1.294(1) 1.299(1)	1.409(1) 1.407(1)	1.422(1) 1.422(1)	1.289(1) 1.282(1)	1.30 1.42	1.20 1.08	2.457(1) 2.439(1)	— —
Dibenzoylmethane	1.304(2)	1.382(3)	1.408(3)	1.287(3)	1.28	1.22	2.468(2)	6
Benzoylacetone	1.294(2)	1.376(3)	1.401(3)	1.276(2)	1.40	1.18	2.498(2)	7

Compound	p	q	r	s	t	u	Ref.
Tetraacetylethane	102.8	121.7	117.9	121.6	101.6	154.3	8
5,6-Diacetyl-2,9--dimethyl-4,7-decane-dione	99 100	121.6 121.3	118.2 118.0	121.4 121.4	100 103	160. 156.	— —
Dibenzoylmethane	100.0	120.3	121.1	119.8	100.	159.	6
Benzoylacetone	104.	120.4	121.9	120.6	102.	151.	7

hydrogen bond in this compound [2.498(2) Å] is therefore longer than that in dibenzoylmethane [2.460(2) Å], although not as long as that of the *trans*-enol arrangement of dimedone [2.593(2) Å],[15] where non-bonded intermolecular repulsions preferentially would give an elongation of the O – O distance.

Some other consistencies and trends may also be pointed out. Although only moderate agreement between corresponding bonds is found, all these systems possess a high degree of conjugation in the essentially planar enol rings. The compounds of Table 4 have all asymmetric hydrogen bonds, with deviations from C_{2v} symmetry in the heavy atom skeleton consistent with the asymmetry in the proton positions. As already mentioned in the crystal structure of bis(*m*-bromobenzoyl)methane a crystallographic symmetrical hydrogen bond has been found (R(O---O) = 2.47 Å).[4] In fact, several of the above compounds (Table 4) have hydrogen bonds shorter than that of bis(*m*-bromobenzoyl)methane. Probably small differences in the crystallographic or chemical environment are responsible for the asymmetries found in these compounds. The hydrogen bond geometry in the enol rings of β-diketones is very similar to that found in the neutron diffraction determination of $\{Ni(C_5H_{11}N_2O_2)H\}^+.Cl^-.H_2O$,[16] where it was concluded that the proton potential has one broad, flat minimum which is slightly shifted towards one of the oxygen atoms.

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Secogalioside, an Iridoid Glucoside from *Galium album* Mill. and ^{13}C NMR Spectra of some Seco-iridoid Glucosides

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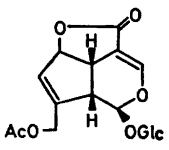
The known glucosides asperuloside and arbutin have been isolated from *Galium album*, in addition to a new iridoid glucoside, named secogalioside. The structure and the absolute configuration have been determined by spectroscopic methods, and by relating secogalioside to the glucoside sweroside, of known absolute configuration. Three other species of *Galium* and a hybrid, *G. verum* × *album*, were found to be devoid of the new compound.

^{13}C NMR spectra of some seco-iridoid glucosides and glucoside acetates have been recorded. Assignments of signals have been made by using general principles, as well as coupled and selectively decoupled spectra.

The genus *Galium* (Rubiaceae) is a known source of iridoid glucosides.[1–3] Asperuloside (*1*), monotropein (*2*) and "Galium glucoside" (*3*) have been reported from the genus. We have found that *Galium album* Mill. (= *G. mollugo* auct) contains asperuloside (*1*) and arbutin (*4*). A third compound, present in amounts equal to those of *1* and *4*, is a novel seco-iridoid glucoside ($C_{17}H_{24}O_{12}$), isolated as an amorphous foam. It is shown that the compound, for which we propose the name secogalioside, has the structure *5*.



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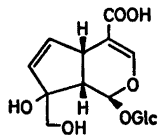

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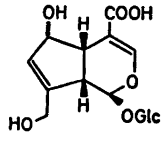

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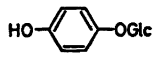

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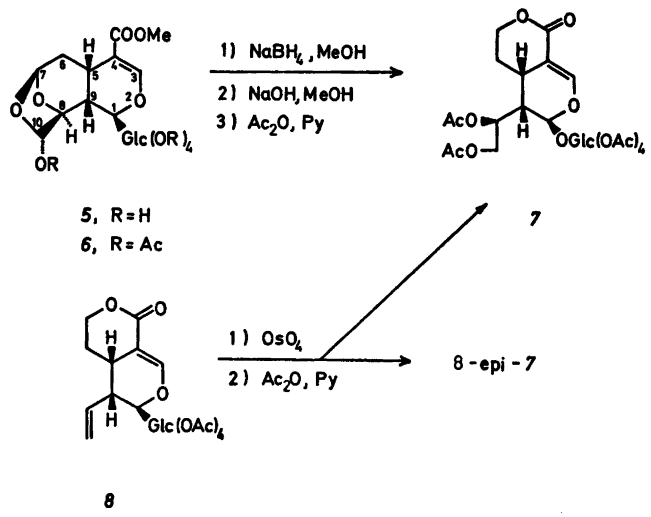
The ^1H NMR spectrum of *5* (Table 1) exhibited absorptions typical of iridoid glucosides. Signals at δ 7.58, 5.59 and 3.73 could be assigned to H-3, H-1 and COOMe, respectively. H-1 appeared as a doublet ($J_{1,9}$ 8 Hz), and successive decoupling experiments led to assignments of H-9, H-5, H-6ax, H-6eq, H-7 and H-8 (see Experimental). A singlet at δ 5.65 could not be assigned at this stage. However, a structure resembling that of 7-β-morroniside [4–6] (*9a*) was likely.

The ^1H NMR spectrum of the crystalline pentaacetate *6* resembled that of *5*; downfield shifts were observed only for the glucose protons (2′ – 6′) and for the unassigned proton (δ 0.64), indicating a position of the latter on a hemiacetalic centre.

The ^{13}C NMR spectra of *5* and *6* (see Table 2) showed four absorptions in the region 90 – 105 ppm, probably arising from as many acetalic (or hemiacetalic) centres in the molecules, *viz.* C-1, C-7, C-1′ and the unassigned carbon atom (see the discussion).

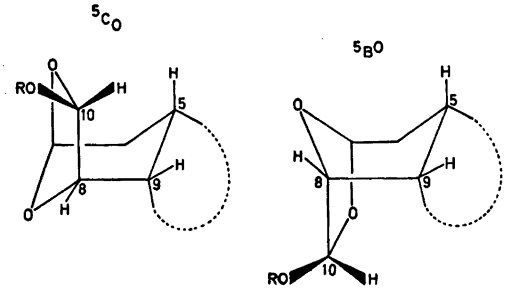
The information obtained seemed best to be accommodated in a structure such as *5*. The absolute configurations at C-1, C-5, C-9 and the presence of a β-D-glucopyranose moiety were established by relating *5* to sweroside tetraacetate (*8*), of known absolute configuration,[7] in the following way: Treatment of *5* with sodium borohydride in methanol converted the masked aldehyde functions at C-7 and C-10 to the corresponding primary alcohols. Saponification followed by neutralization



induced lactone formation, and the crude mixture was acetylated to give, after separation, the crystalline hexaacetate *7*.

Sweroside tetraacetate (*8*) by treatment with osmium tetroxide, yielded an inseparable mixture of the two epimeric diols. However, separation was achieved by chromatography of the acetylated product and two crystalline hexaacetates were obtained. One of these was indistinguishable from *7* derived from *5*.

Only the configurations at C-8 and C-10 now remained to be established. Models showed that two different structures were equally compatible with the information obtained from ^{1}H NMR spectra. The tetrahydropyrane ring could occupy either the ^5C_O or the $^5B^O$ conformation (see formulas below), having C-10 above or below the ring, respectively. These two possibilities imply different absolute configurations at C-8 and because of $J_{8,10}=0$ Hz, also at C-10. By inspection of the two possible



structures, it could be seen that in the ^5C_O conformation H-10 was close in space to H-5 and far removed from H-1, and *vice versa* with $^5B^O$. A ^{1}H NOE experiment carried out on *6*, showed that by irradiation of the H-5 signal an enhancement (15 %, using H-3 and H-7 as references) of the signal intensity of H-10 was obtained. On irradiation of H-1 no enhancement of the H-10 signal was observed.

Thus, the conformation of the tetrahydropyrane ring must be ^5C_O, and consequently the absolute configuration of secogalioside and its derivatives is that shown in *5*, *6* and *7*.

In solution morroniside is a mixture of 7-anomers (*9a* and *9b*, see below). Secogalioside, with a hemiacetalic centre at C-10, should have a similar possibility of anomerization. Nevertheless, the NMR spectra of *5* show the presence of only one anomer and only one acetate is obtained upon acetylation.

Three other *Galium* species and a hybrid have been investigated for the presence of secogalioside, using ^{1}H NMR spectra (Table 1). Only *G. album* (from two locations) contains the compound.

^{13}C NMR SPECTRA

Data of this type have been published only for a few acetates of cyclopentanoid iridoid glucosides.[8] We here report data for the secoiridoid glucosides *5*, *9*, *11*, and *13*, together

Table 1. Examination of *Galium* species for asperuloside (A) and secogalioside (S).

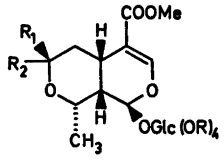
Plant	Location [a]	Voucher No.	S	A
G. album L.	Lundtofte	IOK 18/73	+ [b]	+
(=G. mollugo auct.*)*	Odden	IOK 20/75	+	+
G. verum L.	Odden	IOK 42a/73	− [c]	+
G. verum × *album*	Odden	IOK 43/73	−	+
G. aparine L.	Odden	IOK 15/73	−	+
G. palustre L.	Odden	IOK 46/73	−	+
G. uliginosum L.	Klint	IOK 45/73	−	−

[a] At Sjælland, Denmark. [b] +: present. [c] −: not detected.

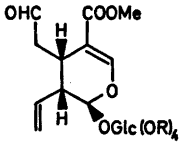
with the (partly corresponding) acetates *6*, *10a*, *10b*, *12*, and *14*. The results are shown in Table 2.

Assignments have been made using principles described by Shilling *et al.*[8] Extensive use has been made of ^1H coupled spectra together with selective decoupling experiments in order to confirm the assignments.

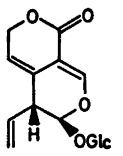
C-1 of the glucoside absorbs between 98.5 and 96.2 ppm; somewhat lower in the acetates. The chemical shifts of C-1′ are found at 100.4 – 99.5 ppm, and 2 – 4 ppm lower in the acetates. No safe assignments of these atoms can thus be made using shift values only. However, C-1′ of β-glucosides consistently shows $^1J_{C,H}$ values within 161 ± 1 Hz.[9] By using this observation we have been able to distinguish between C-1 ($^1J_{C,H}$ = 168 – 177 Hz) and C-1′ ($^1J_{C,H}$ = 161 – 164 Hz). Secogalioside (*5*) and its acetate (*6*) show four absorptions between 95 and 104 ppm; *i.e.* from carbons 1, 7, 10, and 1′. The assignments in *6* were based on selective proton decouplings. Shift values and coupling constants were then used to assign the signals of *5* in analogy to *6*. In aqueous solution morroniside is a mixture of the anomeric compounds *9a* and *9b*, in the proportion of 1:3. Only the B-ring carbon atoms of *9a* and *9b* show different chemical shifts and intensities; thus it is possible to distinguish between (i) the C-7 absorptions *versus* C-1 and C-1′, and (ii) the signals from each of the two anomers.



9a: R = R1 = H, R2 = OH
9b: R = R2 = H; R1 = OH
10a: R = Ac; R1 = H; R2 = OAc
10b: R = Ac; R1 = OAc; R2 = H



11: R = H
12: R = Ac



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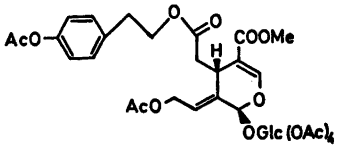

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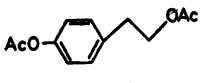

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Table 2. ^{13}C NMR chemical shifts [a] and $^{1}J_{C,H}$ coupling constants [b] for seco-iridoid glucosides and acetates.

C-atom	Compound Secologanin		α-Morroniside		β-Morroniside		Secogalioside		Gentiopicroside	10-OH-Ligstroside Ac_5
	11	*12*	*9a*	*10a*	*9b*	*10b*	*5*	*6*	*13*	*14*
1	97.6 (174)	95.4	96.2 (170x)	94.5 (173)	96.2 (170x)	94.2 (175)	96.3 (174x)	95.7 (168x)	98.5 (177)	92.5 (168)
3	154.0 (194)	150.7	154.9 (192)	151.9 (192)	154.9 (192)	151.9 (192)	151.1 (192)	152.2 (192)	150.4 (196)	152.5 (192)
4	109.6	109.1	110.2	109.9	110.9	110.7	109.8	109.7	104.4	108.1
5	27.5 (134x)	25.1	31.1 (130x)	30.0 (130)	26.8 (130x)	26.1	25.2 (132)	24.6 (132x)	125.2	30.8 (135x)
6	44.6	43.5 [c]	36.1 (130x)	32.9 (130x)	33.4 (130x)	31.4 (130x)	34.6 (130x)	34.4 (130x)	117.8 (169)	39.8
7	206.8 (177)	199.7	95.9 (160x)	93.5 (160x)	91.6 (170)	91.1 (172)	103.3 (174)	103.8 (174)	71.2 (154)	*ca* 170
8	133.8 (156)	131.8	73.8 [c]	73.3 (145x)	65.9	67.1	78.9 (160)	77.2 (162x)	133.9 (161)	124.0 (162)
9	44.6 (132x)	43.1 [c]	38.7 (130)	38.8 (130x)	39.3 (130)	39.2 (129)	37.2 (130x)	36.8 (132x)	45.4 (133)	130.9
10	121.6 (161)	120.6	19.6 (127)	18.8 (127)	19.6 (127)	18.8 (127)	96.7 (176x)	95.1 (178)	119.5 (160)	60.4 (148)
C=O	169.8	166.1	169.8	165.8	169.8	165.9	169.5	166.6	167.5	165.9
OMe	52.6 (147)	51.1	52.6 (147)	51.2 (146)	52.6 (147)	51.1 (146)	52.7 (147)	51.3 (147)	—	51.4 (147)
1′	99.6 (161)	95.4	99.5 (162)	96.5 (163)	99.5 (162)	96.6 (163)	100.4 (162)	98.4 (162)	99.6 (162)	96.8 (164)
2′	73.5	70.4	73.6	70.8	73.6	70.9	73.6	71.0	73.3	70.6
3′	76.6 [c]	72.0	76.8 [c]	72.4	76.8 [c]	72.5	76.7 [c]	72.7	76.5 [c]	72.3
4′	70.5	67.9	70.5	68.3	70.5	68.4	70.4	68.4	70.3	68.0
5′	77.2 [c]	72.0	77.1 [c]	71.8	77.1 [c]	72.0	77.1 [c]	72.1	77.1 [c]	72.1
6′	61.6	61.4	61.6	61.6	61.6	61.5	61.6	61.9	61.6	61.5

[a] In ppm from TMS; ± 0.1. [b] In Hz; ± 2 Hz; if the value is followed by an "x": ± 5 Hz. [c] Interchangeable, in the same vertical column.

With the aid of selective proton decoupling we have assigned C-9 and C-5 of *6* and found the former to absorb at the lowest field, in agreement with the results (two examples) of Shilling *et al.*[8] This criterion was then used for the corresponding assignments of the remaining compounds. Among the high field absorptions, that of C-6 is easily recognized, since this carbon atom is the only one which is coupled to two protons. An exception is *14*, which contains a *p*-acetoxyphenyl-ethyl moiety. The absorptions arising from this part

of the molecule were assigned by comparison with the spectrum of tyrosol diacetate (*15*).* The remaining absorptions arising from methylene carbons, *viz.* 60.4 ppm and 39.8 ppm were assigned to C-10 and C-6, respectively. Due to conjugation with the carboxymethyl group, C-3 and C-4 here absorb at lower field than in decarboxylated compounds.[8]

Except for C-1′, the absorptions arising from the β-D-glucopyranose moiety in both the glucosides and the acetates are consistently very close to published values [8,9] for analogous compounds and apparently not seriously affected by the nature of the aglucone. ^{13}C NMR thus appears to be a complementary way to identify the sugar part of simple iridoid glucosides.

EXPERIMENTAL

Melting points are corrected and determined in a capillary tube in a heated bath. ^1H NMR spectra were recorded at 90 MHz on a Bruker HX-90E instrument in D_2O or $CDCl_3$ with DSS or TMS, respectively, as internal references. ^{13}C NMR spectra were obtained on a Bruker WH-90 instrument at 22.63 MHz using D_2O or $CDCl_3$ with dioxan (5 %, $\delta = 67.4$) or TMS (1 %), respectively, as internal references. Samples were prepared as *ca.* 25 % solutions in 10 mm tubes. A pulse width of 20 μs (90° flip angle), 1 s repetition time, spectral width 5000 Hz, and 8K data points were generally used, resulting in a digital resolution of 1.22 Hz/pt. Broad band noise decoupling was used for the decoupled spectra, and for the selective decoupling experiments a decoupling power giving $\gamma H_2/2\pi = ca.$ 1000 Hz was used. Coupled spectra were recorded in the gated mode using a decoupling time of 1.1 times the sampling time. The ^1H NOE experiment was made on the same instrument at 90 MHz in the FT mode using gated technique as described by Rowan *et al.*[10] 50 Scans (90° pulses – 8 μs) were accumulated with the homodecoupler positioned at the H-5 resonance; a reference spectrum was obtained after moving the homodecoupling frequency 200 Hz upfield. Subtraction of the two spectra indicated the proton signal enhanced by the NOE and showed a very good cancellation of the other signals, except for the irradiated proton. A quantitative measure of the enhancement was obtained from the computer output.

*	α	β	1″	2″	3″	4″
15:	64.1	33.9	135.0	129.2	121.0	148.9
14:	64.8	34.2	134.9	129.5	121.3	149.1

Analyses were performed at Bernhardt, Mikroanalytisches Laboratorium, West Germany.

Isolation of glucosides. Fresh plant material (510 g, collected near the laboratory in July 1973) was homogenized in EtOH and worked up as previously described.[11] The resulting Me_2CO eluate (3.0 g) was crystallized from EtOH to give *asperuloside* (*1*, 925 mg, m.p. 126–129 °C) identified by its ^1H NMR spectrum. The mother liquors were passed through activated carbon to remove a fluorescent compound (if this is omitted, difficulties arise in the following separations). TLC (SiO_2, EtOAc:Bz:EtOH, 4:1:1) gave as the faster moving fraction *arbutin* (*4*, 755 mg, 0.15 %) identified by its ^1H NMR spectrum. The slower band appeared to be a mixture of two compounds and was chromatographed as above ($CHCl_3$:MeOH, 3:1) to give a further amount of *1* (205 mg, total 0.2 %) as the faster moving fraction, together with *secogalioside* (*5*, 650 mg, 0.13 %). Rechromatography, followed by passing a solution of the compound in MeOH through activated carbon, gave the analytical specimen as a colourless syrup $[\alpha]_D^{21} -82°$ (*c* 0.2, EtOH); UV [abs. EtOH (log ε)] : 238 (4.05). ^1H NMR spectrum: δ 7.58 (s, H-3), 5.80 (dd, *J* 2 and 0.5 Hz, H-7), 5.65 (s, H-10), 5.59 (d, *J* 8 Hz, H-1), 4.60 (d, *J* 1.5 Hz, H-8), 3.73 (s, OMe), 2.97 (m, *J* 5, 5 and 12 Hz, H-5), *ca.* 2.1 ($H\text{-}6_{eq}$ and H-9), 1.49 (m, *J* 0.5, 12 and 14 Hz, $H\text{-}6_{ax}$). Anal. $C_{17}H_{24}O_{12}$: C, H.

Secogalioside pentaacetate (*6*). Acetylation of *5* (170 mg) with Py (1 ml) and Ac_2O (0.5 ml) for 2.5 h gave crude *6*. Chromatography (TLC, SiO_2; EtOAc:$CHCl_3$, 1:1) followed by crystallization from EtOH (19 ml) gave the pure, crystalline compound (130 mg) m.p. 171–172.5 °C, $[\alpha]_D^{24} -73°$ (*c* 0.3, $CHCl_3$); UV [abs. MeOH (log ε)]: 236 (4.04). ^1H NMR spectrum: δ 7.46 (s, H-3), 6.29 (s, H-10), 5.77 (dd, *J* 0.5 and 1.5 Hz, H-7), 5.44 (d, *J* 9 Hz, H-1), 4.68 (d, *J* 2 Hz, H-8), 3.73 (s, OMe), 3.09 (m, *J* 5, 6 and 11.5 Hz, H-5), 2.26 (m, *J* 1.5, 6 and 14 Hz, $H\text{-}6_{eq}$), 1.88 (m, *J* 2, 5 and 9 Hz, H-9), 1.37 (m, *J* 0.5, 11.5 and 14 Hz, $H\text{-}6_{ax}$). Anal. $C_{27}H_{34}O_{17}$: C, H.

Conversion of secogalioside to 7. *5* (144 mg) was dissolved in MeOH (10 ml) and $NaBH_4$ (40 mg) was added. After 5 min TLC showed that the reaction was not complete, and additional $NaBH_4$ (20 mg) was added after 30 min. When no more *5* was present (TLC, 45 min), water (5 ml) and KOH (100 mg) was added. Stirring was continued for 10 min, whereafter the mixture was neutralized with AcOH, and taken to dryness. Acetylation of the crude mixture gave, after chromatography, the lactone hexaacetate *7* (37 mg). *7* was crystallized from Et_2O: m.p. 171–172 °C; $[\alpha]_D^{24} -110°$ (*c* 0.4, $CHCl_3$); UV [abs. MeOH (log ε)] : 243 (3.92). ^1H NMR spectrum: δ 7.54 (d, *J* 2.5 Hz, H-3), 5.56 (d, *J* 1.5 Hz, H-1), *ca.* 3.0 (m, H-5), 2.50 (dt, *J* 6.5 and 1.5

Hz, H-9) and 1.97–2.12 (6×AcO); the remaining signals were in accord with those expected from *7*. Anal. $C_{28}H_{36}O_{17}$: C, H.

OsO_4 treatment of sweroside tetraacetate (8). *8* (180 mg) was partly dissolved in ether (50 ml) and Py (0.2 ml). OsO_4 (86 mg) in ether was then added at room temperature during 30 min with stirring. After stirring for 5 days a brown precipitate had formed, but solid *8* was apparently still present. EtOH (10 ml) was added, and the osmate cleaved by bubbling H_2S through the mixture for 1.5 h to give a black precipitate. After filtration, the solvents were removed *in vacuo*. The residue (153 mg) was chromatographed (EtOAc) to give unreacted *8* (31 mg) and a slower moving fraction (69 mg). According to the ^1H NMR spectrum this fraction contained 2 iridoids (peaks at 7.60 and 7.50, H-3) in a proportion of *ca.* 3:2, but it proved impossible to separate them. However, after acetylation complete separation was achieved by chromatography using 4 elutions with Et_2O. The faster moving band (19 mg) was crystallized from Et_2O and was identical (m.p., m.m.p. and ^1H NMR) with *7* derived from secogalioside. The other fraction (35 mg) gave: 8-epi-*7*, m.p. 133.5–134.5 °C; $[\alpha]_D^{24}$ −97° (*c* 0.2, $CHCl_3$); UV [abs. MeOH (log ε)]: 244 (3.87); ^1H NMR spectrum: δ 7.54 (d, *J* 2.5 Hz, H-3), 5.65 (d, *J* 1.5 Hz, H-1), *ca.* 3.0 (m, H-5), 2.34 (dt, *J* 6 and 1.5 Hz, H-9) and 1.95–2.14 (6×AcO). Anal. $C_{28}H_{36}O_{17}$: C, H.

Qualitative examinations of crude plant extracts were done by ^1H NMR spectroscopy. An absorption at 7.58 (in D_2O) indicated the presence of secogalioside, one at 7.46 the presence of asperuloside. The results are shown in Table 3.

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Reactions of Aromatic Anion Radicals and Dianions. V. Protonation and Solvation of Aromatic Dianions by Hydroxylic Compounds and the Consequences on Disproportionation Equilibria of Radical Anions

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The kinetics of the protonation of perylene dianion (PE^{2-}) and anthracene dianion (AN^{2-}) by MeOH in THF was studied by linear sweep voltammetry. Rate constants were calculated from the anodic shifts of the peak potentials for reduction of the anion radicals from the reversible values. At 10 °C, the second order rate constants were found to be equal to 7.5×10^4 and 1.5×10^7 M^{-1} s^{-1} for PE^{2-} and AN^{-2}, respectively. At [MeOH] greater than 0.1 M the calculated rate constants for the protonation of PE^{-2} became increasingly larger with increases in [MeOH] indicating a change in ΔE°, the difference in standard potentials for the first and second charge transfers. The latter effect could not be investigated for AN since the more reactive $AN^{\cdot-}$ undergoes protonation at (MEOH] of about 0.1 M during cyclic voltammetric experiments. Benzophenone (BP) ions were found to be even more sensitive to the presence of low concentrations of MeOH with large shifts in ΔE° being observed at millimolar concentrations of MeOH. Shifts in ΔE° in the presence of MeOH were attributed to association of the dianions with the hydroxylic compound.

Our recent report of the reversible reduction of anion radicals of aromatic hydrocarbons to the corresponding dianions in aprotic solvents [1] opened the door to direct measurements of the kinetics of the protonation of the dianions. If the reversible potential for an electrode process is known, the rate constant for a reaction following electron transfer can readily be determined by the kinetic shift of the voltammetric peak potential.[2] Disproportionation mechanisms have recently been proposed to account for some of the protonation reactions of aromatic anion radicals.[3] In evaluating such mechanisms, K_{disp} (eqn. 1) determined in aprotic solvents in the absence of proton donors, is used to determine the second order rate constant for the dianion plus proton donor reaction. Implicit in this treatment is the assumption that low concentrations of the hydroxylic proton donors have no effect upon the difference in formal potentials for the first and second electron transfers (ΔE°) to the aromatic compound and hence on the disproportionation equilibrium constants. Here we report the first direct measurement of dianion protonation kinetics and the observation of a profound effect of hydroxylic compounds upon the magnitude of disproportionation equilibrium constants of anion radicals of aromatic compounds.

$$2\pi^{\cdot-} \underset{}{\overset{K_{disp}}{\rightleftharpoons}} \pi + \pi^{2-} \qquad (1)$$

In THF, ΔE° for the ions of perylene (PE) is equal to 520 mV at 11 °C, which corresponds to a value of 6×10^{-10} for K_{disp}. In order to measure the reversible potential for the second charge transfer, it was necessary to conduct the measurements in the presence of activated alumina.[1] Measurements of the peak potential differences (ΔE_p) for determination of the rate constant for the protonation of PE^{2-} with

Table 1. Pseudo first order and second order rate constants for the protonation of perylene dianion by methanol in THF.[a]

[MeOH]/M	ΔE_p/mV [b]	k_1/s^{-1} [c]	k_2/M^{-1} s^{-1} [d]
0.0	25	5.2×10^1	–
0.010	65	6.7×10^2	6.7×10^4
0.026	80	2.3×10^3	8.8×10^4
0.079	90	5.2×10^3	6.7×10^4
0.243	110	2.7×10^3	1.1×10^5
0.52	120	1.1×10^5	2.1×10^5
1.87	165	2.0×10^6	1.0×10^6

[a] 0.2 M Bu_4NBF_4, +10 °C, hanging mercury drop electrode. [b] Anodic shift from the reversible peak potential measured at a scan rate of 154 mV s^{-1}. [c] Pseudo first order rate constants calculated from three different scan rates. [d] Second order rate constants.

methanol were conducted in THF which had been passed over active neutral alumina just prior to use. These data are summarized in Table 1. Peak potentials were determined at three different sweep rates for each value of [MeOH]. Pseudo first order rate constants (k_1) were then calculated from the shift in peak potential (ΔE_p) from the reversible value for the second charge transfer. Cyclic voltammetry showed that the first charge transfer remained reversible at all concentrations of MeOH used showing that protonation of $PE^{\cdot-}$ is slow during the time scale of the voltammetric measurements. The value of k_1 in the absence of methanol is that for the reaction of PE^{2-} with residual water in the solvent-electrolyte system.* At [MeOH] from 0.01 to 0.1 M the second order rate constant (k_2) for reaction (2) derived from

$$PE^{2-} + MeOH \xrightarrow{k_2} PEH^- + MeO^- \qquad (2)$$

k_1 values determined from peak potential data according to the method of Nicholson and Shain [2] were observed to be equal to 7.5×10^4 M^{-1} s^{-1} which is 150 times smaller than the estimate of Szwarc based on the apparent anion radical protonation rates.[4] At [MeOH] greater than 0.1 M, the calculated k_2 values became increasingly larger with increases in the MeOH concentration.

The rate of protonation of anthracene (AN) dianions in THF by MeOH was investigated by means of the shift of the voltammetric peak potential from the reversible value. The observed second order rate constant was 1.5×10^7 M^{-1} s^{-1} at [MeOH] ranging from 0.01 to 0.07 M. The reaction of the dianion with residual proton donors made determination of the rate constant at lower [MeOH] impossible while protonation of the anion radical occurred at higher [MeOH]. The protonation of $AN^{\cdot-}$ was complete (no reverse current during cyclic voltammetry at 1 V s^{-1}) at [MeOH] of about 1.0 M whereas no reaction was observed for $PE^{\cdot-}$ under the same conditions.

Results obtained when the same procedure was applied to the protonation of benzophenone (BP) dianion in DMF were more dramatic. A large shift from the reversible potential was observed even before adding MeOH showing that BP^{2-} is rapidly protonated by residual water. The calculated values of k_2 were not constant and increased markedly with increasing [MeOH], in all cases being greater than the maximum possible value for a diffusion controlled reaction (Table 2).

The results presented above indicate that ΔE° for PE and AN are unaffected by the presence

Table 2. Apparent pseudo first order and second order rate constants for the protonation of benzophenone dianion by methanol in DMF.[a]

[MeOH]/M	ΔE_p/mV [b]	k_1/s^{-1} [c]	k_2/M^{-1} s^{-1} [d]
0	110	5×10^4	–
0.0037	275	1×10^{10}	3×10^{12}
0.015	355	5×10^{12}	4×10^{14}
0.041	410	5×10^{14}	2×10^{16}
0.109	450	2×10^{16}	2×10^{17}
0.271	470	1×10^{17}	4×10^{17}
0.850	505	1×10^{18}	2×10^{18}

[a] 0.02 M Me_4NBF_4 + 0.2 M Bu_4NBF_4, +10 °C, hanging mercury drop electrode. [b] Anodic shift from the reversible peak potential measured at a scan rate of 65 mV s^{-1} (Rate constants were calculated from three different scan rates and found to be independent of scan rate). [c] Apparent pseudo first order rate constants. [d] Apparent second order rate constants.

* Second order rate constants are not listed due to the fact that the water concentration is not known.

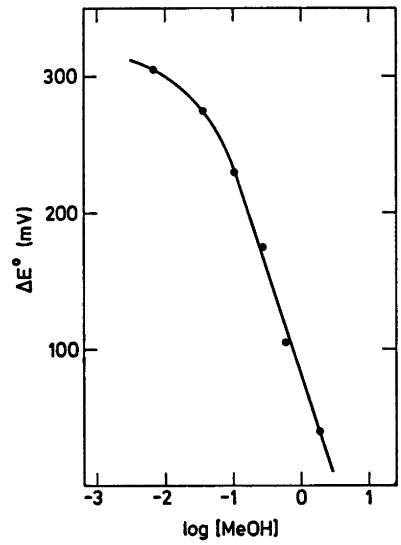

Fig. 1. The dependence of $\Delta E°$ for the two consecutive charge transfers to *p*-dinitrobenzene in THF on the concentration of added methanol.

of MeOH in THF up to concentrations of about 0.1 M and that at higher [MeOH], $\Delta E°$ decreases. In the case of BP, $\Delta E°$ appears to be even more sensitive to [MeOH], changing drastically even at the millimolar level.

In order to firmly establish that low concentrations of MeOH in aprotic solvents can indeed cause substantial shifts in $\Delta E°$ for aromatic compounds in aprotic solvents, the reduction of *p*-dinitrobenzene (DNB) in THF containing MeOH was investigated. This compound was chosen because the dianion is very stable even in the presence of high concentrations of proton donors. In fact, it has recently been observed that addition of water to DMF causes a substantial shift in $\Delta E°$ for DNB.[5] The effect of added MeOH on $\Delta E°$ for DNB in THF is illustrated in Fig. 1. A large change in $\Delta E°$ was observed with changes in [MeOH].

The effect of the hydroxylic compound, methanol, on the voltammetry of *p*-dinitrobenzene in DMF is demonstrated by the cyclic voltammograms shown in Fig. 2. In the absence of methanol, $\Delta E°$ for the two charge transfers was found to be of the order of 650 mV (Fig. 2a) and decreased to about 400 mV at [MeOH] equal to 0.96 M (Fig. 2b). Further increases in [MeOH] were accompanied by the merging of the two distinct peaks into a single quasi-reversible voltammetric peak (Fig. 2c).

The association of dianions of aromatic compounds with cations is a well-established phenomenon.[6] It has been pointed out that the association with small dianions such as those from benzoquinone is much stronger than with larger dianions such as those derived from polynuclear aromatic hydrocarbons.[7] The latter has been explained in terms of increasing delocalization of charge as the size of the dianion increases, thus lowering the requirements for strong pairing with counter ions.

The association of dianions of aromatic hydrocarbons with hydroxylic compounds has previously escaped detection, because until recently [1] it was not possible to obtain reversible potentials from cyclic voltammetry due to the great reactivity of the dianions to trace im-

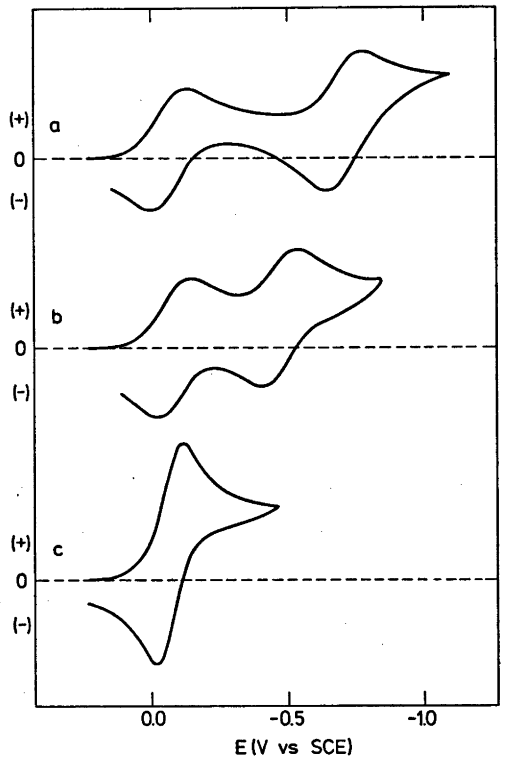

Fig. 2. Cyclic voltammograms of *p*-dinitrobenzene in DMF containing Bu_4NBF_4 (0.1 M) and Me_4NI (0.01 M) recorded at +10 °C at a voltage sweep rate of 65 mV/s. Solvent was DMF containig methanol: (a) 0, (b) 0.96 M and (c) 7.2 M in methanol.

purities in the aprotic solvents, and the other technique, potentiometric titration,[8] cannot be applied to systems containing water or alcohols.

The sensitivity of BP dianions to association with MeOH as compared to the PE dianions can readily be explained in terms of the lower degree of charge delocalization in BP^{2-}. A similar effect has been observed on ΔE° for BP in the presence of tetraalkylammonium ions of varying size.[9]

The results presented here are very pertinent to the study of protonation reactions of aromatic anion radicals, a topic of active current interest.[3,4,10] Studies on the kinetics of protonation of aromatic hydrocarbons dianions are continuing and will be presented in detail later.

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Cyclic Pentapeptides of Sarcosine in Combination with Either Alanine or Glycine. Syntheses and Conformational Processes Studied by NMR Spectroscopy

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A series of cyclic pentapeptides has been synthesised. Cyclic dipeptides, tetrapeptides and partially racemised tetra- and pentapeptides were isolated as by-products. The formation of the smaller rings is due to cleavage of the peptide chain during the cyclisation reaction.

NMR studies of the cyclic pentapeptides in $CHFCl_2$ showed that several conformational changes took place when solutions prepared by dissolving crystals at −70 °C were gradually heated to +20 °C. The conformers present in the final equilibrium, usually reached below room temperature, varied for the different cyclic pentapeptides and the initial crystal conformer was hardly present.

The driving force behind these conformational changes is suggested to be replacement of external hydrogen bonds in the crystal conformer with internal hydrogen bonds to form more stable conformers in solution. This requires conversion of *cis* amide bonds to *trans*.

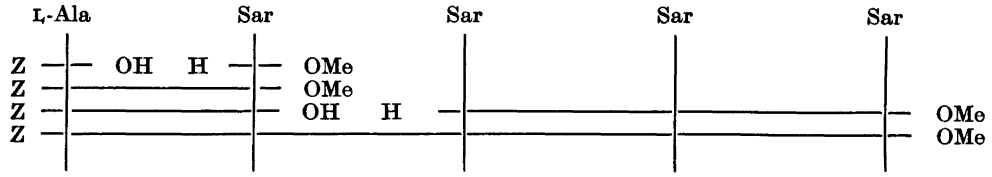
Attempts were made to distinguish between *cis* and *trans* amide bonds by the following NMR methods: differential solvent shifts, different band widths of the *N*-methyl lines, shifts induced by complexation with Eu, and shift differences in ^{13}C NMR, but no conclusive results could be obtained. Addition of benzene, was, however, helpful in resolving overlapping lines.

The majority of conformational studies on cyclic peptides have been carried out on cyclic dipeptides and cyclic hexapeptides, only a few isolated investigations have been reported for cyclic pentapeptides.[1] A difference was found in the yield on cyclisation of glycylleucyldiglycylleucine and of glycyldileucylglycylglycine and also higher yields when both D- and L-leucine was used when compared to the L,L-isomer.[2,3] Dielectric increment measurements showed a shorter distance between the ends of the linear peptides containing the L,D-form and this would favour the cyclisation. Five cyclic pentapeptides[4] such as cyclo-glycyl-L-alanyldiglycyl-L-prolyl and similar compounds were studied by NMR spectroscopy in dimethyl sulfoxide solution and found to be present in two conformations, about 70 % of the major and 30 % of the minor conformer. It was suggested that the major conformer contained only *trans* amide bonds whereas the minor conformer had the amide bond involving the proline nitrogen in the *cis* form. Both conformations contained intramolecular hydrogen bonds.

Our work with cyclic homologues of sarcosine[5,6] has shown that these cyclic peptides have a high degree of conformational homogeneity and most of the rings are present in solution in only one conformation. It was also found that a series of cyclic tetrapeptides[7,8] of sarcosine, in combination with one or two alanine or glycine residues, take the same conformation as the parent compound, cyclotetrasarcosyl. In these, the configuration of the four amide groups are alternately *cis* and *trans*. This skeleton was adopted even in those cases where an NH-amide had to take a *cis* configuration. The corresponding cyclic pentapeptide, cyclo-pentasarcosyl, when studied by NMR spectroscopy in deuteriochloroform, revealed one major and one minor (less than 10 %) conformation. Low-temperature NMR studies showed the major conformation to be the same as that in the crystal with the con-

Scheme 1. Routes to the different linear pentapeptides.

For cyclo-L-alanyltetrasarcosyl



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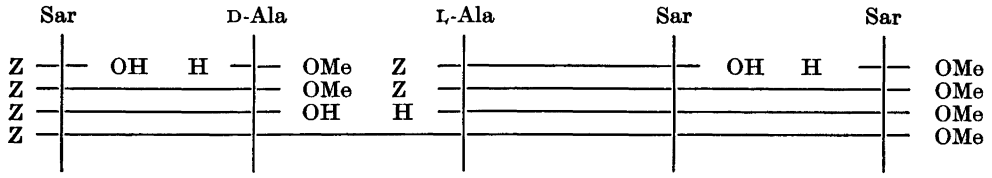

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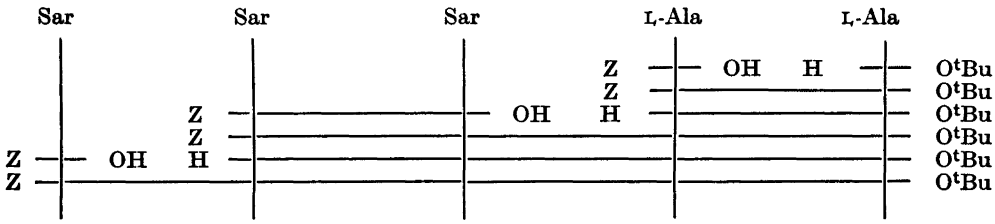

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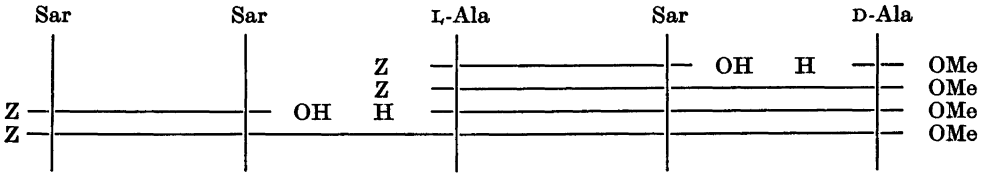
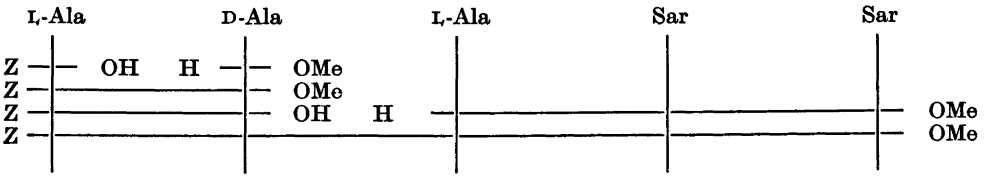
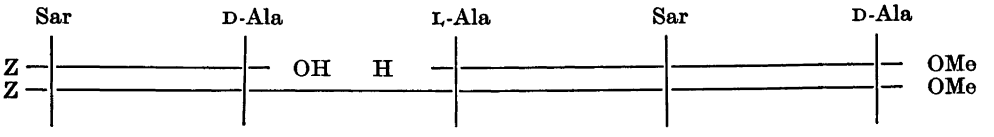


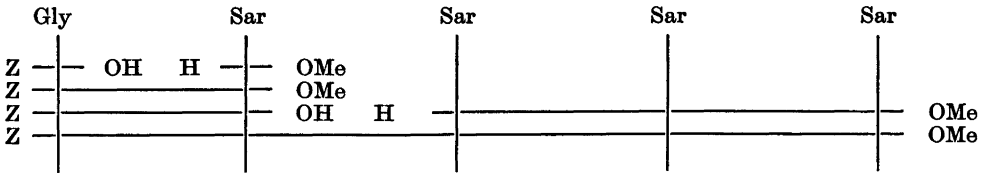
For cyclo-L-alanyl-D-alanyl-L-alanyldisarcosyl



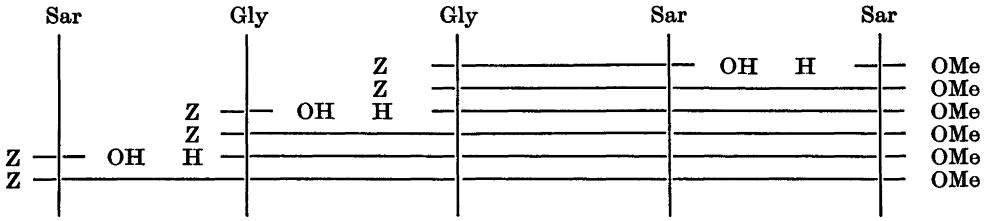
For cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl



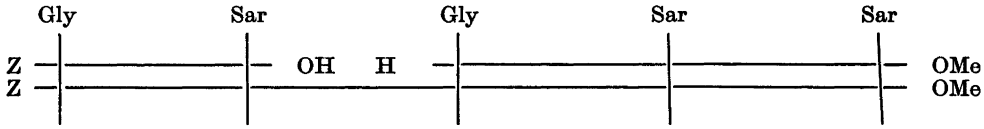
For cyclo-glycyltetrasarcosyl



For cyclo-diglycyltrisarcosyl



For cyclo-glycylsarcosylglycyldisarcosyl



figurational sequence *cis,cis,cis,trans,trans*.[9,10,6] It was therefore considered to be of interest to study a series of cyclic pentapeptides of sarcosine combined with alanine or glycine to see whether these possessed the same degree of conformational homogeneity as found for the analogous tetrapeptides. The compounds prepared and studied were six cyclic pentapeptides of sarcosine combined with alanine:
cyclo-L-alanyltetrasarcosyl (c-L-AlaSar$_4$), cyclo-D-alanyl-L-alanyltrisarcosyl (c-D-Ala-L-AlaSar$_3$), cyclo-di-L-alanyltrisarcosyl (c-L-Ala$_2$Sar$_3$), cyclo-L-alanylsarcosyl-D-alanyldisarcosyl (c-L-AlaSar-D-AlaSar$_2$), cyclo-L-alanyl-D-alanyl-L-alanyldisarcosyl (c-L-Ala-D-Ala-L-AlaSar$_2$), cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl (c-D-Ala-L-AlaSar-D-AlaSar),
and three cyclic pentapeptides of sarcosine in combination with glycine:
cyclo-glycyltetrasarcosyl (c-GlySar$_4$), cyclo-diglycyltrisarcosyl (c-Gly$_2$Sar$_3$), cyclo-glycylsarcosylglycyldisarcosyl (c-GlySarGlySar$_2$).

SYNTHESES

The different linear peptides were synthesised as shown in Scheme I. The benzyloxycarbonyl group was used for protection of the amino function, the methyl ester (in one case the *t*-butyl ester) for the carboxyl group and *N,N'*-dicyclohexylcarbodiimide as the coupling reagent. For cyclisation the methyl ester was hydrolysed by alkali (the *t*-butyl ester by trifluoroacetic acid) and the carboxyl group converted to the 2,4,5-trichlorophenyl ester, the benzyloxycarbonyl group was removed by hydrogenolysis and the peptide active ester cyclised in pyridine. After passage through a strong cat- and an-ion exchange column to remove noncyclised material, the cyclic peptides were purified by chromatography on silica gel, fractional crystallisation and sublimation. Not only the desired cyclic pentapeptide was isolated, but also dipeptides, tetrapeptides, decapeptides and racemised products (Table 1).

Formation of cyclic dipeptides was observed during cyclisation of peptides, especially those containing sarcosine as the second residue in the peptide chain. This was very prominent in a series of tetrapeptides [11] and was also observed in the series of homologous sarcosine peptides.[6] The free amino group attacks the second carbonyl carbon, with cleavage of the chain. Cycli-

sation of alanyltrisarcosylsarcosine trichlorophenyl ester gave together with the expected product two cyclic dipeptides, cyclo-alanylsarcosyl and cyclo-disarcosyl (Table 1). Here the cleavage of the peptide chain has occurred twice as was suggested to be the case in some of the sarcosine peptides.[6] However, from cyclisation of the closely related glycyltrisarcosylsarcosine only the corresponding cyclic pentapeptide was isolated. This shows that cleavage of the peptide chain occurs, but less generally in pentapeptides than in tetrapeptides.[11]

The formation of cyclic tetrapeptides was observed only in the cyclisation of disarcosyl-L-alanylsarcosyl-D-alanine trichlorophenyl ester where two cyclic tetrapeptides of sequence AlaSarAlaSar were isolated. These differed in solubility, sublimation temperature, IR and NMR spectra, and were assigned as cyclo-L-alanylsarcosyl-D-alanylsarcosyl (major product) and cyclo-L-alanylsarcosyl-L-alanylsarcosyl by comparison with the cyclic tetrapeptides previously studied.[8] The possibility that these arise by transannular reactions from the two cyclic pentapeptides formed (see below for racemisation) was excluded as these were recovered unchanged after boiling in pyridine for 9 h. Most probably these tetrapeptides were again formed by cleavage of the peptide chain, but as in both cases it is the *N*-terminal sarcosine which is lost, the attack must be from the carbonyl end. At first sight it is difficult to rationalise the preference of the carbonyl carbon of the second residue over the free amino group. However, the relatively low yield of cyclic pentapeptide and relatively high yield of cyclic tetrapeptide would suggest that the linear molecule exists in a conformation which favours the formation of the smaller ring. Cyclo-L-alanylsarcosyl-D-alanylsarcosyl is known from a previous study to take up the highly favoured *cis,trans,cis,trans* configuration with the C_α-methyl groups in favoured orientations.[8] This fits very well for folding the chain so that the amide nitrogen comes close to the active ester, and formation of a cyclic intermediate as shown in Fig. 1 is possible. The cleavage of the active ester may occur in several ways, either by the departure of a phenolate ion or elimination of phenol if the α-H of the D-alanine residue is removed. Subsequent loss of the *N*-terminal

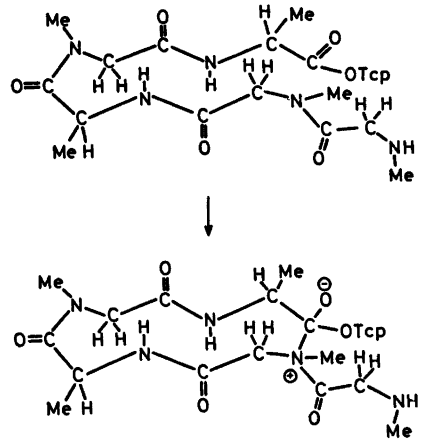


Fig. 1.

sarcosine unit leads to the two cylic tetrapeptides which are isolated on cyclising this specific pentapeptide.

Cyclic decapeptide formation can be very predominant from pentapeptides as shown in a systematic study on analogues of gramicidin S.[12–14] Earlier studies with homologous sarcosine peptides [6] have shown that this doubling reaction is not very dominant even on cyclisation of disarcosylsarcosine, and a similar situation has now been observed for the pentapeptide series. In most cases the presence of the cyclic decapeptide could only be inferred from the TLC. Attempts to isolate these small quantities did not succeed as they showed no tendency for crystallisation and were bound strongly to silica gel. Only for alanyltrisarcosylsarcosine was the cyclic decapeptide isolated by precipitation.

Racemisation was shown to have occurred by the isolation of pairs of pentapeptides (and tetrapeptides) from some of the reactions. Thus disarcosyl-L-alanylsarcosyl-D-alanine trichlorophenyl ester gave two cyclic pentapeptides (identical *m/e* 355) which had different melting points and different R_F values on TLC and could be separated on silica gel. A similar situation was found on cyclising sarcosyl-D-alanyl-L-alanylsarcosyl-D-alanine trichlorophenyl ester where two cyclic pentapeptides were isolated with different melting points and R_F values. As the two linear pentapeptides both contain D-alanine as the C-terminal amino acid it is likely that on cyclisation in the basic

medium at the elevated temperature (115 °C) some racemisation of the C-terminal amino acid takes place. The two cyclic pentapeptides formed in the former case, are therefore most likely cyclo-L-alanylsarcosyl-D-alanyldisarcosyl, m.p. 274 °C (major product) and cyclo-L-alanylsarcosyl-L-alanyldisarcosyl, m.p. 224 °C. This is in good agreement with the two cyclic tetrapeptides which also were formed in this cyclisation. The products in the second case have most likely an analogous relationship: cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl, m.p. (subl.) 317 °C (major product), and cyclo-D-alanyl-L-alanylsarcosyl-L-alanylsarcosyl, m.p. 260 °C. From each of the other cyclisation reactions only one cyclic pentapeptide was isolated, even though the X-ray analysis of the expected cyclo-L-alanyltetrasarcosyl showed that the crystal contained the racemate.[15] It is likely that the racemate crystallised more easily than each of the optical antipodes and that the crude cyclised product is not fully racemised. The optical rotation is low, but this can depend on the conformations of the cyclic peptides. The same was noted on crystallising the analogous cyclic tetrapeptide cyclo-L-alanyltrisarcosyl where the racemate was found in the crystal structure.[16] Thus with both the penta- and the tetrapeptide partial racemisation has taken place at some stage during the reaction sequence. Recently, it has been reported that racemisation does occur in reactions which had been thought to be racemisation free.[17–19]

PHYSICAL AND SPECTRAL PROPERTIES OF THE CYCLIC PENTAPEPTIDES

In contrast to the cyclic oligomers of sarcosine which have sharp melting points,[6] several of the cyclic pentapeptides, after being isolated by column chromatography, melted over a wide range. However, the melting points became sharper after recrystallisation from a suitable solvent (*e.g.* acetone) by slow evaporation in contact with the air. The infrared spectra, showing broad absorptions, gave little information. The mass spectra showed the molecular ions as intense peaks and very similar fragmentation patterns. The NMR spectra were generally more complex than in the sarcosine series but again gave the most informative picture of the conformational situation. When dissolved in $CDCl_3$ at room temperature, the number of lines showed the presence of two or more conformations in equilibrium. The conformational interconversion barriers are high, as temperatures of around 100 °C are needed to obtain coalescence to a single set of lines. This means that at low temperatures (about −70 °C) the conformers, at least as defined by a given *cis,trans*-sequence, will have life-times of hours. It should therefore be feasible to observe the conformation present in the crystal when crystals are dissolved at low temperature (−70 °C) and the NMR spectrum recorded at this temperature.* Slow warming of the sample in the NMR tube to room temperature and recording of the NMR spectra will reveal if any conformational changes take place.

When the initially isolated cyclic pentapeptides of broad melting range were dissolved at −70 °C, the NMR spectra were complex and showed that several conformations were present, while the sharply melting crystals when dissolved at −70 °C showed only one conformer (called A) at this temperature (Figs. 2–5). On allowing the latter samples to warm slowly to about −30 °C, new lines due to a second conformer (called B) began to appear. On further warming a third conformer (C) developed whilst the concentration of the crystal conformation A diminished. The full thermodynamic equilibrium, normally reached already below room temperature, consisted of a mixture of B and C and in some cases a fourth conformer D. Only in a few cases was the crystal conformer present at room temperature. The changes are most easily seen in the *N*-methyl region, the methylene protons, however, do not often give well resolved lines, but in those cases where the quartets are recognized the coupling constants are grouped around 14 and 18 Hz indicating that the CC_α-torsional angles take up the *anti*, *gauche* pattern as seen for the oligopeptides of sarcosine.[6] The NH protons could not be ob-

* Rotation about the single bonds is not expected to be slowed down sufficiently at these temperatures, but in a series of related cyclic peptides, cyclic oligomers of sarcosine, both the CC_α- and the NC_α-torsion angles take up only a limited number of values. The CC_α-torsion angles are either 180 or 60° and the NC_α-torsion angles are grouped around 90°.

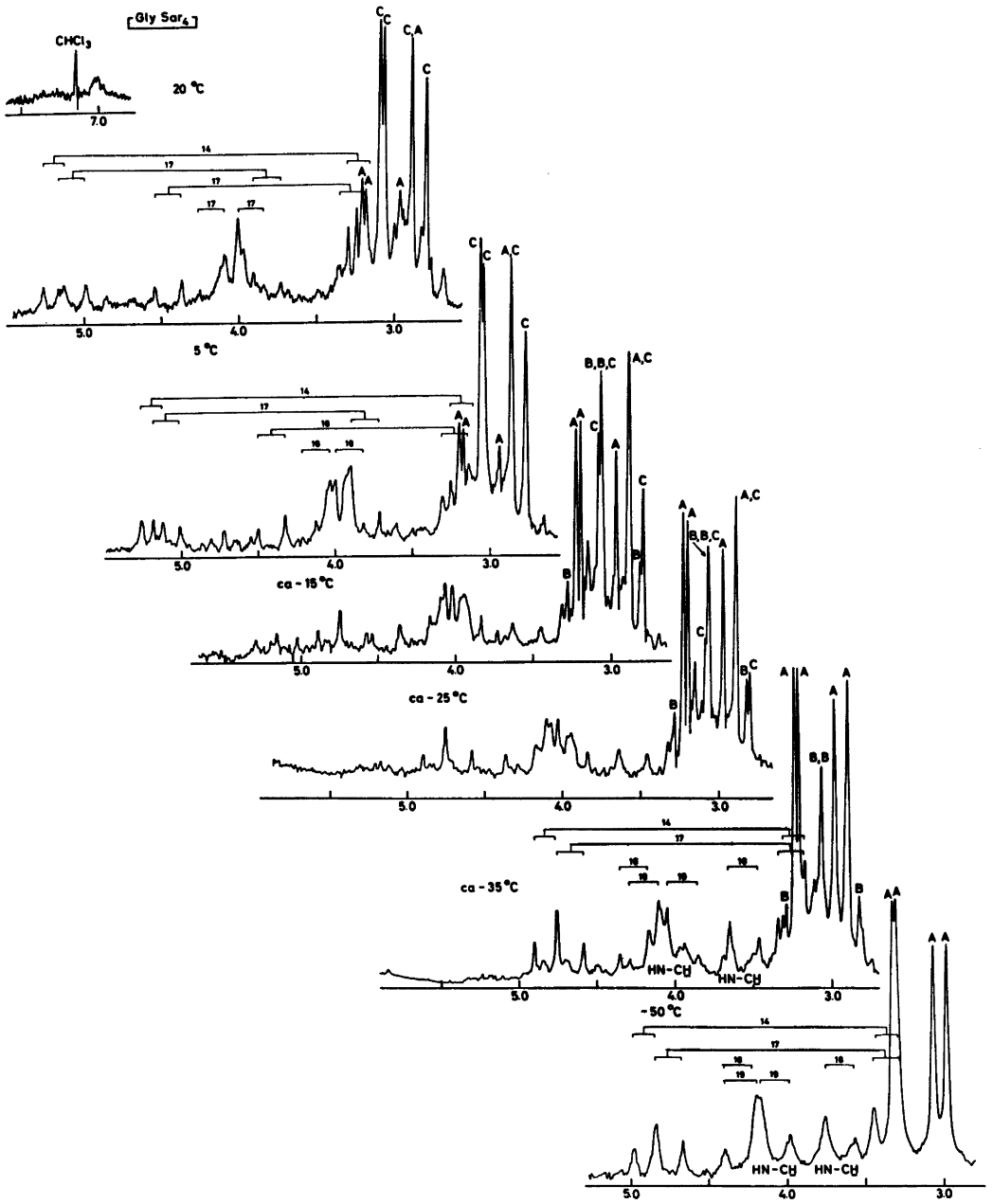


Fig. 2. The 100 MHz NMR spectrum of c-GlySar$_4$. Crystals were dissolved in $CHFCl_2$ at −50 °C (lower spectrum) and the solution allowed to warm up in the probe (NH-region left out). $CDCl_3$ was used as the solvent at the highest temperature (20 °C).

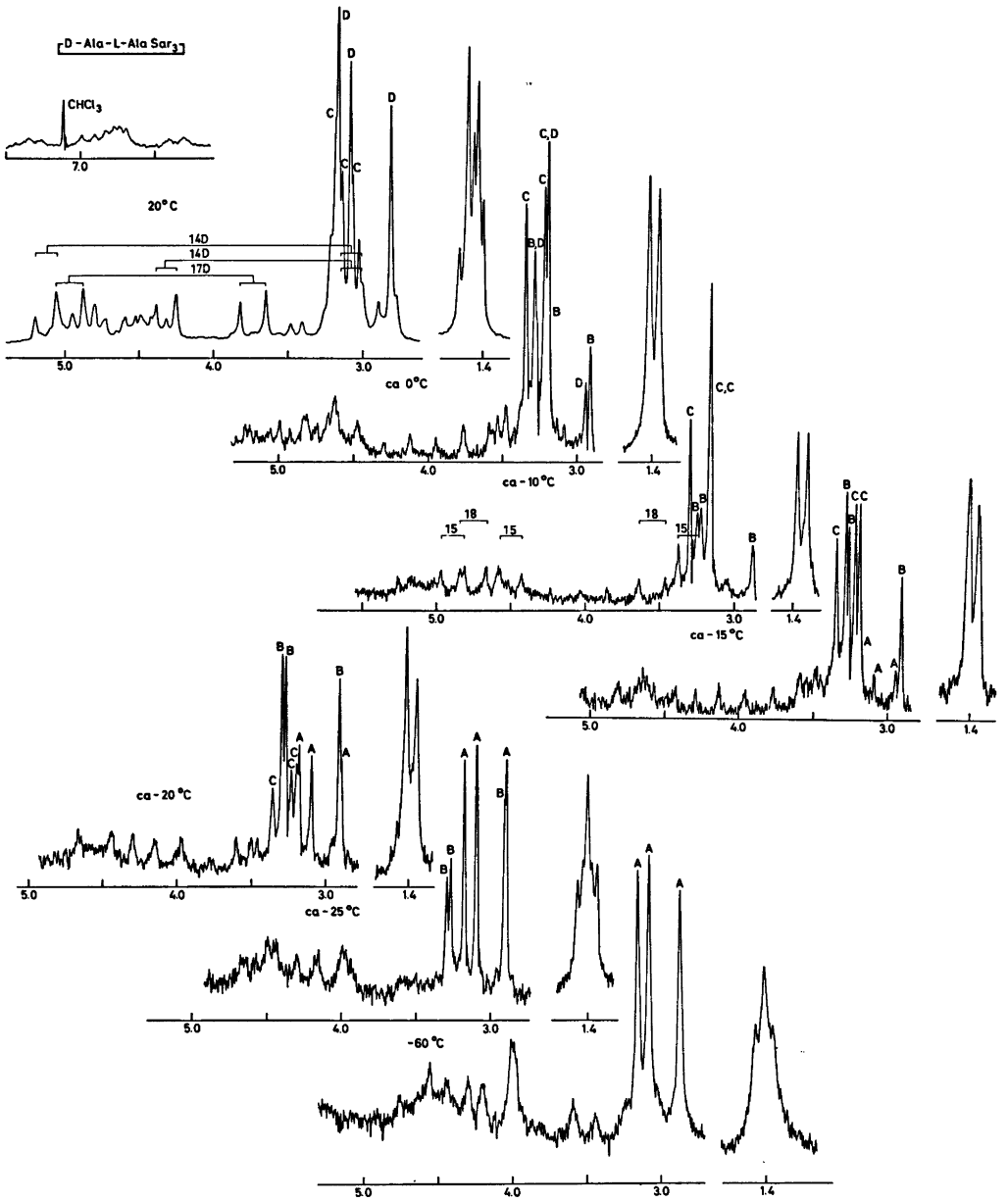


Fig. 3. The 100 NMR spectrum of c-D-Ala-L-Ala-Sar_3. Crystals were dissolved in $CHFCl_2$ plus traces of TFA at -60 °C (lower spectrum) and the solution allowed to warm up in the probe (NH-region left out), $CDCl_3$ was used as the solvent at the highest temperature (30 °C).

served due to the chemical shifts of the $CHFCl_2$ proton, the solvent used in these NMR studies, either pure or together with a small amount of trifluoracetic acid to increase the solubility. When studied in deuteriochloroform at room temperature the NH protons are close to the chloroform peak and the spin-spin coupling to the methine protons, which are hardly resolved, varies from 1 to 10 Hz.

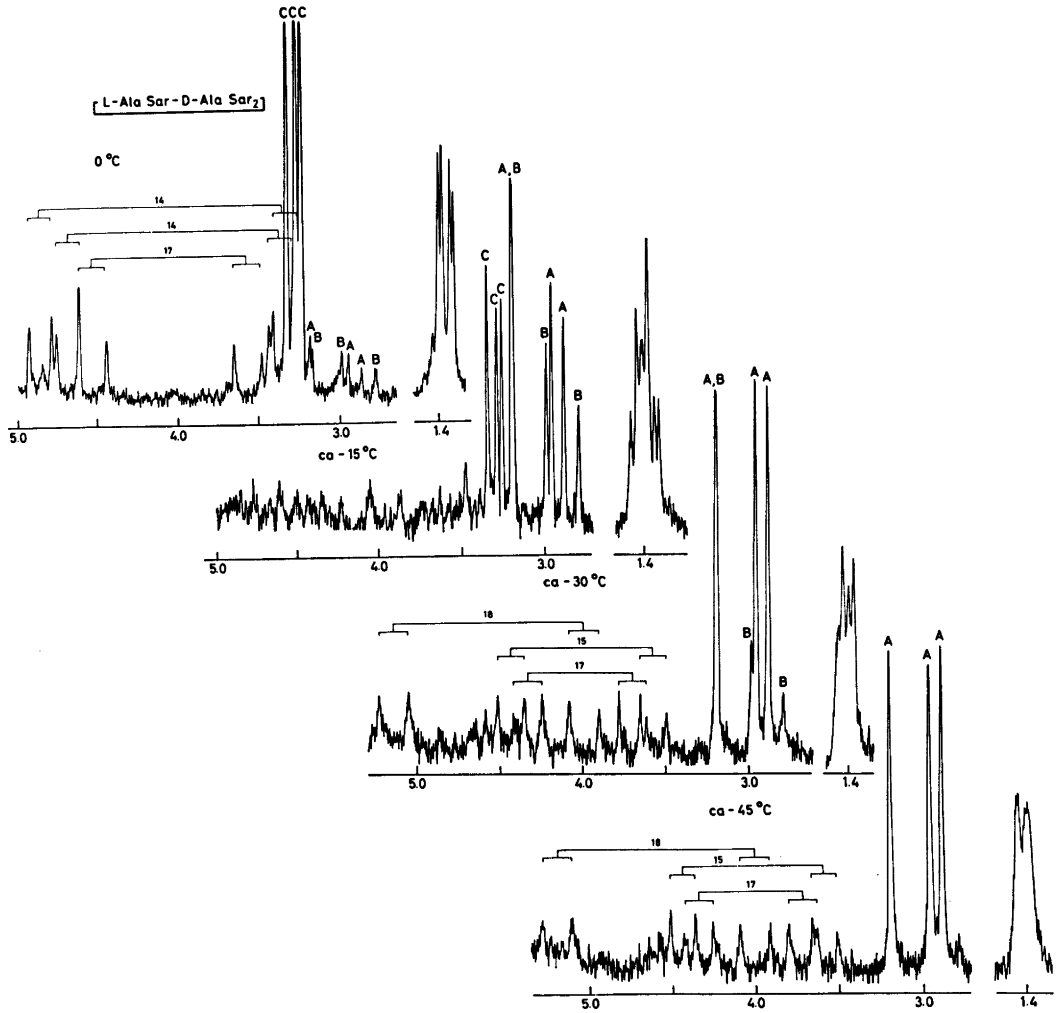


Fig. 4. The 100 MHz NMR spectrum of c-L-AlaSar-D-AlaSar$_2$. Crystals were dissolved in $CHFCl_2$ at −45 °C (lower spectrum) and the solution allowed to warm up in the probe (NH-region left out).

The conformational changes of the individual cyclic pentapeptides are closely related, but the concentration of the transient conformers differ and also the conformers present at equilibrium. c-GlySar$_4$ (Fig. 2) shows when dissolved at −50 °C four N-CH_3 lines due to the presence of one conformer, A. The CH_2-quartets are partly resolved. At about −35 °C a second conformer B appears, its concentration is always less than that of A and decreases when a third conformer C appears. At room temperature the conformer mixture is dominated by C (80 %) with a small amount of A but hardly any of B. This equilibrium mixture was warmed further to observe coalescence phenomena. The CH_2-quartets of conformer C started to broaden at 30 °C, at about the same temperature (40 °C) as was found [6] for c-Sar$_5$. Thus conformer C possesses the same high barrier to ring inversion as found in c-Sar$_5$.

c-AlaSar$_4$ (Fig. in Ref. 32) shows at −60 °C four N-CH_3 lines, but the C-CH_3 is split into two doublets. This indicates the presence of two conformers having N-CH_3 groups of iden-

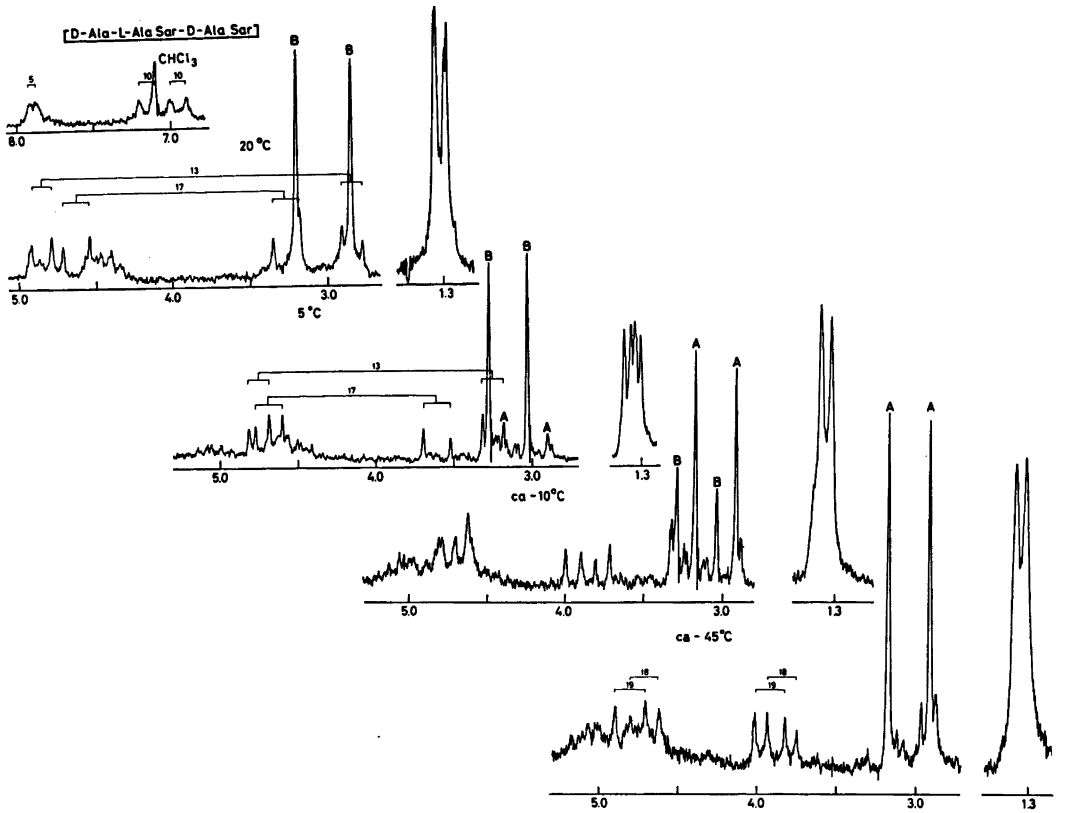


Fig. 5. The 100 MHz NMR spectrum of c-D-Ala-L-AlaSar-D-AlaSar. Crystals were dissolved in $CHFCl_2$ plus traces of TFA at −45 °C (lower spectrum) and the solution allowed to warm up in the probe (NH-region left out). $CDCl_3$ was used as the solvent at the highest temperature (20 °C).

tical chemical shifts. One of the conformers may represent the crystal conformation whereas the other may occupy the same ring skeleton, but with the *C*-CH_3-group inverted. The new set of four *N*-CH_3 lines due to conformer B becomes in this molecule equal to A in concentration before conformer C appears, and at room temperature C dominates (60 %), together with B (40 %), but only traces of A are present.

c-D-Ala-L-$AlaSar_3$ (Fig. 3) and c-Gly_2Sar_3 exhibit identical conformational changes, conformer B approaching A in concentration before C increases to become the dominating conformer. At about 0 °C a fourth conformer D develops and at room temperature the mixture consists of mostly D (70 %) and some C (30 %). The closely related c-L-Ala_2Sar_3 is the only compound in the series which shows a complex low temperature spectrum with several conformers present. On warming the sample, the picture became even more complex.

c-L-AlaSar-D-$AlaSar_2$ (Fig. 4) and c-L-AlaSar-L-$AlaSar_2$ show identical NMR spectral changes with rising temperature. Conformer A has three sharp *N*-CH_3 signals and three well resolved CH_2-quartets. B does not here approach A in concentration before the third conformer C appears. C increases to become the only visible conformation (> 90 %) at room temperature. This indicates that the conformational transformations of these molecules go through the same ring conformations, and that the C_α-methyl groups derived from the C-terminal alanine (D or L) have coincident chemical shifts. Both c-GlySarGlySar_2 and c-L-Ala-D-Ala-L-$AlaSar_2$ undergo analogous conformational changes with C as the dominating conformer in the final equilibrium mixture.

Conformer A of c-L-Ala-D-Ala-L-AlaSar$_2$ has two sharp N-CH_3 lines and two resolved CH_2-quartets both with J of 18 Hz and conformer C has two narrow split N-CH_3 lines and two well resolved CH_2-quartets with J of 18 and 14 Hz. The CH_2-quartets of c-GlySarGlySar$_2$ are not resolved and started to coalesce at *ca.* −5 °C.

c-D-Ala-L-AlaSar-D-AlaSar (Fig. 5) shows at low temperature two N-CH_3 lines and two CH_2-quartets. In this case no conformational change takes place before at about −15 °C when a second conformer B develops. On further warming, A diminishes as B increases in concentration, but no third conformer C appears, and at room temperature pure B is present. This is the only compound in the series where only one conformational transformation is observed. The closely related c-D-Ala-L-Ala-Sar-L-AlaSar shows, however, a different conformational behavior. One major conformation A is present at low temperature. The second conformer B becomes equal to A in concentration before the third conformer C develops at about −30 °C. In the equilibrium mixture there is hardly any B while A and C are present in equal amounts. These compounds do not adopt the same ring skeletons nor do the same conformational transformations occur, although the only difference is that one α-CH_3 group in one is inverted to the other. Steric interaction of the three α-methyl groups may stabilise different conformations.

ATTEMPTS TO APPLY NMR METHODS FOR DISTINGUISHING BETWEEN *cis* AND *trans* AMIDE IN CYCLIC PEPTIDES

In order to understand the conformational changes it was important to know if *cis-trans* amide interchange was involved. Some attempts were therefore made to investigate if it might be possible to distinguish between the N-CH_3 of *cis* amide bonds and those of *trans* in the sarcosine peptides. Several methods have been used to distinguish between *cis* and *trans* amides, such as differential solvent shifts, different band width of the N-methyl lines, shifts induced by complexation with paramagnetic metal ion and shift differences in ^{13}C NMR. These effects have been discussed for simple amides and small peptides. The N-methyl groups in N,N-dimethylformamide [20] and related compounds differ in chemical shift, being highest for the methyl group *cis* to the carbonyl due to the anisotropy of this group. A similar difference is observed in N-acetylsarcosine methyl ester [21] and in N-acetyl-N-methyl-L-alanine methyl ester [22] where the N-methyl protons of the preferred *trans* conformation are shifted to lower field than the *cis* N-methyl protons. This assignment has been extended to polysarcosine [21] where the conclusion is drawn that both *cis* and *trans* amides are present and to helical poly-N-methyl-L-alanine which shows only *trans* amides.[22] In an aromatic solvent, like benzene, the N-methyl peaks are shifted upfield, but to different extents due to a specific solvent/solute interaction. The N-methyl *trans* to the carbonyl shows a greater upfield shift than that *cis* to the oxygen.[23] This effect appears to be general for simple amide systems as it is observed in several N,N-dialkylamides,[24] some small, medium and large sized N-methyllactams [25] and in small peptides such as N-acetyl-N-methyl-L-alanine methyl ester.[26] In addition to chemical shift differences a broadening of the N-methyl peaks *cis* to the carbonyl is observed due to a small long range spin coupling through the amide bond to the C_α-protons.[27] This coupling is largest when the N-methyl group is *anti* to a C_α-proton. In a series of dipeptides with N-methylated amino acid as the second residue the N-methyl peak which belongs to *cis* amides is broader than that of *trans* amides.[28] The different effects described for simple flexible systems were applied to our cyclic sarcosine peptides which differ firstly in being relatively small rings with several amide groups present and secondly in being rigid systems. The most suitable molecule with which to check these techniques was cyclo-tetrasarcosyl where the NMR parameters are known. Cyclo-tetrasarcosyl is a centrosymmetric molecule with the amide sequence alternately *cis* and *trans*. Replacement of one sarcosine residue with alanine [7] showed that the upfield N-methyl peak belongs to a *trans* amide group in contrast to what is usually found in the simple amide systems. Furthermore, the upfield peak of the N-methyl *trans* to the carbonyl is significantly broader and exhibits a small coupling of about 0.5 Hz. Spin decoupling showed that this is

Table 1. Yields (%) of cyclic peptides from pentapeptide trichlorophenyl ester.

Starting material	Dipeptides	Tetrapeptides	Pentapeptides	Decapeptides
HCl.H-AlaSar$_4$-OTcp	c-Sar$_2$ (20 %) c-AlaSar (14 %)		c-AlaSar$_4$ (46 %)	c-(AlaSar$_4$)$_2$ (3 %)
HCl.H-Sar-D-Ala-L-AlaSar$_2$-OTcp			c-D-Ala-L-AlaSar$_3$ (38 %)	c-(D-Ala-L-AlaSar$_3$)$_2$ (traces)
HCl.H-Sar$_3$-L-Ala$_2$-OTcp			c-L-Ala$_2$Sar$_3$ (36 %)	c-(L-Ala$_2$Sar$_3$)$_2$ (traces)
HCl.H-Sar$_2$-L-AlaSar-D-Ala-OTcp	c-Sar$_2$ (10 %)	c-L-AlaSar-D-AlaSar (11 %) c-L-AlaSar-L-AlaSar (3 %)	c-L-AlaSar-D-AlaSar$_2$ (11 %) c-L-Ala-Sar-L-AlaSar$_2$ (6 %)	
HCl.H-L-Ala-D-Ala-L-AlaSar$_2$-OTcp			c-L-Ala-D-Ala-L-AlaSar$_2$ (28 %)	c-(L-Ala-D-Ala-L-AlaSar$_2$)$_2$ (traces)
HCl.H-Sar-D-Ala-L-AlaSar-D-Ala-OTcp			c-D-Ala-L-AlaSar-D-AlaSar (55 %) c-D-Ala-L-AlaSar-L-AlaSar (5 %)	
HCl.H-GlySar$_4$-OTcp			c-GlySar$_4$ (53 %)	c-(GlySar$_4$)$_2$ (traces)
HCl.H-SarGly$_2$Sar$_2$-OTcp			c-Gly$_2$Sar$_3$ (6 %)	
HCl.H-GlySarGlySar$_2$-OTcp	c-GlySar (8 %)		c-GlySarGlySar$_2$ (4 %)	

Table 2. The chemical shifts of the NCH_3 protons of cyclic peptides in $CDCl_3$.

	$(NCH_3)_1$		$(NCH_3)_2$		$(NCH_3)_3$		$(NCH_3)_4$		$(NCH_3)_5$		ml C_6D_6 added to 0.5 ml $CDCl_3$
	δ	$\Delta\delta$(Hz)[a]	δ	$\Delta\delta$(Hz)	δ	$\delta\Delta$(Hz)	δ	$\Delta\delta$(Hz)	δ	$\Delta\delta$(Hz)	
c-Sar$_4$	3.0	(19)*cis*	3.0	(19)*cis*	2.83	(36)*trans*	2.83	(36)*trans*			0.4
c-Sar$_5$	3.14	(49)	3.01	(36)	2.93	(4)	2.93	(2)	2.91	(18)	0.4
c-Sar$_6$	2.96	(20)	2.96	(35)	2.91	(10)	2.91	(31)			0.35
c-(Sar$_3$OGl)$_2$	2.98	(30)	2.92	(13)	2.90	(56)					0.35
c-AlaSar$_4$(B)	3.30	(58)	3.25	(49)	3.12	(57)	2.83	(21)			1.2
c-AlaSar$_4$(C)	3.18	(80)	3.07	(105)	2.92	(23)	2.68	(38)			1.2
c-D-Ala-L-AlaSar$_3$ (C)	3.20	(52)	3.15	(30)	3.07	(47)					0.8
c-D-Ala-L-AlaSar$_3$ (D)	3.17	(41)	3.10	(27)	2.84	(16)					0.8
c-L-AlaSar-D-AlaSar$_2$ (C)	3.25	(27)	3.16	(54)	3.11	(27)					0.5
c-L-Ala-D-Ala-L-AlaSar$_2$ (C)	3.26	(51)	3.11	(64)							0.8
c-D-Ala-L-AlaSar-D-AlaSar (B)	3.2	(68)	2.85	(22)							0.9
c-GlySar$_4$ (C)	3.10	(59)	3.09	(90)	2.89	(26)	2.81	(35)			0.8

[a] $\Delta\delta$ represents the chemical shift differences in Hz when the given quantity of C_6D_6 is added in chloroform. The letters B, C and D represent the different conformations formed from the crystal conformation. OGl stands for the glycolic acid residue, $-OCH_2CO-$.

due to coupling to one of the C_α-protons adjacent to the nitrogen, that one which is *anti* to the *N*-methyl group (across a w-path). No coupling through the amide bonds was observed. The structure [7] shows none of the C_α-protons across the amide bonds to be *anti* to the *N*-methyl groups which had been observed to give the largest coupling constant.[28] Dropwise addition of benzene moved the peaks upfield, the *trans* *N*-methyl more than the *cis* (Table 2), which is in accord with the results from the simple amides. These effects were further checked using the higher cyclic sarcosine homologues with known conformation. Cyclo-pentasarcosyl which contains two *trans* and three *cis* amide bonds shows in chloroform solution two "low field" *N*-methyl peaks and three at higher field. On addition of benzene the former moved upfield to a greater extent than the latter indicating that these low field *N*-methyl peaks belong to the two *trans* amides. Of the other three signals two are virtually unaffected by the benzene addition and one shifts slightly upfield (Table 2). Cyclo-octasarcosyl with the amide sequence *cis,cis,trans,trans, cis,cis,trans,trans* and two-fold symmetry in the molecule has two different *trans* and two different *cis* *N*-methyl groups. In the NMR spectrum in chloroform solution two *N*-methyl peaks are resolved, but on addition of benzene four peaks appear. One of these is shifted slightly upfield, one significantly upfield, and the remaining two are intermediate. The same situation was found in the related depsipeptide, c-$Sar_3OGlSar_3OGl$,[29] which takes the same conformation but with one *trans* *N*-methyl peak lacking (Table 2). Peak broadening was difficult to observe in the larger molecules mostly due to the complexity of the spectra. This shows that solvent effects which seem to be general for open chain peptides cannot be extended to cyclic peptides. The folding of the chain into a ring may lead to situations where the chemical shift of an *N*-methyl group is influenced also by carbonyl groups further along the chain but close in space.

Lanthanide shift reagents which induce pseudocontact shifts by interaction with the lone pair of the carbonyl oxygen, usually shift a methyl group *cis* to the carbonyl more downfield than one being *trans*.[30] When $Eu(fod)_3$ was added to a chloroform solution of cyclotetrasarcosyl, the *cis* *N*-methyl group was shifted more than *trans*, but in cyclo-pentasarcosyl one of the two "low-field" *N*-methyl signals assigned to *trans*, showed the largest shift together with one of the three high-field signals, assigned to *cis*. The other signals were also shifted, but to a lesser extent. These downfield shifts are not consistent with the upfield shifts found in benzene, but as the Eu-complex, when situated at a given carbonyl group, will to a greater extent than benzene influence all protons of the molecule, it will be difficult to predict the different shifts when several amide groups are involved.

Carbon-13 magnetic resonance has been used to distinguish between *cis* and *trans* amides in peptides where the nitrogen of proline is part of the peptide bond.[31] Distinct differences in chemical shifts especially for the γ-carbon in the proline ring were found such that the minor *cis* isomer was shifted about 2 ppm upfield relative to the *trans* isomer and that the chemical shifts were the same for several compounds of this type. ^{13}C resonance spectra of cyclo-tetrasarcosyl, cyclo-alanyltrisarcosyl and cyclo-pentasarcosyl, for which the conformations are known, and of cyclo-glycyltetrasarcosyl were recorded at 15 MHz in chloroform solution. Cyclo-tetrasarcosyl showed *cis* and *trans* peaks, separated by 2 – 4 ppm for each of the three types of carbon atoms. Cyclo-alanyltrisarcosyl showed four carbonyl carbons where two belong to *cis* amide bonds and two to *trans*, but these did not form two distinct groups, the *N*-CH_3 *cis* and *trans* were separated by only 1 ppm. In cyclo-pentasarcosyl four of the five carbonyl carbons were resolved, but again not in two groups and the *N*-CH_3's were hardly resolved. These preliminary ^{13}C investigations showed no clear differences in the chemical shifts due to *cis* and *trans* amide bonds. The conformationally unknown cyclo-glycyltetrasarcosyl showed a similar picture with no well separated *cis-trans* peaks.

Thus, none of the different NMR methods used to distinguish between *cis* and *trans* amide bonds in small linear peptides showed convincing results when applied to the cyclic homologues of sarcosine and derivatives. Although the benzene addition showed some evidence that the *N*-CH_3 of a *trans* amide group

moves more upfield than N-CH_3 of a *cis* amide group, the difference in values can be small and cannot be relied upon diagnostically. Benzene addition to the cyclic pentapeptides was, however, useful to resolve overlapping signals since the conformation does not change on changing solvent. Table 2 shows the chemical shifts of the N-methyl protons in $CDCl_3$ solution and the upfield $\Delta\delta$ shifts when C_6D_6 is added mainly for the conformations B and C (D). Addition of benzene was also attempted at low temperature to the A-conformation but with little success due to the difficulty in keeping the temperature low enough so that the B and C conformers did not develop.

DISCUSSION

The NMR spectra clearly show that in these cyclic pentapeptides the crystal conformations are not the same as those favoured in solution. The conformational transformations might well go through additional transient unpopulated conformers not visible in the NMR spectra, but this seems hardly likely and will not be further considered. Conformational transformations seem to be prominent for cyclic pentapeptides containing NH-amide groups. Only the parent compound, c-Sar_5, retains the same conformation in solution as in the crystal.[6] Replacement of one or more sarcosine units with NH-amino acids as glycine or alanine lead to a mixture of conformations in solution with the crystal conformation hardly present. The ability of the NH-amides to form hydrogen bonds has already been proposed as the driving force for these conformational changes.[32] External hydrogen bonds stabilise the crystal conformers while intramolecular hydrogen bonds stabilise the conformers in solution, and the conformational changes must involve stepwise transformations of *cis* amide bonds to *trans*. Among the NH containing cyclic pentapeptides only c-$AlaSar_4$ [15] (Fig. 6A) has had its crystal conformation determined by X-ray methods. It is found to be identical to that of c-Sar_5,[10] both having the amide sequence *cis,cis,cis,trans,trans* (NH). The NH-amide group which occupies one of the two *trans* positions makes an external hydrogen bond with the carbonyl oxygen of the other *trans* amide group of a neighbouring molecule. On dissolution, the intermolecular hydrogen bonds are lost and the new conformer B may be favoured by a hydrogen bond across the ring. This is only possible if one of the *cis* amide groups becomes *trans*, and the sequence *cis,cis,trans,trans,trans* (NH) is suggested [32] (Fig. 6B). The more stable conformer C may arise by transformation of yet another *cis* amide bond to *trans*, to the sequence *trans,cis, trans,trans,trans* (NH) (Fig. 6C). Both B and C resemble the conformations found by X-ray studies of c-Gly_6 [33] and c-Ala_2Gly_4 [34] which contain both inter- and intramolecular hydrogen bonds.

It is likely that all cyclic pentapeptides which can easily fit the crystal conformation of c-$AlaSar_4$, without having any NH-amide group *cis*, will adopt this skeleton in the crystal. Thus, c-$GlySar_4$ may take this skeleton with the NH-amide in one of the two *trans* positions, but not necessarily the same position as in c-$AlaSar_4$ since the α-substituent is now lacking. The same conformational transformations may take place, but in this case conformer B is less stable compared to C as the mixture at equilibrium hardly contains any

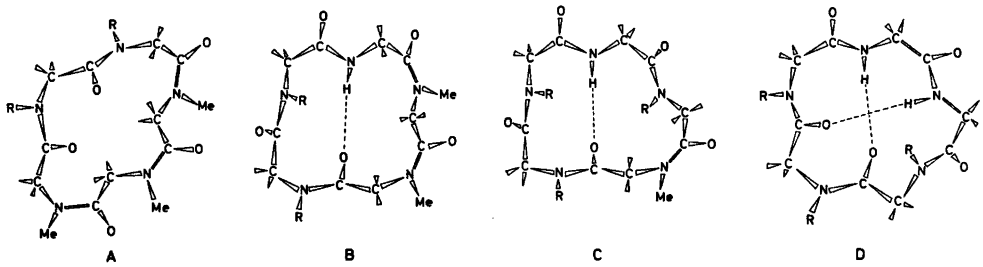


Fig. 6.

B. Both c-D-Ala-L-AlaSar$_3$ and c-Gly$_2$Sar$_3$ fit the crystal skeleton of c-AlaSar$_4$ with the two NH-amides in the *trans* positions, and the same conformers B and C can be formed also here. The last transformation to D seems less clear but may well correspond to the conversion of the remaining *cis* amide bond to *trans*, giving a conformation with all amide bonds *trans* as the most stable. Two intramolecular hydrogen bonds can then be formed as shown in Fig. 6D which is just one example among several possibilities for internal hydrogen bonding.

c-L-AlaSar-D-AlaSar$_3$ and c-L-AlaSar-L-AlaSar$_3$ which have identical NMR spectra both at low and high temperatures cannot easily adopt the crystal conformation found for c-AlaSar$_4$ which would require one NH-amide bond to be *cis*. A crystal structure of a related molecule, actinomycin, which has two identical cyclic pentapeptides, (16-membered ring) shows the amide sequence *cis,cis,trans,trans,trans* with the nitrogens of proline and sarcosine as part of the *cis* amide bonds and a strong hydrogen bond between the pentapeptides.[35] Two adjacent *cis* amide bonds are also found in some sarcosine peptides[6] and it seems therefore likely that the crystal conformation of c-L-AlaSar-D-AlaSar$_3$ has the amide sequence *cis,cis,trans* (NH), *trans,trans* (NH), the same sequence as suggested for conformer B of c-AlaSar$_4$, and with one intra- and one intermolecular hydrogen bond. The conformational transformations may occur as for c-AlaSar$_4$ where the conformer C (Fig. 6C) corresponds to conformer B in c-L-AlaSar-D-AlaSar$_3$ with the amide sequence *trans,cis,trans* (NH), *trans*, *trans* (NH) and one intramolecular hydrogen bond. The second transformation leads to conformer C which should have all the amide bonds *trans* and be similar to that in Fig. 6D. c-GlySarGlySar$_3$ and c-L-Ala-D-Ala-L-AlaSar$_3$ can go through the same conformational transformations and take the same ring conformations without ever having any NH amide bond in *cis*, and the NMR spectra which show closely related changes support this picture.

c-D-Ala-L-AlaSar-D-AlaSar which undergoes only one conformational change from A to B (Fig. 5) cannot easily adopt any of the two aforementioned skeletons for the crystal conformation, but is believed to have a ring conformation close to that in Fig. 6C with the amide sequence *cis,trans* (NH), *trans* (NH), *trans*, *trans* (NH). The transformation of conformer A to B may then correspond to a conversion of the remaining *cis* amide bond to *trans* (Fig. 6D). c-D-Ala-L-AlaSar-L-AlaSar which shows two conformational changes may have the amide sequence *cis,trans* (NH), *trans* (NH), *cis*, *trans* (NH) for conformer A. B may originate by transformation of one of the two *cis* amide bonds to *trans*, giving the sequence *trans,trans* (NH), *trans* (NH), *cis,trans* (NH) and further transformation of the last *cis* amide bond leading to conformer C.

In this series of cyclic pentapeptides a conformation with all amide groups *trans* seems to be the most stable in chloroform solution as the number of observed transformations corresponds to the initial number of *cis* amide bonds present. The only exceptions are c-AlaSar$_4$ and c-GlySar$_4$ which can form only one intramolecular hydrogen bond. Of course, these arguments imply that the barrier for each of the successive transformations is higher than the preceding one.

More definite conclusions about these conformational changes and the nature of the different conformers which are formed must await further detailed NMR studies (^{13}C) and more X-ray structural studies.

EXPERIMENTAL

Solvents used in the reactions were of analytical grade, the light petroleum had b.p. 40 – 60 °C. The monobeded ion-exchange resin, Amberlite, MB-1, analytical grade, was used, and the eluent was methanol. The fully protected linear peptides could all be prepared in methylene chloride. After removal of the benzyloxycarbonyl group was the residual peptide ester (confirmed by NMR) used immediately on isolation.

Many of the peptide derivatives were either viscous oils or non-crystalline solids. Their identity and purity were confirmed by NMR spectroscopy (Varian 60 A) and thin-layer chromatography performed on silica gel G in various solvent systems: ethyl acetate – chloroform 1:2 (A), 5 % methanol – chloroform (B), 10 % methanol – chloroform (C), 15 % methanol – chloroform (D), 20 % methanol – chloroform (E), acetic acid – methanol – chloroform 2:8:90 (F), acetic acid – methanol – chloroform 5:15:80 (G), acetic acid – water – ethanol 10:10:80 (H).

Samples for elemental analysis of the Z-peptide esters were purified on a silica gel column eluting with chloroform, otherwise stated. For the pentapeptides the chloroform elution was followed by chloroform added 2 % methanol, and for the cyclic peptides 2 – 10 % methanol was added to the chloroform. The tendency for the cyclic pentapeptides to contain water even after extensive drying as shown by NMR spectroscopy rendered elemental analysis relatively uninformative.

Abbreviations: Et_3N = triethylamine, DCC = dicyclohexylcarbodiimide, HOTcp = 2,4,5-trichlorophenol, DMF = *N*,*N*-dimethylformamide.

Cyclo-L-alanyltetrasarcosyl

General method for preparation of benzyloxycarbonyl-peptide ester (Z-Sar_2-OMe).[6]

Z-L-AlaSar-OMe. Sarcosine methyl ester hydrochloride[36] (35 g = 250 mmol) was suspended in CH_2Cl_2 (500 ml), Et_3N (25.5 g = 250 mmol) added and the solution filtered before combined with benzyloxycarbonyl-L-alanine[37] (56 g = 250 mmol) in CH_2Cl_2 (100 ml), cooled to – 15 °C and DCC (53.5 g = 260 mmol) added in portions. The reactions mixture was allowed to attain room temperature during 15 h and worked up as described.[6] The residual oil was dissolved in ether and precipitated with light petroleum to leave a viscous oil (72 g = 93 %), homogeneous by TLC, R_F 0.5 (A).

General method for preparation of the benzyloxycarbonyl-peptide acids (Z-Sar_2-OH).[6]

Z-L-AlaSar-OH. Z-L-AlaSar-OMe (70 g = 227 mmol) was hydrolysed in 1 N NaOH (230 ml) for 2 h and the mixture concentrated to ca. 40 ml and extracted twice with CH_2Cl_2. The aqueous layer was then acidified, extracted with CH_2Cl_2, washed with water and dried. The resultant oil was treated with ether and light petroleum and crystallised from acetone on addition of ether (56 g = 84 %), TLC, R_F 0.5 (F), m.p. 137 °C, $[\alpha]_D^{22}$ – 26.3° (*c* 2, MeOH). (Found: C 57.17; H 6.33; N 9.66. $C_{14}H_{18}N_2O_5$ requires C 57.13; H 6.17; N 9.52).

Z-L-AlaSar$_4$-OMe. Z-L-AlaSar-OH (6.5 g = 22 mmol), H-Sar_3-OMe[6] (5.3 g = 21.6 mmol) and DCC (5 g = 24.2 mmol) in CH_2Cl_2 (100 ml) resulted in a white solid (9 g = 80 %). TLC, R_F 0.45 (C), m.p. 65 °C, $[\alpha]_D^{22}$ – 5.7° (*c* 2, MeOH). (Found: C 54.95; H 6.75; N 13.46. $C_{24}H_{35}N_5O_8$ requires C 55.26; H 6.76; N 13.43).

Z-L-AlaSar$_4$-OH. Z-L-AlaSar$_4$-OMe (7.8 g = 15 mmol) and NaOH (16 ml) was reacted for 2 h. After acidification the acid was extracted into chloroform, dried and evaporated. The resulting peptide acid (7 g) was purified by extraction from a CH_2Cl_2 solution into an alkaline aqueous layer, acidification with 2 N hydrochloric acid and extraction into chloroform, followed by drying and evaporation. This afforded a white powder (6.7 g = 88 %), TLC, R_F 0.45 (G), softened at 95 °C, $[\alpha]_D^{22}$ – 5.9° (*c* 2, MeOH). (Found: C 54.42; H 6.49; N 13.76. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55; N 13.80).

General method for preparation of benzyloxycarbonyl-peptide 2,4,5-trichlorophenyl ester (Z-Sar_3-OTcp).[6]

Z-L-AlaSar$_4$-OTcp. Z-L-AlaSar$_4$-OH (3.7 g = 7.3 mmol), HOTcp (2.2 g = 11.1 mmol) and DCC (1.6 g = 7.7 mmol) in CH_2Cl_2 (50 ml) gave a white solid (4.5 g = 90 %), TLC, R_F 0.6 (C). An analytical sample (0.3 g) was further purified on a small silica gel column, softened at 75 °C. (Found: C 50.98; H 5.05. $C_{29}H_{34}N_5O_8Cl_3$ requires C 50.70; H 4.99).

General method for preparation of cyclic peptides (c-Sar_3).[6]

Cyclisation. Z-L-AlaSar$_4$-OTcp (1 g = 1.4 mmol) was hydrogenated for 50 min in methanol (80 ml) containing conc. HCl (0.1 ml) and 5 % Pd-C (0.3 g). The resulting yellowish semi solid (0.8 g = 1.36 mmol = 94 %) of HCl.H-L-AlaSar$_4$-OTcp was dissolved in DMF (50 ml) and added dropwise to stirred pyridine (500 ml) at 115 °C over a period of 1 h and stirred for another 1.5 h. After evaporation the residue was dissolved in methanol (100 ml), passed through an ion-exchange column and eluted with methanol. The first eluate (200 ml) contained most of the cyclic peptides. This was evaporated to dryness, treated with acetone (5 ml) and the undissolved material cyclo-alanyltetrasarcosyl filtered off. The filtrate was combined with the remaining column eluate (400 – 600 ml), which contained further cyclic peptides together with some DC-urea, this total solution evaporated to dryness and purified on a silica gel column, eluting with chloroform followed by chloroform-methanol (2 – 10 %). Yields of cyclic compounds: Cyclo-alanyltetrasarcosyl (45.5 %), TLC, R_F 0.45 (D), m.p. 245 – 250 °C, *m/e* 355, $[\alpha]_D^{22}$ – 2.3° (*c* 2, MeOH), cyclo-disarcosyl (20 %), cyclo-alanyl-sarcosyl (14 %), cyclo-alanyltetrasarcosyl-alanyltetrasarcosyl (3 %), m.p. 195 °C, *m/e* 710. The cyclo-alanyltetrasarcosylalanyltetrasarcosyl was not isolable on the column but was isolated by precipitation from methanol-ether.

Cyclo-D-alanyl-L-alanyltrisarcosyl

Z-Sar-D-Ala-OMe. D-Alanine methyl ester hydrochloride[38] (14 g = 100 mmol), Et_3N (10.5 g = 104 mmol), benzyloxycarbonylsarcosine (22.7 g = 102 mmol) and DCC (22 g = 107 mmol) gave a semi solid (27 g = 87 %), TLC, R_F 0.5 (A). A sample was purified. (Found: C 58.63; H 6.67; N 8.90. $C_{15}H_{20}N_2O_5$ requires C 58.43; H 6.54; N 9.09).

Z-Sar-D-Ala-OH. Z-Sar-D-Ala-OMe (13 g = 42.2 mmol) and NaOH (43 ml) (1.5 h) gave a solid, recrystallised from acetone (10.6 g = 86 %), TLC, R_F 0.55 (F), m.p. 115 – 118 °C, $[\alpha]_D^{22}$ – 6.1° (*c* 2, MeOH). (Found: C 57.15;

H 5.70; N 9.38. $C_{14}H_{18}N_2O_5$ requires C 57.13; H 6.17; N 9.52).

Z-L-AlaSar$_2$-OMe. Sarcosine methyl ester hydrochloride (15 g = 108 mmol), Et_3N (11 g = 109 mmol), Z-L-AlaSar-OH (30 g = 102 mmol) and DDC (23 g = 110 mmol) gave an oil (34 g = 88 %). TLC, R_F 0.5 (C).

Z-Sar-D-Ala-L-AlaSar$_2$OMe. Z-Sar-D-Ala-OH (10 g = 34 mmol), H-L-AlaSar$_2$-OMe (8.5 g = 34.7 mmol) [obtained by hydrogenation of Z-L-AlaSar$_2$-OMe (13.5 g = 35.6 mmol)], DCC (7.7 g = 37.4 mmol) resulted in a foamy solid (15.2 g = 86 %), TLC, R_F 0.5 (C). An analytical sample softened at 70 °C, $[\alpha]_D^{22}$ +18.2° (*c* 2, MeOH). (Found: C 55.05; H 6.83; N 13.66. $C_{24}H_{35}N_5O_8$ requires C 55.26; H 6.76; N 13.43).

Z-Sar-D-Ala-L-AlaSar$_2$-OH. Z-Sar-D-Ala-L-AlaSar$_2$-OMe (11 g = 21 mmol) and NaOH (22 ml) (2 h) resulted in a solid, recrystallised from acetone (9.3 g = 87 %), TLC, R_F 0.5 (G), softened at 90 °C, $[\alpha]_D^{22}$ +5° (*c* 2, MeOH). (Found: C 54.37; H 6.50; N 14.08. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55; N 13.80).

Z-Sar-D-Ala-L-AlaSar$_2$-OTcp. Z-Sar-D-Ala-L-AlaSar$_2$-OH (5 g = 9.86 mmol), HOTcp (2.8 g = 14.2 mmol) and DCC (2.2 g = 10.7 mmol) gave a semi-solid (5.4 g = 80 %), TLC, R_F 0.55 (C). An analytical sample softened at 70 °C. (Found: C 50.76; H 5.22. $C_{29}H_{34}N_5O_8Cl_3$ requires C 50.70; H 4.99).

Cyclisation. Z-Sar-D-Ala-L-AlaSar$_2$-OTcp (1.2 g = 1.75 mmol) was hydrogenated and the white solid (0.9 g = 1.53 mmol = 88 %) of HCl.H-Sar-D-Ala-L-AlaSar$_2$-OTcp cyclised and worked up as described earlier. The residue after passage through an ion-exchange column was taken into small amount of acetone and undissolved material filtered off; a further precipitate formed when kept at 0 °C overnight. The solid consisted mainly of cyclo-D-alanyl-L-alanyltrisarcosyl with some DC-urea which was removed by sublimation (heated to 210 °C/0.01 mmHg). The cyclic pentapeptide sublimed at 230 °C, but tended to decompose at this temperature. The acetone solution, which contained further cyclic pentapeptide, was evaporated, the residue dissolved in chloroform (2 ml) and passed through a small silica gel column. Yield of cyclic compound: Cyclo-D-alanyl-L-alanyltrisarcosyl (38 %), TLC, R_F 0.45 (D), m.p. 270 °C (subl.), *m/e* 355, $[\alpha]_D^{22}$ +14° (*c* 1, MeOH).

Cyclo-di-L-alanyltrisarcosyl

Z-Sar$_2$-L-Ala$_2$-O^{t}Bu. Z-L-Ala$_2$-O^{t}Bu [39] (12 g = 34.5 mmol) (prepared from Z-L-Ala-OH and H-L-Ala – O^{t}Bu [40] using DCC) was hydrogenated and the oil of H-L-Ala$_2$ – O^{t}Bu (7 g = 32.4 mmol) reacted with Z-Sar$_2$-OH [6] (9.5 g = 32.3 mmol) and DCC (7.0 g = 34 mmol) in CH_2Cl_2 (300 ml) resulted in a solid (13 g = 81 %), TLC, R_F 0.6 (C), purified m.p. 90 °C, $[\alpha]_D^{22}$ – 52° (*c* 2, MeOH). (Found: C 58.56; H 7.32; N 11.58. $C_{24}H_{36}N_4O_7$ requires C 58.52; H 7.37; N 11.38).

Z-Sar$_3$-L-Ala$_2$-O^{t}Bu. Z-Sar-OH [41] (1.8 g = 8.1 mmol), H-Sar$_2$-L-Ala$_2$-O^{t}Bu (2.9 g = 8.1 mmol) [obtained by hydrogenation of *Z*-Sar$_2$-L-Ala$_2$-O^{t}Bu (4 g = 8.15 mmol)] and DCC (1.8 g = 8.7 mmol) in CH_2Cl_2 (50 ml) gave a white solid (4 g = 88 %), TLC, R_F 0.35 (C). An analytical sample softened at 70 °C, $[\alpha]_D^{22}$ – 47.6° (*c* 2, MeOH). (Found: C 57.39; H 7.32; N 12.46. $C_{27}H_{41}N_5O_8$ requires C 57.53; H 7.33; N 12.43).

Z-Sar$_3$-L-Ala$_2$-OH. Z-Sar$_3$-L-Ala$_2$O^{t}Bu (3.6 g = 6.4 mmol) was dissolved in ice-cooled trifluoroacetic acid (20 ml), stirred at room temperature for 40 min, evaporated and poured into ether. The ether was decanted, the solid dissolved in acetone and precipitated with ether (2.6 g = 80 %), TLC, R_F 0.4 (G), softened at 85 °C. (Found: C 54.65; H 6.53. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55).

Z-Sar$_3$-L-Ala$_2$-OTcp. Z-Sar$_3$-L-Ala$_2$-OH (1.7 g = 3.35 mmol), HOTcp (1.2 g = 6.1 mmol) and DCC (0.85 g = 4.1 mmol) resulted in a semi-solid (2 g = 87 %), TLC, R_F 0.55 (C). A sample for analysis was crystallised from acetone – ether, softened at 80 °C. (Found: C 50.97; H 5.52; N 10.16. $C_{29}H_{34}N_5O_8Cl_3$ requires C 50.70; H 4.99; N 10.19).

Cyclisation. Z-Sar$_3$-L-Ala$_2$-OTcp (1.9 g = 2.77 mmol) was hydrogenated and the HCl.H-Sar$_3$-L-Ala$_2$-OTcp (1.5 g = 25.6 mmol = 92 %) cyclised. The residue after passage through an ion-exchange column was taken into water to remove undissolved DC-urea and the evaporated residue which showed one main spot on TLC belonging to a cyclic pentapeptide and one minor spot which presumably belongs to the corresponding cyclic decapeptide was chromatographed on a silica gel column. Yield of cyclic compound: Cyclo-di-L-alanyltrisarcosyl (35.4 %), TLC, R_F 0.45 (D), m.p. 225 – 230 °C, *m/e* 355, $[\alpha]_D^{22}$ +20° (*c* 1, MeOH).

Cyclo-L-alanylsarcosyl-D-alanyldisarcosyl

Z-L-AlaSar-D-Ala-OMe. D-Alanine methyl ester hydrochloride (5.8 g = 41.7 mmol), Et_3N (4.3 g = 42.5 mmol), Z-L-AlaSar-OH (12 g = 41 mmol) and DCC (9 g = 43.5 mmol) gave a viscous oil (13 g = 84 %), TLC, R_F 0.6 (C). A sample was purified. (Found: C 57.15; H 6.64; N 10.99. $C_{18}H_{25}N_3O_6$ requires C 56.98; H 6.64; N 11.08).

Z-Sar$_2$-L-AlaSar-D-Ala-OMe. Z-Sar$_2$-OH (5 g = 17 mmol), H-L-AlaSar-D-Ala-OMe (4.2 g = 17.1 mmol) [obtained by hydrogenation of Z-L-AlaSar-D-Ala-OMe (6.5 g = 17.2 mmol)], DCC (3.9 g = 18.9 mmol) gave a foamy solid (7.2 g = 81 %) which on further purification was homogeneous by TLC, R_F 0.4 (C) softened at 65 °C, $[\alpha]_D^{22}$ +5.6° (*c* 2, MeOH).

Z-Sar$_2$-L-AlaSar-D-Ala-OH. Z-Sar$_2$-L-AlaSar-D-Ala-OMe (7 g = 13.4 mmol) and NaOH (14

ml) gave a solid (6.1 g = 90 %) TLC, R_F 0.5 (G), softened at 70 °C, $[\alpha]_D^{22}$ − 5.05° (*c* 2, MeOH). (Found: C 54.36; H 6.69; N 13.96. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55; N 13.80).

Z-Sar$_2$-L-AlaSar-D-Ala-OTcp. Z-Sar$_2$-L-AlaSar-D-Ala-OH (4 g = 7.9 mmol), HOTcp (2.3 g = 11.7 mmol) and DCC (1.8 g = 8.7 mmol) gave a solid (5.1 g = 94 %), TLC, R_F 0.45 (C), crystallised from acetone – ether, softened at 80 °C. (Found: C 51.10; H 5.12. $C_{29}H_{34}H_5O_8Cl_3$ requires C 50.70; H 4.99).

Cyclisation. Z-Sar$_2$-L-AlaSar-D-Ala-OTcp (1 g = 1.46 mmol) was hydrogenated and the HCl.H-Sar$_2$-L-AlaSar-D-Ala-OTcp (0.8 g = 1.36 mmol = 94 %) cyclised. After evaporation of the pyridine the residue was taken into methanol (5 ml) and undissolved material filtered off. This was pure cyclo-L-alanylsarcosyl-D-alanylsarcosyl. The solution was kept at 0 °C overnight and filtered. The precipitate now consisted of some DC-urea and a cyclic tetrapeptide different from that isolated above, and assigned to cyclo-L-alanylsarcosyl-L-alanylsarcosyl (see synthesis). The filtrate was diluted with methanol (100 ml) and passed through an ion-exchange column. The remaining solid after removal of methanol was dissolved in acetone (5 ml) and kept at 0 °C overnight. The precipitate, which showed one spot on TLC (D), melted sharply at 224 °C and had *m/e* = 355 which is the required molecular weight for the cyclic pentapeptide. The filtrate contained more of this cyclic pentapeptide plus two other compounds which were separated by column chromatography on silica gel. The first eluted compound was cyclo-disarcosyl, and the third was the cyclic pentapeptide described above. The second eluted compound, the major product, showed *m/e* = 355 and a fragmentation pattern identical to that of the cyclic pentapeptide isolated above. The latter melted over a wide range, but became more crystalline when dissolved in acetone (2 ml), allowing the solvent to evaporate by leaving the solution open to the air and finally washing with a small amount of acetone. The dried product melted sharply at 274 °C. Yields of cyclic compounds: The major cyclic pentapeptide is suggested to be: Cyclo-L-alanylsarcosyl-D-alanyldisarcosyl (11.3 %). TLC R_F 0.45 (D), m.p. 274 °C, *m/e* 355, $[\alpha]_D^{22}$ − 7° (*c* 1, MeOH) and the minor cyclo-L-alanylsarcosyl-L-alanyldisarcosyl (6.2 %), TLC R_F 0.5 (D), m.p. 224 °C, *m/e* 355, $[\alpha]_D^{22}$ 0° (*c* 0.5, MeOH). The NMR spectra are identical. Further were isolated cyclo-L-alanylsarcosyl-D-alanylsarcosyl (11 %) sublim. temp. 290 °C/0.01 mmHg, m.p. > 350 °C, *m/e* 284, cyclo-L-alanylsarcosyl-L-alanylsarcosyl (2.7 %), sublim. temp. 220 °C/0.01 mmHg, m.p. 340 °C, *m/e* 284 and cyclo-disarcosyl (10.3 %).

Cyclo-L-alanyl-D-alanyl-L-alanyldisarcosyl

Z-L-Ala-D-Ala-L-AlaSar$_2$-OMe. Z-L-Ala-D-Ala-OH (6.2 g = 21.1 mmol) (prepared from Z-L-Ala-OH and HCl.H-D-Ala-OMe using DCC, the protected dipeptide methyl ester was hydrolysed), H-L-AlaSar$_2$-OMe (5.8 g = 23.6 mmol (obtained by hydrogenation of Z-L-AlaSar$_2$-OMe (9.3 g = 24.6 mmol)) and DCC (4.8 g = 23.3 mmHg) in CH_2Cl_2 (70 ml) resulted in a foamy solid (9 g = 82 %), TLC, R_F 0.45 (C). An analytical sample softened at 80 °C, $[\alpha]_D^{22}$ + 3.2° (*c* 2, MeOH). (Found: C 55.25; H 6.66; N 13.38. $C_{24}H_{35}N_5O_8$ requires C 55.26; H 6.76; N 13.43).

Z-L-Ala-D-Ala-L-AlaSar$_2$OH. Z-L-Ala-D-Ala-L-AlaSar$_2$-OMe (7.2 g = 13.8 mmol) in NaOH (15 ml) (2 h) gave a solid which was crystallised from acetone – ether (6.5 g = 93 %), TLC, R_F 0.4 (G), softened at 85 °C, $[\alpha]_D^{22}$ − 1° (*c* 2, MeOH). (Found: C 54.60; H 6.65; N 13.92. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55; N 13.80).

Z-L-Ala-D-Ala-L-AlaSar$_2$-OTcp. Z-L-Ala-D-Ala-L-AlaSar$_2$-OH (3 g = 5.9 mmol), HOTcp (1.7 g = 8.6 mmol) and DCC (1.35 g = 6.55 mmol) resulted in a white powder (3.6 g = 89 %), TLC, R_F 0.4 (C), softened at 85 °C.

Cyclisation. Z-L-Ala-D-Ala-L-AlaSar$_2$-OTcp (0.9 g = 1.3 mmol) was hydrogenated and the HCl.H-L-Ala-D-Ala-L-AlaSar$_2$-OTcp (0.7 g = 1.2 mmol = 91 %) cyclised. The residue after passage through an ion-exchange column was dissolved in acetone (5 ml) and kept overnight at 0 °C. The precipitate contained only a few mg of the cyclic pentamer and some DC-urea which was removed by sublimation, to 200 °C at reduced pressure. The filtrate was evaporated and purified on a silica gel column to give a white compound homogeneous by TLC, but which melted over a wide range. The solid became crystalline when dissolved in acetone (2 ml) and allowed to evaporate to dryness by keeping the sample open in a refrigerator. The residue, cyclo-L-alanyl-D-alanyl-L-alanyldisarcosyl, was washed carefully with acetone and the white powder isolated (28 %) possessed a relatively sharp melting point, m.p. 208 – 210 °C, TLC R_F 0.6 (D), *m/e* 355, $[\alpha]_D^{22}$ = 0° (*c* 2, MeOH).

Cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl

Z-Sar-D-Ala-L-AlaSar-D-Ala-OMe. Z-Sar-D-Ala-OH (5 g = 17 mmol), H-L-AlaSar-D-Ala-OMe (4.2 g = 17.1 mmol) [obtained by hydrogenation of Z-L-AlaSar-D-Ala-OMe (6.5 g = 17.2 mmol)] and DCC (4 g = 19.4 mmol) resulted in a foamy solid (7 g = 79 %), TLC, R_F 0.45 (C). An analytical sample softened at 50 °C, $[\alpha]_D^{22}$ + 39.6° (*c* 2, MeOH). (Found: C 54.92; H 6.91; N 13.68. $C_{24}H_{35}N_5O_8$ requires C 55.26; H 6.76; N 13.43).

Z-Sar-D-Ala-L-AlaSar-D-Ala-OH. Z-Sar-D-Ala-L-AlaSar-D-Ala-OMe (7.3 g = 14 mmol) in NaOH (13 ml) (3 h) gave a solid, recrystallised from acetone – ether (6.6 g = 93 %), TLC, R_F 0.5 (G), m.p. 86 °C, $[\alpha]_D^{22}$ + 36.7° (*c* 2, MeOH). (Found: C 54.54; H 6.50. $C_{23}H_{33}N_5O_8$ requires C 54.43; H 6.55).

Z-Sar-D-Ala-L-AlaSar-D-Ala-OTcp. Z-Sar-D-Ala-L-AlaSar-D-Ala-OH (4 g = 7.9 mmol), HOTcp (2.3 g = 11.7 mmol) and DCC (1.8 g = 8.7 mmol) gave a solid (4.6 g = 85 %), TLC, R_F 0.7 (C), softened at 75 °C.

Cyclisation. Z-Sar-D-Ala-L-AlaSar-D-Ala-OTcp (1.4 g = 2 mmol) was hydrogenated and the HCl.H-Sar-D-Ala-L-AlaSar-D-Ala-OTcp (1.1 g = 1.9 mmol = 92 %) cyclised. The powder left after passage through an ion-exchange column consisted of two compounds (I and II) with approximately the same R_F-values (TLC, solvent D). Most of the major compound (I) could be filtered off after suspending the powder in acetone. The filtrate which still contained both compounds was passed through a silica gel column and the isolated compounds (I and II) both had *m/e* 355 which is the molecular ion required for the cyclic pentapeptide. The major compound (I) is assumed to be cyclo-D-alanyl-L-alanylsarcosyl-D-alanylsarcosyl (53 %), TLC, R_F 0.6 (D), m.p. 317 °C (sublim.), *m/e* 355, $[\alpha]_D^{22}$ + 18° (*c* 2, MeOH). (Found: C 50.85; H 6.92; N 19.87. $C_{15}H_{25}N_5O_5$ requires C 50.69; H 7.09; N 19.71). The minor compound (II) is assumed to be cyclo-D-alanyl-L-alanylsarcosyl-L-alanylsarcosyl (3 %), TLC, R_F 0.55 (D), m.p. 260 °C, *m/e* 355, $[\alpha]_D^{22}$ + 10° (*c* 0.4, MeOH).

Cyclo-glycyltetrasarcosyl

Z-GlySar-OMe. Sarcosine methyl ester hydrochloride (14 g = 100 mmol), Et_3N (10.5 g = 104 mmol), benzyloxycarbonylglycine [37] (21 g = 100 mmol) and DCC (22 g = 107 mmol) resulted in a viscous oil (25 g = 85 %), TLC, R_F 0.7 (B). A sample was purified. (Found: C 56.92; H 5.91; N 9.60. $C_{14}H_{18}N_2O_5$ requires C 57.13; H 6.17; N 9.52).

Z-GlySar-OH. Z-GlySar-OMe (27 g = 92 mmol) in NaOH (93 ml) (2½ h) gave a solid, recrystallised from acetone (20 g = 78 %), TLC, R_F 0.5 (F), m.p. 95 – 97 °C. (Found: C 55.26; H 5.62; N 10.01. $C_{13}H_{16}N_2O_5$ requires C 55.71; H 5.75; N 10.0).

Z-GlySar$_4$-OMe. Z-GlySar-OH (6.5 g = 23.2 mmol), H-Sar$_3$-OMe [6] (5.7 g = 23.2 mmol) and DCC (5.2 = 25.2 mmol) gave a foamy solid (9.2 g = 78 %), TLC, R_F 0.3 (C), softened at 70 °C.

Z-GlySar$_4$-OH. Z-GlySar$_4$-OMe (7.5 g = 14.8 mmol) in NaOH (16 ml) (3 h) gave a solid (6.2 g = 85 %), TLC, R_F 0.6 (H), softened at 80 °C. (Found: C 53.5; H 6.36; N 14.19. $C_{22}H_{31}N_5O_8$ requires C 53.54; H 6.33; N 14.19).

Z-GlySar$_4$-OTcp. Z-GlySar$_4$-OH (3 g = 6.1 mmol), HOTcp (2 g = 10 mmol) and DCC (1.5 g = 7.3 mmol) gave a solid, recrystallised from acetone (3.8 g = 90 %), TLC, R_F 0.65 (D), m.p. 135 °C. (Found: C 50.04; H 4.90; N 10.25. $C_{28}H_{32}N_5O_8Cl_3$ requires C 49.98; H 4.79; N 10.41).

Cyclisation. Z-GlySar$_4$-OTcp (2 g = 2.98 mmol) was hydrogenated and the HCl.H-GlySar$_4$-OTcp (1.6 g = 2.8 mmol = 93 %) cyclised. The residue after passage through an ion-exchange column was suspended in water to remove the DC-urea by filtration, the water was removed from the filtrate and acetone (5 ml) added, some insoluble material was filtered off. This was pure cyclo-glycyltetrasarcosyl and a further crop crystallised out when the solution was kept in a refrigerator. A small amount of cyclo-glycyltetrasarcosyl-glycyltetrasarcosyl (TLC) was present in the filtrate but could not be isolated by these methods. Yield of cyclic compound: cyclo-glycyltetrasarcosyl (52.2 %), TLC, R_F 0.25 (D), R_F 0.3 (E), m.p. 230 °C, *m/e* 341.

Cyclo-diglycyltrisarcosyl

Z-GlySar$_2$-OMe. HCl.H-Sar-OMe (7 g = 50 mmol), Et_3N (5.5 g = 54 mmol), Z-GlySar-OH (14 g = 50 mmol) and DCC (11 g = 53 mmol) resulted in an oil (14 g = 77 %), TLC, R_F 0.7 (C) which did not crystallise from acetone on addition of ether. A sample was purified. (Found: C 55.84; H 6.39; N 11.42. $C_{17}H_{23}N_3O_6$ requires C 55.88; H 6.35; N 11.50).

Z-Gly$_2$Sar$_2$-OMe. Z-GlySar$_2$-OMe (10 g = 27.4 mmol) was hydrogenated and the oil of H-GlySar$_2$-OMe (6.3 g = 27 mmol) dissolved in CH_2Cl_2 (200 ml) together with Z-Gly-OH (5.6 g = 27 mmol) and DCC (6.2 g = 30 mmol) and the resulting solid (9 g = 82 %) recrystallised from acetone, TLC, R_F 0.45 (C), m.p. 120 °C. (Found: C 54.81; H 6.59; N 13.11. $C_{20}H_{28}N_4O_7$ requires C 55.03; H 6.47; N 12.84).

Z-SarGly$_2$Sar$_2$-OMe. Z-Sar-OH (4.2 g = 18.8 mmol), H-Gly$_2$Sar$_2$-OMe (5.4 g = 18.8 mmol) [obtained by hydrogenation of Z-Gly$_2$Sar$_2$-OMe (8 g = 19 mmol)] and DCC (4.3 g = 20.8 mmol) gave a solid (7.9 g = 85 %), TLC, R_F 0.6 (D). An analytical sample softened at 65 °C. (Found: C 53.46; H 6.42; N 13.92. $C_{22}H_{31}N_5O_8$ requires C 53.54; H 6.33; N 14.19).

Z-SarGly$_2$-Sar$_2$-OH. Z-SarGly$_2$Sar$_2$-OMe (7.2 g = 14.6 mmol) was hydrolysed for 2 h in NaOH (16 ml). Extraction of the acidified aqueous layer with chloroform afforded only 4.2 g of the corresponding acid. In order to isolate more of the acid, the aqueous layer was evaporated nearly to dryness, and extraction with chloroform gave a further 1.8 g (6 g = 86 %), TLC, R_F 0.7 (H), softened at 90 °C.

Z-SarGly$_2$Sar$_2$-OTcp. Z-SarGly$_2$Sar$_2$-OH (2 g = 4.18 mmol), HOTcp (1.3 g = 6.6 mmol) and DCC (1 g = 4.86 mmol) gave a solid, recrystallised from acetone (2.4 g = 87 %), TLC, R_F 0.7 (D), softened at 90 °C. (Found: C 49.27;

H 4.69. $C_{27}H_{30}N_5O_8Cl_3$ requires C 49.22; H 4.59).

Cyclisation. Z-SarGly$_2$Sar$_2$-OTcp (1.5 g = 2.28 mmol) was hydrogenated and the HCl.H-SarGly$_2$Sar$_2$-OTcp (1.2 g = 2.15 mmol = 94 %) cyclised. The residue after passage through an ion-exchange column was dissolved in methanol (1 ml) and the solvent allowed to evaporate by keeping the sample open in a refrigerator, the residue was washed carefully with methanol to give a white powder of cyclo-diglycyltrisarcosyl (5.7 %), TLC, R_F 0.35 (E), m.p. 209–211 °C, *m/e* 327.

Cyclo-glycylsarcosylglycyldisarcosyl

Z-GlySarGlySar$_2$-OMe. Z-GlySar-OH (5 g = 17.8 mmol), H-GlySar$_2$-OMe (4 g = 17.3 mmol) and DCC (4 g = 19.4 mmol) gave a foamy solid (7.4 g = 87 %), TLC, R_F 0.3 (C). An analytical sample softened at 65 °C. (Found: C 53.42; H 6.36; N 13.91. $C_{22}H_{31}N_5O_8$ requires C 53.54; H 6.33; N 14.19).

Z-GlySarGlySar$_2$-OH. Z-GlySarGlySar$_2$-OMe (6.4 g = 13 mmol) in NaOH (14 ml) (2½ h) gave the acid (5.5 g = 88 %), TLC, R_F 0.65 (H), softened at 80 °C. (Found: C 52.46; H 5.86. $C_{21}H_{29}N_5O_8$ requires C 52.60; H 6.10).

Z-GlySarGlySar$_2$-OTcp. Z-GlySarGlySar$_2$-OH (3 g = 6.3 mmol), HOTcp (1.8 g = 9.1 mmol) and DCC (1.5 g = 7.28 mmol) gave a solid, crystallised from acetone; (3.7 g = 90 %), TLC, R_F 0.65 (D), m.p. 145–147 °C. (Found: C 49.07; H 4.68. $C_{27}H_{30}N_5O_8Cl_3$ requires C 49.22; H 4.59).

Cyclisation. Z-GlySarGlySar$_2$-OTcp (1.35 g = 2.05 mmol) was hydrogenated and the HCl.H-GlySarGlySar$_2$-OTcp (0.9 g = 1.6 mmol = 79 %) cyclised. The residue after passage through an ion-exchange column was chromatographed on a silica gel column and two cyclic compounds were isolated, cyclo-glycylsarcosyl (8.3 %) and cyclo-glycylsarcosylglycyldisarcosyl, the latter melting over a wide range. The cyclic pentapeptide became more crystalline by dissolving in methanol (1 ml) and allowing the solvent to evaporate by keeping the sample open in a refrigerator. The residue was washed carefully with methanol and the white powder (4 %) of cyclo-glycylsarcosylglycyldisarcosyl was homogeneous by TLC, R_F 0.3 (E), had *m/e* 327 and melted at 222–230 °C.

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Reactions of Aromatic Anion Radicals and Dianions. VI. The Contribution of Homogeneous Electron Transfer Reactions to Electrochemically Irreversible Electrode Processes

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The voltammetric reduction of 3,8-dimethyl-2-methoxyazocine (DMMA) was studied in THF, DMF, and acetonitrile in the presence of Bu_4NBF_4 and Me_4NBF_4. When the counter ion was Me_4N^+, the reduction was observed to be a quasi-reversible process in DMF providing that proton donors were excluded. In DMF and THF, both containing traces of water, or in dry acetonitrile, the very basic $DMMA^{2-}$ was rapidly protonated. In the presence of Bu_4N^+, the reduction of DMMA is best described by the following equations:

$$DMMA + e^- \rightleftharpoons DMMA^{\cdot -} \quad \text{(slow at the electrode)}$$

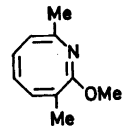
$$DMMA^{\cdot -} + e^- \rightleftharpoons DMMA^{2-} \quad \text{(fast at the electrode)}$$

$$DMMA^{2-} + DMMA \rightleftharpoons DMMA^{\cdot -} \quad \text{(fast in solution)}$$

Simulated cyclic voltammograms for this mechanism were nearly identical to the experimental voltammograms. It was concluded that the reason for the slow electron transfer to DMMA at the electrode is due to an electrolyte effect rather than to the activation energy necessary to bring DMMA from the tub conformation into a planar or near planar transition state for electron transfer.

The electrochemically irreversible reduction of cyclooctatetraene (COT) to the anion radical has been related to the activation energy necessary to flatten the tub conformation of COT into a planar transition state.[1] Characteristic of such irreversible electrochemical processes are cyclic voltammograms in which the reduction peak for the substrate and the corresponding oxidation peak for the ion radical are separated by more than the theoretical amount for a one electron process, 57 mV.[2] The peak separation for the $COT - COT^{\cdot -}$ couple in DMF containing Bu_4NBF_4 is of the order of 200 mV at room temperature.[3] A much larger peak separation and hence a much slower electron transfer has been reported for a related compound, 3,8-dimethyl-2-methoxyazocine (*1*, DMMA). A peak separation of about 800 mV and a heterogeneous rate constant for electron transfer of 10^{-6} cm/s were reported.[4]

Here we report the results of a voltammetric study of the reduction of DMMA which show that the data previously reported [4] were obtained under conditions where the DMMA anions were rapidly protonated and are thus irrelevant to the discussion of electron transfer kinetics.



1

The cyclic voltammogram of DMMA in carefully purified THF containing Bu_4NBF_4 (0.2 M) was essentially the same as that previously reported [4] and consisted of a broad two electron reduction peak and a corresponding oxidation

peak on the reverse scan, the peak to peak separation being about 1 V. When the last traces of water or other proton donors were removed by adding activated alumina to the cell,[5,6] the voltammogram was characteristic for a quasi-reversible redox reaction with a peak separation of about 300 mV. Thus, the voltammogram recorded in the absence of alumina and that previously reported [4] were due to the electron transfer protonation reaction (1).

$$\mathrm{DMMA} + 2e^- + \mathrm{HOH} \rightleftharpoons (\mathrm{DMMA\text{-}H})^- + \mathrm{HO}^- \quad (1)$$

The rate of electron transfer between organic molecules and an electrode in aprotic media has been shown to be greater in the presence of tetramethylammonium ion than when larger supporting electrolyte cations are used.[3,6a,7] The voltammogram for the reduction of DMMA in DMF in the presence of Me_4NBF_4 is illustrated in Fig. 1. The 2e redox couple shows a peak separation of 80 mV. The two electron reduction peak could not be resolved into consecutive 1e steps by cooling to -60 °C or by heating the voltammetric solution. Consequently, it appears that $E°$ for reaction (2) is at more negative potential than that for (3) and that the product of the electrode reaction is $DMMA^{2-}$. That the reduction is a direct 2e process was

$$\mathrm{DMMA} + e^- \rightleftharpoons \mathrm{DMMA}^{\cdot -} \quad (2)$$

$$\mathrm{DMMA}^{\cdot -} + e^- \rightleftharpoons \mathrm{DMMA}^{2-} \quad (3)$$

further demonstrated by the sweep rate dependence of the reduction peak potential. A plot of the peak separation as function of the logarithm of the voltage sweep rate was linear at sweep rates higher than 100 mV s^{-1} with a slope of 65 mV/decade. The peak potential shift for an irreversible electron transfer reaction is $30/\alpha n_\alpha$ mV/decade,[8] *i.e.* the peak potential separation for an irreversible reduction coupled to an irreversible oxidation increases $60/\alpha n_\alpha$ mV/decade at 25 °C. From the symmetry of the observed cyclic voltammogram we conclude, that α must be close to 0.5, and n_α must then be 2, *i.e.* it is a direct two electron reduction. The mechanism of the reduction under these conditions can be described as DMMA diffusing to and $DMMA^{2-}$ diffusing from the electrode without the intermediate formation of $DMMA^{\cdot -}$ (4). Under comparable

$$\mathrm{DMMA} + 2e^- \rightleftharpoons \mathrm{DMMA}^{2-} \quad (4)$$

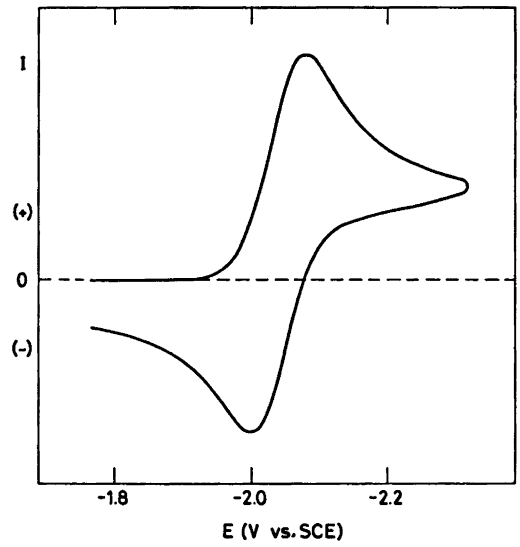


Fig. 1. Cyclic voltammogram of DMMA in DMF containing Bu_4NBF_4 (0.2 M) and Me_4NBF_4 (saturated). Voltage sweep rate 240 mV/s.

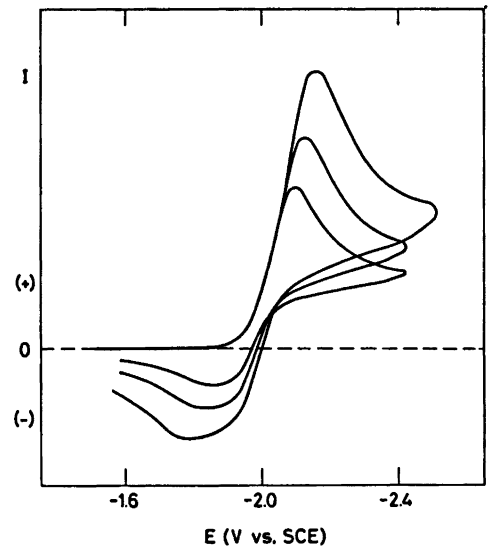


Fig. 2. Cyclic voltammograms of DMMA in DMF containing Bu_4NBF_4 (0.2 M). Voltage sweep rate for voltammograms of increasing currents; 153, 310 and 620 mV/s.

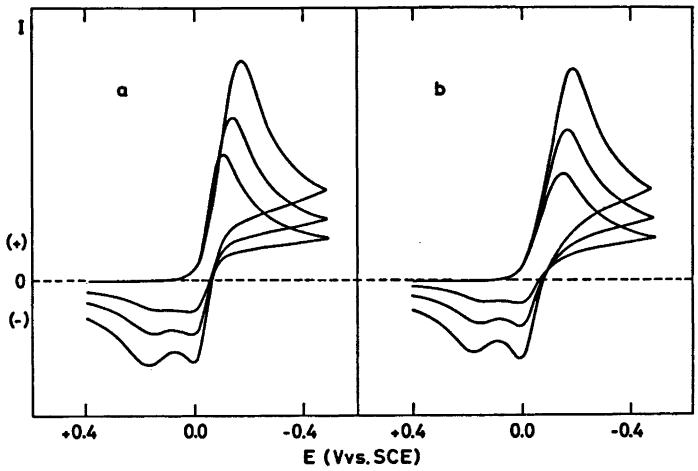


Fig. 3. Simulated cyclic voltammograms for the reduction of DMMA assuming that DMMA + $DMMA^{2-} \rightleftharpoons 2DMMA^{\cdot-}$
(a) is fast in solution and (b) is slow in solution. The simulated mechanism is:

$DMMA + 2e^- \rightleftharpoons DMMA^{2-}$; $E_1° = 0$; $k_s = 5 \times 10^{-3}$ cm s^{-1}; $\alpha = 0.5$
$DMMA^{\cdot-} + e^- \rightleftharpoons DMMA^{2-}$; $E_2° = -30$ mV; fast reversible
$DMMA + DMMA^{2-} \rightleftharpoons 2\ DMMA^{\cdot-}$; (a) $k_f = 1.24 \times 10^4$ M^{-1} s^{-1}
$k_b = 3.64 \times 10^3$ M^{-1} s^{-1}
(b) $k_1 = k_b = 0$

$DMMA^{2-} \rightarrow$ protonated products; $k = 0.248$ s^{-1}
Sweep rates 620, 310 and 155 mV s^{-1}; temperature +11 °C. These values are calculated assuming that all diffusion coefficients are equal to 9×10^{-6} cm^2 s^{-1}.

conditions, COT is reduced in two consecutive steps with a $\Delta E°$ of about 250 mV. A further contrast between COT and DMMA is the relative stability of the corresponding dianions. COT shows two reversible 1e reductions leading to COT^{2-} in acetonitrile[3,8] even at very low voltage sweep rates. On the other hand, no reverse current was observed during cyclic voltammetry of DMMA at voltage sweep rates up to 1 v/s. We therefore conclude that $DMMA^{2-}$ is so basic that it is rapidly protonated by acetonitrile.

When Bu_4N^+ instead of Me_4N^+ was the counter ion, the mechanism of the reduction of DMMA in DMF is far more complicated than (4). The cyclic voltammograms (Fig. 2) show that the reduction peak is sharp while the oxidation gives rise to two broad overlapping peaks. The initial part of the reduction peak is independent of the voltage sweep rate, *i.e.* the current is determined *only* by the potential and *not* by the sweep rate. Voltammograms such as those in Fig. 2 have previously been reported[1,3] for COT and have been attributed[1] to the slow electron transfer to COT due to the activation energy for ring flattening. If the latter were the case, homogeneous electron transfer would also be slow since the same forces must be overcome. The expected cyclic voltammograms, calculated by digital simulation,[9] for such a case are shown in Fig. 3b. The significant differences between the observed (Fig. 2) and the calculated (Fig. 3b) voltammograms are the initial slopes of the reduction curves and the widths of the reduction peaks. The form of the experimental voltammograms (Fig. 2) are very closely reproduced by simulation (Fig. 3a) for the following reaction scheme (5 – 7). While the heterogeneous electron

$DMMA + e^- \rightleftharpoons DMMA^{\cdot-}$ (slow at the electrode) (5)

$DMMA^{\cdot-} + e^- \rightleftharpoons DMMA^{2-}$ (fast at the electrode) (6)

$DMMA^{2-} + DMMA \rightleftharpoons 2\ DMMA^{\cdot-}$ (fast in solution) (7)

transfer (5) to DMMA is slow due to the presence of the bulky Bu_4N^+ ions, the homogeneous electron transfer to DMMA from the dianion (7) is not inhibited to the same degree. Therefore, the primary cause of the slowness of (5) cannot be the activation energy for bringing DMMA from the tub conformation into a planar or near planar transition state for electron transfer, as was previously proposed for DMMA [4] and originally for COT.[1] Although the experimental (Fig. 2) and calculated (Fig. 3a) voltammograms are nearly identical in shape, schemes other than (5 – 7) may possibly also fit the data.

More detailed studies of the voltammetric reduction of COT, DMMA, and related systems will appear later.

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Reaction of Sugar Esters with Hydrogen Fluoride. XV. Ring Contraction of some Hexopyranose Derivatives

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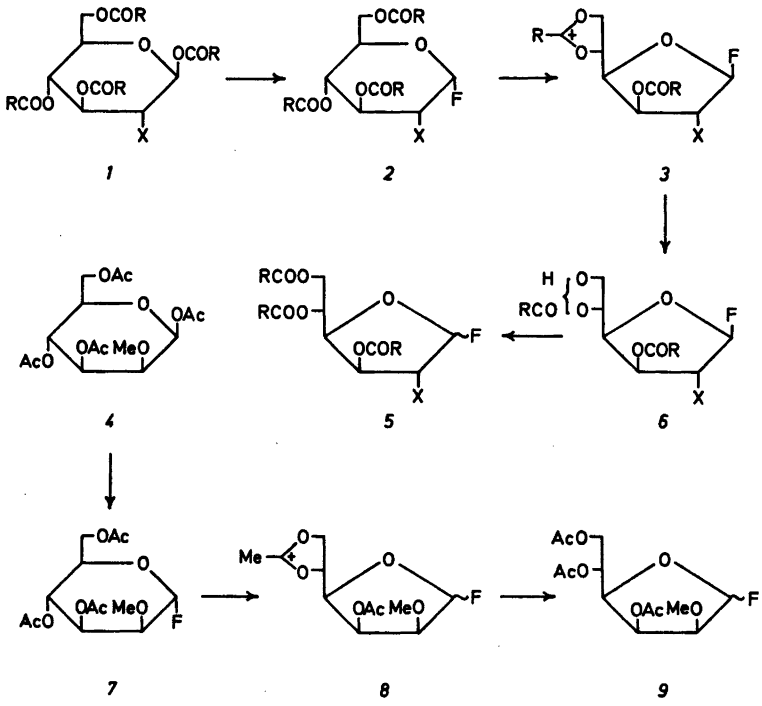
Prolonged treatment of 2-*O*-methyl-D-glucopyranose and -D-mannopyranose derivatives with anhydrous hydrogen fluoride leads to ring contraction and formation of the corresponding furanosyl fluorides. Similar results are obtained with 2-chloro- and 2-bromo-2-deoxy-D-glucopyranose derivatives.

Previously reported results on the treatment of tetra-*O*-benzoyl-2-*O*-methyl-β-D-glucopyranose (*1b*) with anhydrous hydrogen fluoride (HF) at room temperature showed that ring contraction to furanose derivatives took place to a certain extent.[1] Later experiments showed that acylated 2-*O*-methyl-D-xylose derivatives gave furanoses with HF whereas acylated 2-*O*-methyl-D-arabinose derivatives gave pyranoses.[2] In these two cases the nature of the final products is determined by the formation of stable dioxolanylium ions in HF solution.

In the previous study of the reaction of 2-*O*-methyl-glucose derivatives with HF the longest reaction time used was arbitrarily chosen to be 24 h.[1] However, when it was later found that ^{1}H NMR spectra could be measured on HF solutions the progress of the reaction of sugar esters with HF could be followed. It was then shown that dioxolanylium ions were the final products formed in anhydrous HF.[3,4] Using this technique the reaction of 2-*O*-methyl-D-glucose derivatives with HF has now been reinvestigated. Furthermore, the behaviour of 2-*O*-methyl-D-mannopyranose derivatives and of 2-chloro- and 2-bromo-2-deoxy-D-glucopyranose derivatives has also been studied.

When the reaction of tetra-*O*-benzoyl-2-*O*-methyl-β-D-glucopyranose (*1b*) with anhydrous HF was followed by ^{1}H NMR spectroscopy it was found that the fluoride (*2b*) was formed within *ca.* 20 min. On further reaction at room temperature (*2b*) slowly disappeared and a complex spectrum was observed. After 9 days no further changes were observed. Work up of the HF solution at this stage followed by benzoylation gave the anomeric furanosyl fluorides (α- and β-*5b*) in yields slightly higher than those obtained previously when 24 h reaction was used.[1] Only a few percent of the pyranosyl fluoride (*2b*) could be isolated.

Because of the slow reaction of (*1b*) the corresponding acetate (*1a*) was studied. Brief treatment of (*1a*) with anhydrous HF gave the α-pyranosyl fluoride (*2a*) in 80 % yield as previously reported.[5] On further reaction with HF the initially formed (*2a*) underwent ring concentration and after 48 h at room temperature NMR spectra of the HF solution showed that the dioxolanylium ion (*3a*) was present as virtually the only product. A 3 H signal at δ 2.86 shows that an acetoxonium ion is formed.[3,4] A 6 H signal at δ 2.56 arises from 2 equivalents of acetic acid and a 3 H signal at δ 2.32 from the 3-acetoxy group of (*3a*). A doublet centered at δ 6.18 with a splitting of 60 Hz (J_{1F}) and $J_{1,2} \simeq 0$ (H1-H2 *trans*) shows that the product is a β-furanosyl fluoride.[4] Further spectral data of (*3a*) are given in Table 1. Work up of the HF solution at this stage gave a mixture of partially acetylated furanoses (*6a*), which was immediately acetylated. Chromatography then yielded 63 % tri-*O*-acetyl-2-*O*-methyl-β-D-glucofuranosyl fluoride (β-*5a*) and 20 % of the corresponding α-anomer (α-*5a*). The latter was not observed in



a; R = CH_3, X = OMe c; R = CH_3, X = Br

b; R = C_6H_5, X = OMe d; R = CH_3, X = Cl

the NMR spectrum of the HF solution and is probably formed by anomerisation of (*β-5a*) during work up.

The structures of the acetylated furanosyl fluorides (α- and *β-5a*) are derived from their ^1H, ^{19}F, and ^{13}C NMR spectra (Table 1 and Ref. 6). The ^1H spectra are quite similar to those of the corresponding benzoates the structures of which were established by chemical means.[1]

Treatment of tetra-*O*-acetyl-2-*O*-methyl-*β*-D-mannopyranose (*4*) for 10 min with anhydrous HF at 0 °C gave a 63 % yield of tri-*O*-acetyl-2-*O*-methyl-α-D-mannopyranosyl fluoride (*7*).[5] On more prolonged reaction at room temperature (*7*) also underwent ring contraction, and after 48 h it was completely converted into the acetoxonium ion (*8*) as seen from the ^1H NMR spectrum in HF (Table 1). Work up and acetylation gave 40 % of tri-*O*-acetyl-2-*O*-methyl-*β*-D-mannofuranosyl fluoride (*β-9*) and 11 % of the corresponding α-anomer (*α-9*). The structures of these products were derived from their ^1H, ^{19}F, and ^{13}C NMR spectra (Table 1 and Ref. 6).

When gluco- or mannopyranose pentaacetates are treated with HF, they undergo inversion at C2 and C3 to give manno- and altropyranose derivatives.[7] Furanoses are formed to a minor extent only. In the absence of a 2-*O*-acyl group, as in the 2-*O*-methyl-pyranoses, inversion cannot take place and ring contraction therefore becomes predominating, leading to formation of the stable 5,6-dioxolanylium ions *3* and *8*. The pyranose derivatives might form 4,6-acetoxonium or benzoxonium ions; such ions have, however, not been observed in hydrogen fluoride solution. 4,6-Acetoxonium ions are formed when tetra-*O*-acetyl-*β*-D-glucopyranosyl chloride is treated with antimony pentachloride; with this reagent ring contraction does, on the other hand, not take place.[8]

Table 1. ^1H and ^{19}F chemical shifts and observed first order coupling constants of some hexofuranosyl fluorides and of dioxolanylium ions.

Compound, solvent	H1	H2	H3	H4	H5	H6	H6'	J_{12}	J_{23}	J_{34}	J_{45}	J_{56}	$J_{56'}$	$J_{66'}$	J_{1F}	J_{2F}	J_{4F}	Φ_F
α-*5a*, $CDCl_3$	5.88	3.89	5.46	4.59	5.24	4.54	4.12	3.8	3.5	5.0	9.0	2.5	5.2	−12.5	62.5	14.5	~1	−64.6
β-*5a*, $CDCl_3$	5.69	3.89	5.35	4.62	5.36	4.60	4.20	0	0	5.0	9.0	2.5	4.8	−12.5	62.0	5.0	6.0	−45.5
α-*5b*, $CDCl_3$	5.98	4.18	5.92	5.0	5.8	4.95	4.6	3.8	5.0	6.0	9.0	2.5	5.0	−12.5	62.5	16.5	~1	−63.3
β-*5b*, $CDCl_3$	5.83	4.10	5.74	5.07	5.88	5.08	4.75	0	0	5.5	9.0	2.5	4.5	−12.5	62.5	5.3	6.5	−45.3
α-*9*, $CDCl_3$	5.76	4.05	5.53	4.53	5.31	4.58	4.14	2.2	5.0	3.9	8.7	2.4	5.4	−12.2	64.7	16.1	2.0	−48.1
β-*9*, $CDCl_3$	5.72	3.82	5.72	4.44	5.36	4.61	4.24	3.4	5.2	4.8	9.5	2.3	4.7	−12.3	65.8	22.6	7.0	−53.8
β-*5c*, $CDCl_3$	5.96	4.28	5.56	4.85	5.41	4.70	4.24	0	0	4.5	9.0	2.5	4.7	−12.5	64.5	6.0	6.5	−25.5
3a, HF	6.18	4.43	6.1		5.2	——5.8		~0	~0						60	5.3		
3c, HF	6.13	4.43	6.08		5.4	——5.7		~0	~0						62	6.5		
3d, HF	6.12	4.52	6.0		5.3	——5.8		~0							56	~5		
8, HF	6.12	4.38	6.02		5.1	——5.7		3.5	5	~5						23		

Other acylated hexopyranoses without an *O*-acyl group at C2 would also be expected to undergo ring contraction on treatment with HF. To investigate this tetra-*O*-acetyl-2-bromo-2-deoxy-β-D-glucopyranose (*1c*) was reacted with HF. Brief treatment gave tri-*O*-acetyl-2-bromo-2-deoxy-α-D-glucopyranosyl fluoride (*2c*),[9] which slowly underwent further reactions in HF. After 2 weeks at +5 °C *1c* was completely converted into the acetoxonium ion (*3c*) as seen from the ^1H spectrum (Table 1). Work up and acetylation gave the 2-bromo-2-deoxy-furanosyl fluoride (β-*5c*).

The corresponding 2-chloro-2-deoxy compound (*1d*) behaved in the same way. After reaction with HF for 4 days at room temperature it was completely converted into the acetoxonium ion (*3d*) as seen from the ^1H NMR spectrum (Table 1). The HF solution was not worked up in this case.

Tetra-*O*-acetyl-2-chloro- or 2-bromo-2-deoxy-D-mannopyranose decomposed on prolonged treatment with anhydrous HF. This is probably analogous to the reaction of 2-chloro-2-deoxy-D-mannose derivatives with hydrochloric acid, which yields an unsaturated compound.[10] The latter would decompose on treatment with HF.[11]

It might be expected that acylated 2-deoxy-hexopyranoses, having no 2-*O*-acyl group, would behave analogously to the compounds described above. Previous work has, however, shown that tetra-*O*-benzoyl-2-deoxy-D-glucopyranose reacts in a completely different way when treated with anhydrous HF.[11]

The reaction of 3-*O*-methylated pyranoses with HF will be described in a forthcoming paper.

EXPERIMENTAL

^1H NMR spectra and thin layer chromatography was performed as described previously.[7] ^{19}F NMR spectra were measured at 94.10 MHz on a Varian HA-100 instrument. Positions of signals (ΦF) are given in ppm relative to internal methyl trifluoroacetate (5 %).

*Reaction of tetra-*O*-acetyl-2-*O*-methyl-β-D-glucopyranose (*1a*) with HF.* A solution of (*1a*)[12] (558 mg) in anhydrous HF (1 ml) was kept for 48 h at room temperature. It was then diluted with dichloromethane and poured on ice. The organic phase was washed twice with aqueous sodium hydrogen carbonate, dried

($MgSO_4$) and evaporated. The product (440 mg) was acetylated with acetic anhydride in pyridine to give 450 mg of a material which was separated into two fractions by preparative TLC using benzene-ether (1:1) as eluent. The fast-moving fraction gave 315 mg (63 %) of tri-*O*-acetyl-2-*O*-methyl-β-D-glucofuranosyl fluoride (*β-5a*) as a syrup, $[\alpha]_D^{20}$ −15.5° (*c* 4.9, $CHCl_3$). Anal. $C_{13}H_{19}FO_8$: C, H. The next fraction yielded 100 mg (20 %) of the syrupy α-anomer (*α-5a*), $[\alpha]_D^{20}$ +59.2° (*c* 0.9, $CHCl_3$). Anal. $C_{13}H_{19}FO_8$: C, H.

*Tetra-*O*-acetyl-2-*O*-methyl-β-*D*-mannopyranose (4) and HF.* Treatment of *4* [13] (583 mg) with anhydrous HF (1.5 ml) for 48 h at room temperature followed by work up and acetylation gave a crude product (350 mg) which was separated into two fractions by preparative TLC with ether-pentane (1:1) as eluent. The fast-moving fraction yielded 55 mg (11 %) of tri-*O*-acetyl-2-*O*-methyl-α-D-mannofuranosyl fluoride (*α-9*) as a syrup, $[\alpha]_D^{23}$ +45.7° (*c* 1.8, $CHCl_3$). Anal. $C_{13}H_{19}FO_8$: C, H. The slow-moving fraction gave 210 mg (40 %) of the corresponding β-anomer (*β-9*), which was crystallized from ether, m.p. 103–104 °C, $[\alpha]_D^{23}$ +11.1 (*c* 2.0, $CHCl_3$). Anal. $C_{13}H_{19}FO_8$: C, H.

*Reaction of tetra-*O*-acetyl-2-bromo-2-deoxy-β-D-glucopyranose* (1c) *with HF.* A solution of *1c* [14] (506 mg) in anhydrous HF (2 ml) was kept for 14 days at +5 °C. Work up and acetylation as described above gave 305 mg of crude product. Preparative TLC (ether-pentane 2:1) yielded 278 mg (61 %) of tri-*O*-acetyl-2-bromo-2-deoxy-β-D-glucofuranosyl fluoride (*β-5c*) as a syrup $[\alpha]_D^{20}$ +57.4° (*c* 2.8, $CHCl_3$). Anal. $C_{12}H_{16}BrFO_7$: C, H, Br.

Microanalyses were carried out by NOVO analytical laboratory.

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Structural Analogues of GABA. Synthesis of 5-Aminomethyl-3-isothiazolol (Thiomuscimol)

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The synthesis of 5-aminomethyl-3-isothiazolol zwitterion (*12*), a thio analogue of muscimol (5-aminomethyl-3-isoxazolol), is described. Reaction of the starting material aminofumaramide (*1*) with excess of hydrogen sulfide gives dithiodisuccinamide (*2*) which is oxidized by bromine to give 3-hydroxyisothiazole-5-carboxamide (*3*). Conversion of *3* into the *O*-methyl derivative *5* by treatment with diazomethane, followed by reduction with diborane gives 3-methoxy-5-aminomethylisothiazole, the hydrochloride *9* of which is transformed into 5-aminomethyl-3-isothiazolol dihydrobromide (*10*). Treatment of *10* with ethanol gives the corresponding monohydrobromide *11* and conversion of *10* into 5-aminomethyl-3-isothiazolol zwitterion (*12*) is accomplished by treatment with triethylamine. The pK_A values of *12* are determined to 6.06 ± 0.03 and 8.85 ± 0.04.

Muscimol (5-aminomethyl-3-isoxazolol), a centrally active constituent of *Amanita muscaria*,[1,2] is a semirigid cyclic analogue of γ-aminobutyric acid (GABA).[3,4] Several muscimol analogues containing various aminoalkyl groups in position 4 or 5 of the 3-isoxazolol nucleus have been synthesized,[5–10] and a structure-activity correlation of this series of conformationally restricted GABA analogues has been made.[11] Other possible structural changes of muscimol are variations of the heterocyclic ring. This paper describes the synthesis of 5-aminomethyl-3-isothiazolol (thiomuscimol) (*12*) by the sequence outlined in Scheme 1. The biological properties of thiomuscimol (*12*) are being investigated.

Aminofumaramide (*1*) is treated with hydrogen sulfide in glacial acetic acid to give dithiodisuccinamide (*2*). A crude specimen of *2* is converted into 3-hydroxyisothiazole-5-carboxamide (*3*) by oxidation with bromine using a procedure analogous with that described by Goerdeler and Mittler.[12] Attempts to reduce the carboxamide group of *3* with diborane give very complex reaction mixtures from which no product can be isolated. The pronounced garlic smell of the mixture indicates degradation of the isothiazole ring of *3* under the conditions used, and protection of the hydroxy group of *3* prior to reduction is considered necessary.

Reactions of *3* with 4-toluenesulfonyl chloride and benzenesulfonyl chloride give *4a* and *4b*, respectively, in moderate yields and without contamination with the product containing an arylsulfonyl group in position 2 which is in agreement with the findings of Chan and Crow for similar reactions.[13] Reactions of *4a,b* with diborane, however, give complex reaction mixtures containing small amounts of ninhydrin-sensitive products, as revealed by

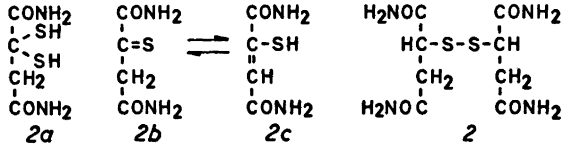


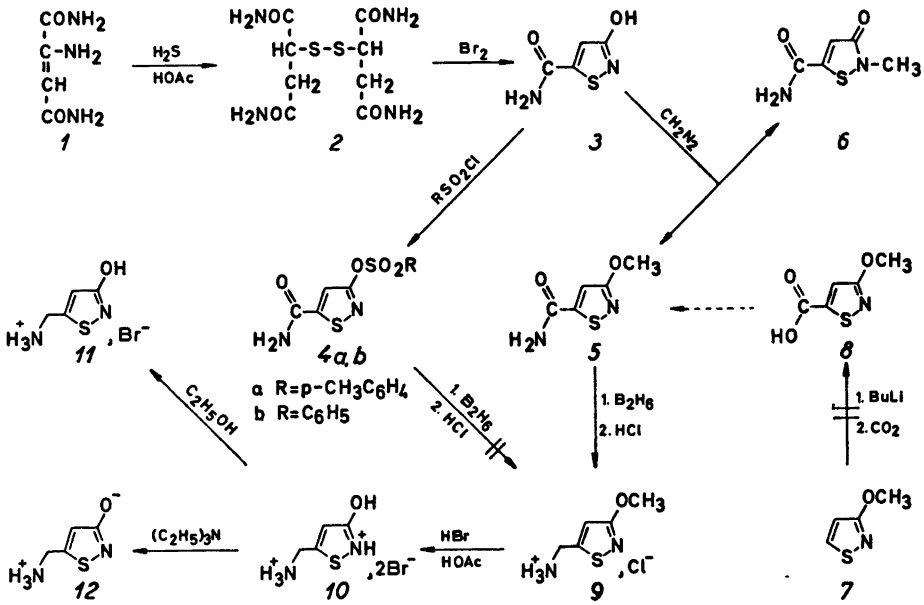
Fig. 1.

TLC, from which no product is isolated.

In another approach masking of the hydroxy group of *3* is accomplished by treatment with diazomethane, which in accordance with the findings for analogous reactions[13] gives a mixture of the *O*- and *N*-methyl derivatives *5* and *6*, respectively, and almost equal amounts are obtained. In an attempt to develop an alternative synthesis of *5* *via* 3-methoxyisothiazole-5-carboxylic acid (*8*), 3-methoxyisothiazole (*7*)[14] is treated with butyllithium followed by addition of carbon dioxide to the reaction mixture. However, *8* cannot be detected in the reaction mixture, which mainly consists of the starting material. Treatment of *5* with lithium aluminium hydride apparently results in a complete destruction of the compound. Finally *5* is reduced with diborane to give a reasonable yield of 3-methoxy-5-aminomethylisothiazole as the hydrochloride *9*. Compound *9* is converted into 5-aminomethyl-3-isothiazolol dihydrobromide (*10*) without purification. In an attempt to crystallize *10* from ethanol-ether the monohydrobromide *11* is obtained. Finally *10* is converted into 5-aminomethyl-3-isothiazolol zwitterion (*12*) by treatment with two equivalents of triethylamine.

A previous and rather inadequate description of the preparation of aminofumaramide (*1*)[15] did not include a structure determination of this compound. The IR, UV, and ^{1}H NMR spectra of *1* provide evidence of the depicted structure *1* of this compound. Reactions of enamines with hydrogen sulfide normally give *gem*-dithiols,[16] but the formations of thiones have been reported in some cases.[12,16] Thus, the structure of the product from the reaction between *1* and hydrogen sulfide was expected to be *2a* or *2b,c* (Fig. 1). These possible structures, however, can be ruled out by the absence of IR absorptions characteristic of thiol groups,[17] and by the absence of a strong thion stretching band in the range 1300 – 1200 cm^{-1} expected for *2b*.[12,18] Finally *2b* and *2c* can be excluded by the absence of UV absorption above 210 nm.[16] If *2b,c* is intermediarily formed, excess of hydrogen sulfide can convert *2b,c* into *2* in agreement with the findings of Bergmann *et al.*[19] This proposal is supported by IR spectroscopy and by elemental analysis, and finally the ^{1}H NMR spectrum exhibits a pattern characteristic of the ABX coupling system present in *2*.

Compound *3* shows absorptions in the range 3600 – 2400 cm^{-1} and at 1560 – 1540 cm^{-1},



Scheme 1.

characteristic of the 3-isothiazolol nucleus.[12] The compounds *5* and *6* can be clearly distinguished by spectroscopic methods. An absorption band of *6* at 1620 cm^{-1}, originating in the carbonyl group in position 3, is absent in the IR spectrum of *5*, and also in that of *3*. Furthermore the UV absorption maxima of *6* and *5* are in agreement with the general findings for *N*-substituted isothiazolin-3-ones and *O*-substituted 3-isothiazololes, respectively.[13,14] In the ^1H NMR spectra of *3* and *5* the protons in position 4 are very similar but they appear at a lower field than the corresponding proton in *6* in accordance with the general findings of Chan *et al.*[13,14] Absorptions in the IR spectra of *4a* and *4b* characteristic of sulfonyloxy groups (1360 and 1170 cm^{-1}) and the absence of absorptions at 1620 cm^{-1} compared with UV and ^1H NMR data provide evidence of *4a* and *4b* being 3-sulfonyloxyisothiazole rather than 2-sulfonylisothiazolin-3-one derivatives.

Compound *12* is the first example of a zwitterion in which a 3-isothiazolol nucleus constitutes the acidic moiety. The structure determinations of *12* and the corresponding mono- and dihydrobromide, *11* and *10* respectively, are based on UV, IR, and ^1H NMR spectroscopy supported by elemental analyses. Broad IR absorptions of *10*–*12* in the range 3600–2200 cm^{-1} are in agreement with the presence of ammonium groups in these compounds, and consequently *12* is a zwitterion. The UV data of *10*, *11*, and *12* are in agreement with the general findings of Chan *et al.*[14] The zwitterion *12* apparently crystallizes with a quarter of a mol of water, the removal of which by heating is accompanied by destruction of the compound.

EXPERIMENTAL

Melting points are corrected and were determined with a hot stage microscope (Mikroskop-Heiztisch, 350 Ernst Leitz, Wetzlar). The recording of IR (KBr technique), UV (methanol solutions), and ^1H NMR spectra, and the performance of microanalyses were accomplished as described in a previous paper.[20] 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 [21] as described in a previous paper.[6]

Aminofumaramide (1). A mixture of 82.4 g (0.4 mol) of diethyl chlorofumarate [22] and aqueous ammonia (400 ml; ϱ 0.88) was stirred for 1 h at 0 °C. Stirring was continued for 18 h at 25 °C, and the precipitate was collected. After drying 45.2 g (88 %) of *1* were obtained; m.p. 188 °C (decomp.) [Ref. 15, m.p. 180 °C (decomp.)]. Recrystallization from water afforded *1* as colourless crystals, m.p. 198 °C (decomp.). λ_{max} 300 nm ($\varepsilon = 6.55 \times 10^3$). IR data ($cm^{-1}$): 3500–2900 (s), 1660–1610 (s), 1550 (s), 1420 (s). ^1H NMR data (DMSO-d_6): δ 7.7–6.7 (four broad signals, total 6 H, $2 \times CONH_2$ and NH_2); 5.05 (s, 1 H, $C=CH-C$).

Dithiodisuccinamide (2). A mixture of 5.2 g (0.04 mol) of *1* and 30 ml of glacial acetic acid was treated with excess of dry hydrogen sulfide during 1½ h at 80 °C. After cooling to room temperature the crystalline solid was filtered off and upon standing at 5 °C for 1 week the mother liquor was filtered to give an additional amount of crystalline product. The two products were combined, and after drying over P_2O_5 (12 h; 50 °C, 12 mmHg) 4.0 g (56 %) of crude *2* were obtained; m.p. 125 °C (decomp.). Recrystallization (water-ethanol-ether) of an analytical sample afforded *2* as colourless crystals, m.p. 176–177 °C (decomp.). (Found: C 32.56; H 4.65; N 18.97; S 19.48. Calc. for $C_8H_{14}N_4O_4S_2$: C 32.64; H 4.79; N 19.03; S 21.79). IR data (cm^{-1}): 3350 (s), 3180 (s), 2920 (w), 1660 (s), 1615 (s), 1410 (s). ^1H NMR data (D_2O): δ 4.62 (s, 8 H, DO*H*); 3.9–3.7 (t, 2 H, $2 \times C-CH-CH_2$); 2.8–2.5 (m, 4 H, $2 \times CH-CH_2-C$).

3-Hydroxyisothiazole-5-carboxamide (3). To a suspension of 2.9 g of crude *2* (10 mmol) in 100 ml of ethyl acetate were added dropwise 1 ml (*ca.* 10 mmol) of bromine in 15 ml of ethyl acetate. After stirring overnight at room temperature, the precipitate was collected and dried to give 2.8 g (97 %) of crude *3*. An analytical sample (300 mg) was taken up in a saturated aqueous solution of sodium hydrogen carbonate (7 ml). Upon extraction with ether (30 ml) the aqueous phase was adjusted to pH 6 with 4 M hydrochloric acid to give 170 mg of *3*, m.p. 220 °C (decomp.). Anal. $C_4H_4N_2O_2S$: C, H, N, S. λ_{max} 290 nm ($\varepsilon = 2.85 \times 10^3$) and 226 nm ($\varepsilon = 8.97 \times 10^3$). IR data ($cm^{-1}$): 3600–2400 (s) (with submaxima at 3320, 3150, 2750, 2675, 2600, 2500), 1660 (s), 1560 (m), 1460 (s), 1400 (m). ^1H NMR data (DMSO-d_6): δ 8.3 and 7.8 (two broad signals, total 2 H, $CONH_2$); 7.20 (s, 1 H, $C=CH-C$).

3-(4-Toluenesulfonyloxy)isothiazole-5-carboxamide (4a). To a solution of 290 mg (2 mmol) of crude *3* in 2 ml of dry pyridine were added 380 mg (2 mmol) of 4-toluenesulfonyl chloride in 2 ml of dry pyridine over a period of 15 min with stirring. Upon standing for 4 h the solution was concentrated *in vacuo* to 1 ml, and upon addition of 50 ml of water 200 mg of TLC-pure *4a* precipitated. The aqueous layer was extracted with ether (30 ml) to give further

70 mg of *4a*. Total yield of crude *4a:* 45 %. An analytical sample was recrystallized (ethanol) to give *4a*, m.p. 123–125 °C. Anal. $C_{11}H_{10}N_2O_4S_2$: C, H, N, S. λ_{max} 262 nm ($\varepsilon = 7.75 \times 10^3$) and 230 nm ($\varepsilon = 27.8 \times 10^3$). IR data ($cm^{-1}$): 3440 (m), 3180 (m), 1680 (s), 1540 (m), 1380 (s), 1360 (s), 1170 (s). ^1H NMR data (DMSO-d_6): δ 8.40 and 8.00 (two broad signals, total 2 H, $CONH_2$); 7.9–7.3 (m, 5 H, C_6H_4 and $C=CH-C$); 2.40 (s, 3 H, CH_3).

3-Benzenesulfonyloxyisothiazole-5-carboxamide (4b). *4b* was prepared as described above for *4a* using 290 mg (2 mmol) of crude *3* and 353 mg (2 mmol) of benzenesulfonyl chloride. Total yield of crude *4b*: 40 %. An analytical sample was recrystallized (ethanol-water) to give *4b*, m.p. 143–145 °C. (Found: C 42.42; H 2.96; N 9.85; S 21.80. Calc. for $C_{10}H_8N_2O_4S_2$: C 42.25; H 2.84; N 9.86; S 22.56). λ_{max} 262 nm ($\varepsilon = 8.20 \times 10^3$) and 222 nm ($\varepsilon = 24.8 \times 10^3$). The IR spectrum of *4b* was almost identical with that described above for *4a*. ^1H NMR data (DMSO-d_6): δ 8.6–7.5 (m, 8 H, $CONH_2$, $C=CH-C$ and C_6H_5).

2-Methyl-3-oxoisothiazoline-5-carboxamide (6) and 3-methoxyisothiazole-5-carboxamide (5). To a solution of 15 g (0.10 mol) of crude *3* in 1000 ml of ether was added with stirring a solution of *ca.* 6 g (0.15 mol) of diazomethane [prepared from 43 g (0.20 mol) of *N*-methyl-*N*-nitroso-4-toluenesulfonamide] in 400 ml of ether. Stirring was continued for 18 h and the remaining diazomethane was destroyed by addition of glacial acetic acid (2.5 ml). 5.9 g (36 %) of crude *6* was filtered off and recrystallized (water) to give 4.3 g of pure *6*, m.p. 215 °C (decomp.). Anal. $C_5H_6N_2O_2S$: C, H, N, S. λ_{max} 318 nm ($\varepsilon = 3.63 \times 10^3$) and 229 nm ($\varepsilon = 8.43 \times 10^3$). IR data ($cm^{-1}$): 3300 (m), 3130 (m), 1690 (s), 1670 (s), 1620 (s), 1555 (m), 1400 (m). ^1H NMR data (DMSO-d_6): δ 8.40 and 8.05 (two broad signals, total 2 H, $CONH_2$); 6.82 (s, 1 H, $C=CH-C$); 3.21 (s, 3 H, $N-CH_3$).

The ethereal filtrate was dried ($MgSO_4$) and evaporated *in vacuo* to give 6.5 g (40 %) of crude *5*, 5.5 g of which were purified by column chromatography [silica gel: 275 g; eluent: methylene chloride-ethyl acetate (4:1)] to give 4.0 g of *5*, m.p. 167–169 °C. Anal. $C_5H_6N_2O_2S$: C, H, N, S. λ_{max} 286 nm ($\varepsilon = 3.15 \times 10^3$) and 224 nm ($\varepsilon = 9.51 \times 10^3$). IR data ($cm^{-1}$): 3360 (m), 3150 (m), 1710 (s), 1690 (s), 1560 (m), 1475 (s), 1390 (s). ^1H NMR data (DMSO-d_6): δ 8.25 and 7.85 (two broad signals, total 2 H, $CONH_2$); 7.29 (s, 1 H, $C=CH-C$); 3.92 (s, 3 H, $O-CH_3$). 1.0 g of crude *5* was purified by sublimation *in vacuo* (12 mmHg) at 140 °C to give 0.7 g of product. Recrystallization from ethyl acetate gave 0.5 g of colourless crystals, m.p. 166–169 °C.

3-Methoxy-5-aminomethylisothiazole hydrochloride (9). To a solution of 1.1 g (7.0 mmol) of 3-methoxyisothiazole-5-carboxamide (*5*) in 100 ml of ice-cooled dry tetrahydrofuran was added diborane, externally generated from 0.72 g (19 mmol) of sodium borohydride in diglyme (60 ml) and 4.3 g (30 mmol) of boron trifluoride etherate in diglyme (40 ml).[23] The mixture was refluxed for 18 h, and after cooling to 25 °C followed by the addition of 4 M hydrochloric acid (15 ml), the tetrahydrofuran was removed *in vacuo*. A 50 % aqueous solution of potassium hydroxide (5 ml) was carefully added to the ice-cooled liquid residue, and the solution was extracted with three 40 ml portions of ether. The combined ether phases were dried and evaporated *in vacuo* to give 0.65 g of an oil. The oily product was dissolved in ethanol (4 ml) and upon addition of an ethanolic solution of hydrogen chloride prepared from ethanol (6 ml) and acetyl chloride (1.3 ml) followed by addition of ether (100 ml), 0.36 g (29 %) of crude *9* precipitated, m.p. 175 °C (decomp.). IR data (cm^{-1}): 3600–2400 (s) (with submaxima at 3425, 2950, 2850), 1950 (m), 1560 (s), 1470 (s), 1390 (s). ^1H NMR data (DMSO-d_6): δ 9.1–8.3 (broad signal, 3 H, NH_3^+, exchangeable with D_2O); 6.97 (s, 1 H, $C=CH-C$); 4.15 (broad signal, 2 H, $NH_3^+-CH_2$); 3.87 (s, 3 H, OCH_3).

5-Aminomethyl-3-isothiazolol dihydrobromide (10). A mixture of 120 mg (0.67 mmol) of crude *9* and 3 ml of glacial acetic acid containing 43 % of hydrogen bromide was heated at 90 °C for a total of 1 h. After reflux for 30 min an additional amount of 2 ml of glacial acetic acid containing 43 % of hydrogen bromide was added. After cooling to room temperature the crystalline solid was filtered off, and after drying over potassium hydroxide (18 h; 40 °C, 12 mmHg) 180 mg (87 %) of *10* were obtained, m.p. 160–163 °C (decomp.). Anal. $C_4H_8Br_2N_2OS$: C, H, Br, N, S. IR data (cm^{-1}): 3500–2300 (m) (with submaxima at 3410, 3120, 2970, 2560, 2470), 1580 (s), 1490 (m). The UV and ^1H NMR (DMSO-d_6) spectra of *10* were identical with those of *11* described below.

5-Aminomethyl-3-isothiazolol hydrobromide (11). Attempts to recrystallize 170 mg of *10* from ethanol-ether afforded 68 mg (55 %) of *11*, m.p. 165–167 °C (decomp.). Anal. $C_4H_7BrN_2OS$: C, H, Br, N, S. λ_{max} 262 nm ($\varepsilon = 4.47 \times 10^3$). IR data ($cm^{-1}$): 3600–2300 (s) (with submaxima at 3425, 3000), 1980 (m), 1620 (s), 1590 (s), 1560 (s), 1480–1450 (s). ^1H NMR data (DMSO-d_6): δ 8.8–8.2 (broad signal, 3 H, NH_3^+, exchangeable with D_2O); 6.82 (s, 1 H, $C=CH-C$); 4.25 [q (*J* 2.5 Hz), 2 H, $NH_3^+-CH_2$].

5-Aminomethyl-3-isothiazolol zwitterion (12). To a solution of 146 mg (0.50 mmol) of *10* in 500 µl of water were added 140 µl (1.0 mmol) of triethylamine to give 60 mg of *12* as a precipitate. Recrystallization (ethanol-water) gave 23 mg (34 %) of *12*, $\frac{1}{4}H_2O$, m.p. 140 °C (decomp.). Anal. $C_4H_6N_2OS, \frac{1}{4}H_2O$: C, H, N, S. λ_{max} 264 nm ($\varepsilon = 4.82 \times 10^3$). IR data ($cm^{-1}$): 3600–3200 (m), 3100–1800 (s) (with sub-

maxima at 2800, 2600, 2200), 1655 (m), 1555 (s). pK_A-values (H_2O, 21 °C): 6.06 ± 0.03, 8.85 ± 0.04.

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Short Communications

Isolation of 1-Hexacosanol, Saturated Higher Fatty Acids, Oleic and Linoleic Acids from *Calamagrostis arundinaceae*

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As far as we know no chemical investigation has previously been undertaken concerning the common perennial grass, *Calamagrostis arundinaceae*. This paper reports on the examination of the lipid constituents of this species. The grass samples were collected in the month of September, only the flower-heads with seeds being used in the investigation. Extraction of the material with light petroleum yielded a coloured, viscous residue on evaporation of the solvent. By saponifying the residue, two fractions, A and B, were isolated as described in the experimental part. The results are of interest because they demonstrate that the main compound of the extractives is 1-hexacosanol (ceryl alcohol).

Identification of 1-hexacosanol. By recrystallization of fraction A (neutral part), a colourless, crystalline substance was obtained. NMR and IR spectra of this substance were consistent with an unbranched saturated aliphatic primary alcohol. HrMS showed the molecular ion at *m/e* 382.4171 (calc for $C_{26}H_{52}O$ 382.4175). A strong peak was seen at *m/e* 364 corresponding to the ion $C_{26}H_{52}$ ($M-H_2O$). The isolated substance was thus identical with 1-hexacosanol (ceryl alcohol). The identity of the C_{26} alcohol was further confirmed by elemental analyses.

Low and high resolution mass spectrometry also demonstrated the presence of small amounts of the higher fatty acids $C_{32}H_{64}O_2$ (dotriacontanoic acid), $C_{30}H_{60}O_2$ (triacontanoic acid), $C_{29}H_{58}O_2$ (nonacosanoic acid), and $C_{28}H_{56}O_2$ (octacosanoic acid). Long-chain saturated alcohols and acids often occur as constituents of plant waxes.[1] In the grass family ceryl alcohol has been found in the blades of *Dactylis glomerata* (cocksfoot) and *Lolium perenne*.[2]

Identification of unsaturated fatty acids. TLC of fraction B (a faintly yellow, viscous liquid) revealed several spots which turned yellow with phosphomolybdic acid in ethanol. Positive permanganate and tetranitromethane tests showed the presence of unsaturated compounds. The mixture was dissolved in a sodium hydroxide solution and oxidized with permanganate by Lapworth's method.[3] Recrystallization of the oxidized product afforded 9,10-dihydroxystearic acid and 9,10,12,13-tetrahydroxystearic acid (sativic acid) as shown by elemental analyses, IR, NMR, MS, and comparison with authentic samples. This provides strong evidence that fraction B was essentially a mixture of oleic and linoleic acids. These acids may be regarded as glyceride components of the investigated material.

Experimental. Elemental analyses and molecular determination were performed by Mikroanalytisches Laboratorium, Elbach, BRD. Chemical shifts in NMR spectra were recorded in δ-values. HrMS was performed by Shrader Analytical, Detroit, USA. The melting points are uncorrected.

TLC of the unsaturated fatty acids was carried out on precoated plates with cellulose as absorbent. Toluene-acetic acid (85 + 15) was used as solvent, and spots were rendered visible with phosphomolybdic acid in ethanol.

Extraction and isolation of the constituents. Dried and ground grass (773 g) was extracted in a Soxhlet apparatus with light petroleum (60 – 80 °C) for 5 h. The solution was evaporated at reduced pressure and the residue refluxed in 450 ml of a 5 % solution of potassium hydroxide in methanol. After dilution with water the insoluble constituent was filtered off and treated with dilute HCl. Recrystallization first from ethanol and then from acetic acid gave fraction A, yield 380 mg, m.p. 72.4 °C. Anal. $C_{26}H_{53}OH$: C, H, O. ^1H NMR (60 MHz, $CDCl_3$): δ 0.88 (aliphatic $C-CH_2$), 1.27 (straight chain CH_2 groups), 1.60 (CH_2CH_2OH), 2.09 (OH), 3.63 (CH_2OH). IR(Nujol, Hostaflon, KBr): 3250 (polym. ass. of OH groups), 2900 (asym. CH stretch vibr.), 1055 (CO stretch vibr. of primary alcohol), 720 cm^{-1} (straight chain CH_2 groups, singlet).

The aqueous alkaline solution after acidification was then extracted with ethyl ether. On evaporation the ethereal solution left a yellowish oily liquid, fraction B, which solidified on cooling. Oxidation of the unsaturated fatty acids in fraction B was carried out in an alkaline solution with 0.2 % aqueous potassium permanganate. After 10 min the solution was acidified with HCl, and $NaHSO_3$ was added until the excess permanganate and manganese(IV) oxide had been converted to manga-

nese(II) sulfate. The oxidized components were removed by filtration. Separation of the components was effected by concentrating the solution in ethanol to about 125 ml. On cooling slowly to 0 °C a crystalline *Substance B1* separated out. The substance was recrystallized from acetic acid, m.p. 158 °C. ^{1}H NMR (60 MHz, DMSO-d_6): δ 0.88 (CH_3), 1.30 (CH_2), 2.20 (CH_2CO_2H), 3.30 ($CHOH$). MS of B1 showed no molecular ion. Prominent peaks were observed at *m/e* 113, 155, 157, 173, and 229 corresponding to the fragments outlined in a previous communication.[4] IR comparison with authentic 9,10,12,13-tetrahydroxystearic acid (sativic acid) demonstrated a complete agreement. Found: C 62.58; H 10.25; O 27.24. Calc. for $C_{18}H_{36}O_6$: C 62.04; H 10.41; O 27.55.

On evaporation of the filtrate above, a crystalline mass remained. This was purified by recrystallization from acetic acid and chloroform. A *Substance B2*, yield 170 mg, m.p. 125 °C, snow-white crystals, was finally obtained. Anal. $C_{18}H_{36}O_4$: C, H, O. MW (osmometric in pyridine): Found 308, calc. 316.5. IR(KBr) and ^{1}H NMR (in DMSO-d_6) spectra agreed completely with those of an authentic sample of 9,10-dihydroxystearic acid.

In the mass spectrum, dominant and characteristic peaks were found. HrMS (*m/e*): 281 ($M-H_2O-OH$), 280 ($M-2H_2O$), 229 ($M-C_6H_{12}-OH$), 185 ($HO^+=CH-CH=CH-(CH_2)_6-CO_2H$), 175 ($HO-CH_2-(CH_2)_7-C(OH)=^+OH$) and 173 ($HO^+=CH-(CH_2)_7-CO_2H$). The base peak at *m/e* 155 corresponded to the fragment $HO^+=CH-(CH_2)_6-CH=C=O$.

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Stable α-Chloro-β-oxosulfenyl Chlorides: a Novel Class of Compounds Formed from Ketones and Thionyl Chloride

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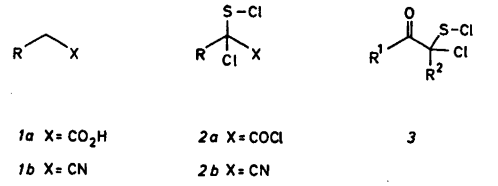
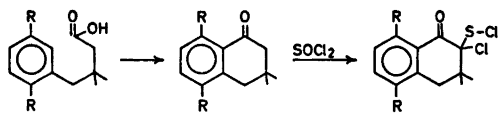
Reaction of monosubstituted acetic acids *1a* or acetonitriles *1b* with thionyl chloride in the presence of a tertiary amine, or Et_2O/HCl, respectively, produces α-chlorosulfenyl chlorides *2a* and *2b*.[1-7] By analogy, α-chloro-β-oxosulfenyl chlorides *3* have been invoked as transient intermediates in reactions between ketones and thionyl chloride leading to 3-thietanones and benzo[*b*]thiophenes.[8,9] The first stable representatives of this class of organic sulfur compounds are described in the following.



1a X = CO_2H *2a* X = COCl *3*

1b X = CN *2b* X = CN



4a R = H *5a* R = H *6a* R = H

4b R = CH_3 *5b* R = CH_3 *6b* R = CH_3

When the hindered ketones *5a* and *5b* are allowed to react with thionyl chloride, without any added catalyst, two products are formed, to which the structures *6a* and *6b* are assigned on the basis of elemental analysis and spectroscopic properties, see Experimental.

Though various pathways to the formation of *6a* and *6b* can be envisioned,[9] the evidence is insufficient to warrant further comments at this stage.

Experimental. ^{1}H NMR were recorded on a Varian 360 instrument, and ^{13}C NMR on a Bruker WH-90 at 22.63 MHz with broad band noise ^{1}H-decoupling. Long relaxation times are indicated by an *a*. The mass spectrum of *6a* was recorded on a Perkin-Elmer 270 instrument; exact measurement was performed on an AEI MS 3074 instrument. The spectrum of *6b* was recorded on a DuPont 21-492 instru-

nese(II) sulfate. The oxidized components were removed by filtration. Separation of the components was effected by concentrating the solution in ethanol to about 125 ml. On cooling slowly to 0 °C a crystalline *Substance B1* separated out. The substance was recrystallized from acetic acid, m.p. 158 °C. ^{1}H NMR (60 MHz, DMSO-d_6): δ 0.88 (CH_3), 1.30 (CH_2), 2.20 (CH_2CO_2H), 3.30 ($CHOH$). MS of B1 showed no molecular ion. Prominent peaks were observed at *m/e* 113, 155, 157, 173, and 229 corresponding to the fragments outlined in a previous communication.[4] IR comparison with authentic 9,10,12,13-tetrahydroxystearic acid (sativic acid) demonstrated a complete agreement. Found: C 62.58; H 10.25; O 27.24. Calc. for $C_{18}H_{36}O_6$: C 62.04; H 10.41; O 27.55.

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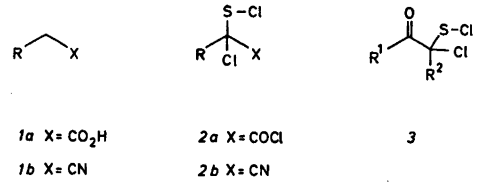

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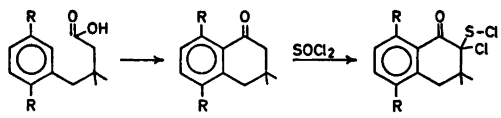

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ment. Melting points are uncorrected. The thionyl chloride used was Fluka *"puriss. p.a. farblos"*. Use of a technical grade gave up to 10 % lower yields of darker coloured products.

The acids *4a* and *4b* were prepared conventionally[10,11] from benzyl chloride and 2,5-dimethylbenzyl chloride,[12] respectively. Cyclization with polyphosphoric acid gave the ketones.[13] The ketone *5b* was also prepared by allowing *4b* (2.20 g) to react with thionyl chloride (0.78 ml; excess thionyl chloride must be avoided, see below) in benzene (2.2 ml) for 18 h at 20 °C. Addition of methanol, removal of the solvents *in vacuo*, and crystallization from light petroleum (3 ml) at −80 °C gave colourless crystals. Yield 1.74 g (86 %), m.p. 58 – 60 °C. ^{1}H NMR (60 MHz, $CDCl_3$): δ 1.03 (6 H, s, aliphatic methyl), 2.22 (3 H, s), 2.42 (2 H, s, methylene α to carbonyl), 2.60 (3 H, s), 2.67 (2 H, s, benzylic methylene), 6.92 (1 H, d, *J* 7.6 Hz), 7.13 (1 H, d, *J* 7.6 Hz). ^{13}C NMR ($DCCl_3$): δ 200.6[a] (C1), 53.9 (C2), 32.5[a] (C3), 41.6 (C4), 28.4 (aliphatic methyl), 19.7 and 23.1 (aromatic methyl), 129.7, 130.5[a], 133.9, 134.2[a] 138.3[a], 141.7[a] (aromatic ring carbon). A selective decoupling experiment showed the protons at δ 2.42 to be coupled to the carbon resonating at 53.9 ppm, confirming the assignment.

2-Chloro-2-chlorosulfenyl-3,4-dihydro-3,3 dimethyl-1-(2H)-naphthalenone 6a and the corresponding *3,3,5,8-tetramethyl derivative 6b*. The ketone *5a* (1.74 g) was dissolved in thionyl chloride (3.6 ml). The temperature rose from 22 to 32 °C and a brisk evolution of gas started after *ca.* 2 min. Crystallization set in after 30 – 45 min, and the mixture was allowed to stand at room temperature for 3 h. Recrystallization from ligroin (80/100, 10 ml) at 0 °C gave yellow crystals of the sulfenyl chloride *6a*. Yield 2.18 g (79 %), m.p. 120 – 122 °C; recrystallization from toluene afforded an analytical specimen, m.p. 124 – 126 °C. Anal $C_{12}H_{12}Cl_2OS$: C, H, Cl, S. ^{1}H NMR δ 1.23 (3 H, s), 1.50 (3 H, s), 3.00 (1 H, d, *J* 18 Hz), 3.36 (1 H, d, *J* 18 Hz), 7.07 – 7.74 (3 H, m), 8.07 – 8.22 (1 H, m). ^{13}C NMR ($DCCl_3$): δ 184.2[a] (C1), 92.7[a] (C2), 44.5[a] (C3), 42.8 (C4), 25.2 and 26.1 (aliphatic methyl), 127.4, 128.8, 129.2[a], 129.2[a], 134.3, 139.2[a] (aromatic ring carbon). MS [*m/e* (% rel. int.)]: 273.9963 (22, M), calc. for $C_{12}H_{12}Cl_2OS$ 273.9986; 239 (26), 207 (7), 203 (30), 171.0824 (43), calc. for $C_{12}H_{11}O$ 171.0810; 153 (45), 152.0067 (100), calc. for C_8H_5ClO 152.0029; 149 (40), 118.0418 (80), calc. for C_8H_6O 118.0419.

The homologous chloride *6b* may be prepared as above, but was obtained also directly from the corresponding acid *4b* (2.20 g) and thionyl chloride (2.6 ml). Addition of pyridine (0.1 ml) catalyzed the reaction and gave a higher yield. Crystallization took place after about 3 h at *ca.* 20 °C. The reaction mixture was allowed to stand for 24 h. It was dissolved in a mixture of ligroin (80/100 °C, 10 ml) and toluene (1 ml) at reflux temperature and decanted from a small amount of a brown oil (mainly pyridine hydrochloride). Crystallization at 0 °C gave yellow crystals of *6b*. Yield 2.56 g (84 %), m.p. 122 – 124 °C. Recrystallization from ethanol produced an analytical sample, m.p. 124 – 125 °C. Anal. $C_{14}H_{16}Cl_2OS$: C, H, Cl, S. ^{1}H NMR (60 MHz, $CDCl_3$): δ 1.25 (3 H, s), 1.46 (3 H, s), 2.23 (3 H, s), 2.59 (3 H, s), 2.95 (2 H, s; no splitting could be detected on a Varian HA-100 instrument), 7.11 (1 H, d, *J* 7.8 Hz), 7.27 (1 H, d, *J* 7.8 Hz).

^{13}C NMR ($DCCl_3$): δ 187.7[a] (C1), 94.9[a] (C2), 43.6[a] (C3), 41.6 (C4), 25.5 and 26.4 (aliphatic methyl), 19.4 and 22.5 (aromatic methyl), 127.9[a], 130.5, 133.9[a], 134.7, 138.1[a], 140.2[a] (aromatic ring carbon). MS [*m/e* (% rel. int.)]: 302.0308 (33, M), calc. for $C_{14}H_{16}Cl_2OS$ 302.0299; 267.0608 (21 [M − Cl]), calc. for $C_{14}H_{16}ClOS$ 267.0610; 235.0881 (54, [M − SCl]), calc. for $C_{14}H_{16}ClO$ 235.0889; 231.0841 (12, [M − Cl_2]), calc. for $C_{14}H_{15}OS$ 231.0843; 199.1085 (24, [M − SCl_2]), calc. for $C_{14}H_{15}O$ 199.1122; 181.0408 (58, rearrangement), calc. for $C_{10}H_{10}ClO$ 181.0420; 177.0365 (43, rearrangement), calc. for $C_{10}H_9OS$ 177.0374; 146.0732 (100, odd-electron acylium ion), calc. for $C_{10}H_{10}O$ 146.0731. The loss of sulfur and chlorine is expected.[1] The base peak of *6a* at *m/e* 152 does not have a counterpart (at *m/e* 180) in the spectrum of *6b*.

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In recent years it has been well established that *N*-hydroxyphenylalkylamines are formed during the metabolism of several phenylalkylamines.[1–4] These aliphatic hydroxylamines are unstable compounds which further metabolize or undergo chemical conversion to various compounds.

During investigations of the chemical and biological properties of *N*-hydroxyphenylalkylamines access to the optical isomers of 2-hydroxylamino-1-phenylpropane (*N*-hydroxyamphetamine, *1*) became desirable. Thus by using optically active substrates information could be gained as to the stereochemical properties of the nitroso compounds formed during autoxidation of *1*.[5] Furthermore pure enantiomers of *1* were needed to assess the influence of its chirality on the enzymatic binding during metabolism.[6]

N-Hydroxyamphetamine was resolved into its (+)- and (−)-enantiomers using (+)- and (−)-tartaric acid, respectively. Five recrystallizations from 5 % solutions in ethanol were required to produce salts with constant physical properties. The absolute configuration of (+)-*1* was established by reduction to (*S*)-amphetamine with $LiAlH_4$. Consequently (+)-*1* can be assigned the (*S*)-configuration.

A synthetic route leading to optical isomers of *1* was recently reported.[7] Optically pure amphetamine was converted into the benzylimine, oxidation of which with *m*-chloroperbenzoic acid, gave the 3-phenyloxaziridine. Subsequent acidic hydrolysis yielded *1*. Although no optical rotations were presented, dextrorotatory amine was claimed to yield dextrorotatory *N*-hydroxylamine. Our results confirm this statement. As *1* easily forms crystalline tartrates resolution is a convenient alternative to the production of the optical antipodes. Racemic *1* can be prepared in reasonable good yield (35 %) by partial reduction of 1-phenyl-2-nitropropene-(1),[8] or in excellent yield (80 %) by a modification[9] of the method of Borch *et al.*[10] utilizing 1-phenyl-2-propanone oxime and cyanoborohydride.

Experimental. Melting points were determined in an electrically heated metal block using open capillary tubes and calibrated Anschütz thermometers. Optical rotations were measured with a Perkin-Elmer 141 spectropolarimeter.

2-Hydroxylamino-1-phenylpropane was prepared in up to 35 % yield by $LiAlH_4$ reduction of 1-phenyl-2-nitro-propene(1).[8] The phenylnitropropene used as the starting material was from seizures and was kindly supplied by the National Laboratory of Forensic Science. The compound contained some impurities and was recrystallized from ethanol (96 %) prior to use.

Resolution of 2-hydroxylamino-1-phenylpropane(1). Racemic *1* (8.0 g, 0.05 mol) was added to a hot solution of (+)-tartaric acid (7.9 g, 0.05 mol) in 300 ml of ethanol (abs.). The solution was kept at room temperature overnight. The salt obtained (11.4 g, m.p. 152–154 °C) required five recrystallizations from 5 % solutions in ethanol (96 %) before it exhibited constant physical properties. Yield 3.5 g (44 %) of resolved (+)-hydrogen tartrate m.p. 163–164 °C, $[\alpha]_D^{23}$ +21.3° (*c* 1.0, H_2O).

Refrigeration of the initial filtrate yielded 2.0 g of pure (−)-*1*-(+)-tartrate, m.p. 143–145 °C, $[\alpha]_D^{23}$ +6.3° (*c* 1.0, H_2O).

The combined filtrates from the above resolution were concentrated *in vacuo* and the residue was dissolved in saturated $NaHCO_3$-solution (25 ml). Extraction with $CHCl_3$ (2 × 25 ml), subsequent drying (Na_2SO_4) and evaporation of the solvent yielded 4.1 g of recovered hydroxylamine. This was added to a hot solution of (−)-tartaric acid (4.4 g, 0.027 mol) in ethanol (96 %). Two recrystallizations from the latter solvent gave 1.3 g of resolved (−)-hydrogen tartrate with constant physical properties, m.p. 164–165 °C, $[\alpha]_D^{23}$ −21.4° (*c* 1.0, H_2O).

The total yield of salts containing resolved (−)-*1* was 3.3 g (41 %).

(S)- and (R)-2-Hydroxylamino-1-phenylpropane. The hydroxylamines were obtained from the resolved (+)- and (−)-hydrogen tartrates by dissolution in saturated $NaHCO_3$-solutions and extraction with chloroform as described above. (*S*)-2-Hydroxylamino-1-phenylpropane, m.p. 79–80 °C (from light petroleum), $[\alpha]_D^{23}$ +1.9° (*c* 1.0, EtOH) +21.2° (*c* 1.0, CH_2Cl_2). (*R*)-2-Hydroxylamino-1-phenylpropane obtained from (+)-tartrate m.p. 79–80 °C $[\alpha]_D^{23}$ −20.6° (*c* 1.0, CH_2Cl_2), obtained from (−)-tartrate m.p. 79–80 °C, $[\alpha]_D^{23}$ −1.9° (*c*

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1.0, EtOH) $-20.4°$ (*c* 1.0, CH_2Cl_2).

Determination of the absolute configuration of 2-hydroxylamino-1-phenylpropane (1). An ethereal solution (25 ml) of (+)-*1* (200 mg, 1.3 mmol) was added dropwise to a well stirred slurry of $LiAlH_4$ (326 mg, 8.6 mmol) in dry ether (40 ml). The mixture was refluxed for 2 h. After cooling the LiAl-complex was decomposed by cautious addition of H_2O (0.3 ml), 15 % NaOH (0.3 ml) and finally H_2O (0.9 ml). The resulting suspension was refluxed for 30 min, then filtered and the precipitate washed with ether. The combined filtrate and washings were dried (K_2CO_3) and the solvent evaporated *in vacuo*, affording 142 mg of the crude base. The base was converted to the sulfate by addition of an equivalent amount of ethereal H_2SO_4 to a cold ethereal solution of the base. Two recrystallizations from diluted ethanol yielded 50 mg of pure amphetamine sulfate, m.p. 318–320 °C decomp. $[\alpha]_D^{23}$ +22.2° (*c* 1.0, H_2O), (Lit.[11] (*S*)-1-phenyl-2-aminopropane sulfate m.p. >300 °C decomp. $[\alpha]_{589}^{23}$ +22.1° (*c* 2.0, H_2O).

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Substituent Effects on ^{13}C Chemical Shifts of Benzoyl Stabilised Phosphonium Ylides

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Recent studies of ^{13}C chemical shifts, *inter alia*, of phosphorus ylides have indicated that the ylide carbon is best described as sp^2 hybridised and carries a significant negative charge.[1,2] Additionally, the existence of $d_\pi - p_\pi$ bonding between phosphorus and the ylide carbon has been recognised, although the extent of this bonding is thought to be moderate;[1] for calculated values of a non-stabilised ylide see Ref. 3. As a result of these studies and in extension of our own studies on ^{13}C chemical shifts in arsenic ylides,[4] we have investigated the ylide, C(7), and carbonyl carbon, C(8), chemical shift variation as a function of substituent, X, in a series of phosphonium ylides containing an *m*-substituted benzoyl group *1–8*. The relevant ^{13}C chemical shifts are given in Table 1.

The present results confirm [1] that the ylide carbon C(7) in stabilised phosphonium ylides absorbs at rather high field; the C(7) shifts correlate with the linear free energy parameter σ_m (from Ref. 5) in the sense that electron-withdrawing substituents cause deshielding. The carbonyl carbons, C(8), are however

Table 1. ^{13}C chemical shifts of exocyclic carbons in metasubstituted acetophenones and phosphorus ylides. ^{13}C chemical shifts (ppm) determined on solutions (0.2 M) in $CDCl_3$ with internal TMS as standard are accurate to 0.05 ppm. σ_m from Ref. 5.

	Ylide C(7)[a]	C(8)[b]	Substituent	Acetophenone C(7)	C(8)	σ_m
1	50.54	184.80	H	26.52	198.00	0.0
2	50.47	185.12	Me	26.54	198.13	−0.7
3	50.79	184.45	OMe	26.63	197.74	0.12
4	51.31	182.95	F	26.59	196.56	0.34
5	51.36	182.79	Cl	26.58	196.60	0.37
6	51.33	182.90	Br	26.55	196.51	0.39
7	52.01	181.91	CN	26.64	196.06	0.68
8	52.33	181.47	NO_2	26.71	195.75	0.71

[a] $^1J_{P-C(7)}$ 111.4–112.4 Hz. [b] $^2J_{P-C-C(8)} = 3.4–3.8$ Hz.

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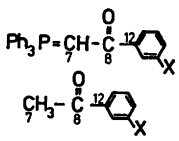
2	50.47	185.12	Me	26.54	198.13	−0.7
3	50.79	184.45	OMe	26.63	197.74	0.12
4	51.31	182.95	F	26.59	196.56	0.34
5	51.36	182.79	Cl	26.58	196.60	0.37
6	51.33	182.90	Br	26.55	196.51	0.39
7	52.01	181.91	CN	26.64	196.06	0.68
8	52.33	181.47	NO_2	26.71	195.75	0.71

[a] $^1J_{P-C(7)}$ 111.4–112.4 Hz. [b] $^2J_{P-C-C(8)} = 3.4–3.8$ Hz.



shielded by electron-withdrawing substituents; again a correlation of the chemical shifts with σ_m is obtained. The alternate response of C(7) and C(8) to substituent change may be rationalised on the basis of substituent induced charge alternation [6,7] with chemical shift changes influenced primarily by charge density variation.[1] See however Ref. 8. We find further, that the substituent induced range of shifts for the carbonyl carbon C(8) is twice that of the more remote ylide carbon C(7). The regular behaviour of C(8) in *m*-methoxy ylide *3* contrasts with that of the *p*-methoxy analogue in both phosphorus [9] and arsenic [4] series and thus indicates a non-enhanced C(8)–C(12) bond order in *3*. Whereas infra-red spectral data [10] indicate a greater polarity of the carbonyl bond in the arsenic ylides, the carbonyl carbon is more deshielded in the parent phosphorus ylide *1* than in its arsenic counterpart [4] by 3.0 ppm. Accordingly, we conclude that it is risky to draw unambiguous conclusions regarding bond polarity from ^{13}C chemical shifts especially in complex multiply bonded systems.

The remaining carbons of the series *1–8* showed unexceptional chemical shift behaviour, however we note a hitherto unreported [11] coupling ^3J[P–CH–C(O)–C(12)] of magnitude 14.4–15.2 Hz.

We have also examined the ^{13}C chemical shifts of a series of *m*-acetophenones (Table 1) and here again carbonyl carbon shieldings correlate with σ_m, although the range of substituent induced shift is now about two-thirds of that found in the ylide series *1–8*, whereas methyl carbon shifts are essentially invariant to substituent variation.

It is possible that this regularity of chemical shift behaviour with substituent variation (Table 1) may be associated more generally with *m*-substituted series. We hope to consider the more involved shift variation in the corresponding *p*-substituted phosphonium ylides in a future publication.

Acknowledgement. We wish to thank Dr. D. S. Rycroft for determination of spectra.

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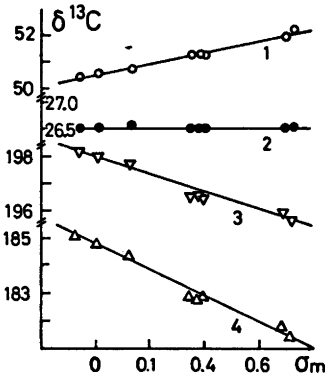

Fig. 1. Plot of ^{13}C shifts in Table 1 against σ_m. 1, ylide C(7); 2, acetophenone C(7); 3, acetophenone C(8); 4, ylide C(8).

Mass Spectra of Some *N*-Methyl-*N*-formylhydrazones

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Though hydrazones are seldom found in nature, they are nevertheless known to have biological activity. Some of them are toxic and synthetic derivatives of hydrazines are frequently used as growth stimulators or growth regulators.[1]

N-Methyl-*N*-formylhydrazones constitute one of the few groups of natural hydrazones. They have been found in the mushroom *Gyromitra esculenta* Pers. Fr., false morel, and accordingly called gyromitrins.[2-4] The toxicity of fresh *Gyromitra esculenta* has been attributed to these *N*-methyl-*N*-formylhydrazones, eight of which (*i.e.*, compounds *2*–*7*, *9* and *12* (*cis* and *trans*) in Table 1) have been identified.[2-5]

N-Methyl-*N*-formylhydrazones are important not only because of their toxicity but because they or their precursors may play a role in the growth regulating system of the exceptionally fast growing mushroom fruit body.

N-Methyl-*N*-formylhydrazones may be present in other natural products in addition to mushrooms and in those products they can be most easily identified on the basis of their specific mass spectral fragmentation. In order to assist their identifications, a series of *N*-methyl-*N*-formylhydrazones were synthesized from those carbonyl compounds common in nature and the spectra were measured.

Results and discussion. In the compounds *1*–*15* where there is the possibility of McLafferty rearrangement (Fig. 1a), the prominent peak $m/e = 59$ is seen in the spectra. Other typical peaks in the spectra of *1*–*15* are at masses $m/e = M - 29$, $M - 58$ and $m/e = 85$ (Fig. 1c).

In the spectra of *6*–*11*, the prominent peak at $m/e = 100$ gradually increases as the aliphatic chain becomes longer. The fragment $m/e = 100$ can be explained as due to the rearrangement schematized in Fig. 1b. This rearrangement is in agreement with the observation of Kleipool *et al.*[6] and Djerassi *et al.*[7] Kleipool *et al.*[6] have found that pentanal and higher aldehyde 2,4-dinitrophenylhydrazones (DNP) display an intensive peak at $m/e = 224$, which they attribute to β-fission of the alkyl chain with the accompanying hydrogen rearrangement. Since butanal DNP does not exhibit an $m/e = 224$ peak, they assume that a hydrogen atom in the δ-position is required, which is transferred to the *p*-nitro group bearing a positive charge. In contrast to the latter Djerassi *et al.*[7] have detected with deuterium labeled DNP that the hydrogen atom at γ-position is rather abstracted in this rearrangement. It was found that a hydrogen atom from a methyl group cannot be abstracted in this process. This explains the failure to observe the peak at $m/e = 224$ in the spectra of butanal DNP and the peak at $m/e = 100$ in the spectrum of butanal *N*-methyl-*N*-formylhydrazone, respectively.

Analogously to the compounds *6*–*11*, the rearrangement (Fig. 1b) in the spectra of *19* and *20* yields the peaks at $m/e = 128$ and $m/e = 126$ (Fig. 1d). The peaks $m/e = 99$ and $m/e = 97$ can be attributed to the fragments $m/e = 128 - 29$ (CHO) and $m/e = 126 - 29$, respectively, and the peaks at masses $m/e = 71$ and $m/e = 69$ to the fragments $m/e = 99 - 28$ ($N{=}CH_2$) and $m/e = 97 - 28$, respectively.

In the spectra of the compounds *16*–*20*, prepared from ketones, there are prominent peaks at masses $m/e = M - R^1$ and $m/e = M - R^2$.

Experimental. Mass spectra were recorded with a Jeol JMS-D 100 mass spectrometer, connected to a gas chromatograph. The compounds were injected through a 50-m FFAP glass capillary column, programmed from 60 to 220 °C, 8 °C per min. Ionizing voltage was 75 eV, ionizing current 300 μA. The temperatures: injection block 200 °C, interphase oven 200 °C

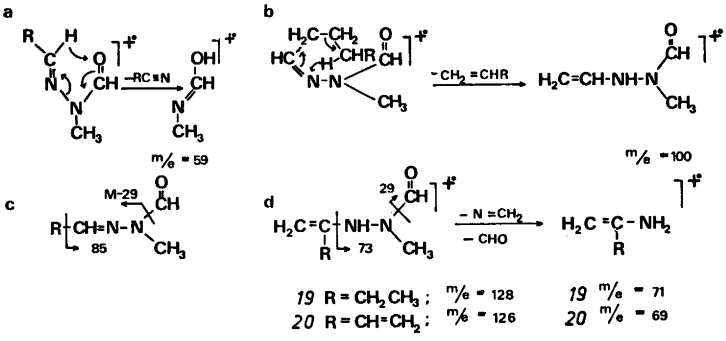
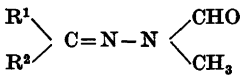


Fig. 1. Mass spectral fragmentation patterns of *N*-methyl-*N*-formylhydrazones.

Table 1. Mass spectra of *N*-methyl-*N*-formylhydrazones.



Compound	R^1	R^2	Mass spectrum
1	H	H	42(30), 43(100), 57(47), 59(100), 72(13), 86(33)
2	CH_3	H	42(60), 57(65), 59(100), 71(30), 85(25), 100(20)
3	C_2H_5	H	43(69), 56(94), 59(90), 71(25), 85(100), 99(3), 114(8)
4	C_3H_7	H	41(52), 43(60), 59(93), 60(85), 70(40), 73(30), 85(100), 128(12)
5	$(CH_3)_2CHCH_2$	H	41(56), 43(80), 57(28), 59(100), 60(19), 71(31), 84(54), 85(89), 100(10), 127(9), 142(13)
6	C_4H_9	H	41(34), 43(50), 57(25), 59(73), 60(19), 67(8), 69(21), 71(25), 84(39), 85(100), 100(48), 113(8), 142(16)
7	C_5H_{11}	H	43(68), 55(25), 59(78), 60(41), 71(20), 85(100), 98(54), 100(57), 113(10), 127(3), 156(12)
8	C_6H_{13}	H	40(88), 43(100), 44(100), 59(85), 71(26), 85(90), 100(78), 113(55), 141(3), 170(15)
9	C_7H_{15}	H	41(60), 43(100), 59(90), 60(43), 69(20), 71(18), 85(78), 100(60), 113(8), 126(28), 155(2), 184(1)
10	C_8H_{17}	H	41(90), 59(75), 60(63), 85(95), 100(100), 114(10), 140(38), 169(5), 198(17)
11	C_9H_{19}	H	41(55), 43(91), 59(60), 60(55), 83(30), 85(90), 100(100), 113(15), 154(40), 212(13)
12	$CH_3(CH_2)_4CH=CH$ (*trans*)	H	41(60), 43(38), 55(37), 59(100), 70(17), 73(27), 124(53), 139(10), 153(6), 182(15)
13	C_4H_3O (furyl)	H	39(93), 43(57), 52(91), 53(82), 59(100), 81(55), 94(55), 95(55), 123(52), 152(25)
14	C_6H_5	H	51(55), 59(75), 77(63), 89(38), 90(41), 104(100), 118(18), 133(30), 162(25)
15	$C_6H_5CH_2$	H	43(18), 51(20), 65(20), 77(16), 85(100), 91(40), 103(10), 117(8), 142(6), 147(5), 176(3)
16	CH_3	CH_3	44(17), 56(38), 71(7), 85(10), 99(100), 114(37)
17	C_2H_5	CH_3	41(39), 42(100), 43(75), 57(30), 59(88), 68(15), 86(25), 99(98), 113(12), 128(17)
18	C_3H_7	CH_3	41(100), 43(100), 56(58), 73(30), 84(15), 99(100), 127(50), 142(35)
19	C_5H_{11}	C_2H_5	43(92), 55(52), 71(63), 73(90), 99(20), 113(100), 128(20), 141(10), 155(67), 184(10)
20	C_5H_{11}	$CH_2=CH$	43(95), 55(47), 60(35), 69(100), 73(87), 83(34), 97(25), 111(80), 124(15), 126(8), 139(20), 153(30), 182(8)

and the ion source 220 °C.

The *N*-methyl-*N*-formylhydrazones were synthesized from *N*-methyl-*N*-formylhydrazine and the carbonyl compounds analogously to the method described by List *et al.*,[2] and the ^1H NMR and IR spectra were in accordance with the proposed structures.

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Binding of Tubulin to Substituted Sepharose

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Tubulin is the main protein constituent of microtubules, which are involved in a number of physiological processes including cell division, cell motility, transport of proteins and support of extended structures. It is entirely unclear how these versatile functions are controlled at a molecular level. There are, however, a number of chemical interactions described for tubulin such as calcium sensitive polymerisation,[1] binding of guanosine nucleotides,[2] colchicine and vinca alkaloids,[3] which may contribute to the physiological functions of tubulin. In this paper another interaction of tubulin is described as it is shown to bind to substituted Sepharose containing aliphatic hydrocarbons. The binding is sensitive to a physiological salt concentration and can be used as a simple purification method for tubulin.

Materials and methods. Rat brains were homogenized in 30 mM sodium phosphate, pH 7.0, containing 10 % glycerol (=PG, about 10 ml/brain) using 20 strokes of a Teflon-glass homogenizer. The homogenate was centrifuged at 100 000 *g* for 60 min and the supernatant was used for further purification.

Brain supernatant was incubated with epoxy-activated Sepharose 6 B containing 12 atom hydrocarbon spacer groups (Pharmacia). Before use the epoxy groups of Sepharose were inactivated by incubating in 1 M Tris, pH 8.0 overnight followed by washes with 0.1 M borate pH 8.0, 0.1 M acetate pH 4.0 and 0.15 M NaCl. After the incubation the mixture of brain supernatant and Sepharose was centrifuged and the supernatant was decanted. Sepharose was taken up in PG and poured into a 2.5 ml syringe. The column was connected to an absorbance monitor (280 nm) and the column was eluted with PG and 75 mM NaCl until no more material came out. Tubulin was eluted with 0.15 M NaCl or with a salt gradient. Aliquots of the samples were taken for the colchicine binding assay,[4] performed at 3.7×10^{-5} M colchicine containing 3.4×10^{-6} M ^{3}H-colchicine (Amersham). All colchicine binding assays were carried out at 0.2 M NaCl. The remaining samples were dialysed against water and used for sodium dodecylsulfate (SDS) gel electrophoresis.[5] Protein was determined according to Lowry *et al.*[6]

Results. When brain supernatant was incubated with Sepharose 6 B containing hydrocarbon spacer groups about 15 % of the protein was observed to bind to it. The binding was ion sensitive and most of the bound protein was removed at 0.15 M NaCl (Fig. 1). The binding increased slightly with increasing incubation time; about 80 % of the maximal binding was observed when the supernatant was passed through the Sepharose column without any preincubation. No binding was observed with

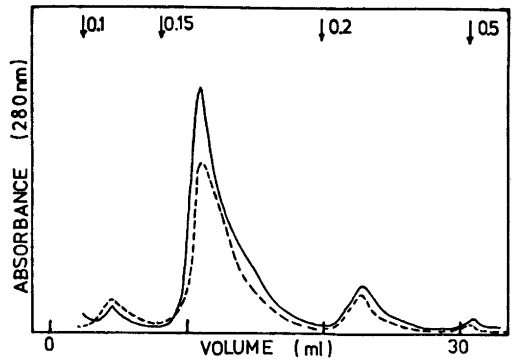

Fig. 1. Elution of protein bound to Sepharose 6 B containing spacer groups with increasing concentrations of NaCl. Rat brain supernatant was incubated with Sepharose at 37 °C for 1 h (———) or immediately passed through a Sepharose column (– – –). The figures on top refer to the molarity of NaCl.

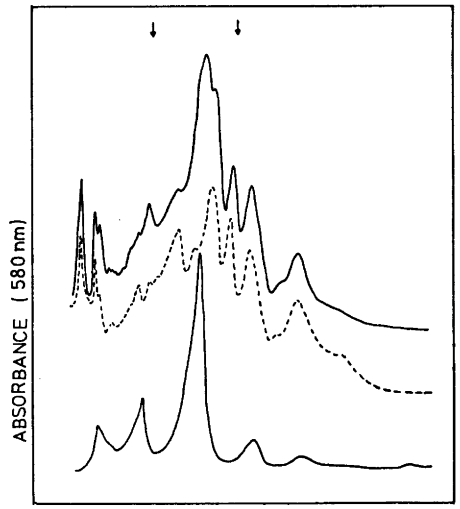

Fig. 2. SDS-polyacrylamide gel electrophoresis of rat brain supernatant protein (top), protein unbound to Sepharose 6 B containing spacer groups and protein bound to Sepharose (bottom). The gels were stained with Coomassie blue. The arrows on top refer to the migration of bovine serum albumin (68 000 daltons) and ovalbumin (43 000 daltons) used as molecular weight standards.

Table 1. Protein content and ^{3}H-colchicine binding of the rat brain fractions bound to Sepharose 6 B containing spacer groups. Means ± SD of 5 determinations.

	Protein content %		Colchicine binding cmp/mg protein × 10^{-3}
	− octanoate	+ octanoate	− octanoate
Supernatant	100	100	23 ± 4
Bound fraction	16 ± 3	0.6 ± 0.3	159 ± 8
Unbound fraction	84 ± 6	99 ± 2	3 ± 2

Sepharose 6 B containing no spacer groups indicating that the substitution of Sepharose was responsible for the binding.

The bound protein was characterized on SDS-polyacrylamide gels (Fig. 2). The binding was selective and efficient: the major component bound displayed a molecular weight of about 55 000 dalton and it was almost completely removed from the supernatant. One of the large molecular weight polypeptides could be a dimer of the 55 000 dalton polypeptide as it had a calculated molecular weight of 110 000 dalton.

Tubulin is a major protein constituent of brain soluble protein.[7] Its molecular weight [8] is similar to that of the bound polypeptide suggesting that this may be tubulin. For this reason colchicine binding assays were performed as this ligand specifically binds to tubulin (Table 1). The colchicine binding activity of the bound protein was enriched about 7-fold over the total homogenate which is almost maximal considering the large amount of tubulin in the supernatant. A corresponding reduction in the colchicine binding activity was noted in the unbound supernatant. The data indicate that the bound protein was markedly purified tubulin. The role of the hydrocarbon chain as the binding component was further suggested by incubating brain supernatant and Sephadex 6 B containing the spacer groups in the presence of an excess (5 %) of octanoic acid (caprylic acid). The added hydrocarbon chains effectively inhibited the binding (Table 1).

Another control experiment was performed to confirm that the bound material was tubulin. Brain soluble protein (100 000 *g* supernatant) was labelled with ^{3}H-colchicine and tubulin was prepared by vinblastine precipitation.[7] A substantial portion of the protein and radioactivity was bound to substituted Sepharose (results not shown).

Discussion. It is shown in this paper that tubulin has affinity to substituted Sepharose containing hydrocarbon chains and a terminal Tris molecule. In order to describe the type of interaction between tubulin and substituted Sepharose experiments have recently been carried out with Octyl-Sepharose CL-4B. As tubulin has no affinity to this preparation (Hemminki, unpublished observation), it is likely that the terminal Tris molecule is responsible for the binding observed in the present article. The sensitivity of this interaction to physiological ion concentrations may be related to cellular functions of tubulin including its association with membranes.[9,10]

The described interaction of tubulin with Tris-Sepharose may be applied for a convenient purification of the protein. About 7-fold purification is obtained. The degree of purity is at least as high as that obtained with any of the conventional one-step purification methods used for tubulin.[4,11] The procedure using affinity chromatography [12] has not replaced the conventional methods due to its cumbersome ligand requirements and other difficulties.

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Synthesis of 2,4,4′,5,5′-Pentachloro-2′-hydroxydiphenyl Ether, a Potential Precursor to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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The toxicity of certain chlorinated dibenzo-*p*-dioxins has initiated intensified research on their formation in the production and use of chlorinated phenols and compounds derived therefrom.[1] In certain cases intermediates have been observed, *e.g.* nonachloro-2-hydroxydiphenyl ether, which undergoes ring closure to octachlorodibenzo-*p*-dioxin under different conditions.[2–5] This paper describes the synthesis of 2,4,4′,5,5′-pentachloro-2′-hydroxydiphenyl ether, an analogue precursor to the particularily toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and therefore of obvious interest for chemical and toxicological investigations. The compound is prepared by demethylation of the corresponding methyl ether, which is obtained from the potassium salt of 3,4-dichloro-6-methoxyphenol on reaction with 1,2,4,5-tetrachlorobenzene in dimethyl sulfoxide. A similar procedure has been used in the direct synthesis of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin from 4,5-dichlorocatechol.[6] The present method seems more suitable than the alternative Ullmann ether synthesis which gave somewhat complicated product mixtures containing, *e.g.*, dechlorination products.

Experimental. 2,4,4′,5,5′-Pentachloro-2′-methoxydiphenyl ether. The potassium salt of 3,4-dichloro-6-methoxyphenol[7] (0.25 g; 1.3 mmol) and 1,2,4,5-tetrachlorobenzene (0.5 g; 2.3 mmol) in DMSO (13 ml) were heated to 100 °C for 60 h. The mixture was cooled to 20 °C and unreacted tetrachlorobenzene was partly removed by filtration. Sodium hydroxide (0.5 M, 50 ml) was added to the solution. The neutral components were isolated after extraction with hexane (3 × 10 ml; 0.24 g). 2,4,4′,5,5′-Pentachloro-2′-methoxydiphenyl ether was isolated by preparative TLC (Silica gel HF, ethyl ether – hexane, 35 – 65, R_F 0.7) and was crystallized from ethanol, m.p. 111 – 112 °C. Anal. $C_{13}H_7Cl_5O_2$: C, H. MS (IP 70 eV; *m/e*): pertinent peaks: 370 (M) and 320 (M – CH_3Cl).[2,4] ^{1}H NMR (100 MHz, $CDCl_3$): δ 3.81 (3 H, s), 6.76 (1 H, s), 7.04 (2 H, two singlets 1.5 Hz apart.), 7.50 (1 H, s). UV [abs. ethanol (log ε)]: 289 (4.10), 296 (sh, 4.05) nm.

2,4,4′,5,5′-Pentachloro-2′-hydroxydiphenyl ether. The crude product mixture mentioned above (100 mg), was dissolved in dichloromethane (2 ml). The solution was cooled in a dry ice-acetone mixture, and boron tribromide (100 mg) in dichloromethane (0.2 ml) was added. The mixture was allowed to reach 20 °C and was kept overnight. Hexane (4 ml) was added and 2,4,4′,5,5′-pentachloro-2′-hydroxydiphenyl ether was isolated by means of sodium hydroxide extraction (2 M, 2 × 10 ml) and acidification. Yield 30 mg, needles from hexane, m.p. 125 – 127 °C, MS [IP 10 eV; *m/e*; (% rel. int.)]: 356 (90, M), 321 (5, M – Cl), 320 (5, M – HCl), 286 (42, M – 2Cl), 180 (100, ether cleavage) 177 (20, ether cleavage). ^{1}H NMR (100 MHz, CCl_4): δ 5.40 (1 H, s), 6.70 (1 H, s), 8.00 (1 H, s), 8.05 (1 H, s), 8.48 (1 H, s) UV [abs. ethanol (log ε)]: 290 (4.04), 297 (sh. 4.00) nm.

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Synthesis of 2,4,4′,5,5′-Pentachloro-2′-hydroxydiphenyl Ether, a Potential Precursor to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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The toxicity of certain chlorinated dibenzo-*p*-dioxins has initiated intensified research on their formation in the production and use of chlorinated phenols and compounds derived therefrom.[1] In certain cases intermediates have been observed, *e.g.* nonachloro-2-hydroxydiphenyl ether, which undergoes ring closure to octachlorodibenzo-*p*-dioxin under different conditions.[2–5] This paper describes the synthesis of 2,4,4′,5,5′-pentachloro-2′-hydroxydiphenyl ether, an analogue precursor to the particularily toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and therefore of obvious interest for chemical and toxicological investigations. The compound is prepared by demethylation of the corresponding methyl ether, which is obtained from the potassium salt of 3,4-dichloro-6-methoxyphenol on reaction with 1,2,4,5-tetrachlorobenzene in dimethyl sulfoxide. A similar procedure has been used in the direct synthesis of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin from 4,5-dichlorocatechol.[6] The present method seems more suitable than the alternative Ullmann ether synthesis which gave somewhat complicated product mixtures containing, *e.g.*, dechlorination products.

Experimental. 2,4,4′,5,5′-Pentachloro-2′-methoxydiphenyl ether. The potassium salt of 3,4-dichloro-6-methoxyphenol[7] (0.25 g; 1.3 mmol) and 1,2,4,5-tetrachlorobenzene (0.5 g; 2.3 mmol) in DMSO (13 ml) were heated to 100 °C for 60 h. The mixture was cooled to 20 °C and unreacted tetrachlorobenzene was partly removed by filtration. Sodium hydroxide (0.5 M, 50 ml) was added to the solution. The neutral components were isolated after extraction with hexane (3 × 10 ml; 0.24 g). 2,4,4′,5,5′-Pentachloro-2′-methoxydiphenyl ether was isolated by preparative TLC (Silica gel HF, ethyl ether – hexane, 35 – 65, R_F 0.7) and was crystallized from ethanol, m.p. 111 – 112 °C. Anal. $C_{13}H_7Cl_5O_2$: C, H. MS (IP 70 eV; *m/e*): pertinent peaks: 370 (M) and 320 (M – CH_3Cl).[2,4] ^{1}H NMR (100 MHz, $CDCl_3$): δ 3.81 (3 H, s), 6.76 (1 H, s), 7.04 (2 H, two singlets 1.5 Hz apart.), 7.50 (1 H, s). UV [abs. ethanol (log ε)]: 289 (4.10), 296 (sh, 4.05) nm.

2,4,4′,5,5′-Pentachloro-2′-hydroxydiphenyl ether. The crude product mixture mentioned above (100 mg), was dissolved in dichloromethane (2 ml). The solution was cooled in a dry ice-acetone mixture, and boron tribromide (100 mg) in dichloromethane (0.2 ml) was added. The mixture was allowed to reach 20 °C and was kept overnight. Hexane (4 ml) was added and 2,4,4′,5,5′-pentachloro-2′-hydroxydiphenyl ether was isolated by means of sodium hydroxide extraction (2 M, 2 × 10 ml) and acidification. Yield 30 mg, needles from hexane, m.p. 125 – 127 °C, MS [IP 10 eV; *m/e*; (% rel. int.)]: 356 (90, M), 321 (5, M – Cl), 320 (5, M – HCl), 286 (42, M – 2Cl), 180 (100, ether cleavage) 177 (20, ether cleavage). ^{1}H NMR (100 MHz, CCl_4): δ 5.40 (1 H, s), 6.70 (1 H, s), 8.00 (1 H, s), 8.05 (1 H, s), 8.48 (1 H, s) UV [abs. ethanol (log ε)]: 290 (4.04), 297 (sh. 4.00) nm.

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Protection of Deoxyribonucleic Acid from Nuclease Action by Histones

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The observation that four of the five main histone proteins present in chromatin do not occur as separate monomeric units, but form an oligomeric structure with equimolar amounts of all these histones,[1] has placed research in this field in a new phase of development. Only the very lysine-rich histone F1 seems to function independently from other histones,[2] while the histones F2A1, F2A2, F2B and F3 form a complex, which apparently has an octameric structure containing two copies of each histone.[1] When chromatin is subjected to the action of DNase, units consisting of the histone octamer and the DNA about 200 nucleotides long are released.[3] The histone octamer is thought to protect the regions of DNA which are associated with it, while DNase is free to cut DNA between the histone complexes.

The arrangement of different histones in the oligomer needs to be investigated further. We have approached this problem by studying the capacity of different histones to protect DNA from nuclease action. The idea behind these experiments was that the histone species that protect DNA best are those that interact physically with DNA in the octamer, while those that protect DNA poorly are possibly not in direct association with DNA. The special properties of histones make these types of reassociation experiments reasonable. Histones are relatively small proteins and for this reason they apparently can, under proper conditions, return easily to their native conformation, which they lose in acidic conditions during purification. This is suggested by some recent studies showing that the complex formation *in vitro* between purified individual histones and also between histones and DNA gives a product identical with, or at least similar to, the product formed *in vivo*.[4,5]

Methods. Both DNA and histones were prepared from calf thymus tissue. DNA was isolated according to the method given by Schwander and Signer.[6] The total histones were extracted with 0.2 N H_2SO_4 from the nuclei, which were prepared by slightly modifying[7] the procedure of Blobel and Potter.[8] This acid extraction was preceded by three washings of the nuclei with 0.14 M NaCl – 0.05 M $NaHSO_3$. The histones were precipitated from the clarified acidic extract with five volumes of ethanol, and the precipitate obtained was washed several times, first with ethanol and then with ether.

The different histone proteins were isolated according to "method 2" of Johns.[9] Four fractions, F1, F2A, F2B, and F3, were obtained. The fraction F2A was then separated into two subfractions, F2A1 and F2A2, by precipitating F2A2 with acetone from an acidic solution.[10] The lysine-rich histone F1 was purified further by the method of Oliver *et al.*[11]

The composition of the histone preparations was analyzed by polyacrylamide gel electrophoresis according to Johns.[12]

The digestion of DNA by DNase in the presence or absence of histones was assayed as follows: DNA plus different histone proteins and DNase were dissolved each separately in 0.1 M CH_3COONa buffer, pH 5.0 with 0.005 M $MgSO_4$. The concentration of DNA was 0.04 mg/ml. A 0.5 ml solution containing varying amounts of histone was added to 1 ml of DNA solution, and the mixture incubated at 37 °C for 10 min. After this preincubation, 0.5 ml of enzyme solution (50 – 100 units/ml) was added, and the reaction allowed to continue at 37 °C for 7 min. The reaction was stopped with 0.5 ml of 3 M CCl_3COOH. The precipitate was removed by centrifugation, and the amount of UV-absorbing material in the supernatant was measured in a spectrophotometer at 260 nm. Electrophoretically purified pancreatic DNase I was purchased from Merck, Darmstadt. The amount of enzyme which gave a 0.001 O.D. unit rise in absorption at 260 nm in 1 min, under the experimental conditions employed with DNA as the substrate, was defined as one enzyme unit.

Protein content was determined according to Lowry *et al.*,[13] and DNA content by a diphenylamine method.[14]

Results. The main histone species were purified from calf thymus. Fig. 1 shows an electrophoretic analysis of these preparations

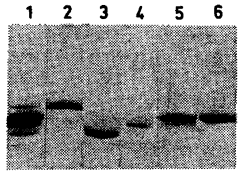

Fig. 1. Polyacrylamide gel electrophoresis of histone preparations. Gel 1, whole histone; gel 2, F1; gel 3, F2A1; gel 4, F2A2; gel 5, F2B; gel 6, F3.

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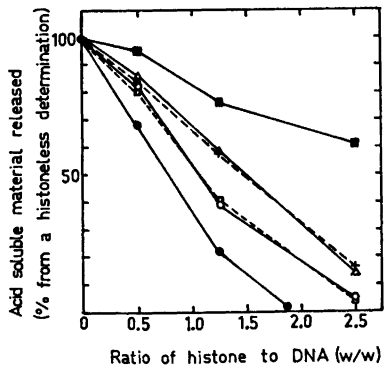

Fig. 2. Protection of DNA by histones from enzymatic digestion by DNase I. In order to allow complex formation between DNA and histones, the DNA was preincubated with different histone fractions at 37 °C for 10 min prior to subjection to DNase action. ○, whole histone; ●, F1; ■, F2A1; □, F2A2; △, F2B; +, F3.

on polyacrylamide gels. Each histone fraction appears to be relatively free of the other histones and nonhistone proteins. These preparations were used to investigate the capacity of histones to protect DNA against DNase digestion.

Increasing amounts of total histones, or each histone protein separately, were incubated with the same amount of calf thymus DNA for 10 min, in order to allow the complex formation between DNA and histones to occur. After preincubation, the susceptibility of the DNA in these complexes to DNase I was examined. As is shown in Fig. 2, the DNA was protected to vaying degrees from enzymatic digestion by DNase, the extent of this protection being dependent on the histone employed. The lysine-rich histone F1 gave the best protection, while the arginine-rich histone protein F2A1 had the weakest effect. The F2A2 histone, which is a slightly lysine-rich protein, inhibited DNase action somewhat more effectively than the histones F2B and F3. The DNA protection capacity of the total histones was very similar to that of F2A2.

Discussion. It is obvious that the histone F1 is directly bound to DNA in chromatin, because it does not appear to interact with the other histones.[2] Its strong inhibitory effect on DNase activity can be well understood on this ground. On the other hand the histone F2A1 may not interact with DNA to any great extent, but rather with other histones in the octamer, because it protected DNA much less than the other histones of the complex. Some interactions with DNA may, however, take place, because the F2A1 protein also has basic terminal segments, which apparently are the primary sites of interaction of the individual histones with DNA.[15] The histones F2A2, F2B and F3 may all interact directly with DNA, because they all protected DNA fairly effectively almost to the same extent. The histone F2A1 may function as a protein which holds the different histones of the complex together. This is also supported by the crosslinking experiments of Thomas and Kornberg,[1] which demonstrated that F2A1 is apparently bound to all three other histones of the complex.

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(Cyanomethyl)phosphines from Trimethylsilylphosphines

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(Cyanomethyl)phosphines, $R_{3-n}P(CH_2CN)_n$, are known for $n=1$.[1] They were prepared from phosphinites and chloroacetonitrile followed by reduction of the product $R_2P(O)CH_2CN$, a method which is not feasible for preparation of (cyanomethyl)phosphines with $n=2$ or 3.

We describe here a more general method for preparation of (cyanomethyl)phosphines, *viz.* reaction of trimethylsilylphosphines with chloroacetonitrile (eqn. 1). Trimethylsilylphosphines

$$Ph_{3-n}P(SiMe_3)_n + nClCH_2CN \rightarrow Ph_{3-n}P(CH_2CN)_n + nMe_3SiCl \quad (1)$$

have been used mostly for preparation of acylphosphines,[2,3] although one reaction with methyl iodide [4] and a few reactions with reactive chloro compounds, *e.g.* diethyl dichloromaleate,[5] are known. Less reactive alkyl chlorides do not react, *e.g.* Ph_2PSiMe_3 is reported not to react with $ClCH_2CH_2Cl$.[5]

We have found that $ClCH_2CN$ reacted in a moderately exothermic reaction with $Ph_{3-n}P(SiMe_3)_n$, $n=1$, 2, or 3, and Me_3SiCl could be isolated in 80–90 % yield. In the cases $n=1$ and 2, Ph_2PCH_2CN and $PhP(CH_2CN)_2$, respectively, were isolated in good yields by distillation, but no $P(CH_2CN)_3$ could be obtained in this way. The reaction in the case of $n=3$ yields predominantly a dark intractable material which is apparently polymeric. It contained, however, some $P(CH_2CN)_3$ (NMR) which could be isolated in low yield by sublimation.

In contrast to Ph_2PCH_2CN, which is oxidized slowly in air,[1] $PhP(CH_2CN)_2$ and $P(CH_2CN)_3$ are stable towards air in the solid state. They are rather easily oxidized in solution or when melted. Their nucleophilic reactivities are very low, as expected.[1] Thus $P(CH_2CN)_3$ is unchanged after 24 h reflux with an excess of EtI in acetone.

The reaction of trimethylsilylphosphines with chloroacetonitrile thus seems to be a convenient method of obtaining cyanomethylphosphines with one or two cyanomethyl groups, but less so with three cyanomethyl groups. We are presently examining other synthetic pathways to cyanomethylphosphines in general and $P(CH_2CN)_3$ in particular.

Experimental. Analyses were carried out by the Microanalysis Department of this laboratory. NMR spectra were obtained on a Bruker HX-90 E Spectrometer. Chemical shifts (ppm) are relative to internal TMS for ^1H data (δ_H) and external 85 % H_3PO_4 for ^{31}P data (δ_P), and are given as positive for low-field shifts. All stated $^1H-^{31}P$ couplings have been verified by decoupling experiments. IR spectra were recorded on a Perkin Elmer 337 Grating Infrared Spectrometer, MS spectra on an AEI-902 Mass Spectrometer at 70 eV, inlet temp. 180 °C.

All reactions with phosphines were performed under nitrogen. The trimethylsilylphosphines were prepared by published methods and their purity checked by ^1H and ^{31}P NMR: Ph_2PSiMe_3,[6] δ_P −57.3 (neat). $PhP(SiMe_3)_2$,[7] b.p. 65–66 °C/0.2 mmHg, δ_P −136.0 ($CDCl_3$) (lit. b.p. 76 °C/0.05 mmHg,[7] and 67 °C/0.01 mmHg [8] could not be corroborated, δ_P −137 (neat)[8]). $P(SiMe_3)_3$,[9] 17 % yield, b.p. 64–65 °C/1.2 mmHg, δ_P −252.0 (neat), −249.9 ($CDCl_3$) (lit. 62 % yield and b.p. 50–52 °C/0.1 mmHg [9] could not be obtained, b.p. 105 °C/16 mmHg,[10] δ_P −251.2 (neat)[11]).

Diphenylphosphinoacetonitrile (Ph_2PCH_2CN). Ph_2PSiMe_3 (12.9 g, 0.05 mol) and $ClCH_2CN$ (3.8 g, 0.05 mol) were mixed and stirred at 25 °C in a flask equipped with a Claisen head with condenser. After the exothermic reaction had subsided the mixture was heated in an oil bath (temp. *ca.* 110 °C), whereupon Me_3SiCl (4.4 g, 80 %) distilled. The red-brown residue gave upon distillation *in vacuo* (same apparatus) Ph_2PCH_2CN (8.0 g, 71 %), b.p. 138–140 °C/0.1 mmHg, δ_P −17.8 ($CDCl_3$) [lit.[1] 146–147 °C/0.35 mmHg, δ_P −17.8 ($CDCl_3$)]. No impurities were observable from its ^1H NMR spectrum.

Phenylphosphinediyldiacetonitrile ($PhP(CH_2CN)_2$). $PhP(SiMe_3)_2$ (5.1 g, 0.02 mol) and $ClCH_2CN$ (3.0 g, 0.04 mol) gave when treated as above Me_3SiCl (3.9 g, 90 %) and $PhP(CH_2CN)_2$ (1.9 g, 50 %), b.p. 148–150 °C/0.25 mmHg, m.p. 59–62 °C. Anal. $C_{10}H_9N_2P$: C, H, N. NMR (*ca.* 2 M in $CDCl_3$): δ_P −31.0. CH_2CN (ABX system): $\delta_{H(A)}$ 2.866, $^2J_{PH(A)}$ 5.9 Hz, $\delta_{H(B)}$ 2.820, $^2J_{PH(B)}$ 5.4 Hz, $^2J_{H(A)H(B)}$ 16.7 Hz. Ph: δ_H 7.4–7.7 (m). IR (KBr): ν_{CN} 2246, 2234 cm^{-1}. $PhP(CH_2CN)_2$ is very soluble in $CHCl_3$ and insoluble in H_2O.

Phosphinetriyltriacetonitrile ($P(CH_2CN)_3$). $P(SiMe_3)_3$ (5.0 g, 0.02 mol) and $ClCH_2CN$ (4.5 g, 0.06 mol) gave when treated as above Me_3SiCl (5.2 g, 80 %) and a dark red residue. ^1H NMR (CD_3CN) on the residue showed as the most prominent signal a doublet, δ_H 2.89, J 5.3 Hz, δ_P −33.5 (determined by ^{31}P decoupling) assigned to $P(CH_2CN)_3$. The amount of $P(CH_2CN)_3$ was estimated as *ca.* 30 % of the theoretical yield by comparison of the $P(CH_2CN)_3$ signal with that from a known amount of added CH_2Cl_2. Attempted vacuum distillation through a Claisen head (0.2–0.5 mmHg, bath up to 220 °C) gave no distillate, and the residue turned black. However, a small amount (*ca.* 0.5 ml) of a liquid consisting mostly of CH_3CN (^1H NMR) had collected in the dry-ice trap. Sublimation of the residue

(0.1 mmHg, 180 °C) gave a small amount (0.3 g, 10 %) of slightly impure (yellow) $P(CH_2CN)_3$, which on resublimation or recrystallization from water (with a small amount of hydroquinone) gave the pure compounds as colourless needles, m.p. 112–113 °C. Anal. $C_6H_6N_3P$: C, H, N. NMR: δ_P −33.4, δ_H 2.88, $^2J_{PH}$ 5.3 Hz (CD_3CN), δ_P −32.9, δ_H 3.12, $^2J_{PH}$ 4.3 Hz [$(CD_3)_2SO$]. IR (KBr): ν_{CN} 2245 cm^{-1}. MS: *m/e* 151 (M^+, 41 %), 111 ($M^+ - CH_2CN$, 100 %), 71 ($M^+ - 2CH_2CN$, 36 %), "metastable" 81.6 (151→111). $P(CH_2CN)_3$ is soluble in DMSO, CH_3CN, and $(CH_3)_2CO$, slightly soluble in CH_3OH and H_2O, and nearly insoluble in $CHCl_3$.

Phenylphosphonoyldiacetonitrile (PhP(O)-$(CH_2CN)_2$). $PhP(CH_2CN)_2$ (1.88 g) in CH_2Cl_2 (35 ml) was oxidized by bubbling NO_2 through the stirred solution until a yellow colour remained. The solution was evaporated to dryness and the residue recrystallized from H_2O. Yield 1.43 g (70 %), m.p. 135.5–136.5 °C. Anal. $C_{10}H_9N_2OP$: C, H, N. NMR[$(CD_3)_2SO$]: δ_P 26.4, CH_2CN: δ_H 3.99, $^2J_{PH}$ 14.2 Hz. IR (KBr): ν_{CN} 2259, 2250 cm^{-1}, ν_{PO} 1200 cm^{-1}. $PhP(O)(CH_2CN)_2$ is very soluble in DMSO, $(CH_3)_2CO$ and CH_3CN, and slightly soluble in $CHCl_3$ and H_2O.

Phosphoryltriacetonitrile ($P(O)(CH_2CN)_3$). To $P(CH_2CN)_3$ (0.15 g) in hot water (3 ml) was added dropwise 35 % H_2O_2 (0.5 ml). The oxide precipitated during the reaction. After cooling to 0 °C the compound was collected by centrifugation, washed with water and dried *in vacuo*. Yield 0.15 g (90 %), m.p. *ca.* 260 °C (dec.). Anal. $C_6H_6N_3OP$: C, H, N. NMR [$(CD_3)_2SO$]: δ_P 27.9, δ_H 3.81, $^2J_{PH}$ 14.8 Hz. IR (KBr): ν_{CN} 2256 cm^{-1}, ν_{PO} 1229, 1225, 1204, 1197 cm^{-1}. $P(O)(CH_2CN)_3$ is very soluble in DMSO, slightly soluble in H_2O and CH_3CN, and insoluble in $CHCl_3$.

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Influence of Ethanol Oxidation Rate on the Lactate/Pyruvate Ratio and Phosphorylation State of the Liver in Fed Rats

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The effect of the ethanol oxidation rate on the interaction between the phosphorylation state (the [ATP]/[ADP] × $[HPO_4]^{2-}$ ratio) and the redox state (the free [NAD+]/[NADH] ratio) of the liver cytosol was studied in intact fed rats. The rate of ethanol oxidation was inhibited to different degrees with pyrazole. The ethanol oxidation rate had no influence on the liver lactate level but correlated significantly with the pyruvate level. Accordingly, a significant correlation was also found between the ethanol oxidation rate and the lactate/pyruvate ratio. The rate of ethanol oxidation correlated significantly with the liver 3-phosphoglycerate level. No change in the glyceraldehyde-3-phosphate level was found. No correlation was found between the ethanol oxidation rate and the glyceraldehyde-3-phosphate/3-phosphoglycerate redox couple. Ethanol administration slightly increased the liver ATP level, but the simultaneous administration of pyrazole eliminated this effect. Other adenine nucleotides and HPO_4^{2-} were not changed. The changes in the rate of ethanol oxidation had no effect on the phosphorylation state in the fed liver. It is assumed that in the fed liver the phosphorylation state is so well stabilized that the redox level has no influence.

Interaction occurs between the ATP-ADP and NAD+-NADH couples in the glycolytic chain.[1] Hohorst *et al.*[2] have shown that this also holds for intact livers with various metabolic conditions. The redox state of the free NAD+—NADH couple in the liver cytosol is very stable during normal conditions, and the redox potential is controlled by the interaction between the phosphorylation state (*i.e.* the ratio [ATP]/[ADP][HPO_4]$^{2-}$) and the redox state of free NAD+-NADH couple.[3,4] Both the phosphorylation state and the level of the redox system of the cytosol can be changed by feeding different types of diets or by giving rats specific substances. It was suggested that these changes were mediated by the glyceraldehyde-3-phosphate—3-phosphoglycerate couple.[5] From this work it could not be stated whether the phosphorylation state influenced the redox level or the redox level influenced the phosphorylation state.

In the liver of the starved rat, ethanol influences the redox level as well as phosphorylation state of the cytosol.[5] In the liver of the fed rat, ethanol has a strong effect on the redox state, but the influence on the phosphorylation state is not so clear.[6] It has been claimed that the rate of ethanol oxidation is influenced by the redox state of the cytosol.[7,8] In two studies the rate of alcohol oxidation from differently treated rats has been compared to the lactate/pyruvate ratios.[7,9] Hillbom showed that the more reduced the redox level was, the lower was the rate of ethanol elimination.[7] Such a relationship was also observed by Smith and Newman [9] in their study on fed and fasted rats. In the present study we wanted to determine if the cytosolic redox changes induced by ethanol are reflected in the cytosolic phosphorylation state, in fed rats where the reaction catalyzed by alcohol dehydrogenase is modified with a specific inhibitor, pyrazole.[10] We also wanted to compare the ethanol oxidation rate and the cytosolic redox state.

EXPERIMENTAL

Animals. Female Sprague-Dawley rats, aged 2—3 months and weighing 200—250 g fed

with the ASTRA-EWOS (Södertälje, Sweden) laboratory diet and tap water *ad libitum* were used in all experiments.

Ethanol elimination rate. The rats were divided into 5 groups, 10 animals in each. The first served as the control group and received saline intraperitoneally (1 ml/kg body weight). The other four groups received pyrazole in saline, 0.1, 0.5, 1.0, or 2.0 mmol per kg body weight. 15 min after saline and pyrazole injections 1.5 g of ethanol in saline per kg body weight was given to all animals. Blood samples were taken from the tip of the tail 1.0, 1.5, 2.0, 2.5, and 3.0 h later and the concentration of ethanol in the blood was determined. The rate of ethanol oxidation was calculated from the slopes of the elimination curves according to Rawat and Kuriyama.[11] The liver was assumed to be 2.79 % of the total body weight.[12]

Determination of metabolites. The groups of rats and doses of pyrazole were the same as for the estimation of the ethanol elimination rate. 15 min after pyrazole administration each group of 12 animals was divided into two groups, one of which received 1.5 g of ethanol per kg body weight intraperitoneally and the other the same volume of saline. The liver samples were taken 1 h after ethanol or saline injections. The animals were anaesthetized with pentobarbital (Nembutal®, 1 % solution in saline, 40 mg/kg body weight) and the livers were quickly frozen *in situ* with aluminium tongs precooled in liquid nitrogen.[13] The powdered frozen liver tissue was suspended in ice-cold perchlorid acid (0.6 M). The protein precipitate was centrifuged down and the metabolites were determined from the neutralized supernatant within a few hours. Lactate and pyruvate were determined according to Hohorst *et al.*,[14] 3-phosphoglycerate according to Czok,[15] dihydroxyacetone phosphate according to Bücher and Hohorst,[16] ATP according to Adam,[17] ADP and AMP according to Adam,[18] and inorganic phosphate according to Bartlett.[19] The glyceraldehyde-3-phosphate concentration was calculated from the dihydroxyacetone phosphate concentration, according to Veech *et al.*[4] 60 % of the inorganic phosphate measured was assumed to be in the form of HPO_4.[2-5]

Ethanol was determined gas chromatographically (Perkin Elmer F 40) by the head space technique. *t*-Butyl alcohol was used as an internal standard.[20]

Statistical analysis. Statistical correlations between the concentrations of metabolites and the ethanol oxidation rate were calculated by linear regression analysis.[21] Statistical differences between different groups were calculated by Student's *t* test.

RESULTS

Rate of ethanol elimination. The rate of ethanol elimination was 4.6 μmol/[(g of liver wet weight) min] in the intact rat (Table 1). This value was similar to that obtained by Veech *et al.*[6] Inhibition of ethanol oxidation by pyrazole was linear with increasing doses and was 59 % with a dose of 2 mmol/(kg body weight). The amount needed for inhibition was much larger than used by Lester and Benson[22] and by Goldberg and Rydberg,[23] but of about the same order as that used by Papenberg *et al.*[24] and by Grunnet and Thieden.[25] Strain differences have been assumed to be responsible for the differences in sensitivity to pyrazole.[26]

Liver lactate/pyruvate and glyceraldehyde-3 phosphate/3-phosphoglycerate ratios. Pyrazole alone had no effect on the liver content of lactate, pyruvate, glyceraldehyde-3-phosphate, and 3-phosphoglycerate, and no change was found in the lactate/pyruvate or glyceraldehyde-3-phosphate/3-phosphoglycerate ratios (Table 2).

Administration of ethanol did not cause any significant change in the liver content of lactate (Table 2), nor was any change found when ethanol and various doses of pyrazole were administered together. There was a significant decrease ($p < 0.05$) in the liver content of pyruvate after administration of ethanol alone (Table 2) and also when ethanol and 0.1 – 1.0 mmol of pyrazole per kg body weight were administered together. With the highest dose of pyrazole [2 mmol/(kg body weight)] the difference to the control group was not significant ($p > 0.05$). The lactate/pyruvate ratio was significantly elevated ($p < 0.05$) when ethanol oxidation was not inhibited and when in-

Table 1. Rate of elimination of ethanol in the intact fed rat. Each figure represents the mean ± standard deviation of 8 – 10 animals. The groups receiving pyrazole were compared with the control group (pyrazole concentration 0) by Student's *t*-test.

Pyrazole administered [mmol/(g body weight)]	Rate of ethanol oxidation μmol/[(g liver wet wt)min]	*p*
0 (Control)	4.6(5)	–
0.1	4.1(5)	<0.050
0.5	3.6(11)	<0.050
1.0	3.4(13)	<0.050
2.0	2.3(13)	<0.001

Table 2. Effects of ethanol and pyrazole on the liver lactate – pyruvate and glyceraldehyde-3-phosphate – 3-phosphoglycerate couples in the intact fed rat. Each figure represents the mean ± standard deviation of 6 animals. Contents expressed in μmol/g of liver fresh weight. Groups receiving ethanol were compared with the corresponding control groups by Student's *t*-test.

Metabolite	Presence of ethanol	Amount of pyrazole administered [mmol/(kg body weight)] 0	0.1	0.5	1.0	2.0
Lactate	–	1.8(5)	1.8(7)	1.7(4)	1.7(7)	1.6(5)
	+	1.9(4)	1.5(4)	1.4(3)	1.7(3)	2.1(3)
Pyruvate	–	0.13(5)	0.14(3)	0.13(5)	0.13(4)	0.13(6)
	+	0.06(2)[a]	0.06(4)[b]	0.07(3)[a]	0.06(5)[a]	0.11(4)
Lactate/pyruvate	–	15(3)	12(4)	14(5)	14(5)	14(5)
	+	36(17)[a]	29(21)[a]	32(21)[a]	30(14)[a]	22(9)
3-Phosphoglycerate	–	0.30(12)	0.25(6)	0.19(5)	0.23(10)	0.19(4)
	+	0.09(3)[c]	0.14(6)[a]	0.13(6)	0.15(4)	0.15(3)
Dihydroxyacetone phosphate	–	0.05(3)	0.05(3)	0.05(3)	0.04(3)	0.05(3)
	+	0.05(2)	0.05(2)	0.04(1)	0.05(2)	0.06(2)
Glyceraldehyde-3-phosphate/3-phosphoglycerate	–	0.02(2)	0.02(1)	0.03(2)	0.02(2)	0.03(2)
	+	0.07(4)	0.04(2)	0.04(2)	0.03(1)	0.04(2)

[a] $p<0.050$. [b] $p<0.010$. [c] $p<0.001$.

hibition by pyrazole was slight [pyrazole doses 0.1 – 1.0 mmol/(kg body weight)]. A pyrazole dose of 2 mmol per kg body weight strongly depressed the rise in the liver lactate/pyruvate ratio caused by ethanol. A significant positive correlation ($r=0.90$, $p<0.05$) was found to exist between the lactate/pyruvate ratio and the rate of ethanol oxidation (Fig. 1).

A highly significant decrease ($p<0.001$) in the content of liver 3-phosphoglycerate was observed after ethanol administration (Table 2). This has been previously found by Williamson *et al.*[27] and also by Veech *et al.*[6] With simultaneous administration of 0.1 mmol of pyrazole per kg body weight the change was less marked and was not significant when 0.5 mmol or higher doses of pyrazole were given together with ethanol. No change in the glyceraldehyde-3-phosphate content was recorded. This may have been due to the very small concentration of this metabolite, which made an accurate determination very difficult. However, ethanol changed the glyceraldehyde-3-phosphate/3-phosphoglycerate ratio significantly ($p<0.05$). The change was due to the decrease in the 3-phosphoglycerate concentration. Administration of 0.1 mmol of pyrazole per kg body weight corrected this shift because there was an increase in the 3-phosphoglycerate concentration. No significant correlation was found between the lactate/pyruvate and glyceraldehyde-3-phosphate/3-phosphoglycerate ratios. This probably depends on the inaccurate ratio for the latter couple.

Liver phosphorylation state. Pyrazole alone had no influence on the contents of liver ATP, ADP, AMP and inorganic phosphate (Table 3). Ethanol administration caused a slight increase ($p<0.05$) in the liver content of ATP,

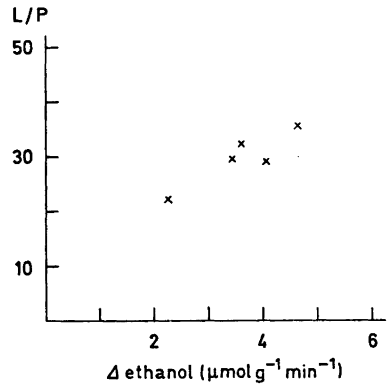

Fig. 1. Variation in the lactate/pyruvate ratio as a function of the ethanol oxidation rate. The scatter plot (with $r=0.90$; $p<0.05$) is drawn from the results of Tables 1 and 2.

Table 3. Effects of ethanol and pyrazole on the ATP, ADP, AMP, and P_i contents of the liver in the intact fed rat. Figures represent the mean ± standard deviation of 6 animals. Contents are expressed as μmol/g of liver fresh weight. Groups receiving ethanol were compared with the corresponding control groups by Student's *t*-test.

Metabolite	Presence of ethanol	Amount of pyrazole administered [mmol/(kg body weight)] 0	0.1	0.5	1.0	2.0
ATP	−	2.5(3)	2.6(3)	2.5(2)	2.5(5)	2.5(2)
	+	2.8(1)[a]	2.7(2)	2.6(3)	2.8(4)	2.8(2)[a]
ADP	−	0.7(1)	0.7(1)	0.7(1)	0.67(4)	0.7(1)
	+	0.7(1)	0.7(1)	0.7(1)	0.7(1)	0.7(1)
AMP	−	0.3(2)	0.3(1)	0.3(1)	0.4(2)	0.3(2)
	+	0.3(1)	0.2(1)	0.3(2)	0.2(1)	0.3(1)
P_i	−	3.6(11)	3.9(8)	3.6(8)	3.6(7)	3.7(8)
	+	3.5(7)	3.5(6)	3.4(7)	3.4(7)	3.5(7)
ATP/	−	1.8(7)	1.7(7)	1.8(5)	1.8(5)	1.6(4)
$ADP \times HPO_4^{2-}$	+	2.0(9)	2.0(6)	1.9(7)	2.0(6)	2.1(6)

[a] $p<0.050$.

but after 0.1 mmol of pyrazole per kg body weight no difference existed between the ethanol-treated and control groups. The concentration of other adenine nucleotides and inorganic phosphate did not change (Table 3). Ethanol did not influence the phosphorylation state of the fed rat liver, nor did the rate of ethanol oxidation influence this state.

DISCUSSION

Alcohol dehydrogenase (EC 1.1.1.1) plays a major role in the oxidation of alcohol in the liver.[28] During normal conditions more of the enzyme is present in the liver than is necessary for the rate of alcohol oxidation and it is, therefore, assumed that the rate of ethanol metabolism is not limited by the amount of this enzyme.[20,28,29] The excess of alcohol dehydrogenase may also explain our finding that rather large doses of pyrazole were needed before an effect on the rate of ethanol oxidation was observed.

Ethanol, when present, is the prime substrate for liver metabolism. The oxidation of ethanol to acetaldehyde takes place in the cytosol and the second reaction, oxidation of acetaldehyde to acetate, is evidently a mitochondrial reaction.[30,31] Only the first reaction is responsible for the change in the lactate/pyruvate ratio which takes place in the cytosol.[20,32] Accordingly, in the present study we found a positive linear correlation between the ethanol oxidation rate and the liver lactate/pyruvate ratio. In this case alcohol dehydrogenase was inhibited, and the decreased rate of ethanol oxidation was responsible for the decrease in the redox state. However, in rats fed with different substances[7] or fasted,[9] a negative correlation between the rate of ethanol oxidation and the lactate/pyruvate ratio was found, *i.e.*, when more ethanol was oxidized, the lactate/pyruvate ratio changed less. In these cases the amount of alcohol dehydrogenase is not rate limiting but rather the redox level of the $NAD^+/NADH$ system or the amount of either NAD^+ or NADH influences the oxidation rate of ethanol.[33] The ethanol oxidation rate can thus be influenced either by directly inhibiting alcohol dehydrogenase or by influencing the processes which affect the redox level of the cytosol.

Measurements of the overall levels of ATP, ADP and P_i in the liver give values which correspond closely to those in the cytosol, since the amounts of these compounds within the mitochondria are small and the mitochondrial matrix space is only about 6 % of the total liver space.[5,34] In the cytosol the phosphorylation state can be influenced by the redox state *via* the glyceraldehyde-3-phosphate – 3-phosphoglycerate couple,[5] by the rate of ATP utilization[35] and also by the interchange of ATP and ADP between cytosol

and mitochondria.[34] The amount of ATP formed by glycolysis in the cytoplasm is so small that it does not need to be taken into account.[36] When this work was begun, the shift in the redox state of the liver cytosol was expected to influence also the phosphorylation state, and we hoped that both of these changes could be correlated with the rate of ethanol oxidation. It was found, however, that only the redox state was influenced and this correlated well with the ethanol oxidation rate. The phosphorylation state was not influenced.

This result differs from those obtained with rats starved for 48 h.[5,6] In the liver of fasted rats ethanol has very strong effects on the lactate/pyruvate ratio [8] and a significant effect on liver phosphorylation state is also seen.[5] During starvation there is a partial shortage of substrate and at the same time intensive utilization of ATP for gluconeogenesis. When ethanol is available, this readily-oxidizable substrate is metabolized first, and, at the same time, ATP utilization for gluconeogenesis is depressed.[35] These effects of ethanol may contribute to the influence on the phosphorylation state of the liver in the fasted rat. In the fed rat the situation is different. Ethanol is utilized instead of other freely available substrates, and the rate of gluconeogenesis is small and not depressed by ethanol.[37] So only the change in the redox state or the interchange of adenosine phosphates with mitochondria can be expected to alter the phosphorylation state of the cytosol. There is no pressure on the adenosine phosphate system and, therefore, no effect of ethanol on the state of phosphorylation as demonstrated by Veech *et al.*[6] and confirmed in the present study. Apparently, in the liver of the fed rat the phosphorylation state is so well stabilized that the oversupply of reducing equivalents and the accompanying alteration of the cytosolic redox state cannot influence the phosphorylation state.

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On the Enzymic Preparation of Decarboxylated Adenosylmethionine

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A method is described for enzymic preparation of *S*-methyladenosylhomocysteamine (decarboxylated adenosylmethionine) with the aid of magnesium-activated adenosylmethionine decarboxylase partially purified from cells of *Escherichia coli*. The procedure involves chromatography on Dowex 50-H^+ column, followed by preparative paper electrophoresis and rechromatography on Dowex 50-H^+. The procedure yields a pure preparation which is suitable for measurements of the activity of spermidine and spermine synthases.

The synthesis of the higher polyamines, spermidine and spermine is accomplished in both eukaryotic and prokaryotic organisms by at least three separable enzymes: (i) a soluble *S*-adenosyl-L-methionine decarboxylase (*S*-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50) that catalyzes the decarboxylation of *S*-adenosyl-L-methionine to yield *S*-methylhomocysteamine (decarboxylated adenosylmethionine) and CO_2,[1-4] (ii) an aminopropyltransferase (spermidine synthase, 5'-deoxyadenosyl-(5'),3-aminopropyl-(1),methylsulfonium-salt:putrescine 3-aminopropyltransferase, EC 2.5.1.16) which transfers the propylamine group from decarboxylated adenosylmethionine to putrescine to yield spermidine, methylthioadenosine and a proton,[4-6] and (iii) a spermine synthase (5'-deoxyadenosyl-(5'), 3-aminopropyl-(1),methylsulfonium-salt:spermidine 3-aminopropyltransferase).[5]

The decarboxylation of adenosylmethionine (Ado-met) is catalyzed by a specific adenosylmethionine decarboxylase (AMDC) which in animal tissues and yeast is strongly activated by putrescine and related diamines [1,3,4,8] but in some lower eukaroytes and in prokaryotic organisms does not require any cofactors [9,10] or shows a stringent requirement for magnesium ions.[3,6,11]

The main, if not necessarily the only function of decarboxylated Ado-met appears to be to serve as the substrate for spermidine and spermine synthases. Decarboxylated Adomet can also function as methyldonor in some transmethylation reactions [12] and it occurs at high concentrations in the *tapetum lucidum* of the catfish (*Arius felis*) [13] for reasons which are not yet known.

In higher organisms, such as in animal tissues and in cells of baker's yeast AMDC is the rate controlling enzyme in the synthesis of spermidine and spermine.[14,15] The measurements of the formation of spermidine and spermine in the presence of Ado-met and the appropriate amine precursor (putrescine or spermidine) in the tissue or cell extracts thus only represents the activity of AMDC and not that of the two propylamine transferases. Accordingly, for the measurements of the actual activities of spermidine and spermine synthases decarboxylated Ado-met is needed as precursor.

In the present communication we describe a method for preparative isolation of decarboxylated Ado-met, synthesized enzymically with the aid of partially purified AMDC from *E. coli*. Some factors that contribute to the yield of the preparative process as well as to the purity and use of the product in the synthesis of spermidine and spermine, are likewise presented.

MATERIALS AND METHODS

Chemicals. Unlabelled *S*-adenosyl-L-methionine was synthesized by the method originally described by Cantoni *et al.*[16] and modified by Pegg and Williams-Ashman.[1]

[1,4-^{14}C]Putrescine (sp. radioactivity 17.5 mCi/mmol), [1,4-^{14}C]spermidine (sp. act. 12.4 mCi/mmol) and [carboxyl-^{14}C]*S*-adenosyl-L-methionine (sp. act. 60 mCi/mmol) were purchased from New England Nuclear Corp. (Dreieichenhain, West-Germany). Radioactive putrescine was purified on a Dowex 50-H^+ column before use.

Cultivation of Escherichia coli. *E. coli* (strain ATCC 4157) was grown aerobically in a supplemented mineral medium the composition of which has been described elsewhere.[8] The cultivation was started by inoculating 25 ml of the medium and incubating the culture at 30 °C for 12 h, after which it was transferred to 500 ml of the same medium and grown for further 12 h. The culture was transferred to 4.5 l of the medium and incubated for 10 h. This culture was finally transferred to a 400 l pilot plant fermentor (Getingeverken, Getinge, Sweden) which contained 100 l of the medium. The mixture was incubated for about 7 h. The cells were harvested in a DeLaval separator at early stationary phase and stored as frozen paste at −25 °C.

Preparation of cell and tissue extracts. Acetone-dried cell powder from cells of *E. coli* was prepared essentially as described by Ellfolk and Soininen.[17] The acetone-treated cells were extracted as described earlier [8] with 25 mM Tris-HCl buffer containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol (referred to below as the standard buffer). AMDC was partially purified from the extracts of *E. coli* by the method of Wickner *et al.*[11] through the ammonium sulfate fractionation and the subsequent chromatography on DEAE-cellulose. The heat step of the cited procedure, however, was omitted. One unit is defined as the amount of the enzyme catalyzing the formation of 1 nmol of CO_2 per 30 min.

Spermidine synthase was partially purified by the method of Hannonen *et al.*[18] (until ammonium sulfate fractionation) from extracts of rat liver.

Cells of baker's yeast (*Saccharomyces cerevisiae*) were obtained from Oy Alko (Helsinki, Finland) and disintegrated by the method of Pösö *et al.*[4] The proteins precipitated between 0.40 − 0.65 saturation of ammonium sulfate were dissolved in the standard buffer, dialyzed overnight against 100 volumes of the same buffer and used as the source of spermine synthase.

Analytical methods. The activity of AMDC from *E. coli* was assayed in the presence of 10 mM $MgCl_2$ essentially as described earlier.[19] The activity of spermidine synthase was assayed in the presence of 0.05 mM decarboxylated Ado-met and radioactive putrescine as described elsewhere.[6,20] The activity of spermine synthase was assayed in the presence of 0.05 mM decarboxylated Ado-met and radioactive spermidine.[21] Protein was measured by the method of Lowry *et al.*[22] The concentration of Ado-met and decarboxylated Ado-met was measured assuming that the molar extinction for the compounds is 15 000 at 257 nm.[23]

Incubation conditions for enzymic preparation of decarboxylated Ado-met. The large scale incubation mixture for the preparation of decarboxylated Ado-met contained, in a total volume of 9 ml, 1 mmol of Tris-HCl buffer pH 7.4 at 37 °C, 0.5 mmol of $MgSO_4$, 10 μmol of dithiothreitol, 5 μmol of Ado-met and the enzyme solution (980 units). Twenty five g acetone-dried cells (equivalent to 100 g of fresh bacteria) were used for the preparation of the enzyme.

The incubation was carried out at 37 °C. The pH was maintained at 7.4 by occasional additions of 1 M Tris-base. After 3.5 h the reaction was halted with 2 ml of cold 50 % trichloroacetic acid. The supernatant solution was separated by centrifugation and the precipitate was washed once with 3.5 ml of cold 10 % trichloroacetic acid.

First Dowex 50-H^+ chromatography. The combined supernatant solutions were applied to a Dowex 50-H^+ column (1 cm × 5 cm) previously washed with 6 M HCl and then with water until the pH approached neutrality. After the application of the sample the column was washed with 1 M HCl until the absorbance at 257 nm in the eluate was below 0.02. The decarboxylated Ado-met and Ado-met were eluted from the column with 6 M HCl. The fractions in which the absorbance at 257 nm was more than 0.2 were pooled. The volume of a typical pool was about 35 ml.

The pooled eluate from Dowex 50 column was then evaporated to dryness (under reduced pressure at temperature below 40 °C) and dissolved in 700 μl of 0.1 M HCl. This solution was used for the paper electrophoresis.

Fig. 1 shows that when AMDC purified until ammonium sulfate fractionation was used as the source of the enzyme in the enzymic preparation of decarboxylated Ado-met, much more 1 M HCl was needed in Dowex chromatography to get the absorbance below 0.02 than when more purified preparations of the enzyme (DEAE-cellulose fraction) were used. This obviously means that the ammonium sulfate fraction contained more contaminating enzyme activities capable of degrading Ado-met than the DEAE cellulose fraction.

The yield of decarboxylated Ado-met was about 20 % greater by employing the DEAE-fraction than by using $(NH_4)_2SO_4$ fraction as the source of AMDC (Table 1).

Preparative paper electrophoresis. Preparative paper electrophoresis by which the carboxylated Ado-met was finally separated from Ado-met

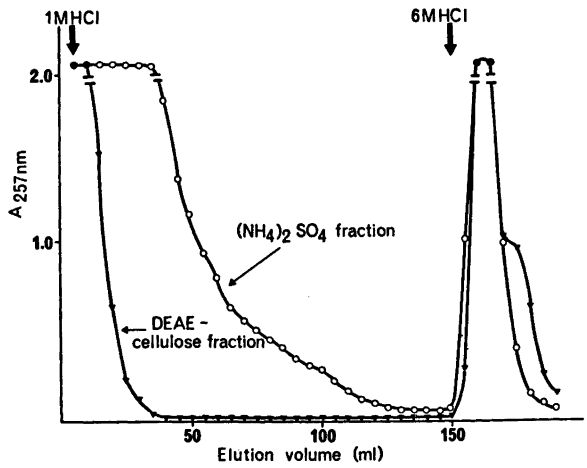

Fig. 1. Elution pattern of the incubation mixture choromatographed for the first time on Dowex 50-H^+. The enzymic preparation of decarboxylated Ado-met was performed as described in the text using either the ammonium sulfate fraction or the more refined DEAE-cellulose fraction as the source of the enzyme (980 units in each case). The arrows indicate the start of the elution either with 1 M HCl or 6 M HCl.

was performed as follows: Whatman No. 1 paper (20 cm × 30 cm) was loaded with 150 µl of solution from the Dowex 50-H^+ eluate containing both Ado-met and decarboxylated Ado-met. The paper was then subjected to electrophoresis at 300 V using 0.05 M citric acid, pH 3.6, as buffer. The running time was 2 h and the migration of both compounds was monitored with the aid of an ultra-violet lamp at 254 nm.

The decarboxylated Ado-met fraction, migrating considerably faster than Ado-met in the paper electrophoresis, was cut into small pieces, and decarboxylated Ado-met was eluted from the paper strips with 1 M HCl overnight at 4 °C. The eluate was separated by filtration and the paper mass was washed several times with 1 M HCl.

Second Dowex 50-H^+ chromatography. The eluate was then applied to a Dowex 50-H^+ column and decarboxylated Ado-met was eluted from the column as described for the first chromatography.

The pooled 6 M HCl-eluate was evaporated to dryness (see above) and dissolved in 0.01 M HCl. The concentration of decarboxylated Ado-met was adjusted to 1 mM and the solution was stored at −20 °C. The overall yield of the decarboxylated Ado-met was usually about 30 % from the added Ado-met (Table 1). Only one UV-absorbing spot was seen after paper electrophoresis.

The whole purification procedure is summarized in the following scheme:

Trichloroacetic acid extract → First Dowex 50-H^+ chromatography (wash with 1 M HCl, elute with 6 M HCl) → Evaporation of the eluate (< 40 °C) → Preparative paper electrophoresis (0.05 M citric acid pH 3.6; 300 V; 120 min) → Second Dowex 50-H^+ chromatography (as in the first chromatography) → Evaporation of the eluate (< 40 °C).

The use of decarboxylated Ado-met from different stages of the preparation as the substrate

Table 1. Effect of enzyme purification on the yield of decarboxylated Ado-met. Five µmol of Ado-met were incubated in the presence of 980 units of AMDC purified until ammonium sulfate fractionation or DEAE-cellulose chromatography as described in the text.

After incubation Ado-met and decarboxylated Ado-met were separated by paper electrophoresis and their amounts measured at 257 nm. The percentages in the parentheses indicate the relative amounts of Ado-met remaining or decarboxylated Ado-met formed from the initial 5 µmol of Ado-met.

	Remaining after incubation (µmol)	
Compound	AS-fraction [a] as enzyme	DEAE-cellulose fraction as enzyme
Ado-met	1.14 (22.7 %)	0.65 (13.0 %)
Decarboxylated Ado-met	0.70 (14.0 %)	1.63 (32.5 %)

[a] AS = ammonium sulfate.

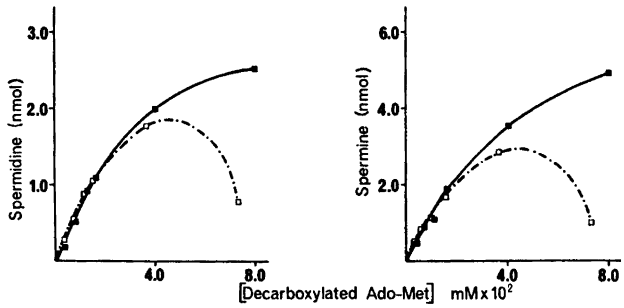

Fig. 2. Effect of the concentration of decarboxylated Ado-met, with or without the final chromatography on Dowex 50-H$^+$, on the activity of spermidine or spermine synthases. Decarboxylated Ado-met was prepared as described in the text and used before (open symbols, dotted curves) or after (closed symbols, solid curves) the second chromatography on Dowex 50-H$^+$ as the substrate for spermidine (left) or spermine (right) synthases.

in the synthesis of spermidine and spermine. Although only one fraction was seen after paper electrophoresis prior to the second chromatography on Dowex 50-H$^+$, it was necessary to subject the preparation to a further ion exchange chromatography prior to use. This was because the decarboxylated Ado-met without the final chromatography on Dowex 50-H$^+$ caused an apparent substrate inhibition when used in spermidine and spermine synthase reactions, as illustrated in Fig. 2.

The inhibition was not due to citric acid, the latter being used as the buffer in the paper electrophoresis, since citric acid (5 mM) added directly into the incubation mixture of spermidine synthase did not inhibit the enzyme activity to any appreciable extent, and a concentration of 10 mM citric acid was only slightly inhibitory (20 %). It appears that the impurities inhibiting the synthases were derived from the paper which was used in paper electrophoresis; these impurities, however, were removed by using the second chromatography on Dowex 50-H$^+$.

DISCUSSION

There are a few reports in the literature describing either enzymic or chemical preparation of decarboxylated Ado-met. The main difficulty in the preparation of pure decarboxylated Ado-met is to separate the decarboxylated compound from Ado-met, especially when enzymic methods have been employed. A separation of the two compounds has been achieved with chromatography on XE-64 columns [23] or employing HCl gradient elution on Dowex 50-H$^+$ columns.[12] Furthermore, the heat stability of the compounds has been reported to be different so that upon heating at 100 °C Ado-met is preferentially decomposed and the remaining decarboxylated Ado-met can easily be separated from the decomposition products of Ado-met.[24] A chemical synthesis of decarboxylated Ado-met has also been described.[25]

The reaction with *E. coli* AMDC stops at about 60 – 90 % completion [1,23] thus necessitating a separation of the decarboxylated product from the substrate. Although reported in literature, we have not been able to achieve proper separation of decarboxylated Ado-met from Ado-met on XE-64 or Dowex 50-H$^+$ columns by employing the conditions described.[12,23]

The chemical synthesis, albeit established, is very complicated containing 14 steps and yielding a racemic mixture that might not be suitable for use in biological systems.

The electrophoretic separation of decarboxylated Ado-met from Ado-met, which already has been used a few times before,[6,20] appears to yield relatively pure decarboxylated Ado-met with reasonable yields and is highly reproducible. As seen in the present paper there are some precautions to be taken into consideration when the preparation is performed with the aid of AMDC from *E. coli*. It is necessary to refine the decarboxylase to some extent, since without previous DEAE cellulose chromatography the enzyme appears to contain all kinds of contaminating enzyme activities including protein methylases.[26,27] Non-enzymic

methylation of macromolecules has also been reported.[28] An extensive methylase activity in the decarboxylase preparation will cause, of course, a rapid breakdown of Ado-met thus lowering the yield and seriously hampering the purification of decarboxylated Ado-met (Fig. 1).

The main function of decarboxylated Ado-met in mammalian tissues appears to be to serve as the precursor of spermidine and spermine molecules, a reaction catalyzed by two specific propylaminotransferases; spermidine and spermine synthases. In most animal tissues the rate limiting reaction in the enzymic synthesis of higher polyamines is the decarboxylation of Ado-met by AMDC.[5,14] The activities of the polyamine synthases markedly exceed that of AMDC.[15,18] Furthermore, the affinity of both spermidine and spermine synthase for decarboxylated Ado-met is very high; the apparent K_m value being less than 10 μM for spermidine synthase from rat ventral prostate (Jänne and Williams-Ashman, unpublished) and 3 – 5 μM for spermine synthase from rat brain.[21]

The high total activity of the polyamine synthases in comparison with AMDC in most tissues together with remarkably high affinity of the transferases for decarboxylated Ado-met explain, at least partly, the earlier misinterpretations that only one enzyme or an inseparable enzyme complex would be responsible for the decarboxylation of Ado-met and the subsequent transfer of the propylamino group to appropriate amine acceptor to yield spermidine or spermine.[29,30] Thus the assay of spermidine and spermine synthase activities in most animal tissues is only possible by employing decarboxylated Ado-met and appropriate amine (putrescine or spermidine, respectively) as substrates.

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Resolution and Absolute Configuration of 1-Ethyl-2-propynylamine and 1-Propyl-2-propynylamine

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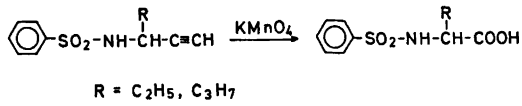
1-Ethyl-2-propynylamine and 1-propyl-2-propynylamine have been resolved and the absolute configurations of the enantiomers have been established by correlation to D-(−)-2-aminobutyric acid and D-(−)-norvaline, respectively.

It has been found in these laboratories that the enantiomers of some *N*-(4-*tert*-amino-1-methyl-2-butynyl)-substituted succinimides and 2-pyrrolidones show strong stereospecificity in blocking the motor effects of the muscarinic agent oxotremorine, *N*-(4-pyrrolidino-2-butynyl)-2-pyrrolidone, and that the pharmacological effect is exerted mainly by the (*R*)-(+)-isomers.[1,2] In continuation of our research in this field we wished to prepare the optical isomers of some analogous compounds substituted by ethyl and propyl groups in the 1-position of the butynyl chain. As starting material for these syntheses we required the enantiomers of 1-ethyl- and 1-propyl-2-propynylamine, and this paper deals with the resolution of these amines and the determination of the configuration of the isomers.

The two acetylenic amines were resolved into their (−)- and (+)-enantiomers using (+)- and (−)-tartaric acid, respectively. The resolution process was followed by measurements of the optical rotation of the benzoyl derivatives, and the optical purities of the enantiomers were determined by NMR spectroscopic analyses of the diastereomeric amides formed by acylation of optically impure amine with (−)-*O*-methylmandelyl chloride.[2,3]

The absolute configurations of the amines were established by oxidation of their benzenesulfonyl derivatives to the corresponding acids.



R = C_2H_5, C_3H_7

Oxidation of the benzenesulfonyl derivative of (−)-1-ethyl-2-propynylamine (neat) with potassium permanganate afforded dextrorotatory *N*-benzenesulfonyl-α-aminobutyric acid, obviously identical with the benzenesulfonyl derivative of (+)-α-aminobutyric acid, since the benzenesulfonyl derivative prepared from (−)-α-aminobutyric acid was found to be levorotatory. Similarly, the benzenesulfonyl derivative of (+)-1-propyl-2-propynylamine (neat) was transformed to the benzenesulfonyl derivative of (−)-norvaline. Since the dextrorotatory isomers of α-aminobutyric acid and norvaline belong to the L-series (*S*-series),[4,5] the (+)-enantiomers of 1-ethyl-2-propynylamine and 1-propyl-2-propynylamine can be assigned the *R* configuration.

EXPERIMENTAL

Melting points were determined in a metal block using open capillary tubes and calibrated Anschütz thermometers. Microanalyses were carried out at the Microanalytical Laboratory, Royal Agricultural College, Uppsala. IR spectra were recorded on a Perkin-Elmer 157 G spectrophotometer and ^1H NMR spectra on a Perkin-Elmer R 12 B spectrometer. Unless otherwise stated optical rotations were measured in absolute ethanol with a Perkin-Elmer 141 spectropolarimeter.

Resolution of 1-ethyl-2-propynylamine. Racemic 1-ethyl-2-propynylamine (10 g, 0.12 mol), prepared as previously described,[6] was added to a hot solution of (+)-tartaric acid (18 g,

0.12 mol) in 800 ml of ethanol-water (10:1). The solution was kept in a refrigerator overnight. The salt obtained (11.7 g, m.p. 181–183 °C) required three recrystallizations from 3 % solutions in ethanol-water (10:1) before constant physical properties of the tartrate, the benzoyl and (−)-*O*-methylmandelyl derivatives were obtained. Yield 7.6 g (54 %) of resolved (+)-hydrogen tartrate, m.p. 186–187 °C, $[\alpha]_D^{22}$ +23.1° (*c* 1.0, H_2O). Anal. $C_9H_{15}NO_6$: C, H, N.

The initial filtrate from the above resolution was concentrated *in vacuo* and the residue dissolved in saturated K_2CO_3-solution. After extraction of the amine with ether and drying of the extract (K_2CO_3), the solution was fractionated through a helix-packed column. The amine fraction [3.8 g, $[\alpha]_D^{22}$ −8.5° (*c* 1.0)] was added to a solution of (−)-tartaric acid in ethanol-water and the salt formed was purified as described above for the enantiomeric salt. The yield, based on recovered amine, of resolved (−)-hydrogen tartrate was 52 %, m.p. 186–187 °C, $[\alpha]_D^{22}$ −23.3° (*c* 1.0, H_2O).

(S)-(−)-1-Ethyl-2-propynylamine. The resolved (+)-hydrogen tartrate (11.0 g, 0.047 mol) was dissolved in saturated K_2CO_3-solution and the pure amine was obtained through the procedure described above, b.p. 104 °C, n_D^{22} 1.442, $[\alpha]_D^{22}$ −20.9° (neat, *d* 0.813), +14.4° (*c* 1.0), yield 2.3 g (58 %), ^{1}H NMR ($CDCl_3$, 37 °C): δ 1.05 (3 H, t, *J* 7.0 Hz, CH_3), 1.40–1.92 (4 H, m, NH_2, CH_2), 2.30 (1 H, d, *J* 2.4 Hz, ≡C*H*), 3.49 (1 H, triplet of doublets, *J* 7.0 and 2.4 Hz, C*H*). *Hydrochloride*: m.p. 198–199 °C (from ethanol-ether), $[\alpha]_D^{22}$ +14.3° (*c* 1.0). Anal. C_5H_9N.HCl: C, H, N. *Benzamide*: m.p. 79–80 °C (from ligroin), $[\alpha]_D^{22}$ −48.6° (*c* 0.7). Anal. $C_{12}H_{13}NO$: C, H, N.

(R)-(+)-1-Ethyl-2-propynylamine was obtained similarly from the (−)-hydrogen tartrate, b.p. 104 °C, n_D^{22} 1.441, $[\alpha]_D^{22}$ +20.3° (neat, *d* 0.813), −13.3° (*c* 1.2), yield 52 %. *Hydrochloride*: m.p. 199–200 °C, $[\alpha]_D^{22}$ −14.4° (*c* 1.0). *Benzamide*: m.p. 79–80 °C. $[\alpha]_D^{22}$ +49.0° (*c* 1.2).

N-[(S)-1-Ethyl-2-propynyl]-(R)-O-methylmandelamide. (*R*)-(−)-*O*-Methylmandelic acid [7] [$[\alpha]_D^{22}$ −148.7° (*c* 0.6)] was converted to its acid chloride with which (*S*)-(−)-1-ethyl-2-propynylamine was acylated according to a method described in the literature,[8] m.p. 66–67 °C (from ligroin), $[\alpha]_D^{22}$ −158.0° (*c* 1.0). ^{1}H NMR (C_6H_6, 37 °C): δ 0.88 (3 H, t, *J* 7.0 Hz, C-CH_3), 1.36–1.89 (2 H, m, CH_2), 1.95 (1 H, d, *J* 2.4 Hz, ≡C*H*), 2.95 (3 H, s, OCH_3), 4.52 (1 H, s, ArC*H*), 4.62–5.06 (1 H, m, N-C*H*). Anal. $C_{14}H_{17}NO_2$: C, H, N.

N-[(R)-1-Ethyl-2-propynyl]-(R)-O-methylmandelamide was prepared similarly from (*R*)-(+)-1-ethyl-2-propynylamine, m.p. 83–84 °C (from ligroin), $[\alpha]_D^{22}$ −4.8° (*c* 1.0). ^{1}H NMR (C_6H_6, 37 °C): δ 0.75 (3 H, t, *J* 7.0 Hz, C-CH_3), 1.18–1.73 (2 H, m, CH_2), 1.98 (1 H, d, *J* 2.4 Hz, ≡C*H*), 2.94 (3 H, s, OCH_3), 4.47 (1 H, s, ArC*H*), 4.65–5.09 (1 H, m, N-C*H*). Anal. $C_{14}H_{17}NO_2$: C, H, N.

(S)-(−)-N-(1-Ethyl-2-propynyl)benzenesulfonamide. To a stirred and cooled (0–5 °C) solution of (*S*)-(−)-1-ethyl-2-propynylamine (1.0 g, 0.012 mol) in pyridine (4 ml) was added dropwise benzenesulfonyl chloride (2.3 g, 0.013 mol) dissolved in pyridine (10 ml). The reaction mixture was left overnight at room temperature and the pyridine was evaporated *in vacuo*. Water was then added and the product extracted with ether, the ether layer washed with 5 % HCl and then with water until neutral reaction. After concentration of the dried ethereal solution *in vacuo*, the product crystallized, m.p. 108.5–110 °C (from ethanol-water), $[\alpha]_D^{22}$ −60.9° (*c* 1.0), yield 2.3 g (84 %). Anal. $C_{11}H_{13}NO_2S$: C, H, N, S.

(S)-(+)-N-Benzenesulfonyl-α-aminobutyric acid. Oxidation of (S)-(−)-N-(1-ethyl-2-propynyl)benzenesulfonamide. The above sulfonamide (1.8 g, 0.008 mol) was oxidized with $KMnO_4$ as described for the oxidation of the 1-methyl-2-propynyl compound.[2] The product slowly crystallized from the acidified solution, m.p. 138–139 °C (from water), $[\alpha]_D^{22}$ +2.0°, $[\alpha]_{365}^{22}$ +30.2° (*c* 1.0), yield 0.8 g (42 %). Anal. $C_{10}H_{13}NO_4S$: C, H, N, S.

(R)-(−)-N-Benzenesulfonyl-α-aminobutyric acid. Acylation of D-*(−)-α-aminobutyric acid.* D-(−)-α-Aminobutyric acid [0.5 g, 0.0048 mol $[\alpha]_D^{22}$ −8.2° (*c* 2.0, H_2O), lit.[9] $[\alpha]_D^{20}$ −7.9° (*c* 5.31 %, H_2O)], was acylated with benzenesulfonyl chloride according to the method described by Wiley *et al.*[10] to give 0.35 g (30 %) of product, m.p. 137–138 °C (from water), $[\alpha]_D^{22}$ −2.2°, $[\alpha]_{365}^{22}$ −31.0° (*c* 1.1). Anal. $C_{10}H_{13}NO_4S$: C, H, N, S.

Resolution of 1-propyl-2-propynylamine. Racemic 1-propyl-2-propynylamine [6] (97 g, 1.0 mol) was added to a hot solution of (+)-tartaric acid (150 g, 1.0 mol) in 600 ml of ethanol-isopropyl alcohol (1:1). The solution was left overnight at room temperature. The salt obtained (105 g, m.p. 120–124 °C) required six recrystallizations from 20 % solutions in ethanol-isopropyl alcohol (1:2) for the separation of the diastereomeric hydrogen tartrates. The resolved (+)-hydrogen tartrate was obtained in 43 % yield (53 g), m.p. 133–134 °C, $[\alpha]_D^{22}$ +25.1° (*c* 1.0, H_2O). Anal. $C_{10}H_{17}NO_6$: C, H, N.

The initial filtrate from the above resolution was concentrated *in vacuo*. The residue was dissolved in 5 N NaOH and the solution saturated with solid K_2CO_3. After extracting the amine with ether and drying the extract (K_2CO_3), the ether was removed through a helix-packed column. The amine was distilled at reduced pressure affording 33 g of product, $[\alpha]_D^{22}$ −8.6° (*c* 1.0), which was converted to the (−)-hydrogen tartrate and purified as described for the enantiomeric salt. The yield, based on recovered amine, of (−)-hydrogen tartrate was 40 % (34 g), m.p. 133–134 °C,

$[\alpha]_D^{22}$ −25.3° (*c* 1.0, H_2O). Anal. $C_{10}H_{17}NO_6$: C, H, N.

(S)-(−)-1-Propyl-2-propynylamine. Resolved (+)-hydrogen tartrate (30 g, 0.12 mol) was dissolved in saturated K_2CO_3 solution and the amine was liberated as described above. Redistillation afforded 10 g (85 %) of the pure amine, b.p. 52–55 °C (40 mmHg), n_D^{22} 1.445, $[\alpha]_D^{22}$ −13.5° (neat, *d* 0.815), +19.2° (*c* 1.7), ^{1}H NMR ($CDCl_3$, 37 °C): δ 0.75–1.1 (3 H, m, CH_3), 1.3–1.7 (6 H, m, NH_2, CH_2CH_2), 2.28 (1 H, d, *J* 2.4 Hz, ≡C*H*), 3.37–3.68 (1 H, m, C*H*). *Hydrochloride:* m.p. 215–216 °C (from ethanol-ether), $[\alpha]_D^{22}$ +19.0° (*c* 1.3). Anal. $C_6H_{11}N.HCl$: C, H, N. *Benzamide*: m.p. 85–86 °C (from ligroin), $[\alpha]_D^{22}$ −42.9° (*c* 1.0). Anal. $C_{13}H_{15}NO$: C, H, N.

(R)-(+)-1-Propyl-2-propynylamine was obtained similarly from the (−)-hydrogen tartrate, b.p. 55 °C (48 mmHg), n_D^{22} 1.444, $[\alpha]_D^{22}$ +13.5° (neat, *d* 0.815), −19.9° (*c* 1.0), yield 81 %. *Hydrochloride*: m.p. 215–216 °C, $[\alpha]_D^{22}$ −19.2° (*c* 1.1). *Benzamide*: m.p. 85–86 °C, $[\alpha]_D^{22}$ +41.9° (*c* 1.0).

N-[(S)-1-Propyl-2-propynyl]-(R)-O-methylmandelamide. (*S*)-(−)-1-Propyl-2-propynylamine was acylated as described above for the 1-ethyl analogue, m.p. 78–79 °C (from light petroleum), $[\alpha]_D^{22}$ −141.6° (*c* 1.0). ^{1}H NMR (C_6H_6, 37 °C): δ 0.60–0.95 (3 H, m, C-CH_3), 1.25–1.65 (4 H, m, CH_2CH_2), 1.93 (1 H, d, *J* 2.4 Hz, ≡CH), 2.95 (3 H, s, OCH_3), 4.53 (1 H, s, ArC*H*), 4.70–5.15 (1 H, m, N-C*H*). Anal. $C_{15}H_{19}NO_2$: C, H, N.

N-[(R)-1-Propyl-2-propynyl]-(R)-O-methylmandelamide was prepared similarly from (*R*)-(+)-1-propyl-2-propynylamine, m.p. 78–79 °C (from light petroleum), $[\alpha]_D^{22}$ −13.3° (*c* 1.0). ^{1}H NMR (C_6H_6, 37 °C): δ 0.50–0.85 (3 H, m, C-CH_3), 1.15–1.55 (4 H, m, CH_2CH_2), 2.03 (1 H, d, *J* 2.4 Hz, ≡C*H*), 2.96 (3 H, s, OCH_3), 4.50 (1 H, s, ArC*H*), 4.75–5.20 (1 H, m, N-C*H*). Anal. $C_{15}H_{19}NO_2$: C, H, N.

(R)-(+)-N-(1-Propyl-2-propynyl)benzenesulfonamide was prepared from (*R*)-(+)-1-propyl-2-propynylamine (2.5 g, 0.026 mol) and benzenesulfonyl chloride (4.2 g, 0.024 mol) in pyridine solution as described above for 1-ethyl-2-propynylamine, m.p. 69–70 °C (from ethanol-water). $[\alpha]_D^{22}$ +47.7° (*c* 1.0), yield 4.1 g (72 %). Anal. $C_{12}H_{15}NO_2S$: C, H, N, S.

(R)-(−)-N-Benzenesulfonylnorvaline. (A) Acylation of D-*(−)-norvaline.* D-(−)-Norvaline (0.5 g, 0.0043 mol), $[\alpha]_D^{22}$ −23.8°, $[\alpha]_{365}^{22}$ −95.8° (*c* 1.2, 1 M HCl), lit.[11] $[\alpha]_D^{20}$ −25.1° (*c* 3.0, 20 % HCl), was acylated as described for D-(−)-α-aminobutyric acid, m.p. 139.5–141 °C (from water), $[\alpha]_D^{22}$ −5.7°, $[\alpha]_{365}^{22}$ −39.9° (*c* 0.9), yield 0.46 g (42 %). Anal. $C_{11}H_{15}NO_4S$: C, H, N, S.

(B) Oxidation of (R)-(+)-N-(1-propyl-2-propynyl)benzenesulfonamide. (*R*)-(+)-*N*-(1-Propyl-2-propynyl)benzenesulfonamide (2.0 g, 0.008 mol) was oxidized with $KMnO_4$ as previously described for the oxidation of the 1-methyl-2-propynyl compound,[2] m.p. 139–140.5 °C. $[\alpha]_D^{22}$ −5.3, $[\alpha]_{365}^{22}$ −39.0° (*c* 1.1), yield 1.0 g (46 %). Anal. $C_{11}H_{15}NO_4S$: C, H, N, S.

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Irradiation-induced Coupling of Enzymes to Solid Phase Carriers

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Coupling of various enzymes to agarose gels, dextran gels, and polystyrene particles has been accomplished by γ-irradiation of a suspension of the carrier in the presence of enzyme. The enzymes studied were trypsin, pepsin, and ribonuclease. The main advantage with the procedure compared to the presently available coupling methods is that it may be used under a wide variety of conditions and with many different materials. The main disadvantages are the low amounts of fixed protein obtained and that some radiation damage occurs.

The effects of irradiation of ribonuclease in an agarose gel suspension and in aqueous solution were compared. In the presence of gel less aggregation of ribonuclease occurred compared to irradiation in free solution.

A great number of methods for the immobilization of biologically active substances have been developed in recent years.[1-3] Most attention has been paid to the development of methods for irreversible covalent fixation and a variety of organo-chemical reactions have been employed. However, the presently available methods have their limitations in one or several respects. The chemical reactions used generally work in a limited range of pH and are dependent on special functional groups in the carrier and the substance to be coupled. Therefore there is still need for more development work in this field.

Recently it was reported [4] that covalent coupling of dyes to macromolecules could be accomplished by γ-irradiation of water solutions containing both components. This tempted us to study the possibility of coupling proteins to solid phase carriers using radiation and further to seek more information about radiation chemistry in gel systems.

We report here a new simple method, whereby the coupling of enzymes to solid phase carriers can be performed by γ-irradiation of the carrier in the presence of enzyme. In this paper we also report some differences obtained upon irradiation of an enzyme in free solution and in a gel system.

MATERIALS AND METHODS

The enzymes trypsin, pepsin, and ribonuclease were obtained from Sigma Chemical Company. Tosylarginine methyl ester (TAME), cytidine-2′,3′-cyclic monophosphate, RNA and hemoglobin (type II) were also from Sigma Chemical Company. Agarose gel (Sepharose 4B) and dextran gel (Sephadex G-150) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and latex particles from Serva Feinbiochemica (Heidelberg, Germany).

Irradiation was performed in a γ-cell containing ^{60}Co. The temperature in the cell was about 20 °C and the dose rate was 30 rad/s. About 10 ml of gel was irradiated in a closed glass tube. Unless otherwise stated the gels were initially in equilibrium with air.

Washing of the gels after irradiation was performed in a small column with various buffers. The pepsin-Sepharose conjugate was extensively washed with the following solutions in the order indicated: distilled water, 0.1 M acetic acid pH 3.0 containing 1 M KCl, 0.1 M sodium acetate buffer pH 5.0 containing 1 M KCl, and 0.002 M acetic acid-sodium acetate buffer pH 4.0. The trypsin-Sephadex and the ribonuclease-Sepharose conjugates were washed with water, 0.1 M acetic acid pH 4.0 containing 1 M KCl, 0.1 M sodium bicarbonate buffer pH 8.5 containing 1 M KCl, and finally with distilled water. The polystyrene latex particles were extensively washed by repeated centrifugation using the following buffers: 0.1 M sodium formate buffer pH 3.0 containing 1 M KCl and 20 % ethanol, 0.1 M sodium bicarbonate buffer pH 9.2 containing 1 M KCl and 20 % ethanol, and finally with distilled water.

Determination of the degree of coupling was made by amino acid analysis. After careful washing with various buffers, the gels were dehydrated by washing with acetone and then dried by heating at 70 °C for 24 h. A suitable amount of dried gel was weighed and then mixed with 6 M HCl and hydrolyzed for 24 h at 110 °C in an evacuated glass tube. The amino acid analyses were performed on a Beckman Model 120 B analyzer.

Leakage and stability tests of the enzyme conjugates were done by the following procedure. The washed conjugate was stored for 1 week at 4 °C in 0.005 M acetic acid – sodium acetate buffer pH 4.7 containing 0.02 M sodium azide. The conjugate was then filtered off and washed. Both the filtrate and the conjugate were then assayed for enzymatic activity to determine the stability of the conjugate.

The activity of trypsin was measured by following the hydrolysis of 0.01 M TAME in 0.02 M $CaCl_2$ – 0.04 M NaCl in a titrator (Radiometer, Copenhagen).

The activity of pepsin was measured by adding the enzyme to a 2 % hemoglobin solution and following the hydrolysis at pH 3.0. The mixture was stirred and aliquots were removed at various times. The protein was precipitated with trichloroacetic acid followed by centrifugation. The absorption at 280 nm of the supernatant was measured.

The activity of ribonuclease was determined by following the hydrolysis of cytidine-2',3'-cyclic monophosphate [5] in 0.1 M KCl at pH 6.9 in a titrator or by the spectrophotometric method of Kunitz [6] using RNA as substrate.

RESULTS

Coupling of trypsin to dextran gel. Swollen Sephadex G-150 gel samples in 0.1 M acetic acid-sodium acetate buffer pH 4.7 containing 0.04 M $CaCl_2$ and various concentrations of trypsin were irradiated with 1 Mrad of γ-radiation. After careful washing, the amount of fixed protein and the esterolytic activity against TAME at different pH-values were determined for each conjugate. It was found that pH for optimum activity was 7.7 for the free enzyme and 9.3 – 9.5 for the enzyme conjugates. This type of pH-shift has earlier been shown to occur with enzymes chemically coupled to gel matrices.[7–9] It probably means that pH-equilibrium is not obtained in the gel particle. The release of H^+ during the reaction is rapid and causes a lower pH in the interior of the gel particle than in the external solution.[8] The ratio of the activity of bound to free enzyme was calculated for each conjugate on the basis of the activity at pH-optimum. The results obtained are shown in Table 1. It is evident that an increased protein concentration during irradiation results in lower amounts of fixed protein, but gives an adduct with a higher specific activity.

Table 1. Coupling of trypsin to Sephadex G-150 gel and the enzymatic activity of the conjugates.

Enzyme concentration (mg/ml)	Amount of fixed protein (mg/g dry conjugate)	Activity ratio bound to free enzyme (%)
2	10	12
10	8	21
30	7	27

In a separate experiment Sephadex G-150 gel was irradiated as described above but in the absence of trypsin. Immediately after irradiation trypsin was added to give a final concentration of 10 mg trypsin/ml gel and the mixture was incubated for 24 h at room temperature. Amino acid analysis of a part of the gel after washing and drying showed that no protein had coupled to the gel.

An experiment was also performed, where Sephadex G-150 gel in the presence of 10 mg trypsin/ml was irradiated as described above but in the absence of oxygen. Deoxygenation was accomplished by allowing a stream of oxygen-free argon to pass through the solution for 2 h. The same degree of coupling and also the same activity of the coupled enzyme were obtained as compared to aerobic irradiation.

Coupling of pepsin to agarose gel. Sepharose 4B gel containing 1.0 mg pepsin/ml gel in 0.1 M acetic acid-sodium acetate buffer pH 4.0 was irradiated with 0.5 Mrad. After careful washing part of the gel was dried and subjected to amino acid analysis. The amount of fixed protein was 7.1 mg pepsin/g of dry gel corresponding to about 16 % of the pepsin present in the gel during irradiation. The enzymatic activity of a part of the gel was determined at pH 3.0 using hemoglobin as substrate. On the basis of the activity for the free and the coupled enzyme at pH 3.0, it could be calculated that the relative activity was 14 %. Stability test on a part of the gel was done after storage and repeated washing. No leakage

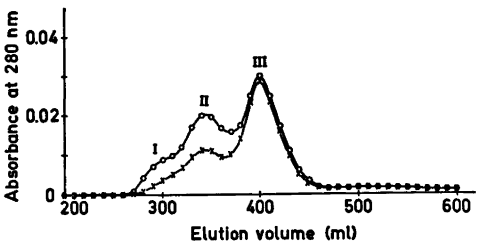

Fig. 1. Elution diagrams of Sephadex G-50 gel filtrations (column 3.2 × 90 cm) of ribonuclease irradiated in 0.1 M Tris-HCl buffer pH 7.4 in the presence of Sepharose 4B gel and in free solution.
Elution patterns: (×) irradiation in the presence of gel: (O) irradiation in free solution.

of enzyme or loss of enzymatic activity of the conjugate could be detected.

Effects of irradiation on the system ribonuclease-agarose gel. In order to obtain more information about the reactions taking place in protein-agarose gel systems upon irradiation, a comparative study was made in which ribonuclease was irradiated in agarose gel suspension and in dilute solution. A sample of 4 ml settled Sepharose 4 B gel in 0.1 M Tris-HCl buffer pH 7.4 containing 1 mg ribonuclease/ml gel and a sample of 4 ml of a solution containing 1 mg ribonuclease/ml of 0.1 M Tris-HCl buffer pH 7.4 were irradiated with 0.5 Mrad of γ-radiation. After irradiation the gel sample was eluted and the eluate was run on a Sephadex G-50 column. The sample irradiated in the absence of gel was also subjected to gel filtration on Sephadex G-50 using 0.1 M ammonium acetate buffer pH 4.7 for elution. Fig. 1 shows the elution diagrams obtained for the two samples. Fraction III in the figure eluted at the same volume as native ribonuclease. By comparison with a calibration curve, it was found that fraction II eluted at the volume expected for the dimer of ribonuclease. The composition of the two samples, with respect to the various products formed, was calculated from the UV-absorption values of the two elution diagrams. The enzymatic activities of the various fractions were determined using RNA as substrate. The specific activities of the various fractions compared to native ribonuclease were calculated and the results obtained are shown in Table 2. The values given are approximate

Table 2. The composition of ribonuclease with respect to the various products formed during irradiation in the presence or absence of Sepharose 4B gel, and the specific activity of the products compared to native ribonuclease.

Product	Fraction of products (%)	Specific activity compared to native (%)
In the presence of gel		
Polymer	6	0
Dimer	27	6
Monomer	67	44
In the absence of gel		
Polymer	10	0
Dimer	37	8
Monomer	53	46

since peak division is difficult and there may be differences in the extinction coefficients of the different types of material. Despite this, it is evident that both dimer and larger aggregates are formed upon irradiation both in the presence and absence of gel, but that more aggregates are formed in the absence of gel. The Sepharose 4B gel from the experiment described above was further analyzed for protein content and enzymatic activity. The gel was extensively washed and a part of it was subjected to amino acid analysis. The results obtained showed that 20 % of the ribonuclease present in the gel during irradiation had been coupled to the gel. Activity determinations on the gel using cytidine-2′,3′-cyclic monophosphate as substrate showed that the activity was 29 % compared to the activity of the same amount of enzyme in free solution. The pH for optimum activity was about the same as for the free enzyme. Stability test of a part of the gel was done after storage and repeated washing. No leakage of enzyme or loss of enzymatic activity of the conjugate could be detected.

Coupling of ribonuclease to polystyrene particles. A 10 % suspension of polystyrene latex particles (diameter 0.81 μm) in 0.1 M Tris-HCl buffer pH 7.4, containing 10 mg ribonuclease per ml of suspension, was irradiated with 1 Mrad of γ-radiation. The latex particles were then extensively washed and the enzymatic activity towards cytidine-2′,3′-cyclic

monophosphate was determined on a part of the latex suspension. It was found that the pH-optimum for particle-bound ribonuclease was about the same as for the free enzyme. A part of the suspension was dried and subjected to amino acid analysis. The result obtained shows that 6 mg ribonuclease was coupled per gram of latex particles. The activity of coupled enzyme compared to the same amount of free enzyme was 14 %. A part of the enzyme conjugate was subjected to stability test after storage and repeated washing. No leakage of enzyme or loss of enzymatic activity of the conjugate could be detected.

In a control experiment a 10 % suspension of latex particles containing 10 mg ribonuclease per ml of suspension was treated as described above but not subjected to irradiation. No enzymatic activity towards cytidine-2′,3′-cyclic monophosphate could be detected in this sample.

DISCUSSION

The results show that it is possible to couple proteins to solid phase carriers using γ-radiation. It had been previously shown that γ-irradiation of solutions containing macromolecules and dyes [4] induced formation of macromolecule-dye adducts. It has also been shown that irradiation of solutions containing mixtures of proteins gives some hybrid molecules.[10] The mechanisms for these coupling reactions are probably very similar. On irradiation of a gel-protein system, radicals will be formed both in the gel matrix and in the protein. These radicals may be primary, that is, the result of direct ionization, or secondary, as the result of a reaction between the component and radicals from the solvent. The radicals formed are usually very reactive and special emphasis can be placed on the radical – radical reactions, which are very fast. It is probable that the actual coupling reaction is a radical – radical reaction as suggested below,[4] where a sequence of reactions is given in simplified form (MH = matrix; PH = protein).

$$MH \rightarrow M\cdot + H\cdot \quad (1a)$$

$$PH \rightarrow P\cdot + H\cdot \quad (1b)$$

$$P\cdot + M\cdot \rightarrow MP \quad (2)$$

The coupling reaction (2) would result in a covalent bond between the protein and the matrix, which is in good agreement with the observed stability of the conjugates formed. The adduct formation according to reaction (2) is, of course, not the only possible fate of the M· and P· radicals. They can, for instance, react with OH radicals from water yielding chemically altered protein or matrix. Reaction (2) can probably be regarded as very rapid but it is also clear that it is strongly dependent on the concentrations of the two radicals. Thus, the intensity of the irradiation should be of importance for the coupling yield. The amino acid residues in the protein participating in the coupling process are probably mainly the radiosensitive, sulfur-containing and aromatic amino acids. The degree of exposure of the amino acid residues to solvent is probably also important.

During the past ten years considerable effort has been made to develop methods for coupling proteins and other substances to solid phase carriers. At present there are many such methods available. However, these are limited in one or several respects, for example a limited range of pH, carrier, *etc.* Irradiation coupling method has the advantage that it can be used under a wide variety of conditions. Radical reactions are usually less sensitive to the environmental conditions than other types of reactions, allowing a broader working range. Another advantage of irradiation coupling is that it probably is possible to use many different carriers. Only a few carriers have been studied in this investigation, but it is likely that irradiation coupling can be performed with most kinds of organic material since formation of radicals by γ-irradiation is a property shared by all organic materials. There are two disadvantages with the method. One is that the coupling yields are fairly low, the other is that a certain amount of radiation damage [11] occurs both to the matrix and to the protein. Those disadvantages may be important for some applications but not for others.

Preparation of immobilized enzymes by use of electron-beam irradiation [12] and also by γ-irradiation [13,14] has been reported earlier. However, in these cases enzymes were entrapped in a gel matrix formed during irradiation and

the possibility of covalent fixation of the enzyme to the gel was not thought to occur.[13] A disadvantage with enzyme conjugates prepared in that way is the low specific activity towards high molecular weight substrates because of restricted diffusion of high molecular weight substances in the gel. Another disadvantage is the difficulty of eliminating enzyme leakage using the entrapment method.[14]

The comparison between irradiation of ribonuclease in solution and in agarose gel shows that less aggregation occurs upon irradiation in a gel. The explanation is most probably that in the absence of gel the formed ribonuclease radicals can react with each other and aggregates are formed. In the presence of gel, however, many ribonuclease radicals will react instead with radicals in the gel matrix. Thus, less aggregates are formed and coupling of ribonuclease to the gel matrix occurs. Less aggregation in the presence of gel may also be due to protection by the carbohydrate against attack by solvent free radicals on the ribonuclease.

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^{1}H NMR Study of L-Fucopyranose and 2,6-Dideoxy-L-lyxo-hexopyranosides in D_2O

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The ^{1}H NMR parameters of α-, and β-L-fucopyranose and of 2,6-dideoxy-α- and -β-L-*lyxo*-hexopyranoses as well as their methyl pyranosides in D_2O have been determined at 300 MHz. The chemical shifts are compared to those of the corresponding D-galactopyranoses and 2-deoxy-D-*lyxo*-hexo-pyranoses.

Recently we have proposed shift increments in order to predict the chemical shifts of the ring protons in aldohexopyranoses relative to those of β-D-glucopyranose. We were able to refine and extend the increment values proposed by Lemieux.[1] Extensions were possible for methyl pyranosides,[2] aldopentopyranoses,[3] D-fructopyranose,[3] and disaccharides.[4] We have further correlated the shift data of the model compound with those in 2-deoxyaldohexopyranoses [5] and rhamnoses.[6] In the present study we report on the results obtained from a more general study of 2,6-dideoxy-aldohexopyranoses. The results were obtained from analysis of the spin systems observed at 300 MHz.

RESULTS AND DISCUSSION

The ^{1}H NMR parameters obtained from the 300 MHz spectra of α- and β-L-fucopyranose and of 2,6-dideoxy-α- and β-L-*lyxo*-hexopyranoses as well as of their methyl pyranosides are shown in Table 1.

The chemical shifts of the aldoses. In order to extrapolate the shift increments from aldohexopyranoses to 6-deoxy aldohexopyranoses it is necessary to introduce a set of corrections resulting from the substitution of CH_2OH (C-6) by CH_3. From a study of the chemical shifts of L-rhamnoses and D-mannoses,[6] this substitution was found to slightly influence H-1, -2, -3, and -5, but H-4 was shielded by −0.23 ppm in the deoxy sugar. Similar results are now observed when the chemical shifts of the ring protons of the fucopyranoses are compared to those of the corresponding anomers of the galactopyranoses, although for H-4 the upfield effect in the 6-deoxy sugar is found to be somewhat smaller (−0.18 ppm) and the downfield effect on H-5 somewhat larger (+0.09 to +0.11 ppm). Thus, when epimerizing an equatorial OH to an axial OH the vicinal axial H-5 undergoes a downfield shift of +0.37 to +0.40 ppm if geminal to a CH_3-group, and of +0.26 to +0.29 ppm [3] if it is geminal to a CH_2OH-group.

In order to evaluate the influence of the substitution of the CH_2OH by a CH_3 upon the chemical shifts of H-2, we have compared the chemical shifts of 2,6-dideoxy-L-*lyxo*-hexopyranose with those of 2-deoxy-L-*lyxo*-hexopyranose [6] (values between brackets in Table 1). An upfield shift is found for H-2_{ax} and H-2_{eq} of, respectively, −0.02 to −0.07 ppm and −0.03 to 0.04 ppm. It is noticeable that no shift difference is observed between H-2_{eq} in rhamnose and mannose, but a slight upfield shift of −0.03 to −0.05 ppm is seen for H-2_{ax} comparing fucose with galactose. We therefore propose a mean value of −0.03 ppm, as the correction for both protons in a 6-deoxy compound.

Table 1. ^{1}H NMR parameters of L-fucopyranose and 2,6-dideoxy-L-*lyxo*-hexopyranoses and their methyl glycosides.

Chemical shifts	H-1	H-2_{ax}	H-2_{eq}	H-3	H-4	H-5	CH_3-6	OMe
α-L-Fucopyranose[a]	5.20 (−0.07)	3.76 (−0.05)	—	3.86 (−0.02)	3.81 (−0.18)	4.20 (+0.11)	1.21	
β-L-Fucopyranose[a]	4.55 (−0.03)	3.45 (−0.03)	—	3.64 (−0.01)	3.75 (−0.18)	3.80 (+0.09)	1.25	—
2,6-Dideoxy-α-L-*lyxo*-hexopyranose[a]	5.35 (−0.05)	1.89 (−0.02)	1.81 (−0.03)	4.09 (−0.01)	3.69 (−0.18)	4.13 (+0.09)	1.21	—
2,6-Dideoxy-β-L-*lyxo*-hexopyranose[a]	4.81 (−0.06)	1.61 (−0.07)	1.98 (−0.04)	3.87 (+0.01)	3.59 (−0.19)	3.66 (+0.06)	1.25	—
Methyl 2,6-dideoxy-α-L-*lyxo*-hexo-pyranoside[a]	4.88 [−0.47]	1.89 [0]	1.83 [+0.02]	4.01 [−0.08]	3.68 [−0.01]	3.96 [−0.17]	1.23	3.35
Methyl 2,6-dideoxy-β-L-*lyxo*-hexo-pyranoside[a]	4.53 [−0.28]	1.59 [−0.02]	1.97 [−0.01]	3.88 [+0.01]	3.60 [+0.01]	3.66 [0]	1.27	3.52
Coupling constants	J(1,2ax)	J(1,2eq)	J(2eq,2ax)	J(2ax,3)	J(2eq,3)	J(3,4)	J(4,5)	J(5,CH_3-6)
α-L-Fucopyranose	3.9	—	—	10.0	—	3.4	~1.0	6.5
β-L-Fucopyranose	7.9	—	—	10.0	—	3.4	~1.0	6.5
2,6-Dideoxy-α-L-*lyxo*-hexopyranose[b]	3.2	1.9	−12.8	11.4	6.2	3.0	0.8	6.5
2,6-Dideoxy-β-L-*lyxo*-hexopyranose[b]	9.8	2.0	−12.2	12.0	4.6	3.4	0.8	6.5
Methyl 2,6-dideoxy-α-L-*lyxo*-hexopyranoside[b]	3.6	1.4	−13.2	11.3	6.6	3.2	0.8	6.6
Methyl 2,6-dideoxy-β-L-*lyxo*-hexopyranoside[b]	10.1	~2.0	−12.2	12.2	~5.0	~3.2	~1.0	6.5

[a] Values between brackets are the increment *vs.* the corresponding anomers of D-galactopyranose, and 2-deoxy-L-*lyxo*-hexopyranose, values between squared brackets are the increments *vs.* corresponding anomers of 2,6-dideoxy-L-*lyxo*-hexopyranoses. Positive values are used for deshielding. [b] Long range couplings are observed between (H=2_{eq}, 4_{eq}), (H-1, H-5) and (H-1, H-3).

The chemical shifts of the aldosides. When we compare the chemical shifts of the ring protons of D-galactopyranose with those of the corresponding anomers of methyl D-galactopyranosides, almost no changes are observed, except for H-5 in the α-form for which an upfield shift of −0.19 ppm is found. This effect of an axial glycosidic OMe group on the axial H-5 has been recognized in previous studies.[2,6] This trend therefore remains valid for 2-deoxy sugars.

The upfield shift of −0.08 ppm for H-3 in the α-form of 2,6-dideoxy-α-L-*lyxo*-hexopyranose is rather unexpected. An upfield shift of −0.26 ppm and −0.43 ppm in, respectively, the β-, and α-form for the glycosidic protons of methyl glycosides has previously been observed[2] for galactopyranosides. The chemical shifts of H-2 in both anomers of the methyl pyranosides of 2,6-dideoxy-L-*lyxo*-hexopyranose have remained unaffected, despite the observation of a "methylation shift" in methoxy cyclohexane *vs.* cyclohexanol.[7] We have previously pointed out[5] that increments are substrate-sensitive, *e.g.* that additional corrections might be necessary if dealing with changes in the skeleton of changes in substitution.

Therefore, if we want to apply the proposed increment rules for aldohexopyranoses[2] on 6-deoxy-aldohexopyranoses (*cf.* Ref. 6), the following corrections are proposed for the α-, as well as for the β-forms): −0.05 ppm for H-1, −0.20 ppm for H-4 and +0.03 or +0.13 ppm for H-5 depending on the equatorial or axial orientation of OH-4. The correction of H-2 is −0.03 ppm, irrespective of its axial or

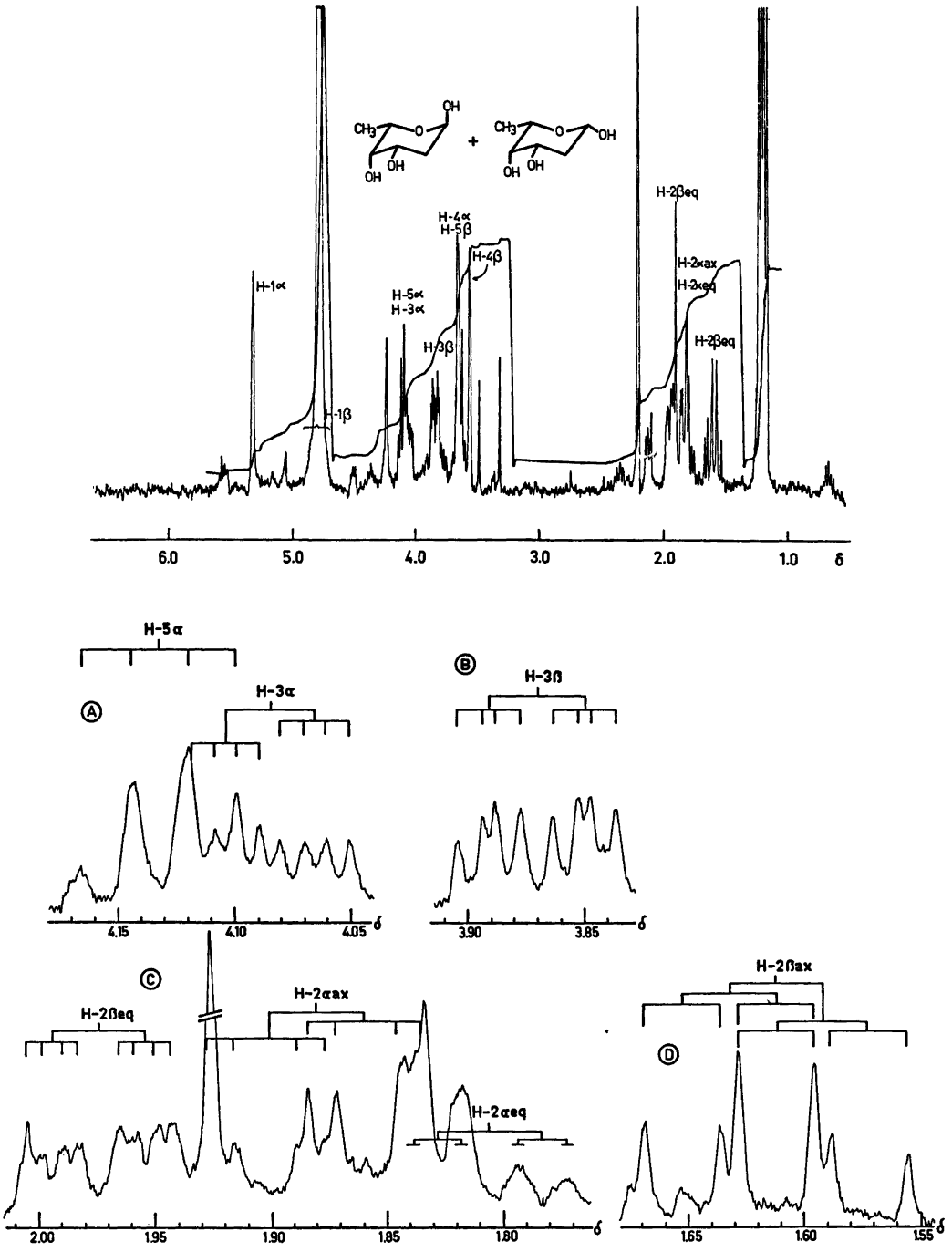

Fig. 1. ^{1}H NMR spectrum at 300 MHz in D_2O (DSS internal) of 2,6-dideoxy-L-*lyxo*-hexopyranoses (top) and extended regions (bottom). Both α- and β-pyranoses are present in appreciable amounts, but not the corresponding furanoses.

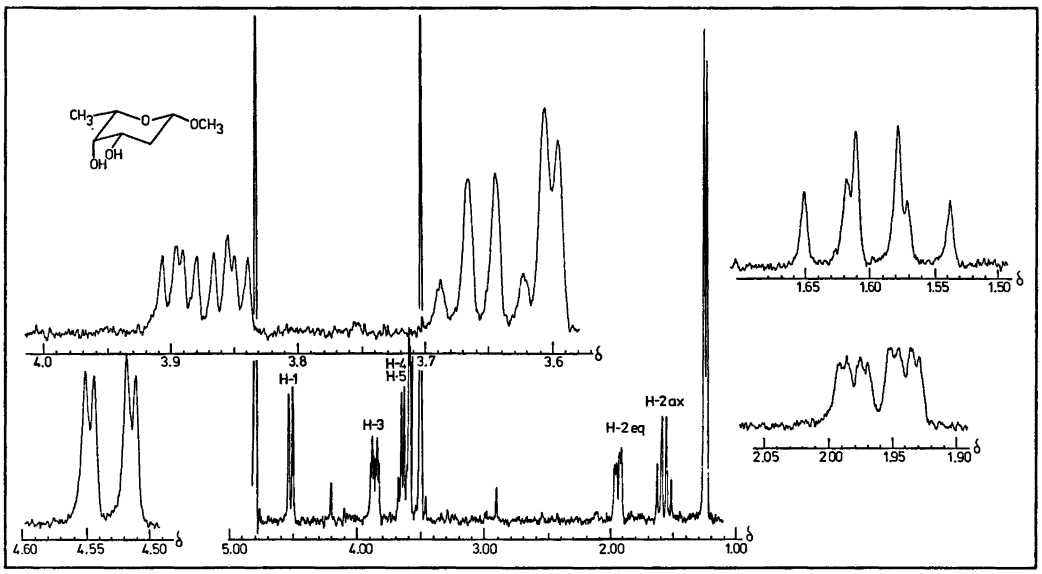

Fig. 2. ^{1}H NMR spectrum at 300 MHz in D_2O (DSS internal) of methyl 2,6-dideoxy-β-L-*lyxo*-hexopyranoside. Inserts are expanded regions.

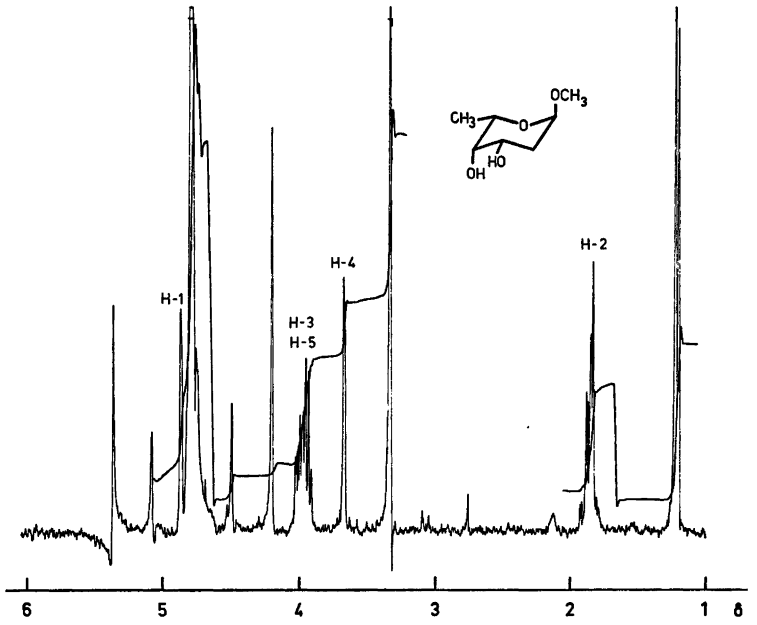

Fig. 3A. ^{1}H NMR spectrum at 300 MHz in D_2O (DSS internal) of methyl 2,6-dideoxy-α-L-*lyxo*-hexopyranoside.

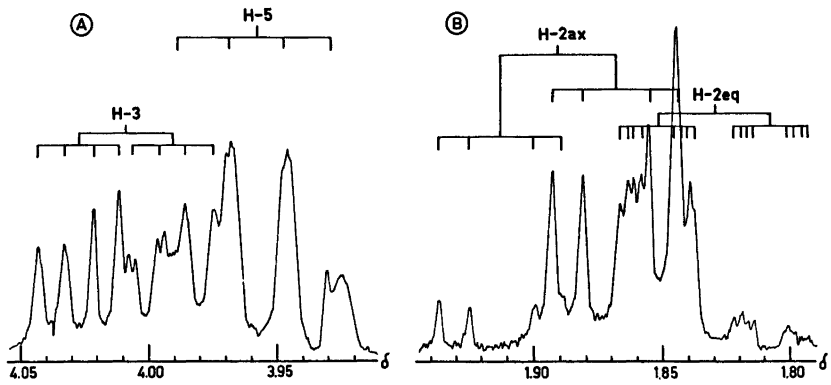

Fig. 3B. Extended regions of the spectrum given in Fig. 3A.

equatorial nature and irrespective of the geminal partner, H or OH.

EXPERIMENTAL

^{1}H NMR spectra were measured at 300 MHz and 19 °C with a VARIAN HR-300 spectrometer equipped with INDOR-facilities (SC 8525-2 unit). Concentrations were *ca.* 25 mg/ml D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (Sic) as the internal standard.

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Phase Diagrams of Systems Containing Cholesterol, Cholesteryl Esters, and Triglycerides

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Binary and ternary systems of cholesterol, the three cholesteryl esters, linoleate, oleate, and stearate and the two triglycerides, triolein and tristearin were studied in order to determine the phase transitions and the conditions for the cholesteric and smectic mesophases. Phase transitions were determined using differential thermal analysis, melting point determination, and polarizing microscopy.

The cholesterol-cholesteryl ester systems studied are of the eutectic type with limited solid solubility. The mesophases, cholesteric and smectic, are monotropic as to the crystalline state and exist up to *ca.* 75 wt. % cholesterol. Ternary systems of cholesterol and two cholesteryl esters show the same general features as the binary systems.

The melting point of cholesterol is depressed by increasing amounts of triglycerides down to an eutectic point at high concentrations of the triglycerides. The solubility of anhydrous cholesterol and cholesterol monohydrate in triolein was found to be the same.

In mixed systems with cholesterol, triglycerides, and cholesteryl esters even low concentrations of the triglycerides removed the cholesteric mesophase typical for cholesteryl ester systems. At higher concentrations of triolein the smectic mesophase was also removed. In systems with cholesterol, a cholesteryl ester and tristearin an apparently smectic mesophase with mosaic texture was exhibited.

Cholesterol, cholesteryl esters, and triglycerides belong to the major components of atherosclerotic deposits and serum lipoproteins. From this standpoint, the phase conditions of these lipids help to understand the physicochemical mechanism of the lipid deposition in atherosclerotic arteries.

Cholesterol, cholesteryl esters and triglycerides are major lipids in atherosclerotic plaques.[1] Correlated morphological and chemical studies have shown that lipids, accumulating as liquid and liquid crystalline droplets in early stages of atherosclerosis, are mainly composed of cholesteryl esters.[2,3] In advanced plaques there is much solid material consisting of crystalline cholesterol and amorphous cholesteryl esters and triglycerides.[3]

Based upon their interactions with water cholesterol and triglycerides are classed as polar insoluble non-swelling amphiphiles and cholesteryl esters as non-polar lipids.[4]

On heating, cholesterol and triglycerides melt from a crystalline form directly to an isotropic liquid while long chain cholesteryl esters exhibit thermotropic mesomorphism.[5]

Regarding the interactions with polar insoluble swelling lipids (*e.g.* phospholipids) the difference between cholesterol, cholesteryl esters and triglycerides is marked. In bulk systems cholesterol can be solubilized in the molar ratio of 1:1 by phospholipids,[5,7] while triglycerides can be solubilized to a much smaller extent,[8] and cholesteryl esters to an almost negligible amount.[9]

This work is part of a program for studying the physical state of, and the interactions between, the different lipid classes which accumulate in the atherosclerotic lesions. Phase diagrams of systems containing cholesteryl esters and triglycerides have been presented in an earlier paper.[10]

MATERIALS AND METHODS

The cholesterol, cholesteryl oleate and cholesteryl stearate used were purchased from E. Merck AG. Cholesteryl linoleate was prepared by a modified acid chloride method.[11]

The cholesterol was recrystallized three times from 1,2-dichloroethane and the monohydrate of cholesterol was prepared by dissolving cholesterol in methanol and recrystallization by addition of water. The cholesteryl esters were recrystallized from pentyl alcohol with subsequent washing in an ethanol-water solution. Triolein and tristearin were purchased from Fluka AG. The triolein was purified by Florisil column chromatography and the tristearin by recrystallization from acetone.

Samples for analysis were prepared by dissolving the weighed components in chloroform which was then removed *in vacuo*. *Ca.* 30 mg of sample were weighed in an aluminium pan which was placed in a Fisher Model 370 Differential Thermal Analyzer (DTA). The heating curves were obtained with a scan speed of 10 °C/min and the cooling curves with a scan speed of 5 °C/min. When examining lipids with unsaturated fatty acid chains, an atmosphere of N_2 gas was used.

The melting point values obtained by DTA measurements were complemented by examinations with a Gallenkamp melting point apparatus. In order to identify the phase changes recorded by the DTA measurements a Wild polarizing microscope equipped with a thermostated stage was used.

RESULTS

Individual lipids. The thermal properties of the three C_{18} cholesteryl esters (stearate, oleate, and linoleate) and the two C_{18} triglycerides (triolein and tristearin) used in this study have been presented in a previous report.[12] Also in this study precautions were taken to obtain the stable higher melting modification of the unsaturated cholesteryl esters and to bring the tristearin into the triclinic β_L form.

On heating, the anhydrous cholesterol showed a reversible endotherm at 38 °C and melted at 148 °C. The 38 °C transition of cholesterol was exhibited also in mixtures with other lipids but is for simplicity omitted from the diagrams. The monohydrate of cholesterol showed no transition at 38 °C, lost its hydration water between 100 and 120 °C and then melted at the same temperature as the anhydrous cholesterol.

Mixtures of cholesterol and cholesteryl esters. The condensed binary phase diagrams of cholesterol and the cholesteryl esters are presented in Fig. 1. The melting point of cholesterol is depressed continuously by increasing amounts of esters down to an eutectic point at high concentrations of the esters.

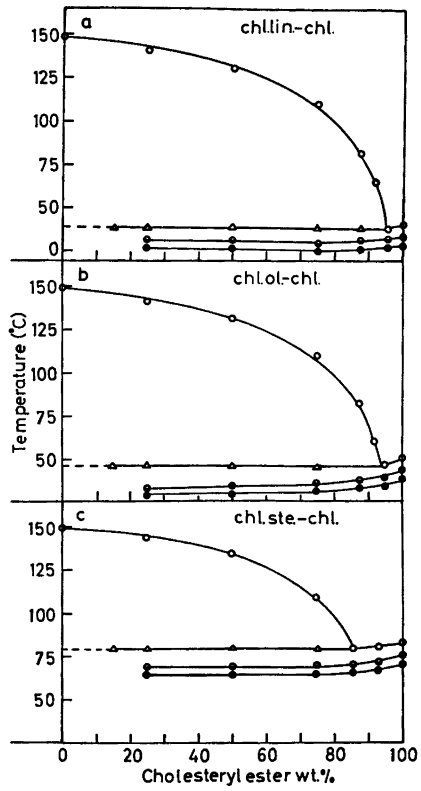

Fig. 1. Condensed binary phase diagrams for the systems (a) cholesterol – cholesteryl linoleate, (b) cholesterol – cholesteryl oleate, and (c) cholesterol – cholesteryl stearate. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, solid-(solid + liquid) △.

On heating, the DTA curves for blends with composition within the miscibility gap have two peaks; the first corresponding to the melting of the eutectic composition and the second one to the melting of the solid portion of the blend. At compositions approaching the eutectic point the second peak diminishes. (Fig. 2a)

On cooling from isotropic melt cholesteric and smectic mesophases were found to exist up to about 75 % cholesterol.

Ternary systems of cholesterol and two cholesteryl esters showed the same general features as the binary systems. This is illustrated in Fig. 3 where the proportion of cholesterol is held constant and the mutual concentrations of the esters are changed. The linear curve for the final melting points of cholesterol shows that

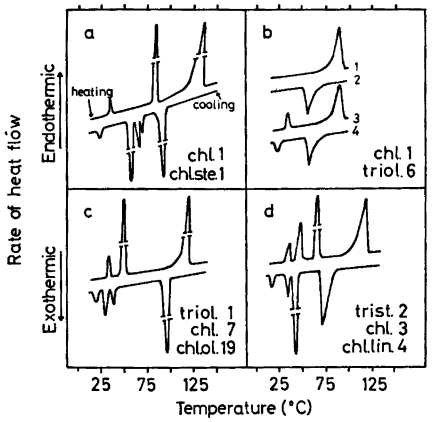

Fig. 2. DTA curves of mixtures of cholesterol, the three cholesteryl esters, linoleate, oleate, and stearate, and the two triglycerides, triolein, and tristearin. In the figures the heating curves are above and the cooling curves below. (a) The heating curve of a 1:1 mixture of cholesterol and cholesteryl stearate showing the 38 °C endotherm of cholesterol and the melting peaks of cholesteryl stearate and cholesterol. The cooling curve shows the crystallization of cholesterol, two mesomorphic transitions (liquid-cholesteric and cholesteric-smectic), the crystallization exotherm of cholesteryl stearate and the reversible crystal transformation of cholesterol. (b) (1) The melting endotherm of cholesterol monohydrate in a 1:6 mixture with triolein. (2) Cooling curve of the same mixture showing the crystallization exotherm of cholesterol monohydrate. (3) Reheating of the same mixture after removing the crystal water of the cholesterol monohydrate by heating to 150 °C. The curve shows the 38 °C and the melting endotherms of anhydrous cholesterol. (4) Cooling curve of the same mixture showing the crystallization and crystal transformation of the anhydrous cholesterol. (c) The heating curve of a 1:7:19 mixture of triolein, cholesterol, and cholesteryl oleate showing the 38 °C endotherm of the cholesterol and the melting peaks of cholesteryl oleate and cholesterol. The cooling curve shows the crystallization peak of cholesterol, two mesomorphic transitions (liquid-cholesteric and cholesteric-smectic) and the crystal transformation of cholesterol. (d) The heating curve shows 38 °C endotherm of cholesterol and the melting peaks of cholesteryl linoleate, tristearin, and cholesterol of a 2:3:4 mixture of tristearin, cholesterol, and cholesteryl linoleate. The cooling curve shows the crystallization exotherms of cholesterol and tristearin, the mosaic phase transition and the cholesterol crystal transformation.

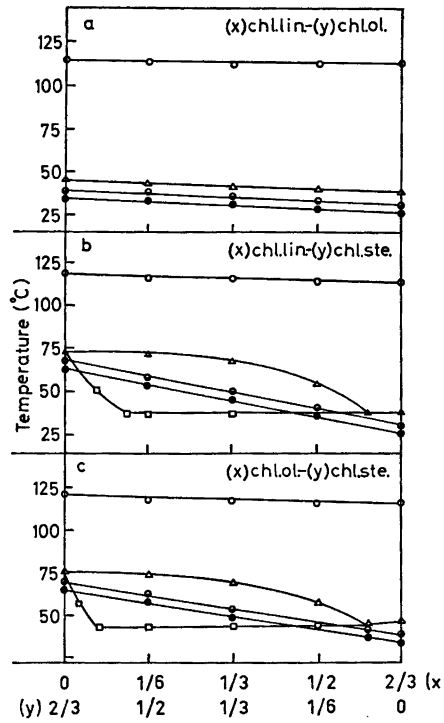

Fig. 3. Phase transitions in the ternary systems with cholesterol (chl) and two of the cholesteryl esters, cholesteryl linoleate (chl. lin.), cholesteryl oleate (chl. ol.), and cholesteryl stearate (chl. ste.). (a) Chl. – chl. lin. – chl. ol., (b) chl. – chl. lin. – chl. ste., (c) chl. – chl. ol. – chl. ste. Proportion of cholesterol is constant at the weight fraction of 1/3 and the proportions of the cholesteryl esters vary between 0 and 2/3. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, solid-(solid + liquid) △ and □.

the melting point depressing properties are additive in an ideal manner. Also the melting point of cholesteryl stearate is depressed by the unsaturated esters. The unsaturated esters show complete miscibility in both the liquid and solid phases.

On cooling from melt, cholesteric and smectic mesophases were found to exist for all mutual concentrations of esters and the fixed weight fraction of 1/3 cholesterol.

Mixtures of cholesterol and triglycerides. In Fig. 4 the phase diagrams of cholesterol-triolein and cholesterol-tristearin are presented. As for the cholesteryl esters increasing amounts of triglycerides continuously depress the melting

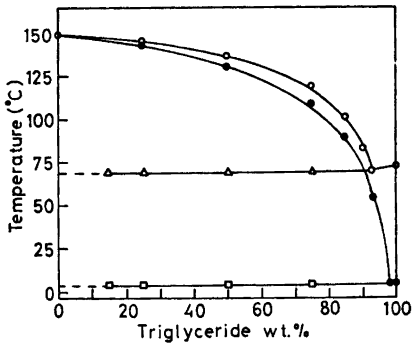

Fig. 4. Condensed binary phase diagrams for the systems (a) cholesterol-triolein, solid-liquid ●, solid-(solid + liquid) □, and (b) cholesterol-tristearin, solid-liquid ○, solid-(solid + liquid) △.

point of cholesterol down to an eutectic point at high concentrations of the triglycerides. The melting point depressing effect of triolein is somewhat greater than that of tristearin.

On heating, the cholesterol monohydrate showed no transition at 38 °C in mixtures with triolein, but if the mixtures were heated above 100 °C and the monohydrate lost its water a transition occurred (Fig. 2 b). No significant differences in solubility of anhydrous cholesterol and cholesterol monohydrate in triolein were found.

Mixtures of cholesterol, triglycerides, and cholesteryl esters. The phase diagrams of cholesterol, triolein, and the three cholesteryl esters respectively are presented in Fig. 5. The concentration of cholesterol is held constant at the weight fraction of 1/3 and the mutual concentrations of triolein and the cholesteryl esters are varied between the weight fractions of 0 and 2/3. The linear curves for the solid-liquid transition show that the melting point depressing effects are additive in an ideal manner. Also the melting points of the cholesteryl esters are depressed by triolein.

On cooling from isotropic melt both cholesteric and smectic mesophases were recorded at low triolein concentrations (Fig. 2 c). The smectic mesophases was recorded to somewhat higher triolein concentrations than the cholesteric one.

In Fig. 6 the phase diagrams of cholesterol, tristearin and the three cholesteryl esters are presented. From the linear liquidus line it can

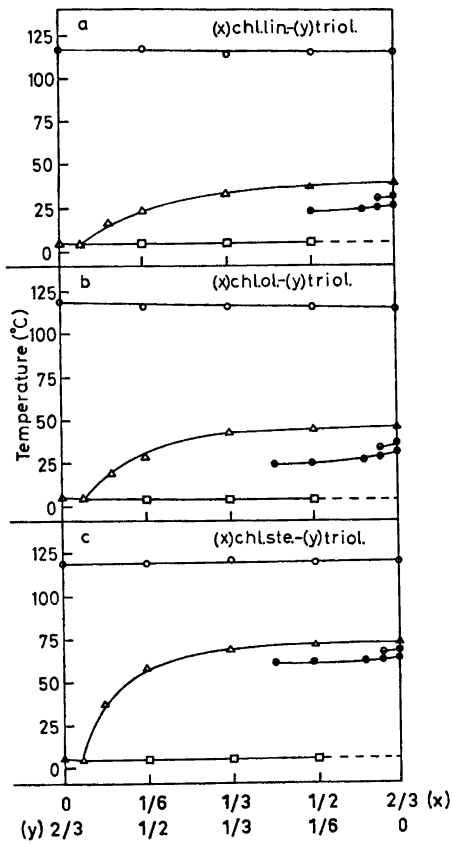

Fig. 5. Phase transitions in ternary systems of cholesterol (chl.), triolein (triol.) and the three cholesteryl esters, cholesteryl linoleate (chl. lin.), cholesteryl oleate (chl. ol.) and cholesteryl stearate (chl. ste.). (a) Chl. – triol. – chl. lin., (b) chl. – triol. – chl. ol. (c) chl. – triol. – chl. ste. Proportion of cholesterol is constant at the weight fraction of 1/3 and the proportions of triolein and the cholesteryl esters vary between 0 and 2/3. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, solid-(solid + liquid) △ and □.

be seen that the melting point depressing effects of tristearin and the cholesteryl esters are additive in an ideal manner. The melting point of tristearin is depressed by the unsaturated cholesteryl esters and the melting point of cholesteryl stearate is depressed by tristearin. On cooling from melt ternary systems with cholesterol, tristearin and either of the cholesteryl esters exhibited both cholesteric and smectic mesophases at low tristearin con-

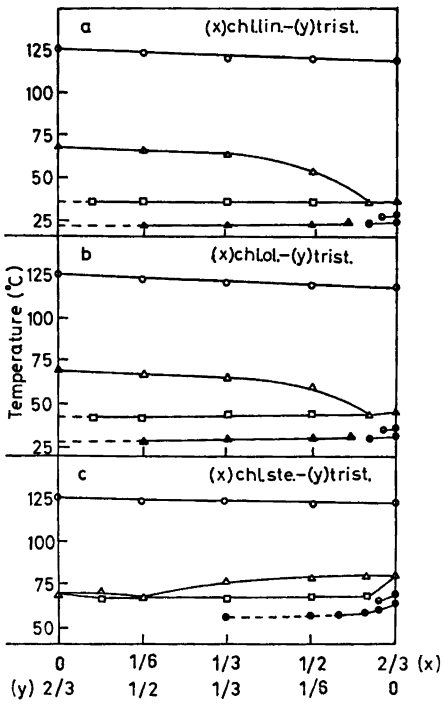

Fig. 6. Phase transitions in ternary systems of cholesterol (chl.), tristearin (trist.), and the three cholesteryl esters, cholesteryl linoleate (chl. lin.), cholesteryl oleate (chl. ol.), and cholesteryl stearate (chl. ste.). (a) Chl. – trist. – chl. lin., (b) chl. – trist. – chl. ol., (c) chl. – trist. – chl. ste. Proportion of cholesterol is constant at the weight fraction of 1/3 and the proportions of tristearin and the cholesteryl esters vary between 0 and 2/3. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, liquid-mosaic smectic ▲, solid-(solid + liquid) △ and □.

centrations. For the unsaturated esters the smectic phase remains at high tristearin concentrations, but the microscopic texture changes considerably; a mosaic texture phase is formed (Fig. 2 d). At low cholesteryl ester concentrations the smectic transition cannot be recorded but the curve obtained by plotting composition *versus* the transition heat for the smectic mesophase indicated that this phase exists down to very low cholesteryl ester concentrations.

The interaction of cholesteryl stearate with tristearin and cholesterol is somewhat different from that of the unsaturated esters. On cooling from melt, cholesteric and smectic mesophases are exhibited at low tristearin concentrations but no mosaic type mesophase is formed at higher tristearin concentrations.

BIOLOGICAL CONCLUSIONS

This work is part of a project to elucidate the factors governing the deposition of lipids in atherosclerotic plaques through studies of the physical state of and interaction between lipids in adequate model systems. This study deals with the phase behaviour of three major atheroma lipids; cholesteryl esters, triglycerides and cholesterol.

All binary systems studied with cholesterol and one cholesteryl ester or triglyceride are of the eutectic type with limited solid solubility. The melting point of cholesterol is depressed to the eutectic point at a high percentage of the cholesteryl ester or triglyceride. The ternary systems show that the melting point depressing capacity is additive in an ideal manner. These facts have to be kept in mind when considering the physical state of lipids in atherosclerotic plaques.

No solid-phase solubility of the cholesteryl esters and triglycerides in cholesterol was detected. This must be due to limited calorimeter sensitivity because complete immiscibility of solid phases in eutectic systems never occurs. The low mutual solid solubility of cholesterol, cholesteryl esters and triglycerides accounts for the fact that crystals of cholesterol, with no chromatographically detectable impurities were found together with amorphous cholesteryl esters in atherosclerotic plaques.[3]

Typical for the early stages of atherosclerosis is a large accumulation of lipids especially cholesteryl esters, in isotropic and birefringent droplets. The polarizing microscopic pattern and freeze-etching electron microscopic pictures indicate a similarity between the anisotropic droplets and the cholesteryl ester suspensions in smectic state. The cholesteric phase has not been found in atherosclerotic lesions. The probable explanation of this fact is the property of triglycerides to remove this mesophase in mixtures with cholesteryl esters.

Besides isotropic and mesomorphic droplets there is also an abundance of solid lipid material in advanced plaques. From a chemical point of view the advanced plaques can be

divided into "cholesteryl ester type" and "cholesterol type".[3]

Typical for the "cholesteryl ester type" lesion is a high cholesteryl ester value and moderate cholesterol, triglyceride and phospholipid values. The plaque is made up of amorphous material which primarily melts between 40 and 50 °C. The melting points of saturated, high melting, cholesteryl esters which occur rather abundantly are thus depressed by unsaturated, low melting, cholesteryl esters and triglycerides.

The "cholesterol type" lesions have high cholesterol values and contain a rather great amount of cholesteryl esters. The triglyceride and phospholipid values are low. Typically enough these plaques contain crystals of cholesterol monohydrate and varying amounts of amorphous cholesteryl esters. The "cholesterol type" plaques typically melt between 50 and 60 °C; thus at a higher temperature than the "cholesteryl ester type" plaques but at a much lower than the melting point of cholesterol. This fact obviously is a result of the melting point depressing effect of the cholesteryl esters and triglycerides on cholesterol.

The anhydrous cholesterol shows a reversible endotherm at 38 °C both in pure form and in mixtures with other lipids. A possible significance of this transition in the atherogenesis has been proposed.[12] This hypothesis is opposed by the fact that the cholesterol in atherosclerotic plaques occurs in the form of cholesterol monohydrate which does not show the 38 °C endotherm. In this study has been shown that cholesterol can crystallize in the form of cholesterol monohydrate from a mixture with triolein. It is thus still unclear whether the cholesterol crystals in atherosclerotic deposits originate from membrane structures oversaturated with cholesterol or from an oily phase.

From a physicochemical point of view one can thus conclude that the most fatal factors for a deposition of solid lipid material in the arterial wall are excessive incorporation of cholesterol and/or saturated, high melting cholesteryl esters giving rise to "cholesterol type" or "cholesteryl ester type" plaques alternatively.

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Reaction of Hydrazonyl Halides with Primary Thioamides; Formation of Thiohydrazides and Hydrazonyl Sulfides

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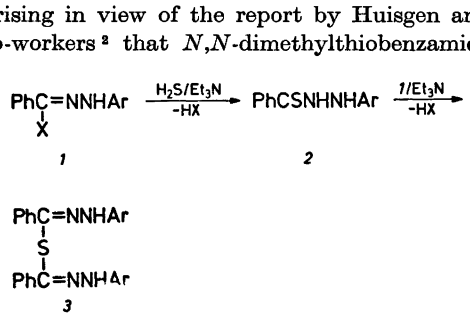
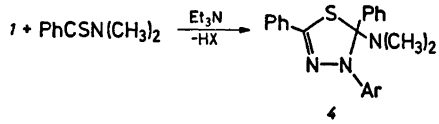
N-Aryl benzohydrazonyl halides react with primary thioamides in the presence of triethylamine to give nitriles and, depending on the relative concentrations of the reactants, thiohydrazides or hydrazonyl sulfides in excellent yields. In the absence of base no reaction is observed. *N,N*-Dimethyl benzohydrazonyl chloride reacts with thioacetamide to give almost quantitatively the hydrochloride of *N,N*-dimethylthiobenzhydrazide, while base (triethylamine) is required for reaction to take place in case of the *N,N*-diphenyl analog. Different reaction mechanisms are discussed in terms of 1,3-dipolar cycloaddition processes and displacement of the hydrazonyl halogen by the thioamide or its anion.

In a recent study [1] of the reaction of hydrazonyl halides (*1*) with hydrogen sulfide in the presence of triethylamine it was found that thiohydrazides (*2*) react with *1* to form hydrazonyl sulfides (*3*), see Scheme 1. This observation is surprising in view of the report by Huisgen and co-workers [2] that *N,N*-dimethylthiobenzamide



Scheme 1.

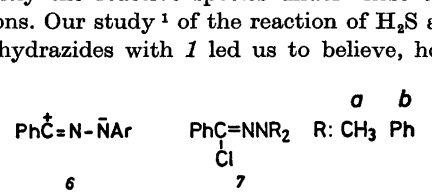
and other compounds with a C=S double bond react with *1* in the presence of triethylamine to give cyclic products such as *4* (Scheme 2). We have therefore examined in more detail



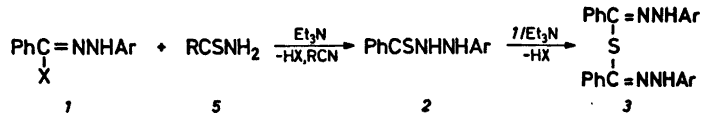
Scheme 2.

the reaction of hydrazonyl halides with various thioamides (for reactions with derivatives of thiourea and thiosemicarbazide, see Ref. 3). The present paper deals in particular with the reactions of primary thioamides ($RCSNH_2$, *5*), while those of secondary and tertiary thioamides will be described in a forthcoming paper.[4]

The thorough studies by Huisgen and collaborators on the reactions of hydrazonyl chlorides in the presence of base have established that 1,3-dipolar species, nitrilimines (*6*), are frequently the reactive species under these conditions. Our study [1] of the reaction of H_2S and thiohydrazides with *1* led us to believe, how-



ever, that nucleophilic displacement of the halogen atom in *1* might in some cases be a viable mechanistic alternative. In order to examine this further we have included in the present study experiments with *N,N*-disubstituted hydrazonyl chlorides (*7*), which are incapable of reacting as 1,3-dipolar species, and experiments with 2,5-diphenyltetrazole, which is known to generate 1,3-diphenylnitrilimine (*6*, Ar=Ph) at elevated temperatures.[6a]



Ar/X
a Ph/Cl
b 2-ClC$_6$H$_4$/Cl
c 2,4-Br$_2$C$_6$H$_3$/Br
d 2-Br-4-ClC$_6$H$_3$/Br
e 2,4,6-Cl$_3$C$_6$H$_2$/Br

R
a CH_3
b Ph
c 3-Pyridyl
d $(CH_3)_2CHO$-

Scheme 3.

RESULTS AND DISCUSSION

Primary thioamides (*5a–c*) and *O*-alkylthiocarbamates (*5d*) react with hydrazonyl bromides and chlorides (*1*) in the presence of triethylamine to form thiohydrazides (*2*), hydrazonyl sulfides (*3*), and nitriles (or alkyl cyanates in the case of thiocarbamates) according to Scheme 3. This reaction provides an easy route to thiohydrazides as well as to hydrazonyl sulfides and compares favourably with previously reported syntheses of these compounds (see Ref. 1 and references cited therein). A thiohydrazide (*2*) is the main product when the reactants *1* and *5* are present in equimolar amounts, whereas a hydrazonyl sulfide (*3*) is formed when an excess of *1* is employed (*cf.* Table 1). The concomitant formation of a nitrile (or cyanate) in these reactions is demonstrated by the appearance of a sharp absorption in the region 2230–2250 cm^{-1} in the IR spectra [7,8] of reaction mixtures from which *3* was subsequently isolated. The nitrile has further been isolated and shown to be identical to authentic material. Nitrile formation by (formal) abstraction of hydrogen sulfide from primary thioamides has been reported to take place through the action of a variety of reagents such as imidoyl chlorides,[9] sulfenyl chlorides in the presence of pyridine,[10] the triphenylphosphine/carbon tetrahalide system,[11] or phenylpropiolamidines;[12] likewise, dehydrosulfurization of *O*-alkylthiocarbamates by mercury(II) oxide to give alkyl cyanates has been described.[13]

Table 1. Yields [a] of thiohydrazides (*2*) and hydrazonyl sulfides (*3*) obtained from PhCXNNHAr (*1*) and RCSNH$_2$ (*5*).

Reactants			Yields in $CHCl_3$		Yields in C_6H_6	
1	*5*	*1/5*	*2*	*3*	*2*	*3*
1a	*5a*	2		90		89
1a	*5b*	2				87
1a	*5c*	2				96
1a	*5d*	2				82
1b	*5a*	2				92
1c	*5a*	2			11	82
1c	*5b*	2				93
1d	*5a*	2				87
1e	*5a*	2				86[c]
1a	*5a*	1[b]	78	19	24	13
1a	*5b*	1		39		60
1c	*5a*	1	93	7	47[c]	26
1c	*5b*	1		14		29
1d	*5a*	1	90, 74[c]	7	81	13
1e	*5a*	1	96, 73[c]	1	80	14

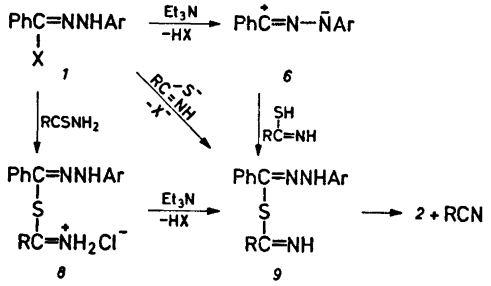
[a] Yields obtained with 5 mmol thioamide in 150 ml solvent. [b] Reaction time 12 h. [c] Crystallized once.

The formation of *2* in preference to *3* when equimolar amounts of *1* and thioacetamide are employed indicates that the thioacetyl group of thioacetamide is more reactive towards *1* (or *6*) than is the thiobenzoyl group of the thiohydrazide. This is further confirmed by the following observations: *2* rather than *3* is formed when triethylamine is added to a mixture of equimolar amounts of a hydrazonyl halide, thioacetamide and a thiohydrazide, and sulfide formation increases significantly at the expense of the thiohydrazide when employing thiobenzamide instead of thioacetamide.

Primary thioamides do not react with *N*-arylhydrazonyl halides in the absence of base; attempted reactions of various hydrazonyl halides with thioacetamide in chloroform (molar

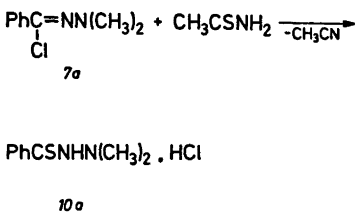
ratio *1*:5 = 1) at room temperature did not produce thiohydrazides in detectable amounts (TLC), and the starting materials were recovered almost quantitatively. Similarly, the chemical shift of the CH_3-group of thioacetamide dissolved in $CDCl_3$ is not changed upon the addition of a hydrazonyl halide to the solution, whereas subsequent addition of triethylamine causes the methyl signal to move upfield to a chemical shift value identical to that of acetonitrile.[14]

The necessity for base to be present for the reaction to proceed is, however, compatible with either of two mechanisms; the triethylamine may dehydrohalogenate *1* [5] (*1*→*6*, see Scheme 4) and the resultant nitrilimine (*6*) then add to the SH group (*6*→*9*), or the halogen atom of *1* may be displaced by the thioamide sulfur atom, in which case the triethylamine would serve to convert the thioamide to the thioamide anion prior to displacement (*1*→*9*) or to deprotonate a possible initial adduct such as *8* (*8*→*9*).



Scheme 4.

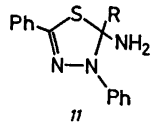
To test the possibility of a displacement mechanism the reaction of *N*,*N*-disubstituted hydrazonyl chlorides (*7*) with thioacetamide was examined. The dimethyl compound (*7a*) gives an almost quantitative yield of the hydrochloride of *N*,*N*-dimethylthiobenzhydrazide (*10a*, see Scheme 5), and the reaction of the diphenyl hydrazonyl chloride (*7b*) with thioacetamide and triethylamine leads, similarly, to *N*,*N*-diphenylthiobenzhydrazide. Base (triethylamine) is not required for the former reaction to take place, since the dimethylamino group of *10a* (or *7a*) in itself is sufficiently basic to serve as proton acceptor. These reactions must occur by nucleophilic displacement of the



Scheme 5.

hydrazonyl halogen atom, 1,3-dipole formation being precluded by the presence of two substituents at the terminal nitrogen atom, and a similar mechanism may indeed be an alternative to reaction *via* 1,3-dipolar species (nitrilimines) for *1*.

To shed light on the possible intermediacy of nitrilimines in these reactions attempts were made to react primary thioamides (*e.g.* thioisonicotinamide) with 2,5-diphenyltetrazole in refluxing bromobenzene, with 1-hexanol added to trap [2,4] any 5-aminothiadiazoline (*11*) formed by addition of the nitrilimine across the C=S double bond of the thioamide.[2]



However, considerable decomposition of the reactants took place and the only identifiable products were ammonia, sulfur and small (4 %) amounts of the corresponding thioanilide (thioisonicotinanilide), presumably formed *via* partial reduction of the tetrazole or the nitrilimine to aniline and subsequent transamidation. These experiments are not regarded as conclusive, because of the rather drastic conditions compared to the reactions described above. Although neither the tetrazole experiment nor the reactions of *1* in the presence of triethylamine gave rise to cyclic compounds such as *11*, nor to possible hydrolysis (or alcoholysis) products formed herefrom, it remains possible that the nitrilimine, if formed from *1*, adds to the thiol form of the thioamide to give *9* (see Scheme 4) in analogy with the reactions of nitrilimines with phenol and thiophenol.[6]

The relative yields of *2* and *3* vary somewhat with the solvent employed, with sulfide (*3*)

formation more favoured in benzene than in chloroform (see Table 1). The reason for this may be that the acidity of thioamides exhibits a significant solvent dependence.[15]

EXPERIMENTAL

Materials

The thioacetamide used was reagent grade (BDH AnalaR). All other thioamides were prepared by literature procedures: thiobenzamide,[16] thionicotinamide,[16] thioisonicotinamide,[16] and *O*-isopropylthiocarbamate.[17] The hydrazonyl halides and the *N,N*-disubstituted hydrazonyl halides were prepared as described previously.[18,20] *N*-(2,4,6-Trichlorophenyl)benzohydrazonyl bromide (*1e*) was prepared according to Chattaway and Walker[21] from benzaldehyde 2,4,6-trichlorophenylhydrazone and bromine in 66 % yield after crystallization from acetic acid as white needles, m.p. 89–90 °C (lit.[22] m.p. 98 °C). (Anal. $C_{13}H_8BrCl_3N_2$: C, H, N). 2,5-Diphenyltetrazole was prepared according to Huisgen *et al.*[6a]

Preparation of thiohydrazides (*2*)

N'-Thiobenzoyl-N-(2-bromo-4-chlorophenyl)-hydrazine (2d). Triethylamine (10.7 mmol) was added to a stirred solution of *1d* (5.0 mmol) and thioacetamide (5.0 mmol) in chloroform (50 ml). After 4 h the solution was washed with aqueous acetic acid and water and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue extracted with boiling ethanol, leaving the sparingly soluble sulfide *3d* (7 %), m.p. 190–192 °C (lit.[19] 188–190 °C). Evaporation of the ethanolic solution yielded the crude thiohydrazide in 90 % yield. (Anal. $C_{13}H_{10}BrClN_2S$: C, H, N, S), m.p. 115–116 °C (benzene–hexane).

The other thiohydrazides were prepared similarly in nearly quantitative yield (see Table 1) and shown to be identical to authentic material (Ref. 1).

N'-Thiobenzoyl-N-(2,4,6-trichlorophenyl)hydrazine (2e) has not been described before; it was prepared as given above from *1e* in 96 % yield. (Anal. $C_{13}H_9Cl_3N_2S$: C, H, N, S), m.p. 139–141 °C (benzene–hexane).

It should be noted that the thioacetamide must be completely dissolved before the addition of triethylamine; otherwise the yield of sulfide increases at the expense of the thiohydrazide. Use of benzene instead of chloroform caused the yields of sulfide to increase (see Table 1). When the thiohydrazide was prepared in larger scale (50 mmol) the chloroform solution was always flushed through with nitrogen before the addition of triethylamine to avoid air oxidation to the corresponding hydrazonyl disulfide.[23]

Attempted reactions with hydrazonyl halides and thioacetamide in the absence of base. Compound *1a* (2.5 mmol) and thioacetamide (2.5 mmol) were dissolved in chloroform (25 ml) and left overnight. TLC of the solution did not show any presence of thiohydrazide (*2a*) and compound *1a* was recovered almost quantitatively, m.p. 127–129 °C. Similarly, *1c* (2.5 mmol) and thioacetamide (2.5 mmol) resulted in 93 % recovery of starting material *1c*.

Compound *1a* or *1e* (*ca.* 15 mg) was added to a solution of thioacetamide (*ca.* 5 mg) in deuteriochloroform (0.50 ml). The addition did not change the chemical shift value (δ 2.57) of the methyl group of thioacetamide; subsequent addition of triethylamine caused the appearance of a new absorption at δ 1.95 (lit.[14] for CH_3 in acetonitrile δ 2.00).

Preparation of hydrazonyl sulfides (*3*)

Bis[α-(phenylhydrazono)benzyl]sulfide (3a). Triethylamine (21 mmol) was added to a stirred suspension of *1a* (10.0 mmol) and thioacetamide (5.0 mmol) in dry benzene (50 ml). After 20 h at room temperature the solution was heated to boiling and filtered to remove precipitated triethylammonium chloride. The sulfide precipitated upon cooling, yield 89 %, m.p. 159–160 °C (lit.[1] 158–160 °C). IR spectrum and R_F-value (TLC) identical to those of an authentic[1] sample. TLC of the mother liquor showed the presence of *2a*.

Compounds *1a*, *1c* and *1d* reacted in a similar manner with other primary thioamides such as thiobenzamide, thionicotinamide, and *O*-isopropyl thiocarbamate to produce the sulfides *3a*, *3b* (m.p. 193–195 °C, lit.[19] 200–202 °C), and *3d* (m.p. 192–194 °C, lit.[19] 188–190 °C) in 80–95 % yield (see Table 1). *3e* was prepared similarly from *1e*, thioacetamide and triethylamine in 85 % yield after recrystallization from benzene. M.p. 183–185 °C (decomp.). (Anal. $C_{26}H_{16}Cl_6N_4S$: C, H, N, Cl).

The yield of *3* may further be increased by reduction of the volume of solvent below that necessary for complete dissolution of the thioamide.

Identification of nitriles

Benzonitrile. (i) Triethylamine (10 mmol) was added to a stirred solution of *1a* (5.0 mmol) and thiobenzamide (2.5 mmol) in chloroform (25 ml). An IR spectrum recorded after 20 h showed a sharp absorption at 2235 cm^{-1}. (ii) An IR spectrum of a mixture of triethylamine (5 mmol), thiobenzamide (5.0 mmol) and triethylamine hydrochloride (5.0 mmol) in chloro-

form (25 ml) showed no absorption after 20 h in the region 2 200 – 2 300 cm^{-1}.

Nicotinonitrile. (*i*) Triethylamine (40 mmol) was added to a stirred suspension of *1b* (20.0 mmol) and thionicotinamide (10.0 mmol) in dry benzene (100 ml). After 12 h at room temperature the solution was heated to boiling and filtered. An IR spectrum showed a sharp absorption at 2230 cm^{-1}. The solution was evaporated under reduced pressure and the crude sulfide *3b* was washed carefully with ethanol, yield 92 %, m.p. 169 – 171 °C (lit.[19] 167 – 168 °C). The ethanolic solution was evaporated to dryness, the residue dissolved in ether and washed with aqueous sodium hydroxide and water, dried over Na_2SO_4 and the ether removed *in vacuo*. The resulting oil was sublimated to give white crystals, m.p. 48 – 50 °C (mixed m.p. 48 – 50 °C). A mass spectrum showed the molecular ion (*m/e* 104, base peak). (*ii*) Triethylamine (1 mmol) was added to a stirred suspension of thionicotinamide (1.0 mmol) and triethylamine hydrochloride (1.0 mmol) in dry benzene (10 ml). After 20 h the solution was filtered and 93 % thionicotinamide was recovered. An IR spectrum showed no absorption in the region 2200 – 2300 cm^{-1}.

Isopropyl cyanate. Triethylamine (2.0 mmol) was added to a stirred solution of compound *1a* (1.0 mmol) and *O*-isopropylthiocarbamate (0.5 mmol) in chloroform (2.5 ml). An IR spectrum recorded after 15 min showed a weak but distinct absorption at 2250 cm^{-1}. After 30 min the intensity had increased, but a new absorption appeared at 2150 cm^{-1}; this absorption (medium intensity) and a weak one at 1700 cm^{-1} were the only important ones in the region 1700 – 2300 cm^{-1} after 4 h.

Pure isopropyl cyanate [24] (kindly donated by Dr. E. Huge-Jensen) and triethylamine were dissolved in chloroform; and IR spectrum recorded after 24 h showed absorptions at 2150 and 1700 cm^{-1} as in the above experiment. These absorptions are probably due to decomposition of the cyanate to a mixture of isopropyl isocyanate and triisopropyl isocyanurate.[24]

Competition experiments with thioamides and thiohydrazides in presence of hydrazonyl halide. Compound *1c* (2.50 mmol), thioacetamide (2.50 mmol) and *2c* (2.50 mmol) were stirred together in chloroform (75 ml) until all thioacetamide had dissolved, whereupon triethylamine (7.5 mmol) was added. After 4 h the solution was washed with aqueous acetic acid and water, and dried over Na_2SO_4. The solvent was removed *in vacuo*, and the residue was extracted with boiling ethanol leaving the sparingly soluble sulfide *3c* (8 %), m.p. 191 – 194 °C. Evaporation of the ethanolic solution to dryness yielded the crude thiohydrazide *2c* in 82 % yield (in excess of added *2c*).

A similar experiment conducted in benzene (75 ml) gave *2c* (71 % in excess) and *3c* (11 %).

Similarly, *1a* (5.00 mmol), *2a* (5.00 mmol), thiobenzamide (5.00 mmol) and triethylamine (15.0 mmol) in chloroform (150 ml) afforded after 24 h a 66 % yield of the sulfide *3a*.

N,N-Disubstituted thiobenzhydrazides (10). N,N-Dimethylthiobenzhydrazide (10a). Compound *7a* (5.0 mmol) and thioacetamide (10 mmol) were mixed together in chloroform (25 ml) at room temperature. After 1 h the reaction mixture was cooled, followed by filtration to give yellow crystals (92 %). Crystallization from ethanol gave the pure hydrochloride of *10a* (82 %), m.p. 145 – 150 °C. (Anal. $C_9H_{12}N_2S.HCl$: C, H, N). Treatment of the hydrochloride with aqueous Na_2CO_3 gave the free *10a*, m.p. 107 – 109 °C (benzene – hexane, 1:1) (lit.[25] 106 – 107 °C).

N,N-Diphenylthiobenzhydrazide (10b). Compound *7b* (2.0 mmol), thioacetamide (4.0 mmol) and triethylamine (8.0 mmol) were refluxed together for 20 h in chloroform (10 ml). Work-up as for thiohydrazides (*2*) gave crude *10b* (57 %). Crystallization from benzene – light petroleum (1:1) gave m.p. 134 – 135 °C (lit.[26] 132 – 133 °C).

Reaction with 2,5-diphenyltetrazole. Thioisonicotinamide (5.0 mmol) and 2,5-diphenyltetrazole (5.0 mmol) were refluxed together in bromobenzene (25 ml) and 1-hexanol (1 ml) for 5 h at 160 °C. The solvent was removed *in vacuo* and the residue treated with ethanol to give thioisonicotinanilide (4 %, after crystallization from benzene), m.p. 180 – 183 °C (lit.[27] m.p. 181 – 182 °C). A control experiment with thioisonicotinamide alone showed that this was stable under the conditions employed.

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Reaction of Hydrazonyl Halides with Secondary and Tertiary Thioamides; Formation of 5-Amino- and 5-Alkoxy-1,3,4-thiadiazolines

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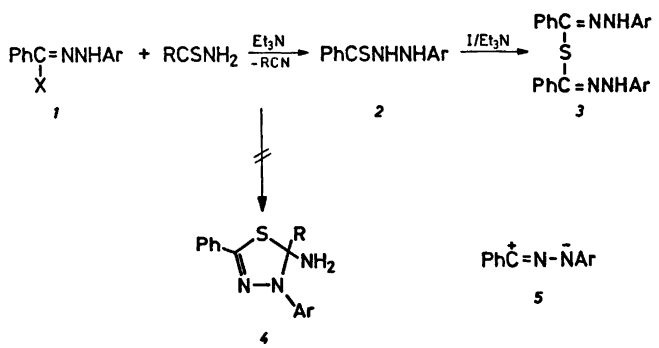
N-Arylbenzohydrazonyl bromides react with *N*-alkyl- and *N*,*N*-dialkylthiobenzamides, but not *N*-arylthiobenzamides, in the absence of base, while hydrazonyl chlorides require base (triethylamine) for reacting. Reactions with *N*-alkylthiobenzamides lead to a mixture of thiohydrazide, hydrazonyl sulfide, and 4-alkyl-1,3,5-triphenyl-1,2,4-triazolium halide. The latter is also formed when treating benzohydrazonyl halides with imidoyl halides in the presence of base. Reactions of benzohydrazonyl halides with *N*-aryl- and *N*,*N*-dialkylthiobenzamides lead to 5-anilino- and 5-dialkylamino-2,4,5-triaryl-1,3,4-thiadiazolines, respectively; alkoholysis of these compounds leads to the corresponding 5-alkoxy-1,3,4-thiadiazolines. Diphenylnitrilimine, generated by thermolysis of 2,5-diphenyltetrazole, adds to *N*-phenyl thiobenzamide to give 5-anilino-2,4,5-triphenyl-1,3,4-thiadiazoline, whereas cycloaddition of diphenylnitrilimine to *N*,*N*-dialkylthiobenzamides does not occur.

In a preceding paper [1] primary thioamides were shown to react with hydrazonyl halides (*1*) to give thiohydrazides (*2*), hydrazonyl sulfides (*3*), and nitriles by (formal) dehydrosulfurization of the thioamide. However, Huisgen and coworkers [2] have shown that tertiary thioamides, such as *N*,*N*-dimethylthiobenzamide, react differently with hydrazonyl halides, producing thiadiazolines.

We now report the results of an examination of the reactions of secondary and tertiary thioamides (*6*) with hydrazonyl halides (*1*) and diphenylnitrilimine (*5a*) (generated by thermolysis of 2,5-diphenyltetrazole [3]). This examination was undertaken to extend our knowledge of the reactions of hydrazonyl halides with the CS "double" bonds of thioacid derivatives, and to examine whether hydrazonyl halides in these systems react by a displacement mechanism or *via* 1,3-dipolar species, nitrilimines (*5*).[4]



Scheme 1.

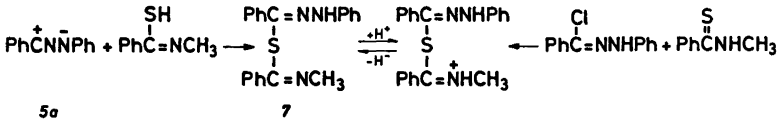
RESULTS AND DISCUSSION

The reactions of hydrazonyl halides with secondary and tertiary thioamides (Scheme 4) were found to be remarkably different, and the reactivity of the hydrazonyl halides was very dependent on the nature of the halogen atom. The reactions of *N*-alkyl- and *N,N*-dialkylthiobenzamides with hydrazonyl bromides proceed readily, whereas with hydrazonyl chlorides base (triethylamine) is required for reaction to take place; base is necessary for *N*-aryl-thiobenzamides to react in either case. It is not clear whether this difference is due to the higher relative reactivity of the hydrazonyl bromides, or if the two types of hydrazonyl halides react in part by different mechanisms.

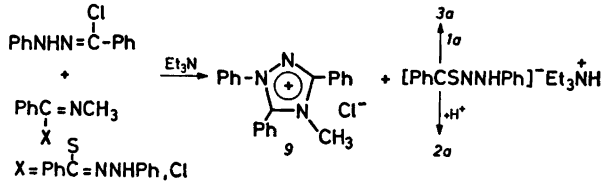
Reactions with N-*methylthiobenzamide.* Hydrazonyl halides react with *N*-methylthiobenzamide under a variety of conditions to give mixtures of thiohydrazides (*2*, detected by TLC), hydrazonyl sulfides (*3*) in yields varying from 20 to 60 %, and 4-methyl-1,3,5-triaryl-1,2,4-triazolium halides (*9*, see Scheme 3). Neither TLC nor NMR provided evidence for the formation of thiadiazolines (*cf.* Ref. 2). The reactions were sluggish and seldom went to completion. Even after prolonged periods of reaction considerable amounts of starting material were present and could be recovered. These findings may be rationalized as outlined in Schemes 2 and 3. The initial step is believed to be formation of a mixed hydrazonylimidoyl sulfide (*7*), which may arise either by displacement of the hydrazonyl halogen atom by the thioamide anion or the thioamide itself (to give a protonated form of the mixed sulfide), or by addition of the nitrilimine (*5*), formed *in situ* by dehydrohalogenation of *1*,[4] to the thiol form of the thioamide (see Scheme 2), in analogy to the reaction of nitrilimines with thiophenols.[3,5] The appearance of an absorption at 1680 cm^{-1} in the infrared spectrum of the reaction mixtures is compatible with the formation of the mixed sulfide, which may be regarded as an imidoyl pseudohalide.[6] Reaction between the hydrazonyl halide or the nitrilimine and the mixed sulfide (or its protonated form) then gives rise to the 1,2,4-triazolium ion and the thiohydrazide anion. The latter may react further with the hydrazonyl halide to produce the hydrazonyl sulfide (see Scheme 3), as discussed recently.[7] The plausibility of the above sequence of reactions is supported by our observation that imidoyl halides react with hydrazonyl halides to give 1,2,4-triazolium halides, *e.g.*, *N*-methylbenzimidoyl chloride reacts with *N*-phenylbenzohydrazonyl chloride (*1a*) and triethylamine (Scheme 3) to give 4-methyl-1,3,5-triphenyl-1,2,4-triazolium chloride. This reaction is analogous to the formation of 1,2,4-triazolines from nitrilimines and Schiff bases.[8]

Reactions with thiobenzanilide and tertiary thioamides. Thiobenzanilide and tertiary thioamides react with hydrazonyl halides in the presence of triethylamine to give 5-amino-2,4,5-triaryl-1,3,4-thiadiazolines (*10*),* which upon alcoholysis produce the corresponding 5-alkoxy-1,3,4-thiadiazolines (*11*, see Scheme 4).

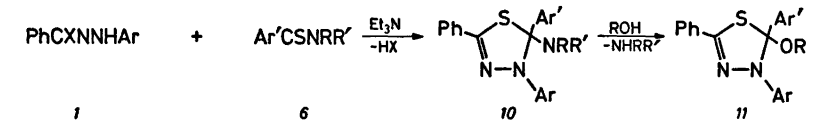
* For numbering see legend to Scheme 4.



Scheme 2.



Scheme 3.



1 6 10 11

X/Ar
a Cl/Ph
b Br/2-Cl-4-BrC_6H_3
c Br/2,4-$Br_2C_6H_3$

Ar'/R/R'
a Ph/H/CH_3
b Ph/H/Ph
c Ph/CH_3/CH_3
d 4-$CH_3OC_6H_4$/CH_3/CH_3
e Ph/-$(CH_2)_5$-
f 4-$CH_3OC_6H_4$/-$(CH_2)_5$-

Scheme 4. Individual 5-amino-thiadiazolines are designated in the text with a double index, *e.g. 10ac*, where the left index (*a*) identifies the starting hydrazonyl halide, and hence the N(4) aryl group, and the right index (*c*) identifies the starting thioamide, and hence the 5-substituents (*10ac* is 5-dimethylamino-2,4,5-triphenyl-1,3,4-thiadiazoline).

Neither thiohydrazides nor hydrazonyl sulfides were formed in these reactions.

N,N-Dimethylthiobenzamide displaces the bromine atom from hydrazonyl bromides, even in the absence of triethylamine, to give 5-dimethylaminothiadiazolines (observed by NMR); decomposition of these compounds by liberation of dimethylammonium bromide occurs in the reaction mixture, decreasing the isolated yield of *10* or, after alcoholysis, *11*.

It was not possible to isolate the 5-anilinothiadiazoline *10ab* from the reaction of thiobenzanilide; only *N'*-thiobenzoyl-*N*-benzoylphenylhydrazine [9] [PhCSNHN(COPh)Ph] was obtained. This compound is presumably formed from the 5-anilinothiadiazoline by elimination of aniline and ring opening. It has been found that the ring of 2,4,5-triaryl-1,3,4-thiadiazolium chlorides is opened by base to give thiohydrazides of the general formula ArCSNHN(COAr')-Ph;[10a] analogously, 2,4,5-triphenyl-1,3,4-oxadiazolium perchlorate reacts with sodium sulfide to give *N'*-benzoyl-*N*-thiobenzoylphenylhydrazine (PhCONHN(CSPh)Ph).[10b]

Of interest in this connection is a recent report [11] that hydrazonyl halides react with β-keto thioanilides in the presence of triethylamine, to give 5-ketonylidene-2,4-disubstituted-1,3,4-thiadiazolines by elimination of aniline from the initial ring closed product. Under more basic conditions a hydrazonyl sulfide was formed as a by-product; a thiohydrazide is most likely formed first by (formal) abstraction of hydrogen sulfide from the β-keto thioanilide; further reaction with the hydrazonyl halide then gives the sulfide (see also Ref. 8).

The 5-dimethylamino-2,4,5-triphenyl-1,3,4-thiadiazoline (*10ac*) suggested by Huisgen and coworkers [2] to be formed in the reaction of the hydrazonyl chloride *1a* with *N,N*-dimethylthiobenzamide and triethylamine was not isolated by these workers. Instead they obtained the corresponding 5-methoxythiadiazoline after methanolysis of the reaction mixture, and only indirect evidence was obtained for the intermediacy of *10*. Our results establish, in support of Huisgen's assumptions,[2] that a 5-dialkylamino group in thiadiazolines is readily replaced by an alkoxy group on treatment with alcohol (see Scheme 4), confirming that ring closure probably precedes displacement of the amino group.

Finally, the reactions of a secondary and a tertiary thioamide, thiobenzanilide and 4-methoxythiobenzpiperidide, respectively, with diphenylnitrilimine (*5a*) (generated by thermolysis of 2,5-diphenyltetrazole in refluxing bromobenzene) were examined. 1-Hexanol (10 %) was added to the bromobenzene to trap any 5-aminothiadiazoline formed. Thiobenzanilide reacts under these conditions (160 °C) to give a 60 % yield of 5-hexyloxy-2,4,5-triphenyl-1,3,4-thiadiazoline, while 4-methoxy-thiobenzpiperidide under similar conditions affords only 4-methoxythiobenzanilide in 14 % yield. The latter is presumably formed in a transamidation reaction preceded by reduction of either the tetrazole or the nitrilimine to aniline. These

results show that thiobenzanilide under these conditions acts as a good dipolarophile, in contrast to the thiobenzpiperidide.

CONCLUSION

The reaction of thioamides with hydrazonyl halides under basic conditions may be divided into those which occur by hydrogen sulfide abstraction leading initially to thiohydrazides, and those which occur by ring closure to thiadiazolines. Generally, primary thioamides,[1] including thioureas and thiosemicarbazide,[12] and certain secondary thioamides are capable of (formally) donating H_2S, and the reactions of these compounds lead to the formation of thiohydrazides and hydrazonyl sulfides. The reactions of tertiary thioamides always lead to thiadiazolines. Reactions of *N*-alkyl- and *N*,*N*-dialkylthiobenzamides deviate in two respects from those of other thioamides: they do not require base (triethylamine) to react with hydrazonyl bromides, and ring closure reactions with (thermally generated) diphenylnitrilimine to give thiadiazolines are not observed; this is also the case for primary thioamides. However, for thiobenzanilide base is required for reaction to take place, and in the reaction with diphenylnitrilimine thiadiazolines are formed.

EXPERIMENTAL

Materials. *N*-Methylthiobenzamide was prepared from carboxymethyl dithiobenzoate[13] and aqueous methylamine, m.p. 81–82 °C (ethanol) (lit.[14] m.p. 79–80 °C). *N*,*N*-Dimethyl-4-methoxythiobenzamide was prepared from carboxymethyl 4-methoxydithiobenzoate[12] and dimethylamine hydrochloride according to Huisgen *et al.*[2] in 94 % yield, m.p. 71–72 °C (abs. ethanol) (lit.[15] m.p. 68–70 °C).

The reactions of N*-methylthiobenzamide (*6a*) with* N*-aryl benzohydrazonyl halides (*1a *and* 1b*).* (i) Triethylamine (10.0 mmol) was added to a stirred solution of *1a*[16] (5.0 mmol) and *6a* (5.0 mmol) in benzene (25 ml). After 20 h the solution was filtered and the precipitate washed carefully with warm benzene, followed by evaporation of the filtrate. Addition of ethanol (5 ml) and filtration gave the sulfide *3a* (19 %), m.p. 158–160 °C (lit.[7] m.p. 158–160 °C). TLC of the residue showed the presence of unreacted *6a*.

(ii) Triethylamine (20.0 mmol) was added to a stirred solution of *1a* (10.0 mmol) and *6a* (5.0 mmol) in chloroform (50 ml). After 4 h the solvent was removed and the residue treated with boiling ethanol, leaving 58 % of the sparingly soluble sulfide *3a*. The filtrate was evaporated to dryness and taken up in ether-chloroform (4:1), filtered to remove insoluble material, and again evaporated to give an oil. An ^{1}H NMR spectrum ($CDCl_3$) of the oil showed the presence of a new compound with a singlet at δ 3.85, in addition to unreacted *6a*. The oil was redissolved in chloroform and addition of hexane caused white needles to precipitate. Recrystallization from chloroform-hexane (10:3) gave 230 mg of 4-methyl-1,3,5-triphenyl-1,2,4-triazolium chloride (*9*). This was identified by conversion to the known perchlorate: treatment of the chloride (230 mg) in ethanol (1 ml) with 4 drops of 70 % perchloric acid afforded an oil, which upon addition of ether crystallized as 4-methyl-1,3,5-triphenyl-1,2,4-triazolium perchlorate (82 %), m.p. 207–208 °C (after one crystallization from acetonitrile) (lit.[17] m.p. 206–208.5 °C).

(iii) Compound *1b*[18] (2.5 mmol) and *6a* (2.5 mmol) were dissolved together in chloroform (10 ml). After 3 days ethanol was added and the solution was taken to dryness. The solid was washed with ethanol leaving the sparingly soluble sulfide *3b* (20 %), m.p. 188–190 °C (lit.[18] m.p. 188–190 °C). TLC of the filtrate showed the presence of unreacted *6a*, but not 5-ethoxy-2,5-diphenyl-4-(2-bromo-4-chlorophenyl)-1,3,4-thiadiazoline.

(iv) In a similar experiment with triethylamine (5.0 mmol) and benzene (10 ml) as solvent the yield of *3b* was 16 %. TLC of the reaction mixture showed the presence of a small amount of the thiohydrazide *2b*.

Independent synthesis of compound 9. *N*-Methylbenzamide (10.0 mmol) and thionyl chloride (15 mmol) were refluxed together (steam bath) until no further evolution of HCl took place. The solution was evaporated under reduced pressure leaving *N*-methylbenzimidoyl chloride as an oil.[19] Triethylamine (20 mmol) and *1a* (10.0 mmol) were added to a solution of this oil in chloroform (10 ml). After 20 h the solvent was evaporated, the residue was taken up in ether-chloroform (2:1, 15 ml) and filtered to remove insoluble material. The filtrate was again taken to dryness and redissolved in ether–chloroform, filtered, followed by evaporation of the filtrate. Chloroform (5 ml) and hexane (2 ml) were added to give an oil which crystallized upon cooling. Recrystallization from chloroform–hexane (10:3) afforded 4-methyl-1,3,5-triphenyl-1,2,4-triazolium chloride. An IR spectrum was identical to that of *9*. Treatment of an ethanolic solution of the chloride with 70 % perchloric acid afforded the corresponding perchlorate salt, m.p. 207–209 °C.

*The reaction of thiobenzanilide (*6b*) with* N*-aryl benzohydrazonyl halides (*1a *and* 1c*).* (i) Triethylamine (21 mmol) was added to a solution

of *1a* (10.0 mmol) and thiobenzanilide [20] (10.0 mmol) in dry benzene (50 ml). After 2 days at room temperature the solution was filtered and evaporated *in vacuo*, leaving an oil, which crystallized by treatment with light petroleum (40 – 60 °C) and cooling (dry ice – acetone). The solid was treated with boiling ethanol to give after crystallization from ethanol 5-ethoxy-2,4,5-triphenyl-1,3,4-thiadiazoline in 91 % yield, m.p. 125 – 128 °C. Recrystallization raised the m.p. to 128 – 129 °C (lit.[2] m.p. 128.5 – 129.5 °C). Attempts to isolate *10ab* yielded small amounts of *N'*-thiobenzoyl-*N*-benzoylphenylhydrazine, m.p. 150 – 152 °C (chloroform) (lit. [9] m.p. 160 °C). MS, *m/e* 332 (M^+). (Anal. $C_{20}H_{16}N_2OS.\frac{1}{3}CHCl_3$: C, H, N, S).

(ii) Compound *1c* [21] and thiobenzanilide gave in a similar manner *5-ethoxy-2,5-diphenyl-4-(2,4-dibromophenyl)-1,3,4-thiadiazoline* in 50 % yield. Anal. $C_{22}H_{18}Br_2N_2OS$: C, H, N, S; m.p. 155 – 157 °C (ethanol-chloroform). In a similar experiment in which the triethylamine was left out no reaction was observed (NMR).

The reaction of N,N-*dimethylthiobenzamide with* N-*aryl benzohydrazonyl halide* (1b). Compound *1b* (2.5 mmol) and *N,N*-dimethylthiobenzamide [2] (2.5 mmol) were dissolved in dry chloroform (5 ml). After 20 h ethanol (5 ml) was added, followed by evaporation. Crystallization from ethanol afforded 33 % of *5-ethoxy-2,5-diphenyl-4-(2-bromo-4-chlorophenyl)-1,3,4-thiadiazoline*. Anal. $C_{22}H_{18}BrClN_2OS$: C, H, N, S; m.p. 146 – 148 °C. NMR spectra of the reaction mixture in $CDCl_3$ showed the formation of 5-dimethylaminothiadiazoline (*10bc*), δ 2.44 [$N(CH_3)_2$], together with dimethylammonium ions (identified by the addition of a few crystals of dimethylamine hydrochloride to the NMR sample).

In a similar experiment with *1a* no reaction had taken place even after 48 h (NMR).

5-Dimethylamino-2,4,5-triphenyl-1,3,4-thiadiazoline (10ac). Triethylamine (10.7 mmol) was added to a solution of *1a* (4.00 mmol) and *N,N*-dimethylthiobenzamide (4.05 mmol) in dry benzene (10 ml). After 2 days at room temperature the solution was filtered and the solvent removed *in vacuo*. TLC showed that the sulfide *3a* was not present in the reaction mixture. Crystallization was induced with light petroleum (40 – 60 °C) and cooling (dry ice-acetone). Recrystallization from light petroleum yielded the title compound as yellow crystals in 84 % yield. Anal. $C_{22}H_{21}N_3S$: C, H, N, S; m.p. 103 – 105 °C.

5-Dimethylamino-2,5-diphenyl-4-(2,4-dibromophenyl)-1,3,4-thiadiazoline (10cc) was prepared in a similar manner from *1c* and *N,N*-dimethylthiobenzamide in 81 % yield. Anal. $C_{22}H_{19}Br_2N_3S$: C, H, N, S; m.p. 128 – 138 °C (hexane).

The product from the reaction of *1a* with *N,N*-dimethyl 4-methoxythiobenzamide was not crystallized, but treated crude with ethanol to give *5-ethoxy-2,4-diphenyl-5-(4-methoxyphenyl)-1,3,4-thiadiazoline* in 83 % yield. Anal. $C_{23}H_{22}N_2O_2S$: C, H, N, S; m.p. 109 – 110 °C (ethanol).

5-Piperidino-2,4,5-triphenyl-1,3,4-thiadiazoline (10ae). Triethylamine (21 mmol) was added to a solution of *1a* (10.0 mmol) and thiobenzpiperidide [13] (10.0 mmol) in dry benzene (50 ml). After 2 days at room temperature the solution was filtered and the solvent removed *in vacuo*. The resulting oil was brought to crystallization with light petroleum (40 – 60 °C). Yield after crystallization from benzene or ethanol 82 %. (Anal. $C_{25}H_{25}N_3S$: C, H, N, S), m.p. 121 – 130 °C (ethanol). A mass spectrum showed only little contamination with the corresponding 5-ethoxy compound. Treatment with boiling propanol gave *5-propoxy-2,4,5-triphenyl-1,3,4-thiadiazoline* m.p. 113 – 115 °C. (Anal. $C_{23}H_{22}N_2OS$: C, H, N, S).

5-Piperidino-2,4-diphenyl-5-(4-methoxyphenyl)-1,3,4-thiadiazoline (10af) was prepared in a similar manner from *1a* and 4-methoxythiobenzpiperidide [13] in 80 % yield. Anal. $C_{26}H_{27}N_3OS$: C, H, N, S; m.p. 126 – 129 °C (light petroleum).

Reactions of thiobenzanilide and 4-methoxythiobenzpiperidide with 2,5-diphenyltetrazole in refluxing bromobenzene – hexanol. The thioamide (5.0 mmol) and 2,5-diphenyltetrazole [3] (5.0 mmol) were refluxed together in a mixture of bromobenzene (25 ml) and hexanol (2 ml) for 5 h. The solvent was removed and ethanol was added and the solution left for evaporation at room temperature. Thiobenzanilide gave *5-hexyloxy-2,4,5-triphenyl-1,3,4-thiadiazoline* in 58 % yield, m.p. 74 – 75 °C (ethanol). (Anal. $C_{26}H_{28}N_2OS$: C, H, N, S). In a similar experiment 4-methoxythiobenzpiperidide gave 4-methoxythiobenzanilide (14 %), m.p. 155 – 158 °C (lit.[22] m.p. 153 – 154 °C).

All 5-aminothiadiazolines decomposed on storage at room temperature. The 5-amino- and 5-alkoxythiadiazolines all exhibited an infrared absorption at 1558 cm^{-1} as described in the literature;[2] the 2,4,5-triaryl-1,3,4-thiadiazolines were further characterized by mass spectrometry.[23]

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Crystal and Molecular Structure of Clonidine Hydrochloride, 2-(2,6-Dichlorophenylamino)-2-imidazoline Hydrochloride

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The crystal structure of the title compound has been determined by X-ray diffraction methods using 3209 observed reflections collected on a counter diffractometer. The crystals are monoclinic, space group $C2/c$ with unit cell dimensions $a=17.957(2)$ Å, $b=11.950(1)$ Å, $c=13.664(1)$ Å and $\beta=128.64(1)°$. The structure was refined to a conventional R-factor of 0.040, the standard deviations for bond lengths and angles involving non-hydrogen atoms were 0.002 Å and 0.1°, respectively.

The planes of the phenyl ring and the imidazoline ring form an angle of 75°; the structure of the molecule may thus to a certain extent be compared to that of phenylethylamines. Bond orders and charge distribution based on CNDO-calculations are discussed, as is the hydrogen bonding in the crystal.

2-(2,6-Dichlorophenylamino)-2-imidazoline (clonidine) is an effective hypotensive drug with action similar to that of methyl-DOPA.[1,2] Several studies seem to indicate that clonidine acts as an agonist on central as well as peripheral α-adrenergic receptors.[3,4]

At this laboratory we have for some time been studying structures of compounds active in adrenergic systems [5–8] and it was considered to be of interest to compare the structure of clonidine to those of the earlier determinations as well as to the model structure discussed by Wermuth.[9]

EXPERIMENTAL

A sample of clonidine hydrochloride kindly given to us by Boehringer-Ingleheim Ltd. was supplied as colourless, nearly hexagonal prismatic crystals which could be used for the X-ray experiments without recrystallization. The specimen selected for data collection was 0.30 mm along the prism edge and 0.35 mm along the diagonals of the hexagon. Data were collected on a SYNTEX P1 four-circle diffractometer using graphite crystal monochromated Mo$K\alpha$ radiation ($\lambda=0.71069$ Å).

The crystals are monoclinic; systematic absences are $h+k$ odd for (hkl) and l odd for $(h0l)$ and the space group is thus Cc or $C2/c$. Cell parameters were determined by a least-squares fit to the diffractometer settings for 15 general reflections.

Intensity data were recorded using the $\theta/2\theta$ scanning mode with a scan speed (2θ) of 2 – 8° min^{-1} depending on the intensity; the scan range was from 0.8° below $2\theta(\alpha_1)$ to 0.9° above $2\theta(\alpha_2)$ and background counts were taken for 0.35 times the scan time at each of the scan limits. All unique reflections with $\sin\theta/\lambda < 0.54$ $Å^{-1}$ were measured, reflections in the interval 0.54 $Å^{-1}$ $< \sin\theta/\lambda < 0.80$ $Å^{-1}$ were measured only if a quick scan gave an intensity larger than a preset value. Out of the 4218 reflections recorded, 3209 with $I > 2\sigma(I)$ were retained for the structure analysis. The standard deviations for the intensities were calculated by $\sigma(I)=[C_T+(0.02\ C_N)^2]^{\frac{1}{2}}$ where C_T is the total number of counts and C_N is the scan count minus background count. The usual corrections were made for Lorentz and polarization effects and also for absorption.

Scattering factors used were those of Doyle and Turner for Cl, Cl^-, N and C,[10] and of Stewart, Davidson and Simpson for H.[11] Descriptions of the computer programs used are given in Refs. 12 and 13. The quantity minimized in the least-squares calculations was $\sum W\Delta F^2$ where W is the inverse of the variance of the observed structure factors.

CRYSTAL DATA

2-(2,6-Dichlorophenylamino)-2-imidazoline hydrochloride (clonidine, HCl), $C_9H_9N_3Cl_2.HCl$, monoclinic, $a=17.957(2)$ Å; $b=11.950(1)$ Å;

$c = 13.664(1)$ Å; $\beta = 128.64(1)°$; ($t = 18 \pm 1$ °C); $V = 2290.2$ Å; $M = 266.56$; $Z = 8$; $F(000) = 1088$; $D_{calc} = 1.546$ g cm^{-3}; $\mu = 7.67$ cm^{-1}. Absent reflections: (hkl) for $h+k$ odd, ($h0l$) for l odd. Space group $C2/c$ (No. 15).

STRUCTURE DETERMINATION

Intensity statistics indicated the presence of centres of symmetry; the space group was thus assumed to be $C2/c$. The structure was solved by direct methods using the program assembly MULTAN.[13] Phases were determined for 300 reflections with $E > 1.56$; an E-map based on the set with the highest absolute figure of merit (1.06) revealed the positions of the three chlorine atoms and a few of the lighter atoms. The remaining non-hydrogen atoms were localized by the use of successive Fourier refinements; the positions of the hydrogen atoms were calculated from stereochemical considerations after a preliminary least-squares refinement. All positional parameters, anisotropic thermal parameters for non-hydrogen atoms, and isotropic thermal parameters for hydrogen atoms were refined by least-squares methods to a conventional R-factor of 0.04 and a goodness of fit, $S = (\sum W\Delta^2/m-n)^{\frac{1}{2}}$, of 2.02. A repeated least-squares calculation where the weights for reflections with $\sin\theta/\lambda < 0.55$ Å^{-1} were multiplied by a factor varying from 0 to 1 as $\sin\theta/\lambda$ increases from 0 to 0.55 Å^{-1} gave a conventional R-factor of 0.040, $R_w = 0.045$, whereas S decreased to 1.59.

The final parameters are listed in Table 1. Tables of observed and calculated structure factors are available from the authors.

The anisotropic thermal parameters were analysed in terms of rigid-body motion both for the organic ion as a whole and separately for the dichlorophenylamine part and the aminoimidazoline part. The results indicated that the latter description justified correction of bond lengths for libration effects.

DISCUSSION

Selected interatomic distances and bond angles are listed in Table 2. Estimated standard deviations calculated from the correlation matrix were about 0.002 Å for distances and 0.1° in angles involving non-hydrogen atoms. Fig. 1 shows the conformation, thermal ellipsoids and bond lengths corrected for libration.

C–H bonds were found in the range 0.91–0.94 Å (mean 0.92 Å) in the phenyl group and 0.92–1.01 Å (mean 0.96 Å) in the imidazoline ring; N–H bonds were found to be 0.79–0.88 Å (mean 0.84 Å).

The dichlorophenyl moiety has a quite normal geometry with the average C–C bond length of 1.389 Å and C–Cl bond length of 1.734 Å. The internal valence angles are slightly exceeding 120° at carbon atoms to which the electronegative chlorine atoms are attached. The imidazoline part is protonized and the two nitrogen atoms are thus chemically equivalent. It has nearly mm symmetry, the C7–N2 and

Table 1. Fractional atomic coordinates and thermal parameters with estimated standard deviations. The temperature factor is given by $\exp-(2\pi^2(U_{11}a^{*2}h^2+\ldots+2U_{12}a^*b^*hk+\ldots))$.

ATOM	x	y	z	U11	U22	U33	U12	U13	U23
CL1	.38246(3)	.04499(3)	.28877(4)	.0394(2)	.0291(2)	.0413(2)	-.0014(1)	.0233(2)	-.0026(1)
CL2	.11145(3)	.09576(4)	.27312(4)	.0348(2)	.0468(2)	.0492(2)	-.0024(2)	.0165(2)	-.0024(2)
CL3	.38934(5)	.39215(5)	.41592(7)	.0743(4)	.0475(3)	.0991(5)	-.0143(2)	.0552(4)	.0041(3)
N1	.19141(12)	.30305(11)	.24426(13)	.0576(8)	.0293(6)	.0376(6)	.0141(6)	.0275(6)	.0061(5)
N2	.18866(12)	.18249(13)	.18596(13)	.0538(8)	.0439(7)	.0329(6)	.0219(6)	.0286(6)	.0064(5)
N3	.09896(12)	.33221(14)	.02918(14)	.0592(9)	.0438(7)	.0424(7)	.0246(7)	.0289(7)	.0163(6)
C1	.25683(11)	.23848(12)	.35331(13)	.0406(7)	.0294(6)	.0302(6)	.0056(5)	.0207(6)	.0013(5)
C2	.22852(16)	.14182(13)	.37769(13)	.0333(6)	.0336(6)	.0318(6)	.0029(5)	.0166(5)	-.0011(5)
C3	.29184(12)	.07879(14)	.48484(16)	.0426(8)	.0398(7)	.0378(7)	.0045(6)	.0232(7)	.0068(6)
C4	.38521(12)	.11311(16)	.57002(15)	.0386(8)	.0540(10)	.0307(7)	.0096(7)	.0164(6)	.0061(7)
C5	.41519(12)	.20938(17)	.54939(16)	.0338(7)	.0539(9)	.0386(8)	-.0015(7)	.0176(6)	-.0077(7)
C6	.35144(12)	.27139(14)	.44185(16)	.0443(8)	.0358(7)	.0463(8)	-.0028(6)	.0294(7)	-.0045(6)
C7	.16182(11)	.27365(13)	.13132(14)	.0393(7)	.0308(6)	.0363(7)	.0087(5)	.0226(6)	.0074(5)
C8	.13999(17)	.17429(18)	-.02736(17)	.0715(12)	.0546(11)	.0362(8)	.0172(9)	.0292(9)	.0051(7)
C9	.07643(15)	.27859(19)	-.08141(17)	.0548(10)	.0589(11)	.0363(8)	.0153(9)	.0244(8)	.0132(7)

ATOM	x	y	z	B	ATOM	x	y	z	B
HC3	.271	.014	.496	4.1	HC4	.428	.072	.644	4.1
HC6	.476	.236	.603	4.1	H1C8	.188	.107	-.058	5.9
H2C8	.182	.174	-.049	5.9	H1C9	.089	.323	-.127	5.9
H2C9	.007	.255	-.148	5.9	HN1	.182	.373	.254	4.7
HN2	.236	.146	.163	4.7	HN3	.972	.386	.027	4.7

Table 2. Distances (Å) and angles (°) in the crystals of clonidine hydrochloride.

Bond	Length	Bond angles	
		C6 – C1 – C2	117.3
C1 – C2	1.391	C1 – C2 – C3	121.5
C2 – C3	1.382	C2 – C3 – C4	119.8
C3 – C4	1.377	C3 – C4 – C5	120.2
C4 – C5	1.371	C4 – C5 – C6	119.8
C5 – C6	1.385	C5 – C6 – C1	121.4
C6 – C1	1.392	C1 – C2 – Cl2	120.0
C1 – N1	1.418	C3 – C2 – Cl2	118.5
C2 – Cl2	1.733	C1 – C6 – Cl3	118.9
C6 – Cl3	1.724	C5 – C6 – Cl3	119.7
N1 – C7	1.328	C2 – C1 – N1	121.4
N2 – C7	1.322	C6 – C1 – N1	121.3
N3 – C7	1.321	C1 – N1 – C7	123.0
N2 – C8	1.450	N1 – C7 – N3	123.1
N3 – C9	1.447	N1 – C7 – N2	125.2
C8 – C9	1.533	C7 – N2 – C8	110.6
		N2 – C8 – C9	103.5
		C8 – C9 – N3	102.6
		C9 – N3 – C7	111.5
		N2 – C7 – N3	111.8

Torsional angles
(positive for a clockwise rotation)

C2 – C1 – N1 – C7	– 76.5
C1 – N1 – C7 – N2	0.0
C1 – N1 – C7 – N3	178.1
C6 – C1 – N1 – C7	105.2

Hydrogen bonds

Cl1 – N1($\frac{1}{2}-x,-\frac{1}{2}+y,\frac{1}{2}-z$) (Å)	3.094
Cl1 – HN1 (Å)	2.25
Cl1 – HN1 – N1 (°)	161.2
Cl1 – N2(x,y,z) (Å)	3.193
Cl1 – HN2 (Å)	2.38
Cl1 – HN2 – N2 (°)	163.4

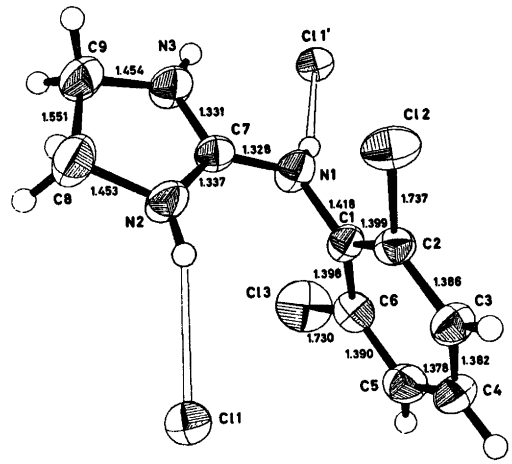

Fig. 1. Perspective view of the molecule showing bond lengths corrected for libration. Hydrogen bonds are indicated. Non-hydrogen atoms are represented by thermal ellipsoids defined by the principal axes of thermal vibration and scaled to include 50 % probability.

C7 – N3 bond lengths being 1.337 Å and 1.331 Å, respectively, and the N2 – C8 and N3 – C9 both close to 1.454 Å. The arrangement of bonds about the bridging atom, N1, is nearly planar with a C1 – N1 – C7 angle of 123.0°.

In order to determine bond orders and net charges on the atoms CNDO calculations were performed for the free organic ion.[14] The π bond order for the three C7 – N bonds is 0.55, for the C1 – N1 bond 0.19, and for the N2 – C8 and N3 – C9 bonds 0.17. The main part of the positive charge is situated on the C7 atom (+ 0.45) and on the three hydrogen atoms bonded to nitrogen (+ 0.17).

The atoms of the six-membered ring are strictly coplanar, as are the atoms of the five-membered ring. The chlorine atoms are situated 0.01 – 0.02 Å out of the benzene plane; the N1 atom is situated 0.02 Å out of the benzene plane and 0.05 Å out of the imidazoline ring plane. The angle between the ring planes is 75°.

The structure of the phenyl-N1 – C7 – N3 part of the molecule is strikingly similar to that of the phenylethylamines previously investigated. The sympathomimetic amines of this kind showing adrenergic activity have without exception been found to crystallize in a conformation with a fully extended side chain. The atoms of the side chain are thus close to a plane which is normally found to be nearly perpendicular to that of the phenyl ring. Wermuth *et al.* propose a structure with the characteristic measures $D = 5.0 - 5.1$ Å (distance from the phenyl ring centre to the projection of the N3 position on the phenyl ring plane) and $H = 1.28 - 1.36$ Å (distance from N3 to the phenyl ring plane); this is in accordance with the figures given by Pullmann *et al.*[15] for favourable distances in α-sympathomimetic amines. The distances for clonidine are slightly overestimated, however, the actual numbers found in the present investigation being $D = 4.89$ Å and $H = 1.02$ Å. Results from previous investigations for 6-hydroxydopamine,[5] 5-hy-

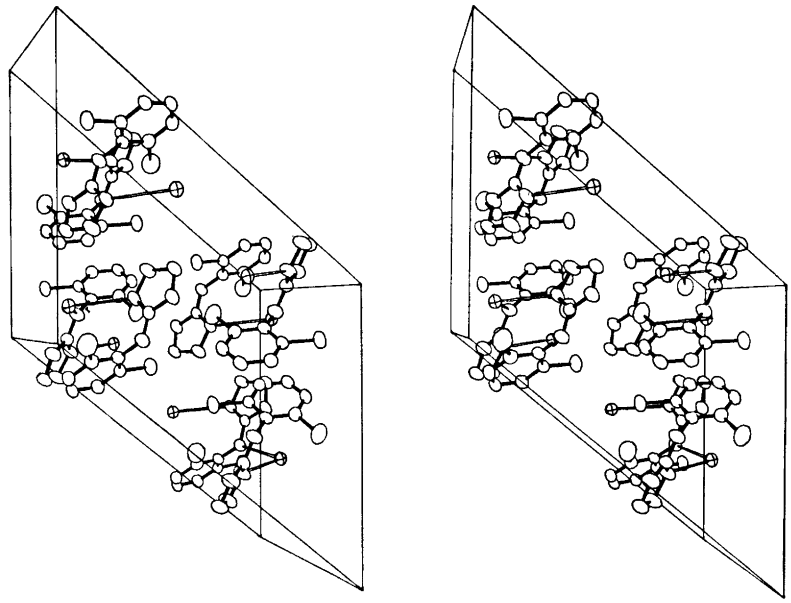

Fig. 2. Stereoscopic view of the crystal structure of clonidine.

droxydopamine,[6] adrenaline,[7] and noradrenaline[8] give D in the range 4.89 – 5.00 Å and H from 1.39 to 1.43 Å.

A stereoscopic drawing of the crystal structure is shown in Fig. 2. There is a strong hydrogen bond between N1 and a chlorine ion (3.094 Å) and a weaker between N2 and another chlorine ion (3.193 Å); N3 is not engaged in hydrogen bonding. Correspondingly, N1 – HN1 was found to be the longest of the N – H bonds and N3 – HN3 to be the shortest of the three.

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Copper(I) Catalysed Reactions between Hydrazines and Isocyanides

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The reaction between isocyanides and hydrazine, *N,N*-disubstituted hydrazines, and trisubstituted hydrazine catalysed by copper(I) chloride has been investigated.

Cyclohexyl isocyanide and *N,N*-disubstituted hydrazines form amidrazones in good yield in a rather slow process. Aromatic isocyanides give formamidrazones in good yield when reacted with trisubstituted hydrazine, while decomposition reactions occur in reactions with *N,N*-disubstituted hydrazines giving complex mixtures. The components of these mixtures have been identified by GLC or GLC-MS. They consist of amines, amidines and amidrazones. The amines corresponding to the isocyanides are formed in good yields in all reactions between aromatic isocyanides and hydrazine or unsymmetrical disubstituted hydrazines.

The formamidrazones prepared have been identified by means of IR, ^{1}H and ^{13}C NMR spectroscopy.

Isocyanides are known to participate in α-addition reactions with compounds such as thiols, alcohols and amines.[1–4] These reactions are catalysed by metal ions, forming derivatives of formamidic acid in high yields. Although a variety of such reactions has been studied, reactions with more complex nitrogen containing compounds such as hydrazones or hydrazines do not appear to have been studied. The reaction between isocyanides and hydrazines may be more complex than the analogous reaction with amines, owing to the very different redox properties and thermal stability of amines and hydrazines. An investigation of this reaction therefore seemed of interest as an extension of the study of the α-addition reactions.

A reaction between isocyanides and di- or trisubstituted hydrazines following a simple α-addition mechanism would give formamidrazones as products.

$$RNC + R'R''NNHR''' \rightarrow RNCHNR'''NR'R''$$

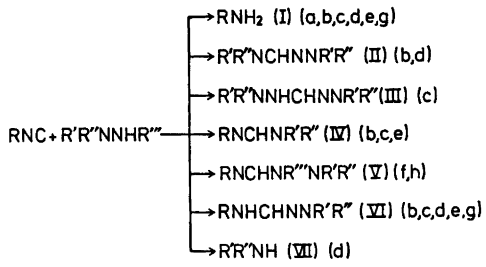
Formamidrazones are often formed in rather poor yields by other synthetic methods.[5–7] In the present investigation formamidrazones were formed in yields varying from 0 to 80 % from the reaction between isocyanides and hydrazines. In addition a variety of other compounds were formed, often in mixtures, from which it was impossible to isolate the different components. The main reaction products were identified by GLC-MS, or the compounds were isolated and identified by their MS, IR and NMR spectral data.

RESULTS

Reaction between aromatic isocyanides and hydrazines (reactions a–f). The reactions were carried out by refluxing isocyanide, hydrazine and CuCl without solvent, until the infrared spectrum from the reaction mixture showed no absorption around 2100 cm^{-1}. The mixture was subsequently distilled and the components isolated, or the composition was determined by GLC or GLC-MS.

In each reaction with unsubstituted hydrazine or *N,N*-disubstituted hydrazines, the main product was the amine corresponding to the isocyanide. Scheme 1 represents the identified products (I–VII) from the reactions (a–h). The product distributions found are summarized in Table 1.

The presence of CuCl in the reaction mixture not only influenced the reaction time (the time



Scheme 1. Identified products from the reactions between isocyanide and hydrazines. (Reaction; R,R',R'',R'''): (a; Ph,H,H,H); (b; Ph,Me,Me,H) (c; Ph,Et,Et,H); (d; Ph,Me,Ph,H); (e; *p*-$CH_3C_6H_4$, Me,Me,H); (f; Ph,Me,Me,Me); (g; C_6H_{11},Me,Me,H); (h; C_6H_{11},Me,Ph,H).

required until the isocyanide absorption had disappeared from the IR spectrum of the reaction mixture) but also the product distribution (*cf.* Table 1).

No obvious dependence on the amount of catalyst was found in the reaction between phenyl isocyanide and *N*,*N*-dimethylhydrazine carried out with CuCl amounts varying from 0.075 to 0.6 mmol (see experimental part).

It is interesting to note that the reaction between *N*-phenylthioformamide and *N*,*N*-diethylhydrazine which was attempted for the preparation of amidrazone (VI c) gave products analogous to those found in the reaction between phenyl isocyanide and *N*,*N*-diethylhydrazine

$$PhNHCHS + Et_2NNH_2 \rightarrow PhNH_2 +$$

$$PhNCHNEt_2 + PhNHCHNNEt_2$$

(IVc) (VIc)

The evolution of hydrogen sulfide and the formation of elemental sulfur was detected. A similar reaction between *O*-ethyl thioformate and *N*,*N*-dimethylhydrazine has been reported by Walter *et al.*[5]

Reaction between aliphatic isocyanides and hydrazines (g–j). The reactions of aliphatic isocyanides and hydrazines differed greatly from those of aromatic isosyanides and hydrazines in reactivity and product distribution. The reaction time was much longer, up to 150 h, and no amine corresponding to the isocyanide was formed. The product arising from α-addition was formed in appreciable yield.

Table 1. Product distribution [a] from the reaction RNC + R'R''NNHR'''. Ratio RNC/R'R''NNHR''' 1/3 and CuCl/RNC 1.5/100.

Reaction	R	R'	R''	R'''	Reaction time/h	RNH_2/%	RNCHNR'''NR'R''/%	RNCHNR'R''/%	R'R''NCHNNR'R''/%
a [b]	Ph	H	H	H	1	38	–	–	–
a	Ph	H	H	H	1(2.5) [f]	70(59) [f]	–	–	–
b	Ph	Me	Me	H	1.5(6) [f]	60(25) [f]	9	27	25
b [b]	Ph	Me	Me	H	1.5(48) [f]	60(25) [f]	0.1	25	–
c [c]	Ph	Et	Et	H	1.5	58	35	7	–
d [d]	Ph	Me	Ph	H	1	38	–	–	21
e	*p*-MeC_6H_4	Me	Me	H	2	60	4	28	–
f [b]	Ph	Me	Me	Me	2	–	80	–	–
f	Ph	Me	Me	Me	2	–	70	–	–
g	C_6H_{11}	Me	Me	H	150	> 3	70	–	–
h [e]	C_6H_{11}	Me	Ph	H	3	< 3	–	–	–
i	C_6H_{11}	Me	Me	Me	350	no reaction			
j	*t*-Bu	Me	Me	H	350	no reaction			

[a] Based on gas chromatographic analyses of the distilled mixtures (± 10 %). [b] Ratio RNC/R'R''NNHR''' 1/1. [c] 5 % of $(Et)_2NNHCHNN(Et)_2$ was isolated. [d] 40 % of PhNHMe was identified. [e] 50 % of PhNHMe was identified. [f] Parentheses indicate data without CuCl catalyst.

Reactions with hydrazones. Two reactions were carried out with phenyl isocyanide and hydrazones (benzophenone hydrazone and acetophenone hydrazone). The reaction time was longer than that found for reactions with hydrazines. From the reaction with benzophenone hydrazone N^2,N^4-bis-(diphenylmethylene)formohydrazide hydrazone (VIII) was isolated (10 % yield).

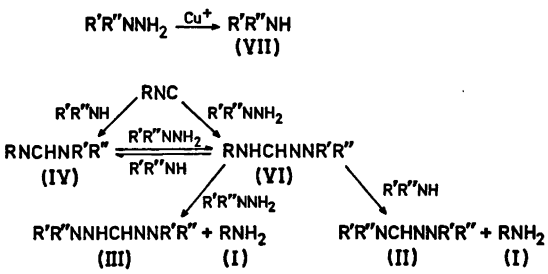
$$(Ph)_2CNNH_2 + PhNC \rightarrow (Ph)_2CNNHCHNN(Ph)_2$$
(VIII)

DISCUSSION

For the reaction between aliphatic isocyanides and hydrazines and the reaction between aromatic isocyanides and trimethylhydrazine, for which the α-addition product is formed in good yield, it seems reasonable to propose that the reaction mechanism is analogous to those proposed for reaction between isocyanides and amines [2] or alcohols.[9] The two possibilities are a reaction in the coordination sphere of an isocyanide – hydrazine – copper complex, or a nucleophilic attack of hydrazine on a copper – isocyanide complex.

Very little is reported about complexes with dialkylhydrazines as ligands.[10] From the long reaction time found and from the lack of reaction in cases where reaction with amines proceeds readily, it seems reasonable to conclude that for the reaction with aliphatic isocyanides, dialkylhydrazines are less efficient nucleophiles or less efficient in coordination to copper than amines. The difference in reaction time found for the reaction between aromatic isocyanides with trisubstituted hydrazines compared to that of aliphatic isocyanides with disubstituted hydrazines might be explained by the difference in strength of the copper-isocyanide complex. Aromatic isocyanides show weak new IR-isocyanide stretch absorptions while in the case of aliphatic isocyanide the absorption of the Cu-isocyanide complex is medium to strong after short time reflux.[11]

In the reaction between aromatic isocyanides and *N*,*N*-disubstituted hydrazines new reactions occur, compared to the analogous reactions with aliphatic isocyanides. Only small amounts of amidrazone from simple α-addition are found, but amidines, amines and amidrazones from more complex reactions are found.



Scheme 2.

This complexity is possibly due to the aromatic isocyanides forming weaker complexes with copper than the aliphatic isocyanides under the conditions used.

In the former case greater amount of metal ion is accessible for the hydrazine, causing cleavage of the NN-bond with formation of secondary amine.[12,13] The products formed thus arise from two competing α-addition reactions of the isocyanide (with amine or hydrazine, respectively) and possible further reactions of the first formed products/complexes. A reaction scheme, including the reaction products found, is presented as Scheme 2.

GLC measurements on reaction b shows that formation of aniline proceeds during the reaction. The lack of *N*,*N'*-diphenylformamidine formation indicates that the reaction between aromatic isocyanides and aniline proceeds slower than the reactions with secondary amine and *N*,*N*-disubstituted hydrazine.

Recently Neunhoeffer *et al.*[14] reported the formation of *N*-unsubstituted hydrazidines from reactions between amidrazones and hydrazine.

Amine formation from reactions with isocyanides has previously only been reported [8] in reactions between *t*-butyl isocyanide and aminoalcohols.

The difference in product distribution between reaction with *N*-methyl-*N*-phenylhydrazine and those with *N*,*N*-dimethylhydrazine (Table 1) can be ascribed to the great lability of the NN-bond in the former, especially when subjected to heating.[13]

EXPERIMENTAL

Microanalyses were carried out in the microanalysis department of Chemical Laboratory II, the H. C. Ørsted Institute. ^{1}H NMR spectra

were obtained on a JEOL JNM MH 60/II instrument with TMS as internal reference. IR spectra were recorded on a Perkin-Elmer model 225 grating spectrograph. Mass spectra were taken on a Finnigan 1015 S/L or an AEI-902 instrument operating at 70 eV. ^{13}C NMR spectra were recorded on a Bruker WH 90 instrument. Melting points were taken on a Büchi melting point apparatus and are uncorrected.

GLC-analyses were carried out on a Perkin-Elmer F 11 gas chromatograph. Columns: Chromosorb 103 or 2 % neopentyl glycol succinate on Chromosorb G (80 – 100 mesh). GLC-MS analyses were carried out on the Finnigan instrument. Columns: 10 % SE 30 on Chromosorb W HMDS (100 – 120 mesh) or 10 % neopentyl glycol succinate on Diatomite CQ (100 – 120 mesh).

Isocyanides were prepared according to the literature.[14]

Reaction a. Phenyl isocyanide (0.1 mol), hydrazine (95 %, 0.1 mol) and CuCl (1.5 mmol) were mixed with external cooling; when the exothermic reaction ceased the mixture was refluxed for 1 h. Distillation *in vacuo* resulted in 3.6 g of aniline identified by IR, ^1H NMR and GLC.

Reaction b. (1) Phenyl isocyanide (0.1 mol), *N,N*-dimethylhydrazine (0.3 mol) and CuCl (1.5 mmol) were refluxed for 1.5 h. The mixture was subsequently distilled. Fraction (1) b.p. 20 – 100 °C, 760 mmHg, fraction (2) b.p. 68 – 108 °C, 10 – 1.2 mmHg. The composition of the fractions was determined by GLC on a Chromosorb 103 column using authentic samples as reference compounds (see below); *ca.* 50 % of the *N,N*-dimethylhydrazine was recovered. The product distribution of the other compounds can be seen from Table 1.

(2) Phenyl isocyanide (0.01 mol), *N,N*-dimethylhydrazine (0.03 mol) and CuCl (see below) were mixed as described under (1); the products were identified by GLC on a neopentyl glycol succinate column using authentic samples as reference compounds.

The alteration in product distribution between aniline, N^3,N^3-dimethyl-N^1-phenylformamide hydrazone and N^1,N^1-dimethyl-N^2-phenylformamidine are summarized below.

mmol CuCl	0.075	0.15	0.3	0.6
% aniline	70	74	82	79
% amidine (IV b)	10	13	6	7
% amidrazone (VI b)	17	12	11	13

N^3,N^3-*Dimethyl*-N^1-*phenylformamide hydrazone (VI b).* (1) Phenyl isocyanide (0.2 mol), *N,N*-dimethylhydrazine (0.6 mol) and CuCl (0.3 mmol) were refluxed for 1.5 h. The mixture was filtered and distilled twice *in vacuo*, b.p. 77 – 78 °C/0.5 mmHg. The distillate crystallised on standing at 0 °C, m.p. 49 – 50 °C, yield 2.1 g (6 %). The spectroscopic data are in accordance with those of an authentic sample.

(2) *N*-Phenylthioformamide[17] (0.05 mol) and *N,N*-dimethylhydrazine (0.05 mol) were refluxed in abs. ethanol (60 ml) for 8 h. After cooling the solvent was evaporated and the residue was distilled *in vacuo* yielding 4.2 g (52 %) liquid (b.p. 71 – 72 °C, 0.15 mmHg) which crystallized on cooling. Recrystallization from hexane, m.p. 49 – 50 °C. Anal. $C_9H_{13}N_3$: C, H, N. MS *m/e* (% of base peak): 164(11), 163(100)M$^+$, 119(13), 106(27), 104(20), 93(67), 77(24), 65(14), 60(67), 59(17), 45(12), 44(23), 42(14) ^{13}C NMR ($CDCl_3$): δ 145.8, 139.8, 129.6, 122.1, 115.5, 46.9. ^1H NMR data are in accordance with literature.[6]

N^1,N^1-*Dimethyl*-N^2-*phenylformamidine (IV b)* was prepared as described.[15] The mass spectrum found was identical with that described in the literature.[16] ^{13}C NMR ($CDCl_3$): δ 153.2, 152.2, 128.9, 122.2, 121.1, 36.2

N,N,N″,N″-*Tetramethylformamidrazone (II b).* *N,N*-Dimethylformamide dimethyl acetal (0.1 mol) was dissolved in methanol (10 ml), *N,N*-dimethylhydrazine (0.1 mol) was added, and the mixture stirred at room temperature for 0.5 h and at reflux temperature for 1 h. B.p. 141 – 143 °C, 760 mmHg, yield 51 %.[7] ^1H NMR ($CDCl_3$): δ 7.6 (1 H, s), 2.79 (6 H, s), 2.42 (6 H, s).

Reaction c. Phenyl isocyanide (0.05 mol), *N,N*-diethylhydrazine (0.15 mol) and CuCl (0.75 mmol) were refluxed for 1.5 h. The mixture was distilled, b.p. 40 – 80 °C, 1 – 8 mmHg; 8.1 g was collected. The mixture was identified by GLC-MS on neopentyl glycol succinate (using authentic samples as reference, see below).

Redistillation b.p. 67 °C, 3 – 8 mmHg yielded a mixture of liquid and crystals. The crystals were isolated and recrystallized from pentane and ether, m.p. 49 °C. These crystals were identified as N^2,N^2,N^4,N^4-*tetraethylformohydrazide hydrazone* (III c). (Found: C 57.61; H 10.89; N 29.52. Calc. for $C_9H_{22}N_4$: C 58.02; H 11.90; N 30.07). MS *m/e* (% of base peak): 187(11), 186(95)M$^+$, 157(11), 116(10), 115(24), 114(21), 100(71), 98(19), 97(6), 88(49), 87(31), 86(29), 74(19), 73(97), 72(71), 71(36), 70(24), 69(12), 60(55), 59(31), 58(100), 57(29), 56(97), 55(14), 45(29), 44(88), 43(21), 42(86), 41(24), 40(12). ^1H NMR ($CDCl_3$): δ 1.05 (6 H, t); 1.13 (6 H, t); 2.68 (4 H, q); 2.77 (4 H, q); 6 – 7 (1 H, broad); 7.62 (1 H, s).

N^3,N^3-*Diethyl*-N^1-*phenylformamide hydrazone (VI c)* was prepared by stirring ethyl *N*-phenylformimidate[18] (0.02 mol) and *N,N*-diethylhydrazine (0.02 mol) for 20 h at room temperature. The mixture was distilled *in vacuo*. B.p. 110 °C, 3 mmHg, yield 60 %. Anal. $C_{11}H_{17}N_3$: C, H, N. ^1H NMR ($CDCl_3$): δ 1.03 (6 H, t); 2.69 (4 H, q); 6.7 – 7.5 (5 H, m); 7.78 (1 H, broad s); 7.8 – 8.4 (1 H, broad). Shaking with D_2O resulted in the disappearance of the broad signal at δ 7.8 – 8.4, and the change of

the broad singlet at δ 7.78 to a sharp singlet. IR (CCl_4 in cm^{-1}): 3460sh, 3420sh, 3350m, 3190w broad, 3052w, 2978m, 2938m, 2875m, 2839m, 1690m, 1635s, 1600s, 1502s, 1444m, 1400m, 1375m, 1360m, 1338m, 1330m, 689s. 1250w, 1192w, 1178w, 1138w, 1075w, 1058w, 689s. MS *m/e* (% of base peak): 192(15), 191(100)M^+, 177(12), 176(96), 147(10), 146(42), 120(15), 119(35), 118(15), 106(54), 104(38), 93(40), 92(22), 78(24), 77(57), 73(15), 65(38), 63(11), 56(10), 52(11), 51(31), 50(13), 44(13), 42(21). ^{13}C NMR($CDCl_3$): δ 148.1, 139.2, 129.0, 121.5, 114.5, 51.7, 16.2.

Attempted preparation of N³,N³-*diethyl*-N¹-*phenylformamide hydrazone*. *N*-Phenylthioformamide[17] (0.04 mol) and *N,N*-diethylhydrazine (0.04 mol) were refluxed in abs. ethanol (60 ml) for 8 h. H_2S evolution was detected. On cooling small amounts of precipitate were formed (sulfur). These were filtered off and the filtrate evaporated *in vacuo* and subsequently distilled, b.p. 46 – 82 °C, 3 – 1 mmHg. The composition of the distillate was determined by GLC on a neopentyl glycol succinate column as: aniline 10 %, *N*¹,*N*¹-diethyl-*N*²-phenylformamidine 10 % and *N*³,*N*³-diethyl-*N*¹-phenylformamide hydrazone 70 %.

N¹,N¹-*Diethyl*-N²-*phenylformamidine (IV c)* was prepared by dropping *N,N*-diethylformamide (0.1 mol) and aniline (0.1 mol) in $CHCl_3$ (20 ml) to a solution of PCl_5 (30 g) in $CHCl_3$ (80 ml). The mixtures were refluxed for 3 h. After standing overnight at room temperature, ether was added until the phases separated. The ether phase was washed with water, the combined water extracts made alkaline with NaOH, and subsequently extracted with ether. The ether layer was dried over $MgSO_4$, and the ether was evaporated. Distillation of the residue gave 88 % of a yellowish liquid, b.p. 87 °C, 3 mmHg.[19–21] ^1H NMR ($CDCl_3$): δ 1.17 (6 H, t), 3.33 (4 H, q), 6.6 – 7.3 (5 H, m), 7.46 (1 H, s). ^{13}C NMR ($CDCl_3$): δ 13.6, 42.0, 121.1, 122.1, 128.8, 151.2, 152.0. MS *m/e* (% of base peak): 176(4)M^+, 175(3), 147(9), 104(27), 93(55), 85(11), 77(100), 72(48), 58(95), 56(33). 51(78).

Reaction d. Phenyl isocyanide (0.014 mol), *N*-methyl-*N*-phenylhydrazine (0.042 mol) and CuCl (0.21 mmol) were refluxed for 1 h. The reaction mixture was subsequently distilled *in vacuo* giving two fractions. (I) b.p. 44 – 47 °C, 1 mmHg, (2.4 g). Identified as a mixture of aniline and *N*-methylaniline. (II) b.p. 47 – 105 °C, 0.1 mmHg, (2.1 g). This fraction consisted of 4 components (GLC on Chromosorb 103); on cooling crystals were formed. Recrystallization from ethanol and light petroleum gave m.p. 112 – 113 °C. The compound was identified as N,N''-*dimethyl*-*N,N''*-*diphenylformamidrazone* (II d),[5] yield 21 %. MS *m/e* (% of base peak): 240(17), 239(91)M^+, 210(23), 209(16), 133(10), 107(47), 106(100), 104(14), 92(10), 78(10), 77(63), 51(21). ^1H NMR ($CDCl_3$): δ 8.29 (1 H, s), 6.8 – 7.6 (10 H, m), 3.50 (3 H, s), 3.23 (3 H, s).

Reaction e. 4-Methylphenyl isocyanide (0.1 mol), *N,N*-dimethylhydrazine (0.3 mol) and CuCl (1.5 mmol) were refluxed for 2 h and subsequently stirred overnight at room temperature. The mixture was distilled (I) b.p. 30 – 80 °C, 760 mmHg, (11 g), consisting mainly of *N,N*-dimethylhydrazine, and (II) b.p. 40 – 100 °C, (12 g). The last fraction was redistilled *in vacuo*, the composition of the fractions was determined by GLC and GLC-MS using a neopentyl glycol succinate column (see Table 1).

MS of N,N-*dimethyl*-N'-*(4-methylphenyl)formamidine (IVe)*. *m/e* (% of base peak): 162 (20)M^+, 161(13), 147(15), 120(34), 118(20), 106(9), 91(59), 80(13), 65(31), 45(24), 44(100), 42(38).

MS of N,N-*dimethyl*-N''-*(4-methylphenyl)formamidrazone (VIe)*. *m/e* (% of base peak): 177(9)M^+, 120(14), 118(14), 107(45), 106(82), 91(36), 60(100).

Reaction f. Phenyl isocyanide (0.095 mol), *N,N,N'*-trimethylhydrazine[22] (0.1 mol) and CuCl (1.5 mmol) were refluxed for 2 h. The mixture was then distilled twice *in vacuo*, b.p. 70 – 73 °C, 0.04 mmHg, yield 42 % of N¹-*methyl*-N²,N²-*dimethylformohydrazide phenylimide* (V f). Anal. $C_{10}H_{15}N_3$: C, H, N. IR (CCl_4, in cm^{-1}): 3060w, 3028w, 3020w, 2995w, 2955m, 2780w, 1690w, 1630s broad, 1590s, 1490m, 1452m, 1328m, 1218m, 1150m, 692s. ^{13}C NMR ($CDCl_3$): δ 154.7, 151.8, 128.9, 122.6, 121.3, 43.5, 24.1. ^1H NMR ($CDCl_3$): δ 7.92 (1 H, s), 6.8 – 7.5 (5 H, m), 3.00 (3 H, s), 2.57 (6 H, s). MS *m/e* (% of base peak): 177(13)M^+, 134(19), 133(19), 104(13), 93(56), 77(48), 74(28), 73(18), 59(100), 51(22), 44(14), 43(13), 42(44).

Reaction g. N³,N³-*Dimethyl*-N¹-*cyclohexylformamide hydrazone (VI g)*. Cyclohexyl isocyanide (0.1 mol), *N,N*-dimethylhydrazine (0.3 mol) and CuCl (1.5 mmol) were refluxed for 6 days. After cooling the mixture was distilled *in vacuo*, b.p. 64 – 65 °C, 0.3 mmHg, it crystallized on cooling, m.p. 48 °C (from hexane), yield 20 %. Anal. $C_9H_{19}N_3$: C, H, N. MS *m/e* (% of base peak): 170(12), 169(100)M^+, 125(19), 124(2), 123(3), 110(7), 98(21), 96(12), 95(10), 87(23), 86(14), 83(13), 67(11), 60(35), 59(23), 56(21), 55(28), 54(17), 46(28), 45(74), 44(63), 43(32), 42(28), 41(35). IR (CCl_4, in cm^{-1}): 3450w, 3365m, 3260w broad, 3015m, 2940s, 2860s, 2820s, 1635s, 1465s, 1448s, 1425m, 1405m, 1365m, 1348m, 1338m, 1265m, 1228m, 1155m, 1148m, 1016m, 956s, 889m. ^1H NMR ($CDCl_3$): δ 6.88 (1 H, d), 5.6 – 5.0 (1 H, broad), 3.3 – 2.8 (1 H, broad), 2.40 (6 H, s), 2.1 – 1.0 (10 H, m broad). ^{13}C NMR ($CDCl_3$): δ 24.1, 24.8, 34.1, 46.0, 52.6, 149.5.

Reaction h. Cyclohexyl isocyanide (0.013 mol), *N*-methyl-*N*-phenylhydrazine (0.040 mol) and CuCl (0.2 mmol) were refluxed for 3 h. The mixture was distilled *in vacuo*; some products were identified by GLC on a chromosorb 103 column, using authentic samples as reference compounds (Table 1).

Reaction with hydrazones. Phenyl isocyanide (0.08 mol), benzophenone hydrazone (0.26 mol) and CuCl (1.5 mmol) were refluxed in 400 ml of benzene for 240 h.

The isocyanide absorption in the IR spectrum had not completely disappeared. The mixture was cooled and the precipitate filtered off. Recrystallization from methanol gave 90 % unreacted benzophenone hydrazone and 10 % of N^2,N^4-bis(diphenylmethylene) formohydrazide hydrazone (VIII), identified by comparison with authentic sample.[23]

Phenyl isocyanide (0.05 mol), acetophenone hydrazone (0.1 mol) and CuCl (0.2 mmol) were refluxed in 25 ml CCl_4 for 24 h. The isocyanide absorption in IR did not disappear completely. Cooling the solution caused the precipitation of yellow crystals, which were isolated and identified as acetophenone azine by comparison with an authentic sample.

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The Reaction of Some Indoles and Indolines with 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone

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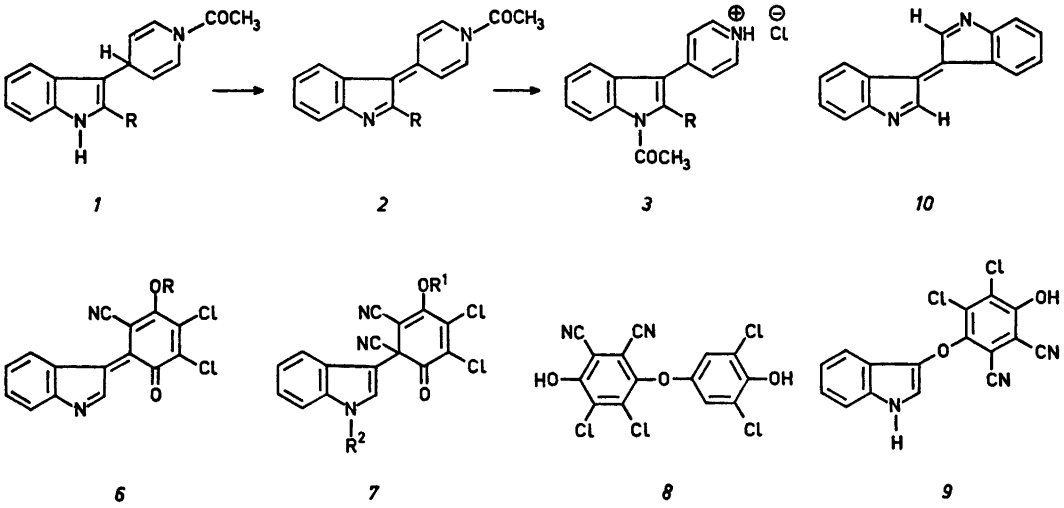
Indole and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) form a 1:1 donor-acceptor complex, which was found to be stable in the crystalline state in dichloromethane. In solvents such as dioxane the complex gave rise to a substitution product (*4*), which on heating eliminated HCN affording the quinone (*5*). 3-Alkyl indoles and DDQ gave 3-alkylidene-3*H*-indoles (which could be isolated as, *e.g.*, sulfates). DDQ dehydrogenated 3-(*N*-acetyl-1,4-dihydro-4-pyridyl) indoles yielding a transacetylated salt (*19*). The dehydrogenation of indolines by DDQ was complicated as the desired indoles could participate in consecutive substitution reactions. Dehydrogenation of *N*-acylated indolines in dioxane at 75 °C followed by hydrolysis was found to be a convenient method for the preparation of indoles.

In the transformation of 3-(*N*-acetyl-1,4-dihydro-4-pyridyl) indole (*1*, R=H) to 4-(*N*-acetyl-3-indolyl)pyridinium chloride (*3*, R=H) by the action of *N*-acetylpyridinium chloride, the 3-alkylidene-3*H*-indole (*2*, R=H) was suggested [1] to be a key intermediate. In order to substantiate this mechanism it appeared desirable to prepare the intermediate *2* by dehydrogenation of *1* under neutral conditions as *2* should be sensitive to basic [2,3] as well as acidic conditions. In this paper the results of the reaction of *1* as well as several model compounds with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) are reported.



Scheme 1. *6 a*, R=H; *b*, R=CH_3; *c*, R=$COCH_3$. *7 a*, R^1=$COCH_3$, R^2=H; *b*, R^1=CH_3, R^2=H; *c*, R^1=R^2=CH_3; *d*, R^1=$COCH_3$, R^2=CH_3.

RESULTS

A. Reaction of 2- and 3-unsubstituted indoles with DDQ. DDQ rapidly reacts (within 3 min) with indole in dichloromethane at 25 °C to give dark-blue crystals (98 % yield) of a stable 1:1 charge-transfer complex. The complex may be recrystallized from hot tetrachloroethylene and was found to be stable for several months. The infrared spectrum of the complex was composed of the spectra of the components, indicating a nonionic, diamagnetic complex. The visible spectrum showed a charge-transfer band at 592 nm. An analoguous, stable 1:1 complex from carbazole and DDQ has recently been reported by several groups.[4–6]

The DDQ-indole complex also formed quickly in dry dioxane, but in this solvent the complex is transformed to an orange-red, acidic (soluble in aqueous $NaHCO_3$ and forming stable pyridine salts) compound in nearly quantitative yield within 2 h. Addition of a small amount of water or methanol reduced the time of conversion to a few seconds. Elemental analysis and spectral data (MS, ^1H NMR, IR) are in agreement with structure *4a*. In refluxing aqueous ethanol or heating above its melting point (*ca.* 130 °C) *4a* eliminated HCN yielding the dark-blue quinone *5a*, which was readily reduced to give the corresponding hydroquinone. Addition of suitable bases (*e.g.* pyridine) facilitated the elimination. The reactions are summarized in Scheme 2.

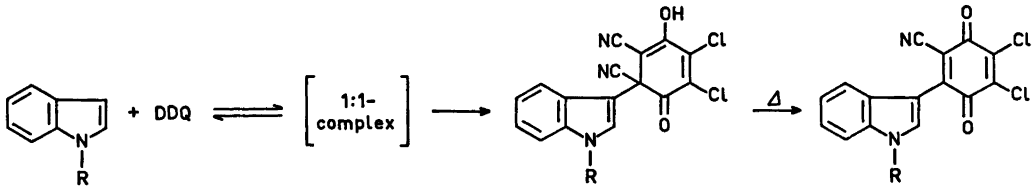
The reaction of indole with benzoquinone probably proceeds similarly, however, in this case only the final product, 3-indolylbenzoquinone, has been isolated.[7,8] Interestingly the reaction of indole and tetracyanoethylene [9–12] also seems to involve a similar sequence as the reaction of indole with DDQ, including elimination of HCN (facilitated by base) to give the final product, 3-tricyanovinylindole.

Since the UV spectra of *5b* (prepared *via* *N*-methylindole and DDQ) and *5a* are somewhat different, a significant contribution from the tautomer *6a* cannot be excluded. Attempts to prepare *6b* and *6c* by elimination of HCN from *7a* and *7b* resulted in hydrolysis to *5a* (when heated in aqueous ethanol) or in the formation of high-molecular tarry products when thermolysed. The reluctance of *7a* and *7b* to eliminate HCN as compared with *4a* is also evident from their mass spectra. While the spectra of *7a* and *7b* showed strong parent ion peaks, that of *4a* was lacking such a peak.

In an extensive study [13–17] of the reactions of phenols with DDQ Becker has isolated products of several types, notably the adduct *8*. The corresponding indole DDQ adduct *9* (isomeric with *4a*) could not be detected along with *4a*, which probably reflects the higher reactivity of indole for electrophilic substitution. No adducts corresponding to *4* have been found in the phenol series.

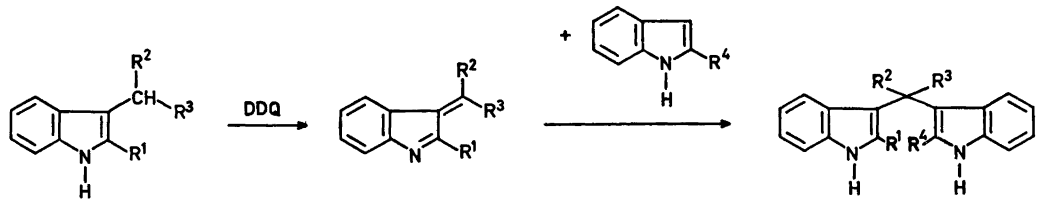
In methanol solution the formation of *4a* from indole and DDQ is completed within 1 min (as compared to 2 h in dioxane). The reaction is, however, less clean and 3,3-biindolyl and the known [18] compound *10* were formed as minor products. Analogous to the oxidative coupling of phenols,[13] DDQ readily dehydrogenated 3,3-biindolyl to give *10*.

B. Reaction of 2- and/or 3-alkyl indoles with DDQ. Several reagents are known [3] to dehydrogenate 3-alkylindoles to the corresponding 3-alkylidene-3*H*-indoles. Accordingly, DDQ was found to effect this transformation under mild conditions. The 3-alkylidene-3*H*-indole formed can, in certain cases, be isolated as a salt. Thus, addition of sulfuric acid to the reaction mixture obtained from 2-methyl-3-isopropyl-



4 *5*

Scheme 2. *4* and *5* *a*, R = H; *b*, R = CH_3.



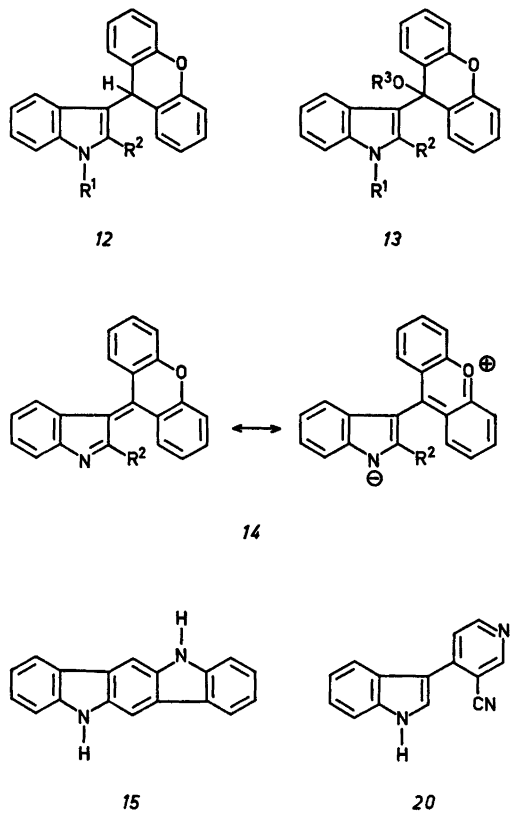
11

Scheme 3.

indole and DDQ in tetrahydrofuran-ether gave the known [19] hydrogen sulfate of 2-methyl-3-isopropylidene-3*H*-indole. The 3-alkylidene-3*H*-indole formed can also be trapped by addition of a 3-unsubstituted indole. Unsymmetrical diindolylmethanes (*e.g.* *11*, $R^1,R^2,R^3=H$, $R^4=CH_3$) may be prepared by this route. By-products (unidentified) are, however, formed and for preparative purposes the conventional technique [20,21] (heating of a gramine with the appropriate indole) is preferred. Mild hydrolysis of the 3-alkylidene-3*H*-indoles formed gave an indole and a carbonyl compound. Thus, treatment of the reaction mixture from 3-(diphenylmethyl)-2-methylindole and DDQ with dilute sodium hydroxide in water/ethanol gave benzophenone and 2-methylindole. Attempts to isolate the free 3-alkylidene-3*H*-indole failed (*cf.* Ref. 2). However, they were readily isolated as salts. Not even the resonance-stabilized 3-alkylidene-3*H*-indole (*14*) could be isolated as a free base. The violet-blue salts of *14* prepared by dehydrogenation of *12* with DDQ slowly absorbed water giving, probably *via* *13a*, xanthone and more complex products (not isolated). Addition of sodium ethylate in ethanol to *12c* and *12d* gave the known [22] compounds *13b* and *13c*, respectively. Hydrolysis of the salts of *14* with sodium hydroxide in water gave as expected xanthone and the appropriate indole.

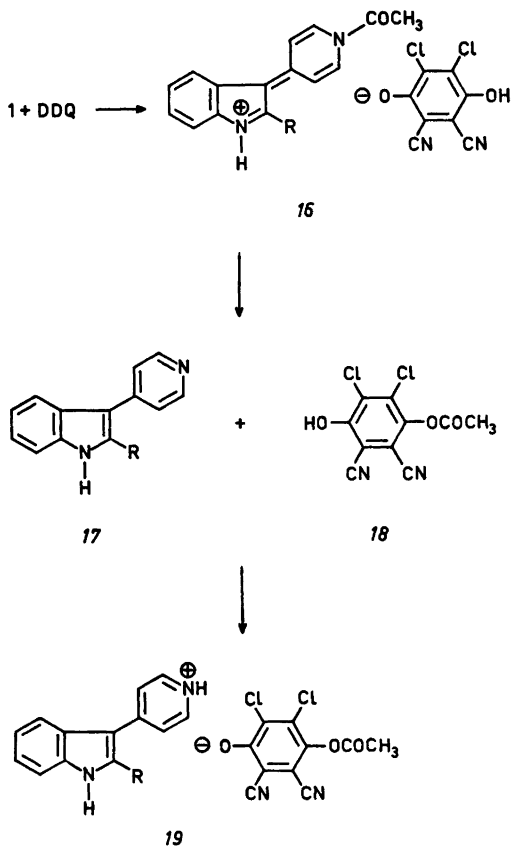
The reactions of 3-unsubstituted 2-alkylindoles with DDQ in methanol were found to be very complex. For example, from the reaction of 2-methylindole with DDQ only the highly insoluble indolo[3,2b]carbazole [23] (*15*) was isolated. Interestingly, Becker [13–17] has described related DDQ-induced C–C couplings in the phenol series.

C. Reaction of 3-(N-acetyl-1,4-dihydro-4-pyridyl)indoles with DDQ. 3-(*N*-Acetyl-1,4-dihydro-4-pyridyl)indoles (*1*) rapidly reacted with DDQ in ethyl acetate forming a brown unstable solid (*16*), which was gradually (0.5 – 2 h) converted to a light-yellow (often crystalline) salt (*19*) in high yield. The structure of the salt (*19*) is evident from an independent preparation from the base (*17*) and the acid (*18*), thus



12 13 14 15 20

Scheme 4. 12 a, $R^1=R^2=H$; *b*, $R^1=H$, $R^1=CH_3$; *c*, $R^1=R^2=CH_3$; *d*, $R^1=H$, $R^2=C_6H_5$. *13 a*, $=R^1=R^3=H$, $R^2=CH_3$; *b*, $R^1=R^2=CH_3$, $R^3=C_2H_5$; *c*, $R^1=H$, $R^2=C_6H_5$, $R^3=C_2H_5$.



Scheme 5.

mimicking the last step in the reaction pathway suggested in Scheme 5.

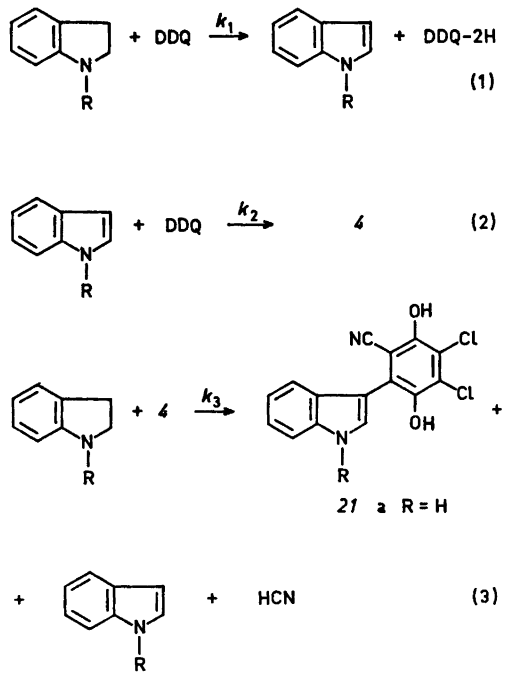
The brown unstable solid (*16*) was found to be a powerful acetylating agent. Thus, phenyl acetate and *N*-acetylindole, were rapidly formed on treatment of *16* with phenol and indole, respectively. The formation of a small amount of 2,3-dichloro-5,6-dicyanohydroquinone diacetate along with *19* is thus not surprising.

By treatment with sodium hydroxide in aqueous ethanol *19* was readily converted into the corresponding free base, (*cf.* Ref. 25). The fact that *16* is an acylation agent supports the earlier formulated transformation (*1*→*3*). In consonance it was found that 3-(4-pyridyl)indole was converted to *N*-acetyl-3-(4-pyridyl)indole by a reagent formed from, *e.g.*, 3-(*N*-acetyl-1,4-dihydro-4-pyridyl)-5-bromoindole and DDQ.

The reaction of 3-(*N*-acetyl-1,4-dihydro-3-cyano-4-pyridyl)indole with DDQ was rather slow and required 1 h at reflux temperature (ethyl acetate). The product formed is readily soluble in this solvent and obviously is not a salt but is probably best described as a complex between *18* and 3-(3-cyano-4-pyridyl)indole (*20*).* The components could readily be separated by column chromatography or by extraction of *18* with sodium acetate (aq., 5.0 %).

D. Some implications of the results. The reaction between indole and DDQ is rapidly performed under mild conditions (25 °C) giving *4a* which seems to be in contradiction to the reported dehydrogenations [27–32] of 3-unsubstituted indolines, *e.g.* 1-(5′-*O*-trityl-2′,3′-di-*O*-acetyl-β-D-ribofuranosyl)indoline to 3-unsubstituted indoles at rather high temperatures (~130 °C, refluxing xylene). For this reason we investigated the reaction between one equiv-

* Compound *20* should be a considerably weaker base than 3-(4-pyridyl)indole. The protonated forms of 3-cyanopyridine and pyridine have the pK_a values [26] 1.5 and 5.2, respectively.



Scheme 6.

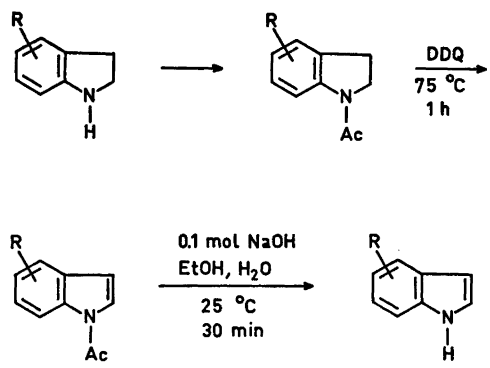
alent indoline with one equivalent DDQ in dioxane at 25 °C.* The main products were indole and 2,3-dichloro-5,6-dicyano-1,4-hydroquinone (DDQ-2*H*) with *21a* as a minor product, showing that *under these conditions* the dehydrogenation (eqn. 1) is faster than the substitution (*i.e.* $k_1 > k_2$). When two equivalents of DDQ were used, *4a* and DDQ-2*H* were the only products.

Eqn. 3 accounts for the formation of the minor product (*21*). In separate experiments it was found that pure *4a* smoothly dehydrogenated indolines in good yields. DDQ-2*H* could not be detected in these experiments, indicating that *4a* or *5a* and not DDQ formed by dissociation of *4a* is the active dehydrogenative agent in this reaction. In a complementary competitive (DDQ *vs* *4a*) dehydrogenation of indoline it was shown that $k_1 \gg k_3$.

The choice of solvent is important for the outcome of the reaction of DDQ with indoline. Thus, when one equivalent of indoline was reacted with one equivalent of DDQ in methanol the predominant products were *21a*, DDQ-2*H*, and indole indicating that in this solvent the substitution (eqn. 2) is faster than the dehydrogenation (eqn. 1) (*i.e.* $k_2 > k_1$). Reaction 3 is slower than reaction 1 in this solvent too, as evidenced from an analogous competitive experiment (*i.e.* $k_1 > k_3$). DDQ has been used extensively [27–31] for dehydrogenation of indolines containing sugar-residues in position 1. From our results with simple indolines we consider it likely that compounds similar to *4* and *21* may be formed, especially if protic solvents are used, along with the desired dehydrogenated products. In this connection it is interesting to note that Preobrazhenskaya *et al.*[27] obtained fractions containing unidentified indoles from the dehydrogenation of 1-(5′-*O*-trityl-2′,3′-di-*O*-acetyl-β-D-ribofuranosyl)indoline with DDQ.

In order to dehydrogenate indolines more cleanly we have developed the following procedure.

The dehydrogenation step is smoothly effected in high yield at 75 °C in dioxane. In contrast tetrachloro-1,4-quinone could not effect dehydrogenation of *N*-acetylated indolines even



Scheme 7.

in refluxing dioxane. In separate experiments it was found that *N*-acetylindoles do not react with DDQ under these conditions. The *N*-acetylindole, which may be isolated, is readily hydrolyzed under mild conditions. By this procedure, *e.g.* 5-iodoindole and 5-acetaminoindole can be conveniently prepared.

DDQ has been suggested [34] as a non-destructive reagent for detection of indoles by taking advantage of the formation of the highly coloured charge-transfer complex. However, in view of the transformation to secondary products now demonstrated, we consider that other reagents suggested such as polynitrofluorenones are more suitable.[35,36] Actually, we could recover indole after treatment with DDQ only if non-polar solvents (CH_2Cl_2, benzene) were used. As materials such as silica gel and cellulose were found to strongly catalyze the conversion of the complex into the adduct (*4*), any work up by chromatography prohibits the use of DDQ as non-destructive reagent.

EXPERIMENTAL

Melting points were determined on a micro hot stage melting point apparatus and are uncorrected. Elemental analyses were carried out by A. Bernhardt, Elbach über Engelskirchen, West Germany, or Centrala Analyslaboratoriet, Uppsala, Sweden. IR spectra were recorded with a Perkin Elmer 421 infrared spectrophotometer, the spectra of solids being determined as KBr discs and oils as liquid films. ^{1}H NMR spectra were recorded with a Varian-A60 instrument or a JEOL JNM-MH-100 instrument. The spectra were obtained on $CDCl_3$ solutions or DMSO-d_6 solutions. Mass

* Interestingly, Baxter *et al.* recently [33] have used diethyl ether as solvent for dehydrogenations of indoline derivatives with DDQ.

spectra were recorded with a LKB 9000 instrument. Unless otherwise stated column chromatography was performed with silica gel (Merck 0.05 – 0.2 mm) using dichloromethane with 5 % methanol as eluent. UVIS spectra were recorded with a Beckmann DK2-instrument.

Indole-DDQ-(1:1)-complex. A solution of indole (1.17 g, 0.01 mol) in dry dichloromethane (10 ml) was added to a stirred solution of DDQ (2.27 g, 0.01 mol) in dry dichloromethane (100 ml). The dark blue crystals formed were collected after 5 – 10 min and dried in a desiccator. Yield 3.38 g (98 %). On heating, the complex decomposed without melting. The complex may, however, be recrystallized from hot tetrachloroethylene or dichloromethane; IR: 3390 (NH), 2232 (w, CN), 1663 (CO), 1553, 1450, 1347, 1243, 1171, 1094, 887, 789, 776, 750, 711, and 666 cm^{-1}. VIS [CH_2Cl_2 (log ε)] 592 (2.03) nm. Anal. $C_{16}H_7Cl_2N_3O_2$: C, H, N.

Reaction of indole with DDQ in dioxane. Synthesis of 4a. A solution of indole (11.7 g, 0.1 mol) in dioxane (50 ml) was added dropwise to DDQ (22.7 g, 0.1 mol) in dioxane (150 ml) at 20 °C without cooling. During the addition the temperature increased to ~30 °C, and the molecular complex precipitated. After 0.5 – 1.5 h. the dark blue complex had been transformed into an orange-red crystalline product (containing crystal-dioxane), which was collected on a filter and dried *in vacuo*. Yield 28.6 g (86 %); m.p. 130 – 135 °C. By addition of light petroleum to the filtrate an additional crop (4.5 g) was obtained. The analytical sample was recrystallized from ether-light petroleum and carefully dried. IR: 3400 (NH), 3115 (broad, OH), 2215 (w, CN), 1684 (CO), 1527, 1448, 1415, 1350, 1228, 1114, 1080, 890, 867, 827, 765, and 740 cm^{-1}. UV, VIS [ethanol(log ε)] 215(4.48), 262(4.31), and 359(3.81) nm. The mass spectrum is identical with that of compound *5a*, due to the ready elimination of HCN. Anal. $C_{16}H_7Cl_2N_3O_2$: C, H, N.

Methylation of 4a *with one equivalent of diazomethane. Synthesis of* 7b. Compound *4a* (0.172 g, 0.5 mmol) in ether (5 ml) was methylated with a solution of diazomethane (0.5 mmol) in ether at 0 °C. The mixture obtained was cooled to – 30 °C and the precipitate collected and washed repeatedly with cool ether. Yield 66 mg (37 %); m.p. 222 – 224 °C. The analytical sample was recrystallized from ethanol; m.p. 231 – 233 °C. IR: 3365 (NH), 2206 (CN), 1702 (CO), 1603, 1559, 1458, 1420, 1348, 1328, 1244, 1235, 1057, 782, 761, and 693 cm^{-1}. UV,VIS [ethanol(log ε)] 214 (4.49), 342 (4.56) and 445 (2.91) nm. MS *m/e* [(% rel.int.)]: 359 (55), 357 (86), 344 (45), 342 (65), 327 (61), 325 (100), 316 (64), 314 (96), 294 (60), 279 (58), 262 (28), 251 (54), 189 (34), 165 (41), and 87 (51). Only peaks stronger than 25 % of the base peak are listed. Anal. $C_{17}H_9Cl_2N_3O_3$: C, H, N.

Acetylation of 4a. *Synthesis of* 7a. A solution of *4a* (344 mg) in acetic acid (5 ml) and acetic anhydride (1 ml) was stirred at 25 °C for 2 h and left overnight. Water (5 ml) was added and after *ca.* 5 min a precipitate separated which was collected, washed with 50 % acetic acid and dried in an exsiccator over KOH. Yield 170 mg (44 %), m.p. 170 – 173 °C. IR: 3425 (NH), 2210 (w, CN), 1790 (CO), 1701 (CO), 1535, 1465, 1422, 1342 1311, 1248, 1234, 1144, 1025, 910, 774, and 748 cm^{-1}. UV,VIS [ethanol(log ε)] 217 (4.58), 264 (4.06), 282 (4.11) and 462 (2.99) nm. Anal. $C_{18}H_9Cl_2N_3O_3$: C, H, N.

Acetylation of 4b. *Synthesis of* 7d. The same procedure as described for the acetylation of *4a* was used. Yield 65 %; m.p. 172 – 176 °C (after crystallization from ethanol). IR: 2212 (w, CN), 1797 (s, CO), 1709 (s, CO), 1535, 1310, 1258, 1240, 1168, 1143, 1040, 1017, 909, 781 and 758 cm^{-1}. UV,VIS [ethanol(log ε)] 217 (4.58), 264 (4.06), 282 (4.07) and 462 (2.99) nm. MS[*m/e*(% rel.int.)]: 401 (32), 399 (45), 359 (66), 358 (42), 357 (100), 356 (34), 334 (39), 332 (76), 330 (42), 322 (32), 315 (42), 313 (61) 294 (76), 165 (47), 131 (30), and 87 (30). Only peaks stronger than 30 % of the base peak and above *m/e* 50 are listed. Anal. $C_{19}H_{11}Cl_2N_3O_3$: C, H, N.

5,6-Dichloro-3-cyano-2-(3-indolyl)-1,4-benzoquinone (5a). *Method A.* Compound *4a* (344 mg, 1 mmol) was heated (140 °C) until the evolution of HCN had ceased. The cooled dark blue reaction mixture was extracted with ether. The extract was concentrated, light petroleum was added and the resulting solution cooled to – 30 °C. The dark blue precipitate obtained was collected. Yield 290 mg (92 %); m.p. 225 – 232 °C. The analytical sample was recrystallized from ethanol; m.p. 232 – 234 °C. IR: 3400 (NH), 2210 (w, CN), 1695 (CO), 1542, 1353, 1208, 1113, 867, 768, 741 and 681 cm^{-1}. UV,VIS[ethanol-(log ε)] 213 (4.57), 277 (4.08), 302 (4.11) and 600 (3.67) nm. MS[*m/e*(% rel.int.)]: 320 (23), 319 (17), 318 (83), 317 (23), 316 (100), 290 (13), 288 (17), 253 (17), 225 (14), 190 (13), 166 (28), 139 (19), 87 (20), and 37 (10). Only peaks stronger than 10 % of the base peak are recorded. Anal. $C_{15}H_6Cl_2N_2O_2$: C, H, N.

Method B. A mixture of *4a* (344 mg, 1 mmol), ethanol (2,5 ml) and water (2.5 ml) was heated at reflux for 1.5 h. After cooling the dark blue solid obtained was collected, dried and recrystallized from ethanol. Yield 240 mg (76 %); m.p. 232 – 234 °C.

*5,6-Dichloro-3-cyano-2-(3-*N*-methyl-indolyl)-1,4-benzoquinone* (5b). Method B (see above) was used. Yield 71 %; m.p. 228 – 230 °C. UV,VIS [ethanol(log ε)]: 216 (4.23), 302 (3.77), 381 (2.66), and 602 (3.45) nm. Anal. calc. for $C_{16}H_8Cl_2N_2O_2$: C 58.0; H 214; Cl 21.5; N 8.5. Found: C 57.5; H 2.5; Cl 20.9; N 8.4.

Reaction of 1-methylindole with DDQ. Synthesis of 4b. Compound *4b* was prepared analogously to *4a*, except that water (200 ml) was added before the collection of the precipitate. The crude product was recrystallized from tetrahydrofuran-light petroleum and dried. Yield 88 %; m.p. 130 – 135 °C, dec. IR: 3390 (NH), 2975, 2880, 2218 (CN), 1705, 1535, 1468,

1362, 1340, 1290, 1257, 1236, 1042, 880, 780, 750 and 690 cm^{-1}. UV,VIS [ethanol(log ε)] 219 (4.65), 263 (4.38), and 380 (3.36) nm. The mass spectrum is identical with that of compound *5b*. Anal. $C_{17}H_9Cl_2N_3O_2$: C, H, N.

Methylation of 4b. *Synthesis of* 7c. The same procedure as described for *7b* was used. Yield 22 %; m.p. 183–186 °C (after crystallization from ethanol). IR: 2203 (CN), 1698 (CO), 1603, 1534, 1465, 1320, 1256, 1240, 1048, 1022, 782 and 748 cm^{-1}. UV[ethanol(log ε)] 217 (4.62) and 283 (4.10) nm. MS[*m/e*(% rel.int.)]: 373 (58), 371 (88), 357 (65), 355 (100), 341 (38), 339 (59), 330 (66), 328 (100), 310 (28), 308 (68), 293 (58), 276 (25), 265 (41), 189 (26), 168 (32), 165 (35), and 87 (32). Only peaks stronger than 25 % of the base peak are listed. Anal. calc. for $C_{18}H_{11}Cl_2N_3O_2$: C, H, N.

Indolo[3,2-b]carbazole (15). DDQ (1.36 g, 6 mmol) was added to a stirred solution of 2-methylindole (262 mg, 2 mmol) in methanol (25 ml). After 3 h at 25 °C the solvent was evaporated and the residue extracted with sodium carbonate (2 × 20 ml, aq. 5 %). The insoluble material was washed with water and extracted with acetic acid. The extract residue after washing with water and recrystallized from pyridine gave indolo[3,2-*b*] carbazole. Yield 28 mg (11 %); m.p. > 400 °C. The IR and MS spectra were identical with those of an authentic [23] sample.

3-(Diphenylmethyl)-2-methylindole. 2-Methylindole (6.55 g, 0.05 mol), diphenyl carbinol (9.2 g, 0.05 mol) and acetic acid (35 ml) were refluxed for 2 h. The crystals formed on cooling were collected and washed with cold methanol. Yield 11.6 g (78 %); m.p. 159–161 °C. Anal. $C_{22}H_{19}N$: C, H, N.

3-(Diphenylmethyl)indole was prepared as described earlier;[37] m.p. 126–127 °C (lit.[37] 126–127 °C).

3-(9-Xanthyl)indole (12a). Xanthydrol (3.96 g, 0.02 mol), indole (2.34 g, 0.02 mol) and acetic acid were mixed and stirred at 30 °C for 0.5 h. After cooling (+10 °C) the solid formed was collected and washed with methanol. Yield 4.1 g (69 %); m.p. 142–146 °C. The analytical sample was recrystallized from methanol; m.p. 146–147 °C. Anal. $C_{21}H_{15}NO$: C, H, N.

The following compounds were similarly prepared.

2-Methyl-3-(9-xanthyl)indole (12b). Yield 76 %, m.p. 194–195 °C. Anal. $C_{22}H_{17}NO$: C, H, N.

1,2-Dimethyl-3-(9-xanthyl)indole (12c). Yield 80 %; m.p. 185–168 °C. Anal. $C_{23}H_{19}NO$: C, H, N.

2-Phenyl-3-(9-xanthyl)indole (12d). The reaction conditions were changed from 30 °C (0.5 h) to 50 °C (1 h). Yield 92 %; m.p. 219–221 °C. Anal. $C_{27}H_{19}NO$: C, H, N.

Dehydrogenation of 2-phenyl-3-(9-xanthyl)-indole, preparation of 2-phenyl-3-(9-xanthylidene)3H-indolium 2,3-dichloro-5,6-dicyano-1,4-hydroquinolate. A solution of DDQ (227 mg, 1 mmol) in ethyl acetate (3 ml) was added to a solution of 2-phenyl-3-(9-xanthyl)indole (373 mg, 1 mmol) in ethyl acetate (5 ml). After 1 h at 25 °C the dark blue crystals formed were collected and dried. Yield 530 mg (88 %); m.p. 228–231 °C. IR: 2222 (CN), 1620, 1600, 1572, 1480, 1425, 1377, 1218, 1138, 1115, 1055, 885, 787, 780, 755, 745, 732, 710, and 701 cm^{-1}. Anal. $C_{35}H_{19}Cl_2N_3O_2$: C, H, N.

2-Phenyl-3-(9-ethoxy-9-xanthyl)indole (13c). To a mixture (prepared as described above) of 2-phenyl-3-(9-xanthylidene)-3*H*-indolium 2,3-dichloro-4,5-dicyano-1,4-hydroquinolate (600 mg, 1 mmol) and ethyl acetate was added sodium ethoxide (prepared from 117 mg, 5 mmol of sodium) in ethanol (8 ml). After 10 min at 25 °C the solvents were evaporated *in vacuo*. The residue extracted with ether gave after recrystallization from ethanol 2-phenyl-3-(9-ethoxy-9-xanthyl)indole. Yield 206 mg (49 %); m.p. 165–167 °C (lit.[22] 166–168 °C). A sample [22] kindly provided by Dr. N. E. Evans was identical with our product.

1,2-Dimethyl-3-(9-ethoxy-9-xanthyl)indole (13b). The method described above was used starting from 1,2-dimethyl-3-(9-xanthyl)indole (*12c*), without isolation of the intermediate xanthylidene salt. Yield 54 %; m.p. 173–175 °C (lit.[22] 174–176 °C). A sample [22] kindly provided by Dr. N. E. Evans was identical with our product.

Hydrolysis of 2-phenyl-3-(9-xanthylidene)-3H-indolium 2,3-dichloro-5,6-dicyano-1,4-hydroquinolate. A mixture of 2-phenyl-3-(9-xanthylidene)-3*H*-indolium 2,3-dichloro-5,6-dicyano-1,4-dihydroquinolate (300 mg, 0.5 mmol), sodium hydroxide (50 ml, aq., 5 %) and ether (80 ml) was stirred for 3 h. The ether phase was dried and evaporated. The residue treated with methanol gave xanthone. Yield 30 mg (31 %); m.p. 174–175 °C. The methanol mother liquor contained 2-phenylindole and several unidentified products as evidenced by TLC (SiO_2, CH_2Cl_2).

Dehydrogenation of 3-(diphenylmethyl)-2-methylindole, preparation of 2-methyl-3-(diphenylmethylene)-3H-indolium chloride. A solution of 3-(diphenylmethyl)-2-methylindole (297 mg, 1 mmol) in dioxane (5 ml). After 2 h at 25 °C the crystals of DDQ-*2H* formed were filtered off and the clear brown solution treated with HCl(g). The brown crystals formed were collected after 2 h and dried; m.p. 205–206 °C (lit.[38] 205–206 °C).

2-Methyl-3-isopropylidene-3H-indolium hydrogen sulfate. Method A. Sulfuric acid (6 ml) was slowly added to a well-stirred solution of 2-methylindole (6.05 g, 0.05 mol) in dry ether (60 ml) and dry acetone (20 ml) at 5 °C. The yellow salt obtained was collected immediately and dried in an exsiccator. Yield 12.4 g (96 %); m.p. 160–165 °C, dec. (lit.[19] 165 °C, dec.)

Method B. DDQ (227 mg, 1 mmol) in THF (5 ml) was added to a stirred solution of 2-methyl-3-isopropylindole (173 mg, 1 mmol) in

ether (5 ml) followed, 5 min later, by sulfuric acid (98.1 mg, 1 mmol). The precipitated salt is identical with that obtained according to method A.

2-Methyl-3-isopropylindole. 2-Methyl-3-isopropylindene-3*H*-indolium hydrogen sulfate (5.38 g, 0.02 mol) was added in portions to a refluxing mixture of lithium aluminium hydride (2.0 g) in ether (250 ml). After completed addition the reflux was continued for 6 h, whereupon water was carefully added. The ether phase was collected, dried and evaporated. Distillation (110 °C/3 mmHg) of the residue gave 2-methyl-3-isopropylindole. Yield 2.95 g (88 %); m.p. 34–36 °C. ^{1}H NMR (100 MHz, DMSO-d_6): δ 1.35 (6, CH_3, d), 2.02 (3, CH_3, s), 3.16 (1, CH, sept.), 6.7–8.0 (4, arom.). Anal. $C_{12}H_{15}N$: C, H, N:

3-Indolyl-(2-methyl-3-indolyl)methane (*11* $R^1=R^2=R^3=H$, $R^4=CH_3$). DDQ (227 mg, 1 mmol) in dioxane (4 ml) was added to 2,3-dimethylindole (145 mg, 1 mmol) in dioxane (3 ml). After 2 min indole (117 mg, 1 mmol) in dioxane (2 ml) was added. The DDQ-*2H* formed was filtered off after 2 h and the solvent evaporated. The residue was separated by preparative TLC on silica gel using CH_2Cl_2 as eluent. Yield 174 mg (67 %); m.p. 140–141 °C (lit.[20] 137–138 °C).

Di(2-methyl-3-indolyl)methane. Following the description given above, 192 mg (70 %) of the title compound was obtained; m.p. 233–235 °C (lit.[39] 235–238 °C).

Dehydrogenation of 3,3-biindolyl.[37] *Preparation of* 10. 3,3-Biindolyl (232 mg, 1 mmol) in hot ethyl acetate (25 ml) was treated with DDQ (227 mg, 1 mmol) in ethyl acetate (5 ml). The mixture was refluxed for 5 min, cooled and filtered through a short column of alumina. The purified (removal of DDQ-2H) solution was concentrated and allowed to crystallize. Yield 154 mg (67 %). The absorption spectrum (EtOAc) was in agreement with that reported.[18]

5-Iodoindole. DDQ (2.27 g, 10 mmol) in dioxane was added to a solution of 5-iodo-*N*-acetylindoline [40] (2.87 g, 10 mmol) in dioxane (80 ml) at 75 °C. After 2 h at this temperature the mixture was cooled, DDQ-2*H* removed, and the residue evaporated under reduced pressure. The residue was dissolved in methanol (20 ml) and water (25 ml) containing sodium hydroxide (1.5 g). After 30 min at 25 °C the solvents were removed under reduced pressure. The residue extracted with ether gave, after evaporation and recrystallization from hexane, 5-iodoindole. Yield 2.2 g (90 %); m.p. 100–102 °C (lit.[41] m.p. 99–102 °C).

5-Bromoindole [yield 82 %; m.p. 88–89 °C (lit.[42] m.p. 89–89.5 °C)] and indole (yield 77 %) were similarly prepared.

5-Bromo-N-acetylindole. Method A. 5-Bromo-*N*-acetylindoline (10 mmol) was treated as described above except that the sodiumhydroxide treatment was omitted. Yield (2.19 g, 92 %). After recrystallization from light petroleum (with final cooling to −25 °C); m.p. 101–102 °C. ^{1}H NMR (100 MHz, $CDCl_3$): δ 2.49 (3, CH_3, s), 6.43 (1, 3-H, d), 7.0–8.2 (4, arom.) MS[*m/e* (% rel.int)]: 239 (25), 237 (25), 198 (8), 197 (95), 196 (11), 195 (100), 179 (34), 117 (6), 116 (64), 115 (20), 114 (7), 89 (20), 88 (12), 63 (9), and 62 (9). Only peaks stronger than 5 % of the base peak are listed. Anal. calc. for $C_{10}H_8BrNO$: C 50.4; H 3.4; Br 33.6; N 5.9. Found: C 50.6; H 3,5; Br 33.1; N 5.7.

Method B. 5-Bromoindole (1.96 g, 10 mmol) and *N*-acetyl imidazole (1.21 g, 11 mmol) in dimethyl sulfoxide (20 ml) were heated at 120 °C for 2 h. To the cooled solution water (80 ml) was added and the product extracted with ether and then treated as in method A. Yield 2.10 g (88 %), m.p. 101–102 °C.

N-(Chloracetyl)indole. Method A. As described for 5-bromo-*N*-acetylindole starting with *N*-(cloracetyl)indoline.[43] Yield 79 %; m.p. 116–117 °C. NMR (100 MHz, DMSO-d_6): δ 5.18 (2, CH_2, s), 6.75 (1, 3-*H*, d), 7.84 (1, 2-*H*, d), J_{23} 4.1 Hz. MS [*m/e* (% rel.int.)]: 195 (9), 194 (3), 193 (27), 130 (4), 118 (9), 117 (100), 116 (17), 90 (12), 89 (22), 88 (3), 77 (5), 69 (3), 68 (14), 67 (5), 51 (4), 50 (3), 49 (6), and 39 (6). Only peaks stronger than 3 % of the base peak are listed. Anal. $C_{10}H_8ClNO$: C, H, N.

Method B. Indole (11.7 g, 0.1 mol) and chloroacetic anhydride (17.1 g, 0.1 mol) in dioxane (90 ml) were refluxed for 3 h. Thereafter cooled water was added and the semisolid mass obtained was crystallized from methanol with final cooling to −20 °C. Yield 14.4 g (75 %); m.p. 116–117 °C.

5-Acetaminoindole. 5-Acetamino-*N*-acetylindoline [44,45] (2.18 g, 0.01 mol) and DDQ (2.27 g, 0.01 mol) in ethyl acetate (100 ml) were refluxed for 3 h. The solvent was removed using reduced pressure and the residue extracted with ether and sodium carbonate (5 %, aq.). The evaporated ether extract recrystallized from acetonitrile gave the title compound. Yield 1.07 g (62 %); m.p. 117–118 °C (lit.[46] m.p. 117–118 °C).

3-(N-Acetyl-1,4-dihydro-4-pyridyl)-2-methylindole. Pyridine (8.1 ml) was added at 20–25 °C to acetyl chloride (3.5 ml) in dry dioxane (60 ml). 2-Methylindole (6.05 g, 0.05 mol) in dioxane (10 ml) was added. After 2 h at 30 °C the mixture was poured into water (300 ml). The light-brown oil obtained was separated, washed with water and triturated with methanol. The crystals formed were recrystallized from methanol. Yield 6.5 g (52 %); m.p. 154–156 °C. ^{1}H NMR (100 MHz, DMSO-d_6): δ 2.21 (3, CH_3, s), 2.34 (3, CH_3, s), 5.48 (1, CH, s), 5.99 (2, CH, t), 7.6–8.8 (arom+CH, 6), 10.72 (1, NH, s). Anal. $C_{16}H_{16}N_2O$: C, H, N.

3-(N-Acetyl-1,4-dihydro-3-cyano-4-pyridyl)-indole. The same procedure as for 3-(*N*-acetyl-1,4-dihydro-4-pyridyl)-2-methylindole was used except that the reaction temperature was kept at 50 °C. Yield 64 %; m.p. 191–192 °C. ^{1}H NMR (100 MHz, DMSO-d_6): δ 2.30 (3, CH_3, s), 4.60

(1, CH, s), 5.14 (1, CH, quart.), 6.3–9.0 (7, arom), 10.98 (1, NH, s). Anal. $C_{16}H_{13}N_3O$: C, H, N.

3-(N-Acetyl-1,4-dihydro-4-pyridyl)-5-bromoindole. The same procedure as for 3-(*N*-acetyl-1,4-dihydro-4-pyridyl)-2-methylindole was used except that the temperature was kept at 45 °C. Yield 71 %; m.p. 188–190 °C. ^{1}H NMR (100 MHz, DMSO-d_6): δ 2.21 (3, CH_3, s), 4.40 (1, CH, s), 6.6–8.1 (6, arom), 9.94 (1, NH, s). Anal. $C_{15}H_{13}BrN_2O$: C, H, N.

4-(2-Methyl-3-indolyl)pyridinium 4-acetoxy-2,3-dichloro-5,6-dicyanophenolate. DDQ (227 mg, 1 mmol) in ethyl acetate (5 ml) was added to a supersaturated solution (25 °C) of 3-(*N*-acetyl-1,4-dihydro-4-pyridyl)-2-methylindole (252 mg, 1 mmol) in ethyl acetate (20 ml). The mixture obtained was stirred at 40 °C for 1 h. The crystals formed were collected and washed with ethyl acetate and recrystallized from methanol (with final cooling to −25 °C). Yield 442 mg (72 %); m.p. 185–188 °C. Anal. $C_{24}H_{16}Cl_2N_4O_3$: C, H, N. Preparative TLC (silica gel, CH_2Cl_2) of the concentrated ethyl acetate mother liquor gave 2,3-dichloro-4,5-dicyano-1,4-hydroquinone diacetate (2 mg, 0.8 %); m.p. 180–181 °C (lit.[47] 181–182 °C).

5-Bromo-3-(4-pyridyl)indole. Method A. Pyridine (8.1 ml) was added at 20–25 °C to acetyl chloride (7.0 ml) in dioxane (80 ml). 5-Bromoindole (9.8 g, 0.05 mol) was then added and the temperature was kept at 50 °C for 3 h. The mixture was filtered and the crude 4-(*N*-acetyl-5-bromo-3-indolyl)pyridinium chloride was collected and hydrolysed by refluxing (1 h) in 2 M sodium hydroxide (75 ml) and ethanol (75 ml). Water slowly added to the cooled solution gave crystals of 5-bromo-3-(4-pyridyl)indole. Yield 5.9 g (43 %); m.p. 218–220 °C. MS [*m/e* (% rel. int.)]: 275 (15), 274 (97), 273 (18), 272 (100), 194 (5), 193 (16), 192 (12), 182.5 (8), 182 (9), 167 (21), 166 (8), 165 (7), 140 (6), 139 (10), 138 (5), 96.5 (10), and 69.5 (5). Only peaks stronger than 5 % of the base peak are listed. Anal. $C_{13}H_9BrN_2$: C, H, N.

Method B. 4-(5-Bromo-3-indolyl)pyridinium 4-acetoxy-2,3-dichloro-5,6-dicyanophenolate was dissolved in a solution of sodium hydroxide in ethanol and water. The solution obtained was slowly diluted with water and the crystals obtained were collected.

2-Methyl-3-(4-pyridyl)indole. DDQ (2.27 g, 10 mmol) in ethyl acetate (40 ml) was added to a supersaturated solution (25 °C) of 3-(*N*-acetyl-1,4-dihydro-4-pyridyl-2-methylindole (2.52 g, 10 mmol) in ethyl acetate (150 ml). The mixture obtained was stirred at 40 °C for 1 h. The salt formed was collected and dissolved in a solution of sodium hydroxide (5.0 g) in ethanol (35 ml) and water (10 ml). The solution obtained was slowly diluted with water and the crystals obtained were collected and recrystallized from acetonitrile. Yield 1.25 g (60 %); m.p. 192–193 °C (lit.[25] 186–187 °C).

3-(3-Cyano-4-pyridyl)indole and 2,3-dichloro-5,6-dicyano-1,4-dihydroquinone monoacetate. DDQ (2.27 g, 10 mmol) in ethyl acetate (25 ml) was added to a hot solution of 3-(*N*-acetyl-1,4-dihydro-3-cyano-4-pyridyl)indole (2.63 g, 10 mmol) in ethyl acetate (50 ml). After 2 h at reflux the solvent was evaporated and the residue extracted with sodium acetate (2 × 25 ml, aq., 8 %). The residue from the extraction was dissolved in hot ethanol (25 ml) containing water (3 ml) and sodium hydroxide (1.0 g). The solution was cooled to 25 °C and water was slowly added giving crystals of 3-(3-cyano-4-pyridyl)indole. Yield 1.36 g (62 %); m.p. 200–202 °C. Anal. $C_{14}H_9N_3$: C, H, N. IR 3330 (NH), 2195 (CN), 1578, 1515, 1470, 1449, 1408, 1387, 1316, 1248, 1177, 820, and 727 cm^{-1}. MS [*m/e* (% rel. int.)]: 220 (18), 219 (100), 218 (17), 217 (5), 192 (6), 191 (8), 165 (7), 164 (13), 138 (5), 82.5 (5), and 63 (5). Only peaks stronger than 5 % of the base peak are listed. The pH of the cooled sodium acetate extract was adjusted to *ca.* 0.5 by addition of conc. HCl. The crystals obtained recrystallized from acetonitrile gave 2,3-dichloro-5,6-dicyano-1,4-dihydroquinone monoacetate; m.p. 185–186 °C (lit.[47] 184–185 °C). IR 3400, 2190 (CN), 1770, 1625, 1553, 1510, 1440, 1340, 1264, 1201, 1166, 1070, and 1008 cm^{-1}. MS [*m/e* (% rel. int.)]: 270 (18), 268 (27), 230 (43), 229 (17), 228 (70), 200 (20), 110 (16), 109 (25), 101 (18), 100 (17), 89 (30), 87 (100), and 86 (22). Only peaks stronger than 15 % of the base peak and above *m/e* 80 are listed.

2,3-Dichloro-5,6-dicyano-1,4-dihydroquinone diacetate. The reaction just described was repeated starting with 1 mmol of the reactants. The reaction mixture was separated by preparative TLC (silica gel) using CH_2Cl_2–CH_3OH (95:5) as eluent. Yield 11 mg (4 %); m.p. 180–181 °C (lit.[47] 181–182 °C).

Reduction of 5a. *Synthesis of* 21a. *Method A. 5a* (344 mg, 1 mmol) in ethanol (5 ml) and sodium sulfite (aq., 5 %, 5 ml) was heated (60 °C) for 30 min. After cooling and acidification with conc. HCl the mixture was evaporated and the dry residue extracted with methyl acetate. The extract obtained was concentrated and toluene was added. The cooled (finally to −25 °C) solution deposited crystals of *21a*. Yield 191 mg (59 %); m.p. 248 °C, dec. MS [*m/e* (% rel. int.)]: 320 (5), 319 (2), 318 (7), 88 (5), 73 (5), 70 (9), 61 (16), 45 (16), 44 (4), 43 (100), 42 (5), 31 (3), 29 (15), 28 (3), 27 (7), 18 (4), and 15 (6). Only peaks stronger than 2 % of the base peak are listed. UV,VIS [ethanol (log ε)] 219 (4.81), 278 (3.95) and 354 (3.98) nm. IR: 3495, 3380, 3225, 2250 (CN), 1587, 1538, 1440, 1419, 1351, 1290, 1230, 1209, 1175, 1135, 895, 880, and 741 cm^{-1}. Anal. $C_{15}H_8Cl_2N_2O_2$: C, H, N.

Method B. To *5a* (344 mg, 1 mmol) in dioxane (4 ml) was added 1-methylindoline (131 mg, 1 mmol) in dioxane (1 ml). After 30 min at 35 °C the mixture was evaporated *in vacuo*. The residue was extracted first with hexane

and then with methyl acetate. After concentration and cooling the methyl acetate solution gave crystals of *10a*. Yield 75 %. The hexane extract was filtered through a short column containing silica gel. Evaporation of the purified extract gave 1-methylindole (104 mg, 79 %).

Method C. 5a (317 mg, 1 mmol) in ethyl acetate (25 ml) was hydrogenated (H_2, Pd/C) until the theoretical amount of hydrogen had been consumed (*ca* 5 min, also indicated by a colour-change; dark blue to light yellow). The catalyst was filtered off and the solution worked up as described above. Yield 97 %.

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Derivatives and Reactions of Glutacondialdehyde. Part 3. 1-Aryl-3-formyl-2(1*H*)-pyridinethiones from the Glutacondialdehyde Anion and Aryl Isothiocyanates

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1-Aryl-3-formyl-2(1*H*)-pyridinethiones have been synthesized from the glutacondialdehyde sodium salt and aryl isothiocyanates. The course of the reaction may be understood on basis of the concept of hard and soft acids and bases.

Ring closure reactions of glutacondialdehyde derivatives have been reported. Thus, pyridines,[1] pyrylium perchlorate,[2] and some nicotinaldehyde derivatives [3] have been prepared from the appropriate reagents. In connection with a current investigation [4] of the properties of the glutacondialdehyde anion (*1*) we report in this paper a high yield synthesis of 1-aryl-3-formyl-2(1*H*)-pyridinethiones (*2*) from *1* and aryl isothiocyanates (*cf.* Scheme 1).

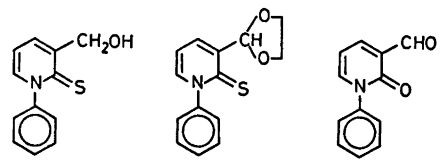
RESULTS

1 *2*

R	R
2a phenyl	*2f* 2′,6′-dimethylphenyl
2b 3′-methylphenyl	*2g* 2′,6′-dichlorophenyl
2c 3′-fluorophenyl	*2h* 1′-naphthyl
2d 4′-fluorophenyl	*2i* 2′-naphthyl
2e 2′-methoxycarbonylphenyl	

Scheme 1.

Due to the low solubility of the salt (*1*) in organic solvents of low polarity, the exothermic reactions were run in *N*,*N*′-dimethylformamide or dimethyl sulfoxide at 25 °C.



3 *4* *5*

Identification. The derivatives *3*, *4*, and *5* were prepared from *2a* by standard methods. By comparing the UV spectra of *3*, *4*, and 1-phenyl-2(1*H*)-pyridinethione it was evident that the same chromophore was present in all three compounds (*cf.* Experimental Section). The IR and MS spectra of *2* further supported the 3-formyl-2(1*H*)-pyridinethione structure. However, based on the information from these spectra and the way of synthesis it seemed impossible to decide definitely whether the formyl group was attached to carbon atom 3 or 6.

^1H NMR spectra. In the ^{1}H NMR spectra of *2* an ABX system was observed (*cf.* Table 1). The magnitudes of the coupling constants $J_{4,5}$ and $J_{5,6}$ were as expected for a 3-substituted 2(1*H*)-pyridinethione or -one.[5] However, both in *2* and *5* the difference between the *ortho* couplings were smaller than predicted [5] and therefore the 6-substituted isomer was not completely excluded.

Table 1. 1-Aryl-3-formyl-2(1*H*)-pyridinethiones from the glutacondialdehyde anion and aryl isothiocyanates $J_{4,5}=J_{5,6}=6.8$ Hz and $J_{4,6}=1.5$Hz for *2a–2i*.

Compound[a]	Yield %	M.p. °C	^{1}H NMR chemical shifts (DMSO-d_6, δ) H(4)	H(5)	H(6)	CHO	Other
2a	95	180–182[b]	7.90	6.98	8.35	10.56	7.3–7.7 (aryl
2b	79	173–175[c]	7.76	6.84	8.20	10.38	7.1–7.3 (aryl), 2.35 (methyl)
2c	97	171–173[b]	7.88	6.93	8.35	10.50	7.2–7.7 (aryl)
2d	82	191–193[c]	7.78	6.86	8.24	10.37	7.3–7.4 (aryl)
2e	52	214–217[c]	7.75	6.95	8.34	10.46	7.4–8.0 (aryl), 3.65 (methyl)
2f	79	190–192[c]	7.99	7.00	8.19	10.54	7.2 (aryl), 2.00 (methyl)
2g	45	259–260[d]	Insoluble				
2h	87	201–204[c]	–	7.06	8.43	10.62	7.4–8.3 (aryl and H(4))
2i	94	195–196[d]	–	6.95	8.43	10.44	7.4–8.1 (aryl and H(4))

[a] *Cf.* Scheme 1. [b] Recrystallized from benzene. [c] Recrystallized from toluene. [d] Recrystallized from 2-methoxyethanol.

The resonance of one of the ring protons was observed within the range δ 8.19–8.35 (*cf.* Table 1). This is in accordance with chemical shift values reported for the 6-proton in 2(1*H*)-pyridinethiones.[5]

^{13}C NMR analyses. The assignments of the chemical shifts for the carbon atoms in *2a* and 1-phenyl-2(1*H*)-pyridinethione were confirmed by selective decoupling experiments (*cf.* Fig. 1). The observed formyl group substituent effects are in agreement with results reported for pyridines and benzenes.[6]

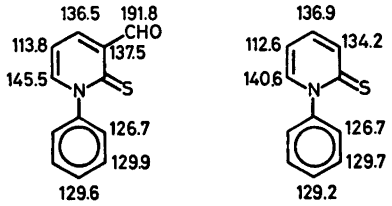


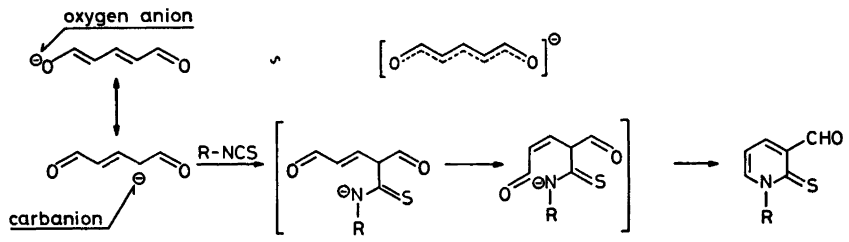
Fig. 1. ^{13}C NMR chemical shifts of 1-phenyl-3-formyl-2(1*H*)-pyridinethione and 1-phenyl-2(1*H*)-pyridinethione (δ, DMSO-d_6).

In the undecoupled spectrum of *2a* a J_{C-H} coupling constant of 183.8 Hz was observed. The magnitude of this coupling constant is in agreement with values reported for $J_{C(6)-H}$ in pyridines [6] and establishes that C(6) in the product *2* is unsubstituted.

DISCUSSION

The reaction of the glutacondialdehyde anion and aryl isothiocyanates gives rise to new and potentially useful pyridine derivatives, which are inaccessible by known procedures; *e.g.* arylation of 2(1*H*)-pyridinethiones is generally not possible.[7] The probable reaction mechanism is shown in Scheme 2.

Ring closure reactions involving isothiocyanates are well-known. However, it may seem surprising that the reaction takes place at carbon number 2 in the glutacondialdehyde



Scheme 2.

anion, but molecular orbital calculations indicate a relatively high electron density at this position.[8]

The reaction course may be understood on basis of the principle of hard and soft acids and bases (HSAB).[9] In the ambident anion, *1*, the carbanion center [C(2)] is a softer base than the oxygen anion center (RO^-, *cf.* Scheme 2). The carbon atom in the isothiocyanato group represents a soft acid. Consequently, the HSAB principle predicts the reaction to take place at C(2). The subsequent cyclization forming the stable 1-aryl-3-formyl-2(1*H*)-pyridinethiones is exceptable.

EXPERIMENTAL

Microanalyses were carried out in the Microanalytical Department of the University of Copenhagen. Satisfactory elemental analyses (±0.3 %) were obtained for all new compounds.

Instrumentation. IR: Perkin Elmer 457. UV: Beckman ACTA III. ^{1}H NMR: Jeol C-60HL and Bruker HX-60. MS: AEI MS-902. ^{13}C NMR: Varian XL-100 F-15FT. The melting points are uncorrected.

General procedure for the preparation of *1-aryl-3-formyl-2(1H)-pyridinethiones (2).* The sodium salt of glutacondialdehyde (*1*) (0.01 mol) in DMSO (10 ml) or DMF and the appropriate aryl isothiocyanate[10] (0.01 mol) were mixed with stirring at room temperature. After 2 h the reaction mixture was added to water (100 ml) and the precipitated orange crystals were isolated, dried and recrystallized (Table 1). In the mass spectra of *2a* to *2i* relative abundant molecular ions were observed. The [M − 29]* ion gave rise to the base peak in all cases. In the IR spectra bands at *ca.* 1100 cm^{-1} (NCS)[11] support structure *2*. UV (ethanol) of *2a*: λ_{max} nm (log ε) = 385 (3.49), 321 (3.99) and 298 sh (3.78).

1-Phenyl-3-hydroxymethyl-2(1H)-pyridinethione (3). 2a (1.0 g) and sodium borohydride (0.5 g) in dioxane (60 ml) were stirred overnight at room temperature. Addition of water (600 ml) and extraction with ether yielded an oil which upon trituration with ether gave *3* (0.42 g). Recrystallization from water gave yellow needles, m.p. 140 − 142 °C.

NMR (DMSO-d_6): δ 7.73 [H(4)], 6.99 [H(5)], 8.04 [H(6)], 7.2 − 7.6 (phenyl), 4.53 (CH_2) and 5.30 (OH); $J_{4,5}$ = 6.9, $J_{5,6}$ = 6.5 and $J_{4,6}$ = 1.8 Hz. UV (ethanol): λ_{max} nm = 368 (log ε = 3.85), 286 (log ε = 4.05) and 238 (sh, log ε = 3.90). IR (KBr) ν_{max} cm^{-1} = 3350 (OH). MS: *m/e* = 217 (100 %, M).

1-Phenyl-3-[2'-(1',3'-dioxalanyl)]-2(1H)-pyridinethione (4). 2a (26.4 g), 1,2-ethanediol (40 g) and *p*-toluenesulfonic acid (0.5 g) in dry benzene (1.2 l) were refluxed (with water separator). When 8 ml water had been collected, the reaction mixture was evaporated *in vacuo* to give *4* (24.3 g). Recrystallization from cyclohexane-benzene yielded yellow needles, m.p. 163 − 165 °C.

NMR (DMSO-d_6): δ 7.73 [H(4)], 6.91 [H(5)] 8.10 [H(6)], 7.3 − 7.6 (phenyl), 6.41 (CH) and 4.03 (CH_2); $J_{4,5}$ = 7.2, $J_{5,6}$ = 6.5 and $J_{4,6}$ = 1.8 Hz. UV (ethanol): λ_{max} nm = 378 (log ε = 3.94), 293 (log ε = 4.33) and 236 (sh, log ε = 4.05). MS: *m/e* = 259 (67 %, M) and 186 (100 %).

1-Phenyl-3-formyl-2(1H)-pyridone (5). 2a (8.6 g) and mercury(II) oxide (19.5 g) were refluxed in abs. ethanol (750 ml) for 5 h. After filtration of the black reaction mixture and concentration *in vacuo* a semicrystalline red compound (8.5 g) was obtained. Trituration with benzene yielded *5* (1.2 g). Recrystallization from toluene yielded *5* as pale yellow crystals, m.p. 155 − 157 °C.

NMR (DMSO-d_6): δ 8.08 [H(4)], 6.57 [H(5)], 8.19 [H(6)], 7.5 (phenyl) and 10.15 (CHO). $J_{4,5}$ = 7.2, $J_{5,6}$ = 6.5 and $J_{4,6}$ = 2.3. UV (ethanol): λ_{max} nm = 360 (log ε = 3.82) and 238 (sh, log ε = 4.01). IR (KBr): ν_{max} cm^{-1} = 2855 (CHO). 1689 (CHO) and 1660 (2(1*H*)-pyridone). MS: *m/e* = 199 (28 %, M) and 171 (100 %).

1-Phenyl-2(1H)-pyridinethione. 1-Phenyl-2(1*H*)-pyridone[12] (0.7 g) was heated with phosphorus pentasulfide at 170 °C for 6 h. Addition of 4 N sodium hydroxide, extraction with chloroform, concentration *in vacuo* and recrystallization from cyclohexane yielded yellow crystals, m.p. 102 − 104 °C (31 %).

NMR(DMSO-d_6): δ 6.85 (H(5)), 7.98 [H(6)], 7.3 − 7.7 [H(3), H(4) and phenyl]; $J_{4,5}$ = 6.0, $J_{5,6}$ = 6.0, $J_{4,6}$ = 1.2 and $J_{3,5}$ = 2.3 Hz. UV (ethanol): λ_{max} nm = 371 (log ε = 3.76), 292 (log ε = 4.01) and 234 (sh, log ε = 3.81). MS: *m/e* = 187 (100 %, M$^+$).

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Chemical Studies on Bryophytes. 17. A New Luteolin Tetraglycoside from *Hedwigia ciliata*

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The moss *H. ciliata* contains twelve flavonoid compounds. One of the flavonoids has been assigned the structure luteolin-7,4′-di-*O*-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) (*1*) by a combination of spectroscopic methods and a sugar sequence analysis by GLC-MS of the methylated alditol acetates.

In this series of chemical studies of liverworts and mosses, the common moss *Hedwigia ciliata* was investigated. This communication describes the isolation and separation of the flavonoids from this moss, and the structural determination of one luteolin tetraglycoside. The structures of the other flavonoids will be reported later.

The ethanolic extract from 4.8 kg of the moss *H. ciliata* after gel filtration, paper and column chromatography, contains twelve flavonoid compounds (Table 1). Four of these flavonoids (*1*, *3*, *10*, and *11*) were obtained in larger quantities than the others. The yields of these main components were each larger than 0.5 g. One of the minor flavonoids (*12*) is probably identical with the biluteolin isolated from *Dicranum scoparium*.[1]

One of the main components (*1*) was purified by gel filtration. By R_F values and UV spectral studies of the products obtained after acid hydrolysis of *1* the aglycone was identified as luteolin and the sugars as glucose and rhamnose. The sugars are linked to luteolin at the 7- and the 4′-position according to UV spectral data of *1* using diagnostic shift reagents.[2]

Partial hydrolysis of *1* gave two intermediate luteolin glycosides, showing that *1* contains at least three monosaccaride molecules. The

Table 1. R_F values of the isolated flavonoids from *H. ciliata* on 0.1 mm pre-coated cellulose TLC plates.

Compound	Solvent		
	TBA	BAW	15 % HOAc
1	0.09	0.15	0.75
2	0.04	0.07	0.66
3	0.04	0.07	0.63
4	0.16	0.28	0.62
5	0.11	0.24	0.52
6	0.10	0.24	0.45
7	0.13	0.20	0.34
8	0.54	0.50	0.32
9	0.30	0.37	0.18
10	0.15	0.22	0.40
11	0.07	0.11	0.25
12	0.92	0.94	0.02

osmometrically determined molecular weight of *1* (mol. wt. 885) indicates that *1* is probably a tetraglycoside of luteolin. Only one luteolin glycoside with more than two monosaccaride molecules has been reported earlier.[3] The latter is a luteolin-triglycoside with sugars attached in the 7- and the 4′-positions.[4]

The ^{1}H NMR spectrum of the TMS ether of *1* (Fig. 1) shows four sugar C-1 proton signals confirming that *1* is a tetraglycoside. The two doublets at 5.14 and 5.45 must be due to two glucosyl C-1 protons, since the coupling constants of about 7 Hz agree with a diaxial coupling between the C-1 and the C-2 protons in a β-linked glucose.[2,5] The two signals at 4.76 and 4.86 correspond to two rhamnosyl C-1 protons.[2] Since the two C-1 proton signals at the lowest field are due to glucose, this

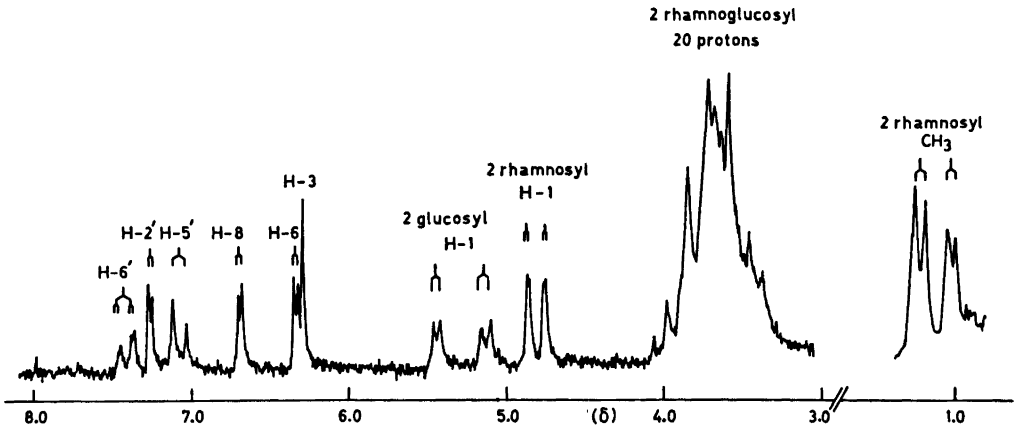


Fig. 1. ^{1}H NMR spectrum of the TMS ether of luteolin-7,4'-di-*O*-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside).

indicates that *1* has two β-glucose molecules attached to luteolin and that the two rhamnose units are linked to the glucose. Treatment of *1* with β-glucosidase (emulsin) gave no hydrolysis, showing that there are no terminal β-glucose units.

Mass spectroscopy of the permethylated *1* gave no molecular peak and only a weak peak for the aglycone (*m/e* 314). A base peak at *m/e* 189 and peaks at *m/e* 157 (189 − CH_3OH) and 125 (157 − CH_3OH) indicated a terminal rhamnose unit. No fragments from a terminal glucose could be detected. The peak at *m/e* 393 indicated a rhamnoglucoside, and the peak at *m/e* 361 that the rhamnose unit is probably linked to the glucose at the 2-position.[6]

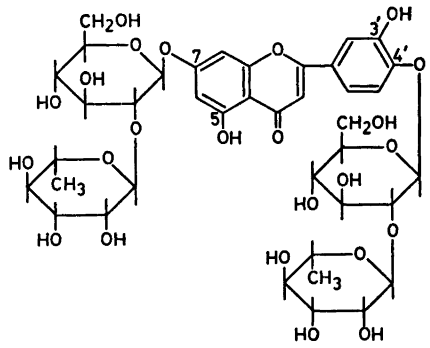
To establish the sugar sequence, a method used for the analysis of polysaccharides was applied.[7–10] This method has also been used for structural determinations of the sugar moieties in flavonoids.[11–13] The luteolin tetraglycoside was permethylated, hydrolyzed and the methylated sugars reduced and acetylated to give partially methylated alditol acetates. Results obtained by GLC-MS analysis of the alditol acetate mixture are given in Table 2. The ratio of the two GLC-peak areas was 1:1 proving that, after methylation and hydrolysis, there are only two different sugar units. The peak with a *T* value of 0.45 corresponds to 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol showing that the rhamnose units are terminal. The peak with a *T* value of 1.97 corresponds to both 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, but the MS data eliminate the second alternative.[10] This result shows that the two rhamnose units are linked to the two glucose units at the 2-position.

The aglycone isolated after permethylation

Table 2. GLC-MS analysis data of the methylated alditol acetates. *T* values are relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Alditol acetate	*T*	*T* [10]	Prominent fragments *m/e*
1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol	0.45	0.46	175, 161, 131, 117, 115, 101, 89, 72, 45, 43
1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol	1.97	1.98	205, 189, 161, 145, 129, 101, 99, 87, 71, 45, 43

and hydrolysis was identified as 5,3′-di-*O*-methylluteolin by means of MS and UV spectral data. Therefore *1* is a luteolin-7,4′-di-*O*-(2-*O*-rhamnopyranosyl-glucopyranoside). The optical rotation of synthetic luteolin-7-*O*-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside is $[\alpha]_D^{22}$ −97.55° (pyridine).[14] To explain the large negative rotation, $[\alpha]_D^{26}$ −192° (pyridine), of the isolated luteolin 7,4′-di-*O*-rhamnoglucoside, *1* must contain two units of β-D-glucose and two units of α-L-rhamnose. Taking these data together, the structure of *1* is proposed to be luteolin-7,4′-di-*O*-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) (*1*).



EXPERIMENTAL

NMR spectra were recorded on a Varian A 60-D instrument, UV-visible spectra on a Bausch & Lomb Spectronic 505 spectrophotometer and on a Zeiss spektralphotometer DMR 10. MS were recorded on a LKB 9000 and an AEI MS 30 instrument. GLC were performed on a Perkin Elmer 990 Gas Chromatograph as described earlier.[11] Gel filtrations were performed on Sephadex G-25 columns with EtOH−H_2O (1:1) as eluent. Molecular weight determination was made with a Knauer vapour pressure osmometer. Solvent systems: BuOH−HOAc−H_2O, 6:1:2 (BAW), *t*-BuOH−HOAc−H_2O, 3:1:1 (TBA).

Isolation. The moss *H. ciliata* was collected in the surroundings of Enköping and Uppsala. Foreign materials were carefully removed and the air-dried moss (4.8 kg) was pulverized and first extracted with $CHCl_3$, air-dried and then extracted with 80 % aqueous EtOH at room temperature. The ethanolic extract was evaporated in vacuum at room temperature and the residue suspended in H_2O and continually extracted with ether. The water phase was concentrated to a small volume and, after gel filtration, gave four flavonoid fractions. The first fraction consisted of nine flavone glycosides (*1*−*9*), the second and third fractions gave two flavone C-glycosides (*10* and *11*) and the fourth fraction gave one biflavone (*12*). PC on Whatman 3 MM paper with BAW and CC on cellulose with 5 % HOAc of the first fraction led to complete separation of the nine flavone glycosides. R_F values, see Table 1.

*Luteolin-7,4′-di-*O*-rhamnoglucoside* (*1*). The crude fraction *1* was further purified by gel filtration. Drying at 100 °C/0.1 mmHg over P_2O_5 gave 0.5 g of a pale yellow compound, m.p. > 360 °C, $[\alpha]_D^{26}$ −192° (*c* 0.27, pyridine). Found: C 48.24; H 5.95. Calc. for $C_{39}H_{58}O_{28}$: C 48.05; H 6.00. Mol. weight, obs. 885, calc. for $C_{39}H_{50}O_{24}$: 902.8. UV (99.9 % CH_3OH): 272, 340; (+$AlCl_3$): 279, 295sh, 354, 380sh; (+$AlCl_3$/HCl): 281, 293sh, 352, 379sh; (+MeONa): 270, 371; (+NaOAc): 271, 338; (+NaOAc/H_3BO_3): 271, 342 nm.

Acid hydrolysis of *1* with 6 % HCl at 100 °C gave luteolin, glucose and rhamnose. Luteolin was identified by MS data, R_F values and UV data. Glucose and rhamnose were identified by PC and TLC by comparison with authentic samples.

Partial hydrolysis of *1* with 10 % HCl at room temperature for 140 h gave two intermediates; *1a* and *1b*. *1a* gave on complete hydrolysis luteolin, glucose and rhamnose, partial hydrolysis with 10 % HCl at room temperature gave *1b*. *1b* gave on complete hydrolysis luteolin, rhamnose and glucose. *1a*: R_F values 0.48 (15 % HOAc) and 0.15 (TBA). UV (99.9 % CH_3OH): 269, 336; (+$AlCl_3$): 279, 294sh, 351, 392sh; (+$AlCl_3$/HCl): 279, 294sh, 349, 392sh; (+MeONa): 276, 380; (+NaOAc): 269, 313sh, 330; (+NaOAc/H_3BO_3): 271, 340 nm. This indicated a luteolin-7,4′-tri-*O*-glycoside. *1b*: R_F values 0.21 (15 % HOAc) and 0.38 (TBA). UV (99.9 % CH_3OH): 266, 348; (+$AlCl_3$): 277, 292sh, 430; (+$AlCl_3$/HCl): 277, 292sh, 358, 381sh; (+MeONa): 276, 409; (+NaOAc): 260, 407; (+NaOAc/H_3BO_3): 260, 370 nm. This indicated a luteolin-7-*O*-glycoside.

The acetate was prepared with Ac_2O in pyridine. M.P. 144−146 °C, Found: C 52.13; H 5.53. Calc. for $C_{67}H_{84}O_{41}$: C 52.07; H 5.48. ^1H NMR (100 MHz, $CDCl_3$): δ 7.73 (H6′), 7.64 (H2′), 7.31 (H5′), 7.03 (H8), 6.72 (H6), 6.58 (H3), 5.4−4.9 (sugar H), 4.3−3.7 (sugar H), 2.41 (aromatic acetyl H), 2.25−1.85 (sugar acetyl H), 1.22 and 1.12 (rhamnose CH_3).

The TMS ether was prepared according to standard procedures.[2] ^1H NMR (100 MHz, CCl_4): δ 7.42 (H6′), 7.26 (H2′), 7.08 (H5′), 6.70 (H8), 6.35 (H6), 6.30 (H3), 5.45 (glucose H1, *J* 6 Hz), 5.14 (glucose H1, *J* 7 Hz), 4.86 (rhamnose H1, *J* 2 Hz), 4.76 (rhamnose H1, *J* 2 Hz), 4.10−3.30 (sugar H), 1.22 and 1.02 (rhamnose CH_3).

The permethyl ether was prepared with NaH, DMSO and CH_3I using Hakomori's procedure.[15] The permethyl ether was purified by CC on silica gel with acetone as eluent. ^1H NMR

(100 MHz, $CDCl_3$): δ 7.44 (H6'), 7.32 (H2'), 7.10 (H5'), 6.70 (H8), 6.60 (H3), 6.53 (H6), 5.42 (glucose H1), 5.33 (glucose H1), 5.00 (rhamnose H1), 4.93 (rhamnose H1), 4.0 – 3.0 (sugar H and OCH_3), 1.33 and 1.25 (rhamnose CH_3). MS [70 eV; *m/e* (% rel. int.)]: 394 (0.5), 393 (1), 391 (0.6), 390 (0.4), 379 (0.4), 363 (0.3), 362 (1), 361 (5), 360 (4), 347 (0.7), 346 (0.4), 332 (0.4), 331 (0.3), 329 (0.3), 328 (0.4), 314 (0.3), 204 (13), 190 (11), 189 (100), 188 (23), 187 (7), 175 (8), 173 (5), 157 (33), 145 (13), 143 (5), 131 (6), 129 (10), 125 (8), 113 (7), 101 (35), 99 (33), 89 (10), 88 (22), 75 (18), 71 (10), 59 (20). Only peaks larger than 5 % (0.3 % *m/e* 300 – 500) of the base peak are given.

Sequence analysis of sugar. The permethylated glycoside was hydrolyzed with 8 % H_2SO_4 at 100 °C for 3 h. 5,3'-Di-*O*-methylluteolin deposited on cooling. The acid solution was neutralized with the anion exchanger IRA-400 in carbonate form and concentrated to 4 ml in vacuum at room temperature. Reduction with $NaBH_4$ and acetylation with Ac_2O in pyridine was performed as described earlier.[7] Retention times and prominent fragments in GLC-MS analysis are given in Table 2.

5,3'-Di-O-methylluteolin. M.p. 273 – 276 °C. UV (99.9 % CH_3OH): 241, 265, 337; (+$AlCl_3$): 241, 265, 337, 412; (+$AlCl_3$/HCl): 241, 265, 301, 340, 414; (+MeONa): 261, 270sh, 317sh, 392; (+NaOAc): 263sh, 270, 313, 384; (+NaOAc/H_3BO_3): 264, 337 nm. MS [70 eV; *m/e*; (% rel. int.)]: 315 (21), 314 (100), 313 (50), 300 (16), 298 (10), 297 (14), 286 (6), 285 (31), 284 (26), 283 (14), 270 (11), 269 (9), 268 (36), 256 (6), 255 (6), 253 (6), 242 (5), 241 (6), 238 (5), 213 (6), 167 (4), 151 (4), 149 (4), 148 (19), 143 (9), 138 (4), 137 (21), 136 (5), 133 (14), 128 (6), 123 (4), 118 (4), 108 (5), 105 (10). Only peaks larger than 4 % of the base peak are given.

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Synthesis of 4-*O*-(β-D-Glucopyranosiduronic Acid)-dopamine

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4-*O*-(β-D-Glucopyranosiduronic acid)-dopamine was synthesized for comparison with a substance of identical properties present in a golden hamster islet cell tumour. The compound was prepared by ion pair alkylation of tetrabutylammonium 2-benzyloxy-4-formylphenolate with methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate followed by nitromethylation of the aldehyde function, catalytic hydrogenation of the nitrostyrene derivative to a saturated amine with simultaneous removal of the benzylic group and a final hydrolysis of the ester functions. The ion pair alkylation afforded a very convenient route to this glycoside.

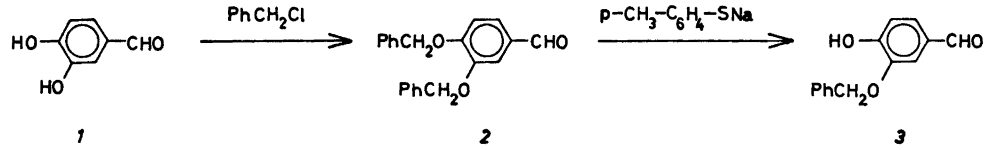
In studies on catechol and indole derivatives in a transplantable islet cell tumour in the golden hamster we have obtained chromatographic and spectroscopic evidence suggesting the presence of an unusual biogenic amine. This substance on treatment with formaldehyde and glyoxylic acid vapour gives a strongly fluorescent derivative with emission at much shorter wavelength than is usual for similar derivatives from biogenic catecholamines. Biochemical and chemical data [1] indicate that the substance is a glucuronide of dopamine with the glycosidic linkage in the 4-position. Monoglucuronides of catecholamines have been found earlier [2] but the position of the conjugation has never been well established and they have never been prepared synthetically before. To obtain more definite proof of the structure and to obtain material for histochemical and pharmacological studies we decided to make a total synthesis of the most probable compound, 4-*O*-(β-D-glucopyranosiduronic acid)-dopamine. The synthetic material showed identical fluorescence spectrum and R_F-value to the compound obtained from the golden hamster islet cell tumour.

RESULTS AND DISCUSSION

The desired product, *8*, has two main components, the sugar and the aglycon. The sugar component was protected as the fully acetylated methyl uronate and this was converted to methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate *4* according to known procedures.[3] In the aglycon part we circumvented the need for nitrogen protection by insertion of the nitrogen part of the molecule after formation of the glycosidic linkage and we protected the 3-oxygen atom with a benzylic group which could easily be removed by catalytic hydrogenation under very mild conditions which would leave the glycosidic bond intact. As a point of attack for insertion of the nitrogen-containing fragment of the molecule we decided to use the easily available aldehyde group.

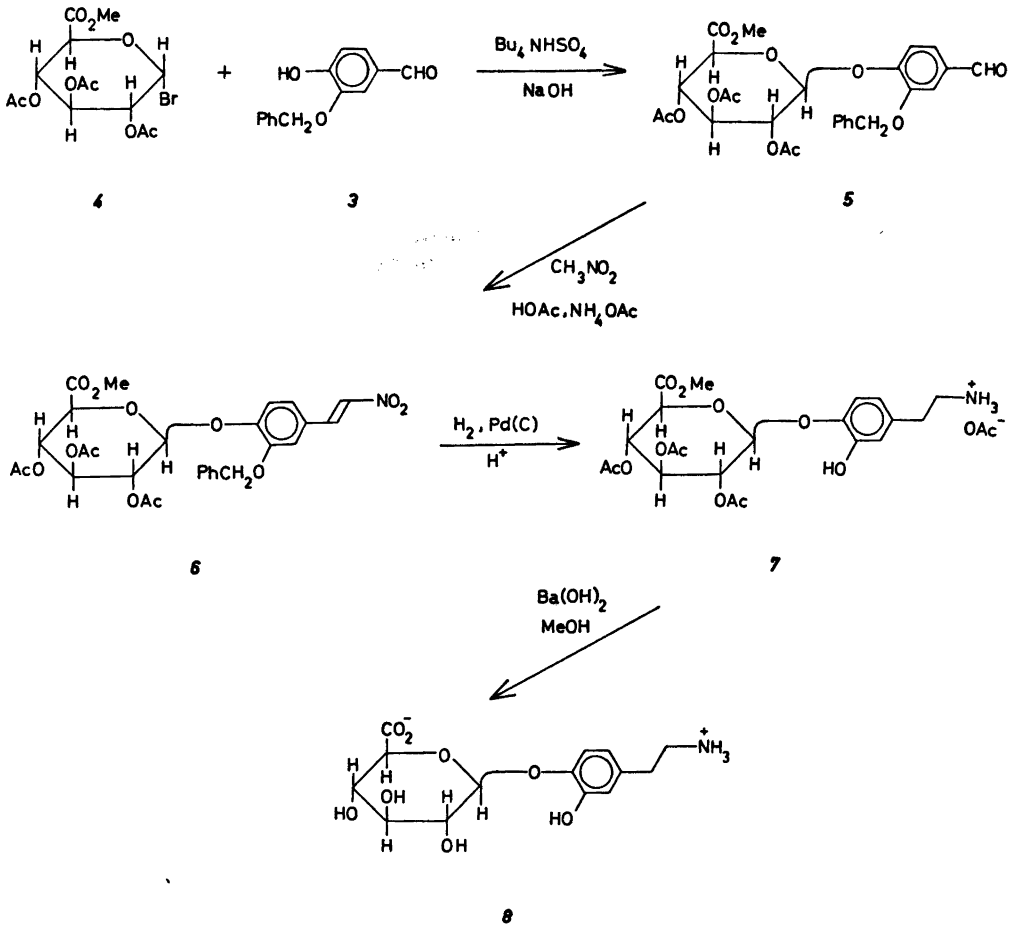
Selective protection of the 3-oxygen of the 3,4-dihydroxybenzaldehyde (*1*) by benzylation in strong alkali [4] was tedious with a difficult isomer separation and furthermore gave a low yield. These problems were, however, avoided by full benzylation to 3,4-dibenzyloxybenzaldehyde *2* and selective debenzylation to 3-benzyloxy-4-hydroxybenzaldehyde *3*. It was carried out by nucleophilic substitution using the very strong nucleophile sodium *p*-thiocresolate to attack the more electrophilic benzyl group in the 4-position [5] (Scheme 1).

The formation of the glycosidic bond presented serious difficulties as most of the usual glycosidation reactions failed entirely or gave



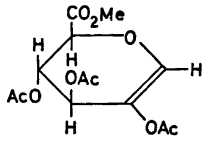
Scheme 1.

very low yields. Thus, coupling of 3-benzyloxy-4-hydroxybenzaldehyde, *3*, with methyl (tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate, *4*, using lithium hydroxide in methanol [6] gave a difficultly separable mixture which contained only about 10 % of the desired product *5*. Cadmium carbonate in toluene has been successfully used as base for a similar glycosidation of steroid phenols [7] but gave in this case about the same yield. However, by performing the glycosidic coupling by ion pair alkylation [8] we succeeded in obtaining the desired product, *5*, in 28 % yield (Scheme 2). To our knowledge this is the first time ion pair alkylation has been used in the synthesis of a glycosidic bond. Tetrabutylammonium 2-benzyloxy-4-formylphenolate was extracted with methylene chloride from an aqueous solution of tetrabutyl-



Scheme 2.

ammonium hydrogen sulfate and excess sodium hydroxide and reacted with methyl (tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate, *4*. The principal byproduct was methyl 2,3,4-tri-*O*-acetyl-1-deoxy-D-arabinohex-1-ene uronate, *9*, (37 %) formed by internal elimination of hydrogen bromide. This elimination product has been reported as the main product on attempted formation of glycosides of certain complex phenols.[9] The starting aldehyde *3* was used in slight excess and 50 % was recovered.



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As the yield of the tetrabutylammonium phenolate might have been lowered by formation of an adduct of free phenol and the tetrabutylammonium phenolate [10] we also tried the use of an equimolar 50 % sodium hydroxide solution with a one-step extraction and alkylation, but this method was inferior to the two-step procedure. It should be noted that this is a rather special case with a very lipophilic phenol and a formyl group in the *para* position.

The ion pair alkylation procedure gave a β-glycosidic linkage since the product, *5*, was identical with products obtained by reactions known to give the β-anomer, for example by use of lithium hydroxide in methanol. The β-linkage was also corroborated by the optical rotations. These comply with Hudson's rule of isorotation for *5* as well as for the subsequent derivatives. Further the IR spectrum showed absorption at 890 cm^{-1}, characteristic for β-anomers, but no absorption at 845 cm^{-1}, characteristic for α-anomers.[11]

The glycosidic aldehyde *5* was reacted with nitromethane in absolute ethanol with acetic acid/ammonium acetate as a catalyst [12] giving the nitrostyrene derivative *6* (76 %). The nitrostyrene *6* was then hydrogenated catalytically in acetic acid with a small amount of concentrated sulfuric acid [13] and a palladium catalyst. The reaction was very fast even at atmospheric pressure and the benzyl group was removed in the same reaction. To minimize acidic cleavage of the glycoside, the mineral acid was removed by passing the reaction mixture through an anion exchanger in acetate form.

Hydrolysis of the dopamine acetate glucuronide *7* with aqueous barium hydroxide in methanol gave the desired 4-*O*-(β-D-glucopyranosyluronic acid)-dopamine, *8*, in 94 % yield. This dopamine glucuronide was compared with material from the hamster transplantable islet cell tumour. Both showed the same R_F-value on paper chromatography and their fluorescent derivatives from reaction with glyoxylic acid showed the same characteristic fluorescence spectrum. The synthetic product, *8*, was cleaved by β-glucoronidase giving free dopamine in the same way as the biological material. The β-configuration was further established by IR data and optical rotation, as for the glycosidic aldehyde *5*. The NMR spectrum of *8* gave a coupling constant of 6.6 Hz between the H-1′ and H-2′ protons and thus according to the Karplus curve must correspond to the β-glycosidic product. The combined evidence confirms the structure of the synthetic product and indicates its equivalence with the substance found in the biological preparation.

EXPERIMENTAL

All melting points are uncorrected. IR spectra (in KBr) were measured with a Perkin-Elmer Modell 257 spectrophotometer. NMR spectra were recorded on a Jeol JNM-MH-100 NMR-spectrometer or a Varian T-60 spectrometer. TLC was performed using Merck Fertigplatten F_{254}, silica gel, dimethyl sulfoxide impregnated silica gel, and cellulose. For visualization of the compounds, UV light, silver nitrate (2 % aq) spray followed by sodium hydroxide (2 M) and 10 % sulfuric acid spray followed by heating at 120 °C for 15 min were used. The following solvent systems were used; A (chloroform – ethyl acetate, 3:1), B (ethyl acetate-light petroleum, 2:1), C (ethyl ether saturated with dimethyl sulfoxide), D (phenol – 0.1 M hydrochloric acid, 1:9). Elemental analyses were performed by Centrala Analyslaboratoriet, Uppsala.

*Methyl(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate* (*4*) was prepared accordingly to Ref. 3.

3-Benzyloxy-4-hydroxybenzaldehyde (*3*). 3,4-Dibenzyloxybenzaldehyde,[14] *2*, (1.58 g) and a slight excess of sodium *p*-thiocresolate (950 mg) were refluxed under nitrogen in sodium-dried toluene (15 ml) with a small amount of HMPA

(1.15 ml) for 20 h. The reaction was monitored on TLC using silica gel plates and solvent system A. When 3,4-dibenzyloxybenzaldehyde had disappeared, methylene chloride (35 ml) was added and 3-benzyloxy-4-hydroxybenzaldehyde was extracted with ten portions of 2 M sodium hydroxide. The aqueous phase was collected and acidified with concentrated HCl to pH 1 and the 3-benzyloxy-4-hydroxybenzaldehyde was reextracted with methylene chloride. The methylene chloride phase was washed with water and dried over sodium sulfate. Concentration under reduced pressure on a Rotavapor gave pure (TLC, NMR) crystals which could be used without further purification or recrystallised from ethanol. Yield 1.10 g (96 %). M.p. 112–113 °C, lit.[4] 114 °C. ^{1}H NMR (60 MHz, $CDCl_3$): δ 5.13 (s, 2 H), 7.04 (d, 1 H, J 8.0 Hz), 7.40 (m, 7 H), 9.80 (s, 1 H). IR: (KBr) 3500–3000, 1660, 1590, 1507 cm^{-1}.

*Methyl(2-benzyloxy-4-formylphenyl 2',3',4'-tri-*O*-acetyl-β-*D*-glucopyranoside) uronate* (*5*). 3-Benzyloxy-4-hydroxybenzaldehyde, *3*, (3.2 g) was dissolved in methylene chloride (50 ml). Solutions of sodium hydroxide (5.6 g) in water (15 ml) and tetrabutylammonium hydrogen sulfate (7.0 g) in water (25 ml) were prepared. The three solutions were mixed and shaken vigorously for 10 min. The methylene chloride phase was carefully separated and diluted with ethyl ether (50 ml). Methyl(tri-*O*-acetylglucopyranosyl bromide) uronate, *4*, (4.0 g) dissolved in methylene chloride–ether 1:1 (100 ml) was added with stirring (1 h at 0 °C). The reaction mixture was left overnight and then concentrated under reduced pressure to 10 ml on a Rotavapor. The residue was chromatographed on silica gel (deactivated with 2.5 % water) using methylene chloride as eluent. The eluate was monitored by a UV-detector. As soon as the starting aldehyde *3* had been eluted, 5 % ether was added to the eluent thus to give the desired product, *5*, in a rather small volume. The product fraction was evaporated to dryness giving a gelatinous residue (2.8 g), which was crystallised by dissolving in a small volume of methylene chloride, adding ether until opalescence appeared and then leaving in a refrigator overnight; this gave white crystals (0.9 g) of pure (TLC, NMR) product *5*. From the mother liquor a second crop of pure product (0.6 g) was isolated, yield 1.5 g (28 %). Recrystallisation from methylene chloride-ether gave m.p. 148.5–149.5 °C. $[\alpha]_D^{25}$ −83.7° (*c* 1, chloroform). ^{1}H NMR (60 MHz, $CDCl_3$): δ 1.80 (s, 3 H), 2.04 (s, 6 H), 3.72 (s, 3 H), 4.05–4.30 (m, 1 H), 5.15 (s, 2 H), 5.35 (m, 4 H), 7.40 (m, 8 H), 9.85 (s, 1 H), IR: (KBr) 1770, 1705, 1600, 1514, 1235, 1090, 1045, 895 cm^{-1}. Anal. $C_{27}H_{28}O_{12}$: C, H.

Evaporation of the mother liquor and recrystallisation of the solid residue from ethanol gave methyl 2,3,4-tri-*O*-acetyl-1-deoxy-D-arabinohex-1-ene uronate *9* (1.2 g, 37 %), m.p. 74–76 °C, lit.[9] 76 °C.

The first fraction of the eluate gave 1.65 g (50 %) recovery of the starting aldehyde *3*.

*Methyl(2-benzyloxy-4-(2-nitrovinyl)phenyl 2',3',4'-tri-*O*-acetyl-β-*D*-glucopyranoside) uronate* (*6*). Methyl(2-benzyloxy-4-formylphenyl-tri-*O*-acetyl-β-D-glucopyranoside) uronate *5* (850 mg) was dissolved in absolute ethanol (25 ml). Ammonium acetate (0.5 g), acetic acid (0.5 ml), and a few drops of acetic anhydride and nitromethane (1.1 ml) were added and the mixture was heated with stirring at 60 °C overnight. Methylene chloride (25 ml) was then added and the solution was washed twice with water. After evaporation to dryness the residue was dissolved in a small volume of methylene chloride and then ether was added until opalescence appeared. The mixture was left in a refrigator overnight giving 600 mg of the desired glycosidic nitrostyrene *6* as yellow crystals. Another crop (90 mg) of the product *6* was collected from the mother liquors. Total yield 690 mg (76 %), m.p. 205–210 °C. After two recrystallisations from methylene chloride-ether, the melting point was 208–210 °C. $[\alpha]_D^{25}$ −71.7° (*c* 1, chloroform), ^{1}H NMR (60 MHz, $CDCl_3$): δ 1.80 (s, 3 H), 2.03 (s, 6 H), 3.70 (s, 3 H), 4.05–4.30 (m, 1 H), 5.10 (s, 2 H), 5.30 (m, 4 H), 7.00–8.00 (m, 10 H). IR (KBr): 1770, 1645, 1610, 1599, 1530, 1517, 1235, 1107, 1045, 898 cm^{-1}. Anal. $C_{28}H_{29}NO_{13}$: C, H, O.

*Methyl(4-(2-ammonioethyl)-2-hydroxyphenyl 2',3',4'-tri-*O*-acetyl-β-*D*-glucopyranoside) uronate acetate* (*7*). Methyl(2-benzyloxy-4-(2-nitrovinyl)-phenyl 2',3',4'-tri-*O*-acetyl-β-D-glucopyranoside) uronate *6* (256 mg) was hydrogenated in acetic acid (5 ml), with concentrated sulfuric acid (75 μl) and palladium (5 % on carbon, 100 mg) which had been previously saturated with hydrogen. With vigorous stirring at atmospheric pressure and room temperature the calculated amount of hydrogen was absorbed within 1 h. After removal of the catalyst, excess acetic acid was evaporated under reduced pressure with continuous addition of ethanol (70 %) to maintain the volume at about 5 ml. The residue was passed through an anion exchange resin, Amberlite CG 4B in acetate form, and eluted with ethanol 75 %. The eluate was evaporated under reduced pressure and the residual syrup was crystallized by adding ether. The white crystals formed were collected and washed with ether giving 165 mg (70 %) of the desired product *7*, m.p. 125–127 °C. $[\alpha]_D^{25}$ −27.1° (*c* 0.2, methanol). ^{1}H NMR (100 MHz, CD_3OD): δ 1.90 (s, 3 H), 2.05 (s, 9 H), 2.84 (t, 2 H, J 6.6 Hz), 3.12 (t, 2 H, J 6.6 Hz), 3.70 (s, 3 H), 4.39 (d, 1 H, J 9.6 Hz), 5.05–5.55 (m, 4 H), 6.62 (m, 2 H), 6.95 (d, 1 H, J 8.1 Hz). IR (KBr): 3700–3200, 1765, 1580, 1515, 1235, 1105, 1050, 895 cm^{-1}. Anal. $C_{23}H_{31}NO_{13}$: C, H. N.

*4-*O*-(β-*D*-glucopyranosiduronic acid)-dopamine* (*8*). The glycosylated dopamine acetate *7* (132 mg) was treated with aqueous barium

hydroxide (5 ml 0.15 M) and methanol (5 ml) at 60 °C for 3 h. Acetic acid (1 ml) was then added and the barium was precipitated by adding concentrated sulfuric acid (100 μl). After centrifugation the supernatant was passed through an anion exchange resin, Amberlite CG-400 in acetate form, and the product was eluted with water. The eluate on evaporation under reduced pressure at 30 °C gave a dried residue of white amorphous material which could not be induced to crystallize, yield 78.7 mg (94 %). $[\alpha]_D^{25}$ −54.0° (*c* 0.8, water). ^{1}H NMR (100 MHz, D_2O): δ 2.83 (t, 2 H, *J* 6.6 Hz), 3.19 (t, 2 H, *J* 6.6 Hz), 3.67 (m, 3 H), 4.15 (d, 1 H, *J* 9.0 Hz), 4.73 (broad s), 5.05 (d, 1 H, *J* 6.6 Hz), 6.76 (m, 2 H), 7.05 (d, 1 H, *J* 8.1 Hz). IR (KBr): 3700 – 3000, 1565, 1415, 875 cm^{-1}. Found: C 50.09; H 6.04; N 3.97. Calc. for $C_{14}H_{19}NO_8$: C 51.06; H 5.82; N 4.25.

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Synthesis of 2-*O*-α-D-Glucopyranosyl-β-D-galactopyranoside Derivatives Suitable for Linking to Proteins

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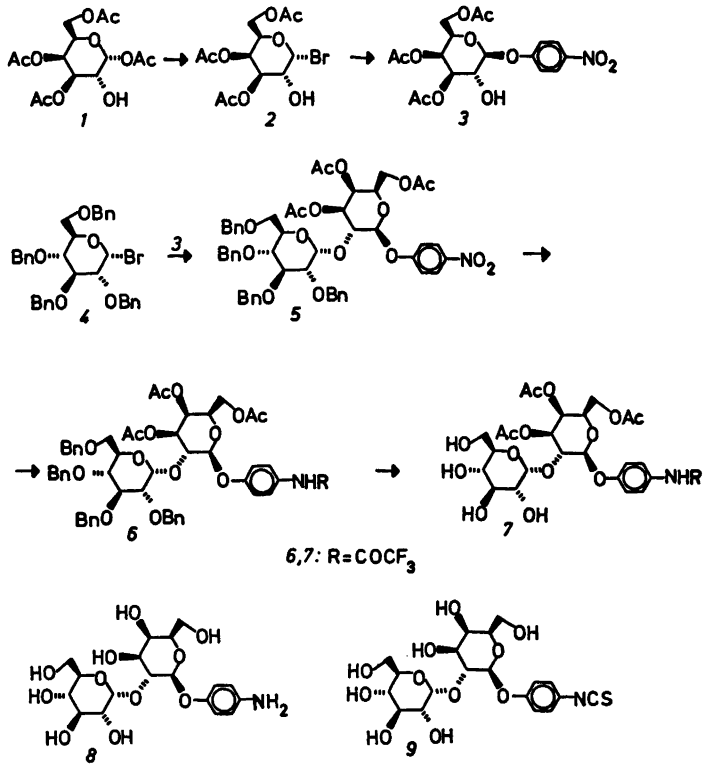
The synthesis of *p*-trifluoroacetamidophenyl 3,4,6-tri-*O*-acetyl-2-*O*-(α-D-glucopyranosyl)-β-D-galactopyranoside (*7*) suitable, after deacylation, for linking of 2-*O*-α-D-glucopyranosyl-β-D-galactopyranoside residues to proteins is described. The disaccharide is of interest in blood platelet agglutination studies.

In connection with studies on human platelet aggregation,[1] a disaccharide glycoside containing a 2-*O*-α-D-glucopyranosyl-β-D-galactopyranoside with an aglycone suitable for covalent linking to peptides or proteins was required. The present paper describes the synthesis of such a disaccharide.

1,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranose [2] (*1*), in dichloromethane, was treated with hydrogen bromide. The α-bromo sugar *2* thus obtained was dissolved in dry acetone and



condensed with *p*-nitrophenol in the presence of potassium carbonate,[3] to give the glycoside 3 with the hydroxyl group in the 2-position free, in an 81 % yield from *1*. Glycosidation of *3*, with 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl bromide (*4*) in dichloromethane containing tetraethylammonium bromide and molecular sieve [4] afforded the fully protected disaccharide derivative *5*, in a 27 % yield from *3*. Hydrogenation of the nitro group in *5* and conversion of the product into the *N*-trifluoroacetyl derivative *6* was followed by hydrogenolysis to give the final product *7* in 70 % yield from *5*. The *N*-trifluoroacetyl derivative *6* is an appropriate derivative to store inasmuch as the free amine *8*, readily produced from *7* by deacylation, and the isothiocyanate *9*, produced from *8* by reaction with thiophosgene,[5] are unsuitable for storage due to their instability. The *p*-isothiocyanato derivative *9* reacts with free amino groups and acidic hydroxyls in peptides and proteins.[5] The use of the disaccharide derivative *9* in platelet aggregation studies will be published elsewhere.

EXPERIMENTAL

General methods. Concentrations were performed at reduced pressure. Optical rotations (*c* 0.5 to 2.0) were determined at room temperature (23 – 25 °C) using a Perkin-Elmer 141 polarimeter. NMR spectra were recorded using a Varian XL-100 instrument, in deuteriochloroform unless otherwise stated. Only pertinent parts of spectra for key compounds are given below. The remaining portion of all spectra was invariably in accordance with the presumed structures. Analytical TLC was performed on "Merck DC-Fertigplatten, Kieselgel F 254" and preparative TLC on 2 mm "Merck PSC-Fertigplatten, Kieselgel F 254". The absorbent for silica gel column chromatography was "Merck, Kieselgel 0.040 – 0.063 mm".

3,4,6-Tri-O-acetyl-α-D-galactopyranosyl bromide (2). A solution of 1,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose (*1*)[3] (4.0 g) in dichloromethane (20 ml) at 0 °C was added dropwise with stirring to a saturated solution of hydrogen bromide in dichloromethane (150 ml) also at 0 °C. The reaction was monitored by TLC (toluene – ethyl acetate, 1:1). After 1 h at 0 °C when no starting material remained, the solution was concentrated to a syrup which was used directly in the next step, $[\alpha]_D$ +188° (chloroform). NMR: δ 2.05 – 2.20 (9 H, OAc), 3.96 (1 H, dd, $J_{1,2}$ 4 Hz, $J_{2,3}$ 10 Hz, H-2), 4.10 – 4.19 (2 H, m, H-6, H-6′), 4.50 (1 H, t, H-5), 5.22 (1 H, dd, $J_{3,4}$ 3 Hz, H-3), 5.48 (1 H, d, H-4), 5.70 (1 H, OH), 6.62 (1 H, d, H-1).

p-*Nitrophenyl 3,4,6-tri-O-acetyl-β-D-galactopyranoside* (3). *p*-Nitrophenol (4 g) and potassium carbonate (4 g) were added to acetone (100 ml) (dried over potassium carbonate) and the glycosyl halide *2* (prepared from the tetraacetate (4 g) and used immediately) dissolved in dry acetone (20 ml) was added. The mixture was stirred at room temperature for 30 min and then refluxed for 30 min.[3] The solution was diluted with 250 ml chloroform and shaken with several portions of ice-cold saturated aqueous sodium carbonate until no further *p*-nitrophenol was removed (absence of yellow coloration). The solution was dried over sodium sulfate, filtered and concentrated to a syrup (4 g). Column chromatography on silica gel (toluene – ethyl acetate, 1:1) yielded chromatographically homogeneous *3* (3.8 g), $[\alpha]_D$ −12° (chloroform), R_F 0.46 (TLC same solvent). (Found: C 51.1, H 5.21, N 2.86. $C_{18}H_{21}NO_{11}$ requires: C 50.6, H 4.95, N 3.28). The NMR assignments were corroborated by spin decoupling experiments. Decoupling irradiation at the broad multiplet at δ 4.15 (4 H, H-2, H-5, H-6 and H-6′) caused the H-1 (d) to collapse into a singlet and the H-3 (dd) to collapse into a doublet. Irradiation at δ 5.45 (H-4) caused the H-3 signal to collapse into a doublet. From chemical shift considerations H-2 could not be acetylated. The following assignments were made: δ 2.08 – 2.16 (9 H, OAc), 2.88 (1 H, OH), 4.15 (4 H, H-2, H-5, H-6 and H-6′), 5.00 (1 H, dd, $J_{2,3}$ 10 Hz, $J_{3,4}$ 3 Hz, H-3), 5.08 (1 H, d, $J_{1,2}$ 8 Hz, H-1), 5.45 (1 H, d, H-4), 7.12 and 8.18 (2 H each, both d, both $J_{H,H}$ 9 Hz, *p*-$NO_2C_6H_4O$ protons).

p-*Nitrophenyl 3,4,6-tri-O-acetyl-2-O-(tetra-O-benzyl-α-D-glucopyranosyl)-β-D-galactopyranoside* (5). The *p*-nitrophenyl 3,4,6-tri-*O*-acetyl-β-D-galactopyranoside *3* (2.2 g) was dissolved in purified dichloromethane (50 ml) which contained tetraethylammonium bromide (2.1 g) and molecular sieve 4 Å (5 g). Tetra-*O*-benzyl-α-D-glucopyranosyl bromide *4* freshly prepared from tetra-*O*-benzyl-1-*O*-(*p*-nitrobenzoyl)-α-D-glucopyranose (3.5 g) was added and the mixture was stirred at room temperature under nitrogen for 5 days.[4] The solids were removed by filtration through a bed of Celite, and the filtrate was diluted with dichloromethane and washed with water, saturated aqueous sodium hydrogen carbonate and finally water. The solution was dried over anhydrous sodium sulfate, filtered and concentrated to a syrup (4.7 g). Column chromatography on silica gel (toluene – ethyl acetate, 2:1) yielded chromatographically homogeneous *5* (1.3 g), $[\alpha]_D$ +13° (chloroform), R_F 0.64 (TLC same solvent). (Found: C 65.7, H 5.93, N 1.83. $C_{52}H_{55}NO_{16}$ requires: C 65.8, H 5.84, N 1.47). The ^1H NMR spectrum was in accordance with that expected for *5*.

p-*Trifluoroacetamidophenyl 3,4,6-tri-O-acetyl-2-O-(tetra-O-benzyl-α-D-glucopyranosyl)-β-D-ga-*

lactopyranoside (6). The foregoing compound *5* (500 mg) was hydrogenated at room temperature and atmospheric pressure in ethyl acetate (25 ml) using Adams catalyst (100 mg). When sufficient hydrogen to account for the conversion of a nitro to an amino group had been consumed, trifluoroacetic anhydride (0.8 ml) and pyridine (1.9 ml) were added and the mixture was kept at 60 °C for 30 min.[6] The catalyst was removed by filtration and the filtrate concentrated. The residue was dissolved in toluene and shaken with water. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated to a syrup (545 mg). Column chromatography on silica gel (toluene–ethyl acetate, 2:1) yielded chromatographically homogeneous *6* (400 mg), $[\alpha]_D$ +27° (chloroform), R_F 0.51 (TLC, same solvent). The ^{1}H NMR spectrum was in accordance with that expected for *6*.

p-*Trifluoroacetamidophenyl 3,4,6-tri*-O-*acetyl-2*-O-*(α*-D-*glucopyranosyl)-β*-D-*galactopyranoside* (7). The syrupy product *6* (290 mg) from the above reaction was hydrogenated, in a Parr apparatus, in ethanol (25 ml) using 10 % palladium on charcoal (150 mg) as catalyst. When hydrogen consumption had ceased, the catalyst was removed by filtration and the filtrate was concentrated to yield *7* as a chromatographically homogeneous syrup (175 mg), $[\alpha]_D$ +34° (acetone). NMR: (acetone-d_6): δ 2.03–2.15 (9 H, OAc), 5.17 (1 H, dd, $J_{2,3}$ 10 Hz, $J_{3,4}$ 3 Hz, H-3 galactose residue), 5.33 (1 H, d, $J_{1,2}$ 8 Hz, H-1 galactose residue), 5.44 (2 H, d, $J_{1,2}$ 4 Hz, H-1 glucose residue, H-4 galactose residue), 7.18 and 7.64 (2 H each, both d, both $J_{H,H'}$ 9 Hz, *p*-$CF_3CONHC_5H_4O$–).

An aliquot of *7* was hydrolysed with 0.25 M aqueous sulfuric acid for 24 h at 100 °C. The product was reduced with sodium borohydride and acetylated.[7] The glucitol hexaacetate and galactitol hexaacetate thus obtained were indistinguishable from authentic standards on GLC.

Another aliquot of *7* was methylated,[8] hydrolyzed, reduced with sodium borohydride and acetylated.[9] The two *O*-methylalditol acetates thus obtained were indistinguishable from authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-galactitol, respectively, on GLC and MS.

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Effect of Metal Chelators on the Absorption Spectrum and on the Chlorpromazine Oxidase Activity of Ceruloplasmin

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In the presence of NADH and other reducing compounds (cysteine, reduced glutathione and ascorbate) the copper enzyme, ceruloplasmin, can bind bathocuproine and neocuproine, probably to one of the non-paramagnetic copper ions. An absorption band centered around 485 and 465 nm was associated with the enzyme-chelator complex in the case of bathocuproine and neocuproine, respectively. Experiments indicated that EDTA could bind to the same site on the enzyme molecule. The chelators investigated increased the chlorpromazine oxidase activity of ceruloplasmin in the presence of NADH, the activating effect increasing with increasing stability of the enzyme-chelator complex [bathocuproine ($K_d \cong 0.2$ μM) > EDTA ($K_d = 8.5$ μM) > neocuproine ($K_d = 19.3$ μM)]. A considerably higher V_{max}-value was obtained in the presence of chelator, while the K_m-value was slightly lowered.

Ceruloplasmin (coeruloplasmin, ferroxidase, EC 1.16.3.1) is a blue serum protein, containing several copper atoms, namely the paramagnetic Type-1 ("blue") Cu(II) and Type-2 ("non-blue") Cu(II) and non-paramagnetic copper, supposed to consist of strongly coupled Cu(II) – Cu(II) ions.[1] Approximately half of the copper in ceruloplasmin is non-paramagnetic. The number of copper atoms bound to the ceruloplasmin molecule is still uncertain: values of six,[2] seven,[3] and eight [4] atoms/molecule have been reported. Chelex-100 treatment of the protein results in the loss of one of the copper atoms.[5] Ceruloplasmin has oxidase activity towards several compounds, such as aromatic diamines and diphenols,[6–8] inorganic Fe(II),[7–10] and drugs of the phenothiazine class.[11] The enzyme is inhibited by the anions, azide and fluoride,[12–14] which bind to Type-2 copper when the enzyme is in the resting state.[13,14] In this communication it is shown that the copper chelating agents, bathocuproine, neocuproine and EDTA, readily bind to ceruloplasmin in the presence of NADH and other reducing compounds, changing the visible absorption spectrum of the enzyme and increasing its chlorpromazine oxidase activity.

EXPERIMENTAL

Materials. Human ceruloplasmin was purchased from AB Kabi and crystallized according to the method of Deutsch.[15] The purified enzyme had an absorbance ratio, A_{610}/A_{280}, of 0.047. Enzyme concentrations were calculated from the 610 nm absorption ($\varepsilon = 10.9$ mM^{-1} cm^{-1}).[15] The enzyme was treated with Chelex-100, which removes one of the protein bound copper atoms [5] without affecting the visible absorption spectrum and chlorpromazine oxidase activity of ceruloplasmin.

NADH, 2,9-dimethyl-1,10-phenanthroline (neocuproine) and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline-disulfonate (bathocuproine) were obtained from Sigma Chem. Co., EDTA and sodium azide from E. Merck AG and chlorpromazine from A/S Dumex. Aqueous solutions were prepared in deionized, glass-distilled water.

Measurement of enzyme activity. The chlorpromazine oxidase activity of ceruloplasmin was measured spectrophotometrically by adding NADH to the reaction mixture and recording the change in absorption at 340 nm, due to the disappearance of NADH, which is spontaneously oxidized by free radicals generated from chlorpromazine.[11] Appropriate corrections were made for a slow non-enzymic oxidation of NADH. Bathocuproine, neocuproine, and EDTA were incubated with enzyme and NADH for 10 min before adding chlorpromazine. Trace iron ions, which activate the reaction

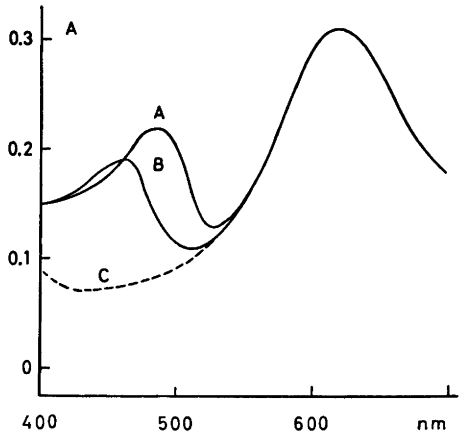

Fig. 1. Effect of bathocuproine (A) and neocuproine (B) on the visible absorption spectrum of Chelex-100 treated ceruloplasmin in the presence of NADH. The solution contained 30 μM ceruloplasmin, 0.5 mM bathocuproine (0.8 mM neocuproine) and 1.6 mM NADH in 0.2 M sodium acetate buffer, pH 5.5. C, Spectrum of ceruloplasmin alone.

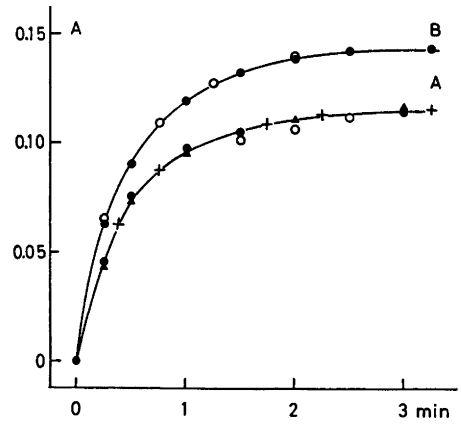

Fig. 2. Time course of the 485 nm (A) and 465 nm (B) absorbance changes observed on mixing bathocuproine and neocuproine, respectively, with Chelex-100 treated ceruloplasmin, incubated with NADH for 15 min. A: The solution contained 25 μM ceruloplasmin, 0.4 mM bathocuproine and NADH (●, 15 mM; +, 10 mM; ○, 8 mM; ▲, 5 mM) in 0.3 M sodium acetate buffer, pH 5.5. B: The solution contained 39 μM ceruloplasmin, 0.8 mM neocuproine and 10 mM NADH (●, ○; two experiments) in 0.3 M sodium acetate buffer, pH 5.5.

between ceruloplasmin and several compounds,[8] did not affect the enzymic oxidation of chlorpromazine.

Apparatus. Kinetic measurements and recordings of absorption spectra were performed in a Beckman DK-1 spectrophotometer, which was equipped with a thermo cell. The temperature was kept at 30 °C in all experiments.

RESULTS

Effect of chelators on the absorption spectrum of Chelex-100 treated ceruloplasmin. Absorption spectra in the visible region, recorded immediately after mixing ceruloplasmin with bathocuproine or neocuproine in the presence of NADH, are shown in Fig. 1. The interaction between ceruloplasmin and bathocuproine leads to the appearance of a new absorption band with a maximum at 485 nm. No significant change of the 610 nm chromophore of ceruloplasmin was observed. The 485 nm band also appeared when ceruloplasmin was incubated for 10 min with 2.8 mM cysteine, reduced glutathione or ascorbate instead of NADH. Cysteine and ascorbate completely reduced the 610 nm chromophore of the enzyme without increasing the 485 nm absorption band in the presence of an excess of bathocuproine. When bathocuproine was substituted with neocuproine an absorption band centered around 465 nm appeared (Fig. 1). Azide (1 mM) did not affect the formation of the new absorption bands, when incubated for 10 min with ceruloplasmin before adding NADH and chelator. Gel filtration experiments showed that the chelators did not remove copper from the Chelex-100 treated enzyme molecule.

Kinetics of the reaction between ceruloplasmin and chelators. Fig. 2 shows the formation of the 485 nm chromophore observed on adding bathocuproine to Chelex-100 treated ceruloplasmin, incubated with NADH for 15 min. Another curve shows the formation of the 465 nm absorption band observed when neocuproine was added instead of bathocuproine. An excess of NADH (≧ 5 mM) was used in order to make all the enzyme molecules available for chelator binding. The time course of the absorbance changes at 485 nm and 465 nm was not affected by variations in the NADH concentration in this case. Titration of ceruloplasmin with bathocuproine or neo-

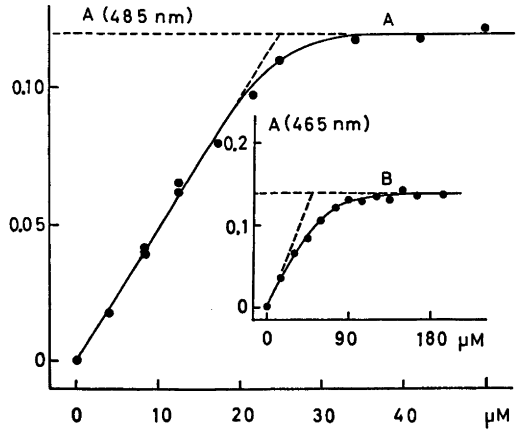

Fig. 3. Titration of Chelex-100 treated ceruloplasmin with bathocuproine (A) and neocuproine (B). Absorbance changes were determined 10 min after each addition of chelator to ceruloplasmin (25 μM (A), 41 μM (B)) and 10 mM NADH in 0.3 M sodium acetate buffer, pH 5.5.

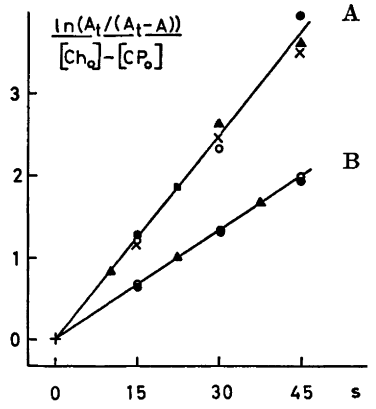

Fig. 4. Plots of $\ln(A_t/(A_t-A))/([Ch_0]-[CP_0])$ *vs.* time for the formation of the ceruloplasmin-chelator complex. A: ●, 25 μM enzyme and 15 mM NADH; ○, 25 μM enzyme and 8 mM NADH; ▲, 25 μM enzyme and 5 mM NADH; ■, 15 μM enzyme and 5 mM NADH; ×, 45 μM enzyme and 8 mM NADH. The bathocuproine concentration was 0.4 mM in 0.3 M sodium acetate buffer, pH 5.5. B: ●, 0.8 mM neocuproine; ○, 0.63 mM neocuproine; ▲, 0.4 mM neocuproine. The solutions contained 38 μM ceruloplasmin and 10 mM NADH in 0.3 M sodium acetate buffer, pH 5.5.

cuproine (Fig. 3) suggests that one chelator molecule is bound to the enzyme molecule. A molar absorption of 4.8 mM^{-1} cm^{-1} (485 nm) and 3.8 mM^{-1} cm^{-1} (465 nm) was calculated in the case of bathocuproine and neocuproine, respectively. The dissociation constant, K_d, for the system

$$CP + Ch \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CP - Ch \qquad (1)$$

(CP = ceruloplasmin, Ch = chelator) was calculated and listed in Table 1. The concentration of ceruloplasmin in its free and chelator bound form was determined from the final absorbance reading at 485 nm or 465 nm, using suitable concentrations of enzyme and chelators. The formation of the enzyme-chelator complex, recorded at 485 nm or 465 mn (Fig. 2), followed second order kinetics initially, as indicated by the linear graphs obtained on plotting ln $(A_t/(A_t-A))/([Ch_0]-[CP_0])$ against time (Fig. 4). The corresponding rate constant, k_1, was calculated from the slope of the line. $[Ch_0]$ and $[CP_0]$ represent concentrations at zero time, while A_t is the maximum absorption obtained with the amount of enzyme present. Several concentrations of enzyme, NADH and chelators (excess) were used. The rate constant for the splitting of the enzyme-chelator complex, $k_{-1} = K_d k_1$, was also estimated. The constants are listed in Table 1.

When EDTA was added to a solution of ceruloplasmin, containing an excess of NADH and neocuproine, after all the enzyme is bound to neocuproine, a reduction of the 465 nm absorption band took place. Eventually a new steady state level was reached. It is assumed that EDTA competes with neocuproine for the binding site on ceruloplasmin. From the new steady state level the fraction of enzyme bound to neocuproine and to EDTA was determined. The amount of free enzyme was negligible, since large concentrations of chelators were used. The dissociation constant for EDTA was calculated from the equation (NC = neocuproine)

$$K_d^{EDTA} = K_d^{NC} \frac{[CP-NC][EDTA]}{[CP-EDTA][NC]} \qquad (2)$$

and listed in Table 1.

Effect of chelators on the chlorpromazine oxidase activity of Chelex-100 treated ceruloplasmin. Bathocuproine, neocuproine, and

Table 1. List of kinetic parameters.

Chelator	K_d/ μM	k_1/ mM^{-1} min^{-1}	k_{-1}/ min^{-1}	K_m/ mM	V_{max}/ [CP_0]/ μM NADH/min
Bathocuproine	~0.2	5.0	~0.001	2.7	380
Neocuproine	19.3± 1.4 [a]	2.6	0.05	2.2	224
EDTA	8.5± 1.5 [b]			2.6	312
No chelator				3.6	54

[a] Average value of 12 determinations ± SEM. [b] Average value of 5 determinations± SEM.

EDTA have an activating effect on the ceruloplasmin-catalyzed oxidation of chlorpromazine in the presence of NADH, the order of effectiveness being bathocuproine > EDTA > neocuproine (Fig. 5). A sigmoidal curve is obtained when the activity is plotted against chelator concentration (Fig. 5). The activating effect increased with increasing NADH concentration, when an excess of chelator was present, suggesting that more NADH makes more enzyme molecules available for chelator binding. In the absence of chelator the reaction rate is independent of the NADH concentration. When NADH is omitted from the reaction solution the activity can be measured spectrophotometrically at 530 nm as the rate of chlorpromazine radical formation.[11] In this case the chelators did not affect the oxidation rate, demonstrating that NADH is necessary for chelator activation. The effect of bathocuproine, neocuproine, and EDTA on the oxidase activity of ceruloplasmin at different chlorpromazine concentrations was investigated and the results are presented in a double reciprocal plot (Fig. 6). The NADH concentration was kept constant in these experiments and maximum activation was obtained with the chelator concentrations used. The apparent Michaelis constants (K_m) and maximum activities (V_{max}) were calculated from the plot in Fig. 6 and listed in Table 1. V_{max} is markedly increased in the presence of chelators, while K_m is slightly decreased.

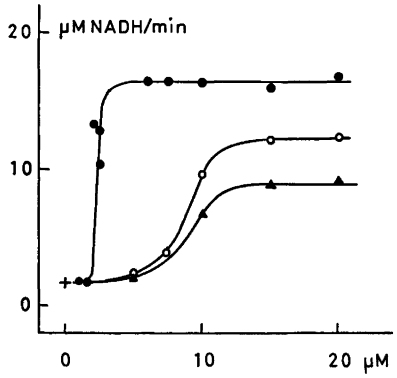

Fig. 5. Effect of bathocuproine (●), EDTA (○) and neocuproine (▲) on the chlorpromazine oxidase activity of ceruloplasmin. The reaction mixtures contained 0.15 μM ceruloplasmin, 1 – 20 μM chelator, 0.25 mM NADH and 1 mM chlorpromazine in 0.25 M sodium acetate buffer, pH 5.5. +, Activity in the absence of chelator.

DISCUSSION

The experiments suggest that one of the ceruloplasmin copper atoms can bind bathocuproine, neocuproine, and EDTA after reduc-

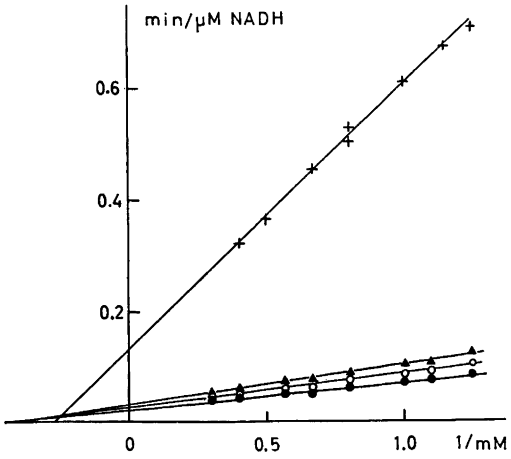

Fig. 6. The reciprocal activity plotted against the reciprocal chlorpromazine concentration in the absence (+) and presence of chelators (●, 25 μM bathocuproine; ○, 25 μM EDTA; ▲, 0.25 mM neocuproine). The reaction mixtures contained 0.14 μM ceruloplasmin, 0.3 mM NADH and 0.8 – 3.3 mM chlorpromazine in 0.3 M sodium acetate buffer, pH 5.5.

tion with NADH, cysteine, reduced glutathione or ascorbate. Studies by Carrico *et al.*[16] demonstrated that ascorbate reduced both Type-1 and Type-2 Cu(II) as well as non-paramagnetic Cu(II). Bathocuproine and neocuproine are Cu(I)-specific complexing reagents, giving rise to yellow chromophores on binding to inorganic Cu(I) or to Chelex-100 treated ceruloplasmin (Fig. 1). The observations that (1) the enzyme-chelator complex can be formed without altering the 610 nm chromophore of ceruloplasmin and that (2) cysteine and ascorbate completely reduce the 610 nm chromophore without increasing the concentration of the enzyme-chelator complex, when an excess of chelator is present, suggest that the "blue" Type-1 copper is not involved in chelator binding.

The Type-2 copper of ceruloplasmin has been reported to be essential for oxidase activity.[1] Kinetic studies indicate that during reaction Type-2 copper is located between Type-1 and non-paramagnetic copper in the electron transport chain.[17] Since the chelators investigated increase the enzyme activity (Fig. 5), and since azide, which binds to Type-2 copper,[13] does not inhibit the formation of the NADH dependent enzyme-bathocuproine (neocuproine) complex, it seems unlikely that the chelators bind to the Type-2 copper. Therefore it is probable that the complexing compounds react with one of the non-paramagnetic copper ions, and that this ion is not involved in the catalytic process.

Ceruloplasmin forms an especially stable complex with bathocuproine; the dissociation of which is characterized by a low rate constant (Table 1). Bathocuproine was also the best activator of the reaction between ceruloplasmin and chlorpromazine. The activating effect of the chelators was found to decrease with decreasing stability of the enzyme-chelator complex (bathocuproine > EDTA > neocuproine) (Table 1). The sigmoidal activation curves shown in Fig. 5 cannot be accounted for at present. This type of curve is usually associated with positive cooperativity of the effector molecules. The increased V_{max}-values obtained for the ceruloplasmin-catalyzed oxidation of chlorpromazine in the presence of chelators and NADH (Table 1), suggest that the chelator bound enzyme molecules have an increased rate of product formation from the enzyme-substrate (product) complex. The lower K_m-values obtained in the presence of chelators indicate that they also increase the enzyme affinity for chlorpromazine.

The observation that bathocuproine readily binds to ceruloplasmin in the presence of ascorbate and SH-containing compounds, could mean that the copper ion on the chelator binding site is reduced, when ceruloplasmin is circulating in the blood.

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Synthesis of 1-Acyl-3-piperidones and Ring Expansion of Methyl 3-Oxopiperidine-1-carboxylate with Ethyl Diazoacetate

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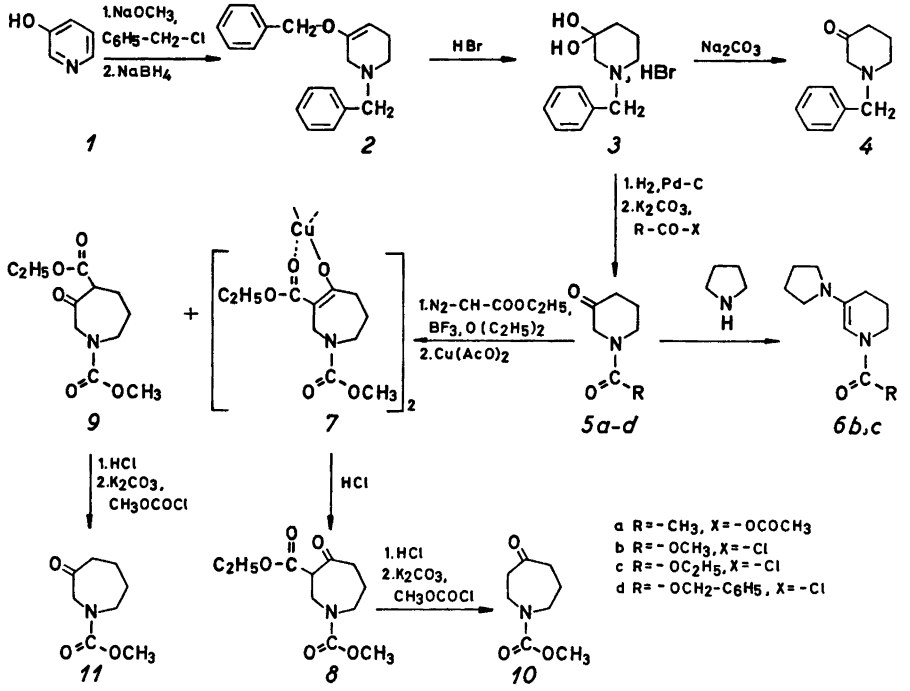
The 1-acyl-3-piperidones *5a–d* have been synthesized from pyridin-3-ol *via* a reaction sequence, which seems to be of general utility for the preparation of 1-acyl-3-piperidones. Pyridin-3-ol was converted into 1-benzyl-3-piperidone hydrobromide hydrate (*3*) *via* 1-benzyl-1,2,5,6-tetrahydro-3-pyridyl benzyl ether (*2*). Hydrogenolysis of *3* followed by treatments with the appropriate acylating agents gave *5a–d*. Compound *3* was converted into 1-benzyl-3-piperidone (*4*). The 1-acyl-3-piperidones *5b,c* were transformed into the corresponding 1-pyrrolidinyl enamines *6b,c*. The boron trifluoride catalyzed reaction of *5b* with ethyl diazoacetate gave a mixture of the two cyclic β-oxoesters ethyl 1-methoxycarbonyl-4-oxoperhydroazepine-3-carboxylate (*8*) and ethyl 1-methoxycarbonyl-3-oxoperhydroazepine-4-carboxylate (*9*), which were separated *via* selective formation of the copper(II) chelate of *8*. The structures of *8* and *9* were finally confirmed by conversion into the ketones methyl 4-oxoperhydroazepine-1-carboxylate (*10*) and methyl 3-oxoperhydroazepine-1-carboxylate (*11*), respectively. The amide rotation in the urethane groups of *5b–d*, *6b,c*, and *11* is discussed on the basis of the ^{1}H NMR spectra.

Nipecotic acid (piperidine-3-carboxylic acid)[1–3] and guvacine (1,2,5,6-tetrahydropyridine-3-carboxylic acid)[3,4] represent a new structural class of potent γ-aminobutyric acid (GABA) uptake inhibitors of neurophysiological and pharmacological interest. In our attempts to evaluate structural analogues of nipecotic acid and guvacine certain 1-acyl-3-piperidone derivatives have proved useful as synthetic intermediates.

The synthetic aspects of 3-piperidone derivatives have not been profoundly investigated. 1-Methyl-[5,6] and 1-benzyl-3-piperidone [7] have been synthesized. 1-*tert*-Butyl-3-piperidone and 1-*tert*-butyl-4-methyl-3-piperidone were formed by rearrangement of the appropriate aminomethylcyclopropylketones.[8] 1-Acetyl-3-piperidone (*5a*)[9] and the corresponding 5*S*-methyl analogue [10] were formed in very small scale by pyrolysis of 1-acetyl-2-benzoyloxypiperidin-3-ols. This paper presents a synthetic procedure, apparently of general utility, for the preparation of 1-acyl-3-piperidones as outlined in Scheme 1. In addition the syntheses of the two isomeric cyclic β-oxoesters *8* and *9* are presented using *5b* as a starting material.

The reaction sequence for the preparation of *3* as shown in Scheme 1 is analogous to that described for the corresponding 1-methyl derivative.[5] Because the parent compound 3-piperidone may be unstable like its 1-methyl [6] and 1-benzyl derivatives, [7] aqueous solutions of the crude reaction products from hydrogenolysis of *3* were treated with the appropriate acylating agents immediately after addition of base to give the 1-acyl-3-piperidones *5a–d*. Attempts to increase the yield of *5b* by addition of methyl chloroformate to the reaction mixture immediately before addition of base were unsuccessful.

Treatment of *5b* with ethyl diazoacetate and boron trifluoride etherate gave as the only products a mixture of the cyclic β-oxoesters *8* and *9*, as established by TLC. Attempts to separate *8* and *9* by distillation *in vacuo* under a variety of conditions were unsuccessful. Repeated column chromatographic treatments of an analytical sample of the concerned mixture, however, afforded *8* and *9* in a pure state. The ^{1}H NMR spectra of *8* and *9* in tetrachloromethane solutions revealed that *8* as regards



Scheme 1.

ca. 25 % is in the enol form, whereas the enol form of *9* could hardly be detected. Thus there was reason to suppose that *8* and *9* might be separated *via* selective copper(II) salt formation of *8*. After treatment of the mixture of *8* and *9* with copper(II) acetate, the copper(II) chelate *7* actually could be isolated. Subsequent treatment of *7* with hydrochloric acid gave *8* in a pure state. Repeated column chromatographic treatments of the residue, which largely contained *9*, gave this compound in a pure state.

The compounds *2*, *3*, *5b–d*, *6b,c*, and *7–11* are new and the structure determinations of all except *7* were supported by elemental analyses. The structure determinations of *2* and *3* were based on ^{1}H NMR and IR spectroscopy. Heating of *3* to 120 °C for 4 h was accompanied by the loss of one mol of water and the appearance of a carbonyl absorption band at 1725 cm^{-1}. Treatment of *3* with sodium carbonate gave *4*. The IR and ^{1}H NMR data of *4* are in agreement with the depicted structure. The 1-acetyl-3-piperidone *5a* [9] and its 5*S*-methyl analogue [10] have been shown by ^{1}H NMR spectroscopy to exist in two conformers at room temperature as a result of slow amide rotation. In the ^{1}H NMR spectra of *5b–d*, however, the signals from the C-2 protons were singlets. These findings compared with the ^{1}H NMR signals from the urethane groups of *5b–d* indicate lower barriers to amide rotation in *5b–d* than in the above-mentioned 1-acetyl-3-piperidones in agreement with the general findings for different types of amides.[11] In the ^{1}H NMR spectrum of *11* the methyl group appeared as a singlet, and the C-2 protons gave rise to a broadened signal probably indicating non-equivalence of the two protons rather than hindered amide rotation.

The enamine-enamide structure of the unstable compounds *6b,c*, as depicted in Scheme 1, was supported by UV absorptions at 250 nm. Simple enamines normally absorb at much lower wavelengths.[12] ^{1}H NMR spectroscopy confirmed the depicted positions of the double bonds in *6b*, *c* and demonstrated the existence of two conformers of both *6b* and *6c* at room temperature, in agreement with the findings for the 1-pyrrolidinyl enamine of *5a*.[9] Thus the

C-2 proton in both *6b* and *6c* gave rise to two slightly broadened singlets.

The structure of the copper(II) chelate *7* was not investigated in detail. As an indication of the purity of *7*, acid cleavage of this compound gave the β-oxoester *8* as the only organic compound. The structure determination of *8* and *9* were based on IR and ^{1}H NMR spectroscopy and finally confirmed by conversion into *10* and *11*, respectively, the structures of which were established by the same spectroscopic methods.

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.[13] Unless otherwise stated TLC and column chromatographic procedures were accomplished using Silica gel GF_{254} plates (Merck) and Silica gel, 0.05–0.20 mm (Merck), respectively. The ketones *4*, *5a–d*, *10*, and *11* were visualized on TLC plates by using a 2,4-dinitrophenylhydrazine (DNP) spraying reagent and the β-oxoesters *8* and *9* by using a DNP spraying reagent and subsequent treatment with iodine vapour followed by heating to 100 °C for 5 s.

1-Benzyl-1,2,5,6-tetrahydro-3-pyridyl benzyl ether (2). To a solution of sodium methoxide prepared from 160 ml of methanol and 8.6 g (0.37 mol) of sodium was added 31.0 g (0.33 mol) of pyridin-3-ol (*1*). Upon addition of 84 g (0.66 mol) of benzyl chloride the solution was refluxed for 7 h. After cooling to room temperature 25 g (0.66 mol) of sodium borohydride was added in portions. The solvent was removed *in vacuo* and the residue was stirred with water (200 ml), potassium carbonate (20 g), and ether (250 ml) for 1 h to give two homogeneous liquid phases. The ether phase was isolated, dried (K_2CO_3), and evaporated *in vacuo* to give a brown oil. To a solution of this oil in ether (9 ml) was added slowly and with vigorous stirring light petroleum (650 ml) and diatomaceous earth (11 g), and stirring was continued for an additional 30 min. After filtration and evaporation of the filtrate *in vacuo* 61.5 g (67 %) of crude *2* was obtained as a pale yellow oil, which crystallized at −20 °C. An analytical sample was recrystallized twice (ether–light petroleum) to give *2* as colourless crystals, m.p. 55.0–56.0 °C. Anal. $C_{19}H_{21}NO$: C, H, N. IR (KBr): 3025 (w), 2945–2745 (several bands, m), 1675 (s), 1500 (m), 1455 (m), 1380 (m) cm^{-1}. ^{1}H NMR (CCl_4): δ 7.17 (10 H, broad s), 4.63–4.43 (m) and 4.58 (s) (a total of 3 H), 3.47 (2 H, s), 2.92–2.70 (2 H, broad s), 2.53–2.27 (2 H, t), 2.23–1.87 (2 H, m).

1-Benzyl-3,3-dihydroxypiperidine hydrobromide (3). A solution of 34.0 g (0.12 mol) of *2* in hydrobromic acid (100 ml; 48 %) was refluxed for 3 h. After cooling to room temperature the reaction mixture was extracted with four 100 ml portions of ether. The aqueous phase was evaporated *in vacuo* to give an oil, which was crystallized (butanone) to give 25.1 g (72 %) of *3* as colourless crystals, m.p. 108.0–109.0 °C. Anal. $C_{12}H_{18}BrNO_2$: C, H, Br, N. Anal. $C_{12}H_{16}BrNO$ (after drying of *3* at 120 °C for 4 h): C, H, Br, N. IR (KBr): 3320–3260 (s), 3230–3170 (s), 2990–2860 (m), 2770–2660 (w), 1505 (w), 1442 (m), 1410 (m) cm^{-1}. IR (KBr) (after drying of *3* at 120 °C for 4 h): 3600–3150 (m), 2980–2830 (m), 2750–2480 (several bands, m), 1725 (s), 1500 (w), 1450 (m), 1422 (m), 1400 (m) cm^{-1}. ^{1}H NMR (D_2O): δ 7.58 (5 H, s), 4.78 (3 H, s), 4.37 (2 H, d), 3.8–2.7 (4 H, m), 2.2–1.7 (4 H, m).

1-Benzyl-3-piperidone (4). To a solution of 11.3 g (40 mmol) of *3* in water (15 ml) was added a solution of 5.3 g (50 mmol) of sodium carbonate in water (15 ml). The mixture was extracted with three 50 ml portions of ether. The combined ether phases were dried (K_2CO_3), filtered, and evaporated *in vacuo*. Distillation of the residue gave 6.4 g (86 %) of *4* as a colourless oil, which rapidly turned brown, b.p. 115–116 °C/20 Pa (Ref. 7, b.p. 89–91 °C/3 Pa). IR (film): 3035 (w), 2950 (m), 2800 (m), 1720 (s), 1500 (w), 1455 (w) cm^{-1}. ^{1}H NMR (CCl_4): δ 7.17 (5 H, s), 3.45 (2 H, s), 2.83 (2 H, s), 2.50 (2 H, t, *J* 6.0 Hz), 2.3–2.0 (2 H, m), 2.0–1.4 (2 H, m).

1-Acetyl-3-piperidone (5a). A solution af 14.8 g (52 mmol) of *3* in an aqueous solution of ethanol (200 ml; 25 %) was hydrogenated (304 kPa) in a PARR hydrogenation apparatus by using 3.5 g of a 5 % Pd-C catalyst. The reaction mixture was filtered and evaporated to dryness *in vacuo*. To an iced solution of the residue in water (15 ml) was added with stirring an iced solution of 17.9 g (130 mmol) of potassium carbonate in water (15 ml) immediately followed by addition of 8.1 g (79 mmol) of acetic anhydride during a period of 10 s. Stirring was continued at 0 °C for 30 min. The reaction mixture was continuously extracted with ether-dichloromethane (4:1) at room temperature for 21 h. The organic phase was dried (Na_2SO_4) and evaporated *in vacuo* to give 6.6 g of crude product, distillation of which gave 5.2 g (72 %) of *5a* as a colourless oil, b.p. 106–108 °C/10 Pa (Ref. 9, 118–120 °C/30 Pa). IR and ^{1}H NMR data were in agreement with those published for *5a*.[9]

Methyl 3-oxopiperidine-1-carboxylate (5b). *5b* was prepared in analogy with the preparation of *5a* described above by using 21.7 g (75 mmol) of *3*, 4.5 g of a 5 % Pd-C catalyst, 25.6 g (185 mmol), of potassium carbonate, and 21.3 g (225 mmol) of methyl chloroformate. However, a crude product of *5b* was isolated by extraction of the reaction mixture with three 100 ml por-

tions of ether. The combined and dried (K_2CO_3) ether phases were evaporated *in vacuo* and distillation of the residue gave 7.2 g (61 %) of *5b* as a colourless liquid, b.p. 85–87 °C/20 Pa. Anal. $C_7H_{11}NO_3$: C, H, N. IR (film): 2955 (m), 2870 (w), 1722 (s), 1710 (s), 1455 (s), 1410 (m) cm^{-1}. ^1H NMR (CCl_4): δ 3.87 (2 H, s), 3.62 (s) and 3.53 (t) (a total of 5 H), 2.5–2.2 (2 H, m), 2.2–1.7 (2 H, m).

Ethyl 3-oxopiperidine-1-carboxylate (5c). *5c* was prepared as described above for *5b* by using 14.8 g (52 mmol) of *3*, 3.0 g of a 5 % Pd-C catalyst, 17.5 g (127 mmol) of potassium carbonate, and 16.9 g (156 mmol) of ethyl chloroformate. Obtained was 4.4 g (50 %) of *5c* as a colourless liquid, b.p. 100–101 °C/40 Pa. Anal. $C_8H_{13}NO_3$: C, H, N. IR (film): 2980 (m), 2875 (w), 1730 (s), 1705 (s), 1480 (m), 1435 (s), 1393 (m) cm^{-1}. ^1H NMR (CCl_4): δ 4.03 (2 H, q, J 6.5 Hz), 3.87 (2 H, s), 3.53 (2 H, t, J 5.5 Hz), 2.5–2.2 (2 H, m), 2.2–1.7 (2 H, m), 1.23 (3 H, t, J 6.5 Hz).

Benzyl 3-oxopiperidine-1-carboxylate (5d). *5d* was prepared as described above for *5c* by using 26.6 g (156 mmol) of benzyl chloroformate. Obtained was 9.5 g (79 %) of slightly impure *5d*, as established by TLC (eluent: benzene–ethyl acetate 3:2), b.p. 154–157 °C/20 Pa. The impurity could not be removed by redistillation. An analytical sample was submitted to column chromatography (eluent: dichloromethane to which increasing amounts of ethyl acetate were added). Appropriate fractions were mixed and evaporated *in vacuo*. The residue was purified by ball-tube distillation at 20 Pa (oven temperature 180 °C) to give pure *5d* as a colourless oil. Anal. $C_{13}H_{15}NO_3$: C, H, N. IR (film): 3030 (w), 2950 (m), 2870 (m), 1720 (s), 1703 (s), 1500 (m), 1420 (s) cm^{-1}. ^1H NMR (CCl_4): δ 7.22 (5 H, s), 4.97 (2 H, s), 3.82 (2 H, s), 3.47 (2 H, t, J 5.5 Hz), 2.4–2.1 (2 H, m), 2.1–1.6 (2 H, m).

Methyl 3-(1-pyrrolidinyl)-1,4,5,6-tetrahydropyridine-1-carboxylate (6b). A solution of 1.57 g (10 mmol) of *5b* and pyrrolidine (5 ml) in benzene (100 ml) was refluxed for 2 h, water being removed by azeotropization with a Dean-Stark apparatus. The reaction mixture was evaporated *in vacuo*. The oily residue was distilled to give 1.7 g (81 %) of *6b* as a colourless liquid, which rapidly turned brown, b.p. 119–121 °C/15 Pa. Anal. $C_{11}H_{18}N_2O_2$: C, H, N. IR (film): 3115 (w), 2950 (s), 2860 (m), 2800 (m), 1700 (s), 1655 (m), 1445 (s), 1395 (s) cm^{-1}. UV [methanol (log ε)]: 250 (3.96) nm. ^1H NMR (CCl_4): δ 5.80 and 5.67 (a total of 1 H, slightly broadened s), 3.62 (3 H, s), 3.6–3.3 (2 H, m), 3.1–2.6 (4 H, m), 2.3–2.0 (2 H, m), 2.0–1.6 (6 H, m).

Ethyl 3-(1-pyrrolidinyl)-1,4,5,6-tetrahydropyridine-1-carboxylate (6c). *6c* was prepared as described above for *6b* by using 1.71 g (10 mmol) of *5c* as starting material. Obtained was 1.5 g (67 %) of *6c* as a colourless liquid, which rapidly turned brown, b.p. 125–127 °C/15 Pa. Anal. $C_{12}H_{20}N_2O_2$: C, H, N. IR (film): 3120 (w), 2970 (s), 2865 (m), 2800 (m), 1695 (s), 1655 (m), 1465 (m), 1420 (s), 1385 (s) cm^{-1}. UV [methanol (log ε)]: 250 (3.99) nm. ^1H NMR (CCl_4): δ 5.75 and 5.63 (a total of 1 H, slightly broadened s), 3.98 (2 H, q, J 7.0 Hz), 3.5–3.3 (2 H, m), 3.1–2.6 (4 H, m), 2.4–2.0 (2 H, m), 2.0–1.6 (6 H, m), 1.22 (3 H, t, J 7.0 Hz).

(±)-Ethyl 1-methoxycarbonyl-4-oxoperhydroazepine-3-carboxylate (8). To a stirred solution of 7.4 g (47 mmol) of *5b* in dry ether (60 ml), maintained at −60 to −50 °C, were added simultaneously and drop by drop 6.7 g (47 mmol) of freshly distilled boron trifluoride etherate and 6.7 g (59 mmol) of ethyl diazoacetate, both of which were dissolved in dry ether (20 ml). The addition of the reagents took 20 min and 35 min, respectively. Stirring was continued at the same temperature for an additional 30 min and subsequently until the temperature of the reaction mixture reached 20 °C. The reaction mixture was shaken with an aqueous solution of potassium carbonate (60 ml; 30 %) and the ether phase was separated. The aqueous phase was extracted with two 50 ml portions of ether. The combined ether phases were dried (K_2CO_3) and evaporated *in vacuo* to give an oil, which was shown to consist of two compounds with $R_F = 0.57$ and $R_F = 0.55$ (eluent: dichloromethane–ether–ethyl acetate (16:3:1)). The oily residue was dissolved in methanol (10 ml) and to this solution was added with vigorous stirring a hot (80 °C) solution of 9.0 g of copper(II) acetate in water (85 ml). After stirring for 16 h at 20 °C the precipitate was filtered off and thoroughly treated with two 20 ml portions of water and subsequently with two 20 ml portions of ether. The precipitate was dried *in vacuo* to give 5.6 g of the copper(II) chelate *7* as a greyish blue powder. IR (KBr): 3700–3100 (m), 2930 (m), 1695 (s), 1585 (s), 1480 (s), 1440 (m), 1404 (w) cm^{-1}. The combined filtrate and wash water was extracted with two 50 ml portions of ether. The combined 2 × 20 ml and 2 × 50 ml ether phases were dried (K_2CO_3) and evaporated *in vacuo* to give 4.6 g of a yellow oil. This was examined as described under the next heading.

5.6 g of *7* was treated with iced hydrochloric acid (4 M; 40 ml) for 30 s, after which the mixture was extracted with three 30 ml portions of dichloromethane. The combined organic phases were dried (Na_2SO_4) and evaporated *in vacuo* to give an oil, which by ball-tube distillation at 55 Pa (oven temperature 160 °C) gave 3.9 g (34 %, calculated on the basis of *5b*) of pure *8* as a colourless oil. Anal. $C_{11}H_{17}NO_5$: C, H, N. IR (film): 2980 (m), 2955 (m), 1735 (s), 1710 (s), 1640 (m), 1480 (s), 1445 (s), 1410 (s) cm^{-1}. ^1H NMR (CCl_4): δ 13.2 (*ca.* 0.25 H, s), 4.3–3.8 (a total of 4 H, q, J 7.0 Hz, overlapped by m), 3.60 (s), 3.53 (s), and 3.8–3.3 (m) (a total of 4 H), 3.3–2.2 (4 H, m), 2.0–1.5 (2 H, m), 1.32 and 1.27 (a total of 3 H, two

overlapping t, both with J 7.0 Hz).

(±)-Ethyl 1-methoxycarbonyl-3-oxoperhydroazepine-4-carboxylate (9). The oily by-product (4.6 g), obtained in the preparation of *8*, was shown by TLC (eluent: dichloromethane – ether – ethyl acetate (16:3:1)) to consist mainly of *9*. Column chromatography [silica gel (Woelm 0.063 – 0.1 mm): 150 g; eluent; benzene to which increasing amounts of ether were added] of this product and rechromatography of appropriate fractions several times gave *9*, which by ball-tube distillation at 55 Pa (oven temperature 160 °C) gave 1.7 g (15 %, calculated on the basis of *5b*) of pure *9* as a colourless oil. Anal. $C_{11}H_{17}NO_5$: C, H, N. IR (film): 2980 (m), 2955 (m), 2870 (w), 1740 (s), 1710 (s), 1475 (s), 1470 (s), 1440 (s), 1410 (s) cm^{-1}. ^1H NMR (CCl_4): δ 13.4 (< 0.05 H, s), 4.07 (s) and 4.03 (q, J 7.0 Hz) (a total of 4 H), 3.65 (3 H, s), 3.6 – 3.0 (3 H, m), 2.1 – 1.6 (4 H, m), 1.25 (3 H, t, J 7.0 Hz).

Methyl 4-oxoperhydroazepine-1-carboxylate (10). A mixture of 400 mg (1.6 mmol) of *8* and 6 ml of hydrochloric acid (4 M) was refluxed for 1½ h. The solution formed was evaporated to dryness *in vacuo*. To an iced solution of the residue in water (1.5 ml) was added with stirring an iced solution of 1.7 g (12 mmol) of potassium carbonate in water (1.5 ml) immediately followed by addition of 450 mg (4.8 mmol) of methyl chloroformate. Stirring was continued at 0 °C for 30 min and subsequently at 25 °C for 30 min. The reaction mixture was extracted with three 10 ml portions of ether. The combined ether phases were dried (K_2CO_3) and evaporated *in vacuo* to give an oil, which by ball-tube distillation at 40 Pa (oven temperature 140 °C) gave 159 mg (56 %) of *10* as a colourless oil. Anal. $C_8H_{13}NO_3$: C, H, N. IR (film): 2950 (m), 2860 (w), 1700 (s), 1690 (s), 1535 (w), 1480 (s), 1440 (s), 1410 (s) cm^{-1}. ^1H NMR (CCl_4): δ 3.60 (s) and 3.7 – 3.4 (m) (a total of 7 H), 2.6 – 2.3 (4 H, m), 1.9 – 1.5 (2 H, m).

Methyl 3-oxoperhydroazepine-1-carboxylate (11). *11* was prepared from 400 mg (1.6 mmol) of *9* as described above for *10*. Obtained was 176 mg (63 %) of *11* as a colourless oil. Anal. $C_8H_{13}NO_3$: C, H, N. IR (film): 2950 (m), 2860 (w), 1710 (s), 1690 (s), 1485 (s), 1475 (s), 1445 (s), 1410 (s) cm^{-1}. ^1H NMR (CCl_4): δ 3.88 (2 H, slightly broadened s), 3.65 (3 H, s), 3.5 – 3.2 (2 H, m), 2.6 – 2.2 (2 H, m), 2.0 – 1.4 (4 H, m).

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Chitinase and β-*N*-Acetylglucosaminidase in the Digestive Juice of *Helix pomatia*

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A β-*N*-acetylglucosaminidase from *Helix pomatia* digestive juice was separated and partly purified by gel chromatography. The optimal pH for the degradation of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide was 3.4. The molecular weight was around 160 000 and the pI = 4.95. In the same gel chromatography run two chitinase active peaks were also obtained. These chitinases, with molecular weights around 26 000 and 13 000, had somewhat different pH activity curves with optima at 4.2 and 4.3. By isoelectric focusing the first peak with molecular weight around 26 000 was divided in two chitinase active regions with pI at 5.7 and 3.8. The second peak with molecular weight around 13 000 had a pI at 7.3.

The digestive juice of the snail, *Helix pomatia* has been used as a source for different enzymes. During studies of lysozyme and glycol chitinase activities in different animal species it was found [1] that *Helix* digestive juice had a strong glycol chitin splitting activity when assayed viscosimetrically and a very weak lysozyme (muramidase) activity when tested as a *Micrococcus*-lysing enzyme. Further studies of the *Helix* juice showed a strong β-*N*-acetylglucosaminidase* activity, earlier reported [2-4] but not characterized. Cellulase [5] and chitinase [2,5] have also been reported in *Helix* juice and a muramidase has been separated from a chitinase from the same material.[6] In the present investigations two glycol chitin splitting enzymes were separated from a β-*N*-acetylglucosaminidase. These enzymes were preliminary studied.

* *Abbreviation.* β-*N*-Acetylglucosaminidase: NAGase. *Enzymes*: NAGase (EC 3.2.1.30), chitinase (EC 3.2.1.14), β-glucosidase (EC 3.2.1.21), lysozyme (EC 3.2.1.17).

MATERIAL AND METHODS

Materials. The *Helix pomatia* digestive juice (batch No. 61310) was purchased from Koch-Light Lab., Colnbrook, Bucks., England. The glycol chitin was from Seikagaku Kogyo Co., Tokyo (lot 4701, mol.wt. 20 000 – 60 000). *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide, grade III, was purchased from Sigma, USA.

Chitinase assay. The activity was determined with glycol chitin as substrate using the viscosimetric method of Hultin [7] as described earlier.[1,8] The chitinase activity is expressed in viscosimetric units called Hultin units (HU) as defined from Hultin's formula.[8] The incubation mixture consisted of 1.0 ml enzyme, 1.5 ml 0.1 M McIlvaine buffer and 1.5 ml 0.35 % glycol chitin in 0.9 % NaCl. The incubation was made at 35.5 °C and pH 4.1 if not otherwise reported.

β-N-*Acetylglucosaminidase* (NAGase) assay was performed according to Verpoorte [9] as follows. Twentyfive μl of the enzyme sample was mixed with 2.0 ml of 2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.05 M Na-citrate buffer, pH 4.5, and incubated at 37 °C. After 10 min the reaction was stopped by increasing the pH to about 10.5 by the addition of 2 ml of 0.5 M glycine-NaOH buffer pH 10.5. The absorbance was measured at 430 nm in a Beckman DB-G spectrophotometer and the NAGase activity expressed as absorbance in the figures. The absorbance, $A_{430}(1\ \text{cm}) = 0.500$, corresponded to 0.33×10^{-3} μkat of *p*-nitrophenol liberated under the conditions of the experiments.

Gel chromatography. The Sephadex gel (Pharmacia Fine Chemicals, Uppsala, Sweden) and the Bio-Gel (Bio Rad, USA) were equilibrated with the elution medium always containing 2 % 1-butanol as sterilizing agent.

Isoelectric focusing. The material was also studied isoelectrophoretically in a 110 ml column, type 8100-1 (LKB-Beckman Instrument AB, S-162 11 Vällingby 1, Sweden). For further details see Ref. 1.

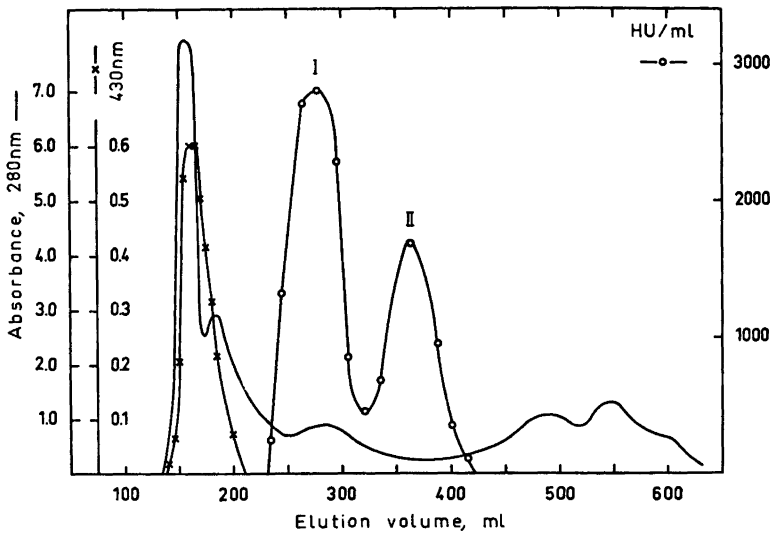

Fig. 1. Gel chromatography of *Helix pomatia* digestive juice. A 1.9 ml sample of the juice was applied to a P-150 column (1.9 × 178 cm) in 0.15 M ammonium acetate, pH 6.0 and 0.5 M NaCl and eluted in the same medium. The effluent was collected in 5 ml fractions at a flow rate of 23 ml/h at + 5 °C. ×, NAGase activity; O, chitinase activity.

RESULTS

Gel chromatography. The *Helix pomatia* digestive juice was run through Sephadex G-100, Sephadex G-150, and Bio-Gel P-150 columns. The best fractionation was obtained in a Bio-Gel P-150 column (Fig. 1). A full separation of NAGase from chitinase was obtained here and the glycol chitin splitting activity appeared in two peaks. The NAGase activity was assayed at pH 4.5 and the (glycol) chitinase activity at pH 4.1. These pH values were chosen from preliminary pH activity curves.

β-*N*-Acetylglucosaminidase

Influence of pH. The optimal pH of NAGase, purified 10 times by means of gel chromatography, was about 3.4 as seen in Fig. 2. At this pH 1.20×10^{-3} μkat of *p*-nitrophenol was liberated per ml reaction mixture. At pH values below 3, the enzymatic activity decreased rapidly, which was also the case for pH values higher than 5.

Kinetics. The effect of time on the NAGase reaction at constant enzyme concentration was linear for incubation times from 10 to 60 min (Fig. 3 A). The effect on the reaction rate of a 50-fold variation in the enzyme concentration was also linear when assayed after a constant time interval (Fig. 3 B). Both these series were studied at pH 4.5.

Isoelectric focusing. Fig. 4 demonstrates an isoelectric focusing of NAGase purified 6 times by gel chromatography. A homogeneous peak of enzymatic activity was obtained at a pI =

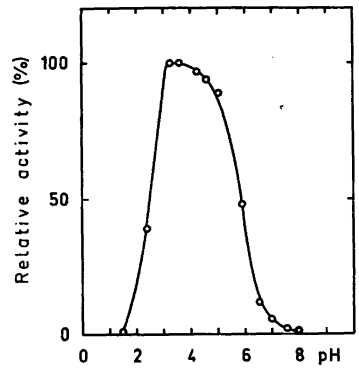

Fig. 2. Effect of pH on the activity of β-*N*-acetyl-glucosaminidase. The *Helix pomatia* NAGase was purified 10 times by gel chromatography.

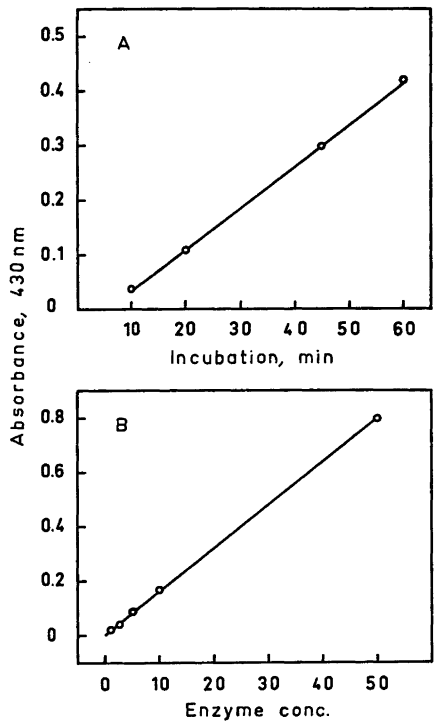

Fig. 3. Activity of NAGase. A. Effect of time of incubation on the degree of hydrolysis. B. Effect of enzyme concentration on the reaction velocity.

4.95 ± 0.1. The procedure resulted in a final 28-fold purification of the enzyme with a recovery of 30 % in the top fraction.

Molecular weight. By means of molecular sieving through a Bio-Gel A-1.5 m column in 0.15 M ammonium acetate, pH 6.0 and 0.5 M NaCl, the molecular weight could be estimated to be around 160 000 ± 20 000 with IgG (Tika), ceruloplasmin (Kabi) and albumin (Kabi) as marker proteins.

Chitinase

Influence of pH. Degradation of glycol chitin by the unpurified *Helix pomatia* digestive juice gave an optimum at pH 4.3 in McIlvaine buffer. The active interval was found between pH 3.2 – 5.7. By means of gel chromatography (Fig. 1) two active peaks were obtained. The effect of pH for each of these active regions is shown in Fig. 5.

Isoelectric focusing. Fig. 6 demonstrates an isoelectric focusing of the purified chitinase I from Fig. 1. One chitinase peak appeared at pI 5.7 ± 0.1 and another peak, at pI 3.8 ± 0.1. Repeated isoelectric focusing of peak I from Fig. 1 gave essentially the same results. The chitinase peak II (Fig. 1) gave one sharp distinct peak (Fig. 7) at pI 7.3. The maximum enzyme activities for these chitinase peaks

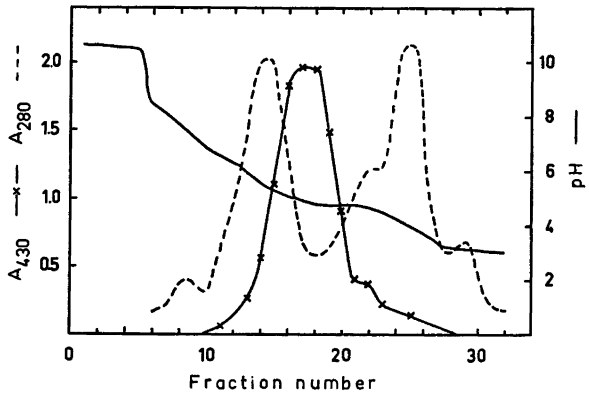

Fig. 4. Isoelectric focusing of *Helix* NAGase. The purified enzyme was dialyzed against 1.0 % glycine for 20 h at +4 °C and the dialyzed solution (20 ml) was applied to the column in 0.85 % Ampholine (LKB No. 8141 pH 3 – 10). The medium was stabilized with a sorbitol gradient (0 – 55 %). After 47 h running at 400 – 1100 V (2.4 – 1.7 W) and at +4 °C the column was drained in 3.3 ml fractions and the pH was measured at +4 °C. After dialysis against 0.9 % NaCl the enzymatic assay was performed.

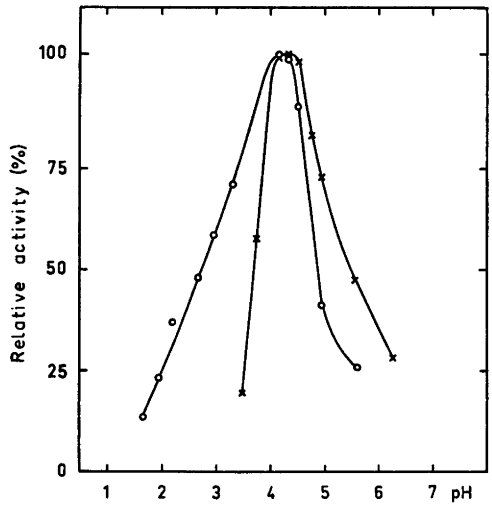

Fig. 5. Effect of pH on the activity of *Helix* chitinase. The purified chitinase pools I and II (Fig. 1) were used. Chitinase from pool I, O; and from pool II, ×.

were 880, 540, and 2480 HU/ml for the peaks with pI values at 5.7, 3.8, and 7.3, respectively. The recovery of the pI 7.3 enzyme (from peak II, Fig. 1) was 77 % of which the top fraction contained 43 % with a purification factor of 72 (Fig. 7, fraction 10).

Molecular weight. The molecular weights for chitinase I and II estimated by gel chromatography were 26 000 ± 2000 and 13 000 ± 1000, respectively. A Bio-Gel A-1.5 m column in 0.15 M ammonium acetate, pH 6.0 and 0.5 M NaCl was used and ovalbumin (Schwartz/Mann), myoglobin (whale skeletal muscle, Sigma, 17 800) and cytochrome *c* (horse heart Sigma) were used as marker proteins.

DISCUSSION

NAGase. For the assay of the enzyme *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide was used as substrate. This assay is convenient and the substrate has been used in studies of NAGase from *inter alia* ram testis,[10] *Helix pomatia* juice,[4] Jack bean meal,[11] beef spleen,[9] human parotid saliva,[12] silkworm [13] and cock spermatozoa.[14] In the present investigation the pH optimum found of purified *Helix* NAGase was 3.25 which is near the pH optimum 3.5 of cock spermatozoa.[14] In earlier studies of *Helix* NAGase,[2-4] pH optima were not reported. For most NAGases the pH optima with this substrate are usually higher, *e.g.*, around 4.5 in many studies,[3,10,12,15,16] 5 – 6 for NAGase from Jack bean,[11] 5.0 from silk worm blood,[12] and 5.8 from moulting fluid of this animal.[13] The isoelectric point for Helix NAGase was 5.0. Very few pI values have been reported for this enzyme. A purified endo-NAGase from *Staphylococcus aureus* [17] possessed a pI = 9.6 ± 0.1 and NAGase from human plasma [18] had a pI = 4.73. Compared to the molecular weight of 160 000

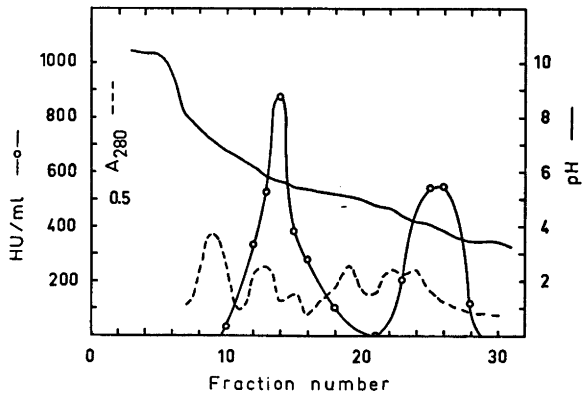

Fig. 6. Isoelectric focusing of *Helix* chitinase I. From peak I (Fig. 1) a pool was dialyzed against 1 % glycine and the dialyzed solution (10 ml) was applied to the column in 0.85 % Ampholine (LKB no 8141 pH 3 – 10). The medium was stabilized with a sorbitol gradient (0 – 55 %). After 94 h running at 380 – 1020 V (2.2 – 1.0 W) and at +4 °C the column was drained in 3.2 ml fractions and the pH was measured at +4 °C. After dialysis against 0.15 M ammonium acetate, pH 6 and 0.5 M NaCl the viscosimetric assay was performed.

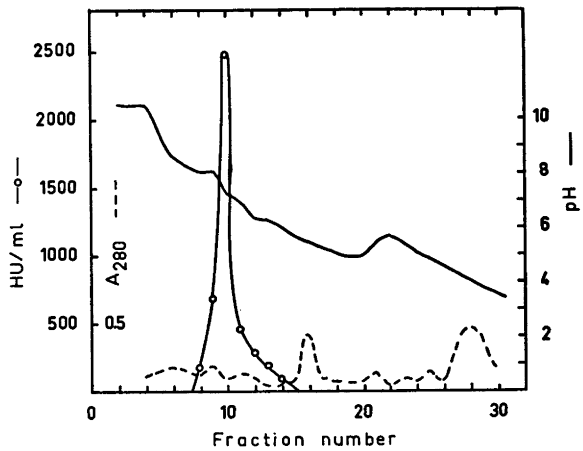

Fig. 7. Isoelectric focusing of *Helix* chitinase II. From peak II (Fig. 1) a pool was dialyzed against 1 % glycine and the dialyzed solution (25 ml) was applied to the column. After 43 h running at 435 – 1000 V (2.6 – 1.8 W) and at + 4 °C the column was drained in 3.2 ml fractions and the fractions dialyzed as described in Fig. 6.

of our NAGase, values of 100 000,[11] 105 000,[18] 140 000,[9] and 153 000 [12] have been reported for other species.

Chitinase. The pH optima in this paper were close to 4.25 for both the separated enzymes, but the curves were not coincident (Fig. 5). This optimum is close to those of *Staphylococcus aureus* chitinase [19] at pH 4.5 and commercial *Streptomyces* chitinase [20] at pH 4.2. More acid pH optima were found for an *Aspergillus niger* extract [21] at 3.6 and for bovine serum chitinase, as studied by the present authors,[22] at pH = 2.0. An extremly low pH optimum of 0.7 was recently found in Leydig's organ (lymphomyeloid oesophageal tissue) from the fish *Raja radiata.*[23]

The activity of chitinase II (Fig. 1) was confined to a narrow peak at pI 7.3, whereas the activity of chitinase I appeared in two peaks, one at pI 5.7, the other at pI 3.8.

For chitinases of different origin, molecular weights of 30 000 for *Streptomyces antibioticus* [24] and 90 000 for chitinase isoenzymes contained in pancreas of the gastric mucosa of the frog [25] were found.

The estimated molecular weights of 26 000 and 13 000 for the two chitinase activities, I and II, see Fig. 1, rises the question whether we are dealing with one or two enzymes. In view of the isoelectrophoretic separation of activity peak I, Fig. 6, into two chitinase active peaks with pI-values of 5.7 and 3.8, suggesting the presence of two isoenzymes, it seems reasonable to regard the two chitinase activity peaks I and II of Fig. 1 to be ascribed to two different enzymes, or perhaps more correctly three enzymes thereby accounting for the two suggested isoenzymes of molecular weight 26 000.

It has been discussed if the *Helix* chitinase is of bacterial origin, but the extensive studies by Jeuniaux [26] have stated that the enzymatic activity derives from the hepato-pancreas.

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Direct and Indirect Electrolysis of 1,2-Dihalo-1,2-diphenylethanes

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1,2-Dihalo-1,2-diphenylethanes (*meso* and *d,l*) have been reduced in *N,N*-dimethylformamide at a mercury cathode and indirectly by means of electrolytically generated anion radicals to a mixture of *cis*- and *trans*-stilbene. The ratio of *cis*- to *trans*-stilbene was found to depend on the kind and concentration of the supporting electrolyte; the *cis/trans* ratio increases with the size and concentration of the cation. These results were interpreted as being caused by ion-pairing between the cation and an anionic intermediate.

Several investigations [1,2] on the electrolysis of *vic*-dihalides have shown that a reductive elimination of the halogen atoms is the usual reduction route; the stereochemistry of the electrolytic reductive elimination [3] of the *d,l*- and *meso*-1,2-dibromo-1,2-diphenylethanes in *N,N*-dimethylformamide (DMF) has been investigated, and *trans*-stilbene was reported to be the only product obtained.

Sodium naphthalene reacts with *vic*-dihalides to give olefins in high yield.[4] The stereochemistry of the dehalogenation of *erythro*- and *threo*-2,3-dibromo-3-methylpentanes by sodium naphthalene in 1,2-dimethoxyethane (DME) [5] and of 2,3-dihalobutanes [6] under similar conditions has been reported; in the latter investigation [6] CIDNP was used to demonstrate the occurrence of radicals during the reaction.

In this investigation comparison is made between the stereochemical results from direct and indirect electrolytic reduction of *d,l*- and *meso*-1,2-dibromo-1,2-diphenylethane (*d,l*- and *meso-1*) and *d,l*- and *meso*-1,2-dichloro-1,2-diphenylethane (*d,l*- and *meso-2*) in DMF; the mechanism is further illuminated by varying the supporting electrolyte which might influence the degree of ion-pairing during the reaction, if anionic species were intermediates.

RESULTS

Direct electrochemical reduction of *meso-1* in DMF/TBABr gave *trans*-stilbene (*trans-3*) with no detectable (NMR, GLC) *cis*-stilbene (*cis-3*) in the product, in agreement with published results.[3]

Table 1. Relative yield of *cis*- and *trans*-stilbene in the electrochemical reduction of *d,l*-1,2-dibromo-1,2-diphenylethane in DMF containing different supporting electrolytes.

Supporting electrolyte	Concentration/M	*cis-3/trans-3*
$(CH_3)_4NBF_4$	0.20	0.47 ± 0.04
$(C_2H_5)_4NBr$	0.10	0.45 ± 0.04
$(C_2H_5)_4NClO_4$	0.11	0.56 ± 0.05
$LiClO_4$	0.10	0.52 ± 0.05
LiBr	0.10	0.56 ± 0.05
$(C_4H_9)_4NBr$	0.10	0.79 ± 0.06
$(C_4H_9)_4NBF_4$	0.1	0.89 ± 0.06
$(C_2H_5)_4NBr$ + $(C_4H_9)_4NBr$	0.01 + 0.10	0.82 ± 0.06
$(C_8H_{17})_4NBr$	0.1	1.27 ± 0.06

Table 2. Relative yield of *cis*- and *trans*-stilbene in the electrochemical reduction of *d,l*-1,2-dibromo-1,2-diphenylethane in DMF containing different concentrations of supporting electrolyte.

Supporting electrolyte	Concentration/M	*cis-3/trans-3*
LiBr	0.012	0.18 ± 0.03
LiBr	0.10	0.56 ± 0.05
LiBr	0.58	0.79 ± 0.06
$(C_4H_9)_4NBr$	0.010	0.37 ± 0.04
$(C_4H_9)_4NBr$	0.10	0.79 ± 0.06
$(C_4H_9)_4NBr$	0.30	0.96 ± 0.06

In DMF with different supporting electrolytes *d,l-1* gave a mixture of *cis-* and *trans-3* at variance with previous results;[3] the product distribution depended on the nature and concentration of the supporting electrolyte. In Table 1 is given the relative yields of *cis-* and *trans-3*. No other products than the stilbenes were detected. In Table 2 is shown the influence of the concentration of supporting electrolyte on the relative yields of *cis-* and *trans-3*.

2 differs from *1* in that both the *meso-* and the *d,l*-forms gave only *trans-3* on direct electrolytic reduction.

Indirect reduction of *d,l-1* by electrolytically generated chloranil anion radical gave a stilbene mixture containing 7 % *cis-3*, whereas indirect reduction by means of quinoxaline anion radical of *d,l-2* gave no detectable *cis-3*. The *meso*-forms gave only *trans-3*.

The absence of *cis-3* from the reduction of *d,l-2* could conceivably be due to instability of *cis-3* under the experimental conditions. In order to check this a solution of *cis-3* in DMF was allowed to be in contact for 10 h with a mercury electrode kept at a potential slightly more negative than that used for the reduction of *2*, but less negative than the reduction potential of *3*; no isomerisation of *cis-3* to *trans-3* was observed. This is in accordance with the results published recently [7,8] which show that the *cis/trans* isomerisation of *3* proceeds through the ion-pair of the dianion of *3* in THF and relatively slowly through the anion radicals of the *cis-* and *trans*-forms in HMPT.

DISCUSSION

The *meso*-forms of both *1* and *2* give *trans-3* in direct and indirect electrochemical reductions, as they do in chemical reductions.[9,10]

d,l-2 gives also *trans-3* on direct electrochemical reduction in DMF/TBAI and by using continuously electrogenerated quinoxaline anion radicals as reducing agents. In both cases a rotation around the central carbon-carbon bond must be allowed during the reaction unless a *syn*-elimination is assumed. There seems little reason to assume that *d,l-2* should react by a *syn*-elimination and *meso-2* by an *anti*-elimination.

d,l-1 gives a fair yield (32 – 56 %) of *cis-3* on electrochemical reduction, contrary to results reported previously.[3] The change in product distribution with the kind of supporting electrolyte, although not dramatic, (Table 1) is parallel to the change in effective size of the cation; the larger the cation the higher is the yield of *cis-3*.

The isolation of a fair yield of *cis-3* shows that there is not time enough during the reaction to establish a rotational equilibrium between the precursors leading to the two alkenes. The lower yield of *cis-3* obtained in the indirect electrolytic reduction is consistent with the larger time interval between the delivery of two electrons by one-electron carriers, such as anion radicals, compared to that in the direct reduction at the electrode.

Different cations could influence the reaction by changing the proton availability in the double layer, the adsorption of the substrate to the electrode, the distance of closest approach of the substrate to the electrode, and the ion-pair formation with a carbanionic intermediate.

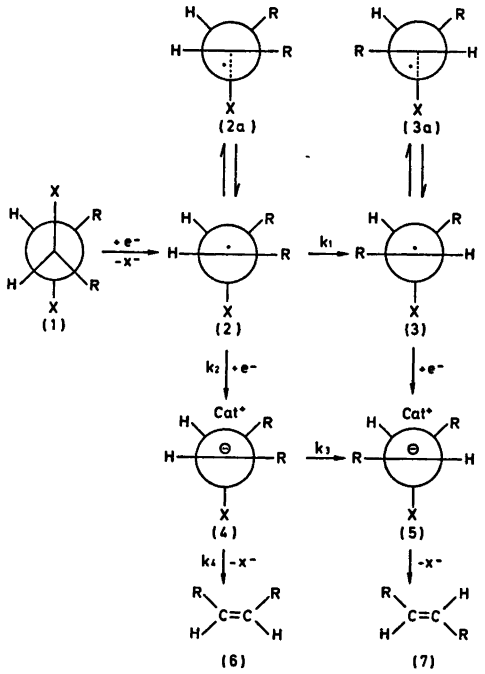
Protons are not important in the reaction, as no other products than *3* have been isolated. Protonation of any intermediates cannot compete with the elimination.

Adsorption of the substrate to the electrode competes with adsorption of cations. Adsorption of tetraalkylammonium ions to a mercury electrode in DMF at −0.9 V (SCE) is probably not very strong, but stronger for tetraethylammonium (TEA^+) than for tetraoctylammonium (TOA^+) ions. If adsorption was important, adsorbed *d,l-1* should be reduced to *cis-3*, contrary to what has been suggested.[3] The same *cis/trans* ratio is obtained by reduction of *d,l-1* at −0.9 V and at −1.9 V (SCE); at the latter potential TBA^+ would be stronger adsorbed than at −0.9 V.

If differences in the closest approach of the substrate to the electrode were important, the half-wave potential of *d,l-1* would be expected to vary so the reduction would be easiest in the presence of Me_4N^+ (TMA^+). This is not the case. Furthermore, reduction in the presence of 0.1 M TBA^+ and 0.01 M TMA^+ gave (Table 1) a *cis/trans* ratio close to that obtained in the presence of TBA^+ alone. It has been shown [11] that even small concentrations

of smaller quaternary cations displace larger cations from the electrode surface.

Change in the *cis/trans* ratio through differences in ion-pair formation could occur, if a carbanionic intermediate was formed as shown in the Scheme, and if the ions come close enough to each other to exert some influence on each other. The Scheme is analogous to that suggested for the reduction of 2,3-dichlorobutane by sodium naphthalene.[6] In the Scheme the intermediate (*4*) is depicted as a carbanion, although it might not be a fully developed carbanion.



The carbanion (*4*) may either rotate to (*5*) (k_3) or lose a bromide ion forming *cis*-stilbene (k_4). The Scheme must thus explain that the nature of the cation (Cat^+) can influence the ratio k_3/k_4. It should be noted that a rapid inversion of the radical or the carbanion, or whether the radical is planar or not,[12,13] is of no consequence for the stereochemistry of the product; only a rotation around the central bond brings about a change. In the scheme it is assumed that the electron uptake takes place with the halogen atoms *anti*-periplanar, although such a conformation probably is not the most favoured one for the *d,l*-forms;[14] the difference in reduction potential for the *d,l*- and the *meso*-forms (*d,l-1*, −0.35 V, *vs.* SCE; *meso-1*, −0.18 V) may be related to differences in the probability of the *anti* periplanar conformation.

In DMF solvation of a large carbanion, such as a benzylic carbanion, is rather loose, and the same is the case for the large tetraalkylammonium ions; solvent separated ion-pairs are commonly found in DMF. A lithium ion is solvated rather firmly, and the effective radius of a solvated lithium ion lies between that of a tetraethylammonium ion and that of a tetrabutylammonium ion.

The cation in an ion-pair partly neutralizes the charge of the anion (and *vice versa*), and the closer the positive and the negative charge can be approached, the more effectively a neutralization can take place. The more effectively the negative charge of the carbanion is neutralized, the slower would the loss of halide ion be expected to occur.

Electrochemical reduction in the presence of an (effectively) small cation would thus be expected to give a product in which the stereochemical integrity would be lost to a higher degree than in the presence of a large cation, as a result of a diminishing of k_4 in the former case.

The Scheme is thus capable of explaining for *d,l,-1* the dependence of the product distribution on the size of the cation; the dependence on the concentration of supporting electrolyte could be explained by the formation at higher concentrations of electrolyte of larger aggregates (*e.g.* $Li^+\cdots Br^-\cdots Li^+$) which act as large cations.

d,l-2 gives *trans-3* on electrochemical reduction. The experimental evidence does not allow conclusions with respect to the different behaviour of the dibromide and the dichloride, but at least two factors could be suggested. The bridging of the radical (*2a*) could be less important for X=Cl than for X=Br which would result in a faster transformation of (*2*) into (*3*); furthermore, as chloride ion even in DMF usually is a poorer leaving group than bromide ion, k_4 would be lower for X=Cl than for X=Br with a more complete transformation of (*4*) into (*5*) as a result.

If the Scheme is correct, a coupling during

an indirect reduction between the anion radical and the radicals (*2*) or (*3*) could be expected. It has recently been shown,[15,16] however, that benzylic radicals are much less apt to couple with anion radicals than are aliphatic radicals. In a reduction of 1,2-dichloroethane with anthracene anion radical, however, a good yield of a mixture of 1,2-di(9,10-dihydroanthranyl-9)-ethane and 2-chloro-1-(9,10-dihydroanthranyl-9)ethane was obtained.[17]

EXPERIMENTAL

The electrochemical cell was an H-cell with two sintered-glass diaphragms separating the catholyte (65 ml) from the anolyte; the area of the mercury cathode was 15 cm^2. The catholyte was kept at ambient temperature by means of a water bath. For potential control a Juul 100 V/3 A potentiostat was used; a Varian A-60 spectrometer was used for the ^1H NMR measurements. All potentials are referred to the aqueous saturated calomel electrode (SCE).

Materials. The *d,l-* and *meso* stilbene dibromides (*d,l-1* and *meso-1*) were prepared from *cis-* and *trans*-stilbene, respectively, by bromination according to Buckles *et al.*[18] *d,l-1* was purified on a column of silica with $CHCl_3$ as eluent, m.p. 113–114 °C (ethanol); $E_{\frac{1}{2}}$ in DMF/0.1 M TEAP = −0.35 V (aq. SCE). *meso-1*, m.p. 237–239 °C (ethanol); $E_{\frac{1}{2}}$ in DMF/0.1 M TEAP = −0.18 V (aq. SCE).

The stilbene dichlorides (*2*) were prepared according to Buckles *et al.*[19] *d,l-2*, m.p. 89–90 °C light petroleum); $E_{\frac{1}{2}}$ in DMF/0.1 M TEAP = −1.89 V (aq. SCE); *meso-2*, m.p. 189–190 °C (ethanol); $E_{\frac{1}{2}}$ in DMF/0.1 M TEAP = −1.80 V (aq. SCE).

Reduction of stilbene dihalides. Stilbene dihalide (100 mg) was reduced at −0.95 V (aq. SCE) for bromides or at −1.9 V for the chlorides in 65 ml of DMF containing the appropriate supporting electrolyte, $n = 2$ F/mol. When the reduction was completed (3–6 h), the catholyte was diluted with 100 ml of water and extracted 3 times with doubly distilled light petroleum (b.p. 50 °C); the organic phase was washed twice with 4 N HCl and twice with water, dried ($CaCl_2$), and evaporated. The residue (yield 70–85 %) was analyzed by GLC and by ^1H NMR spectrometry using the relative intensities of the signals at δ 6.6 (*cis*-stilbene) and δ 7.1 (*trans*-stilbene) as a measure of the *cis/trans* ratio. All results reported are based on at least two reductions under the specified conditions; the reproducibility was good and the spread not greater than is inherent in the NMR measurements.

Indirect reduction of stilbene dihalides. Chloranil (50 mg) was reduced at −0.15 V (SCE) in DMF/0.1 M TBAI in the presence of *d,l-1* (100 mg). When the reduction was completed, the reaction mixture was worked up as described above for the direct reduction; the traces of chloranil left did not influence the NMR measurements. For the indirect reduction of *d,l-2*, quinoxaline (68 mg, reduced at −1.6 V (SCE) in DMF/0.1 M TBAI) was employed as electron transferring agent.

GLC. For the determination of the ratio *cis/3/trans-3* a 2.5 % FFAP-column was used, injection temperature 180 °C, column temperature 140 °C for 8 min, increasing (8 °C/min) to 200 °C.

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Short Communications

The Influence of Added Ethanol on the Acidities of 4-Hydroxy-3-methoxybenzyl Alcohol and 2,2′-Dihydroxy-5,5′-di(hydroxymethyl)-3,3′-dimethoxybiphenyl in Aqueous Solution

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The acidities of phenols and carboxylic acids are generally lower in ethanol than in water.[1] In connection with work on enzymatic oxidation of phenols related to lignin it was of interest to find out whether the addition of ethanol to the oxidation mixture had a stronger influence on the acidity of *o,o′*-dihydroxybiphenyl structures than on monomeric phenols. The acidity of *o,o′*-dihydroxybiphenyls is strongly influenced by intramolecular hydrogen bond formation [2] and therefore the effect of solvent polarity on the dissociation constant was expected to be different from that of monomeric phenols.

The model compounds chosen for this study were 3-hydroxy-4-methoxybenzyl alcohol [3] (*1*) and 2,2′-dihydroxy-5,5′-di(hydroxymethyl)-3,3′-dimethoxybiphenyl [3,4] (*2*). The dissociation constants of *1* and *2* in 0.1 M sodium perchlorate were measured spectrophotometrically at 292 and 308 nm, respectively, in a series of ethanol-water mixtures. Buffer solutions for the measurement of *1* were prepared according to Sørensen's method [5] (0.1 M sodium hydroxide/0.05 M borax) and for the measurement of 2 according to Kolthoff's method [6] (0.05 M borax/0.1 M potassium dihydrogen phosphate).

The hydrogen ion concentrations of the buffered ethanol-water mixtures were measured with a glass electrode. Since the response of the glass electrode in aqueous ethanol was not known, it was determined in a separate series of measurements. For each ethanol–water mixture, which was 0.1 M in sodium perchlorate, the electrode was calibrated by addition of known amounts of 0.1 M hydrochloric acid.

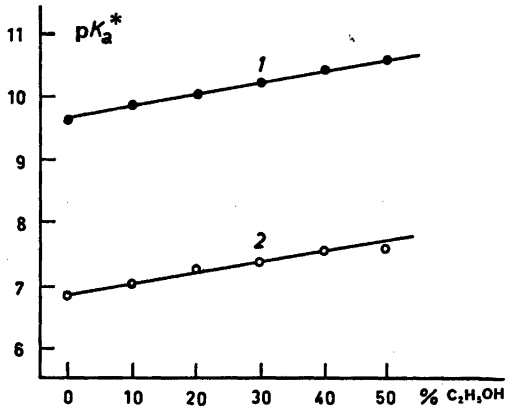
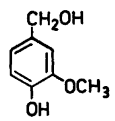


Fig. 1. The acidities of compounds *1* and *2* in ethanol–water mixtures as a function of ethanol concentration in the solvent.

The dissociation constants for *1* and *2* in 0.1 M sodium perchlorate at 25 °C in different ethanol–water mixtures are shown in Fig. 1 as a function of the ethanol concentration in the solvent. Each pK_a*-value, the asterisk indicating a solvent mixture, given in the graph is a mean of measurements in three buffer solutions.

In pure aqueous 0.1 M sodium perchlorate solution the constants obtained were 9.59 for the monomeric phenol, and 6.87 for the biphenyl compound. Throughout the measurements individual results from different buffer solutions did not differ more than 0.04 for the monomer and 0.01 for the dimeric compound. To obtain the thermodynamic dissociation constant 0.20 units should be added to the value at ionic strength 0.1. The value 0.20 originates from measurements of the dissociation constant of *1* in pure aqueous solutions of different ionic strength. After the correction for the influence of ionic strength the obtained



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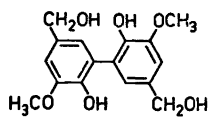

2

values for *1* 9.59 and *2* 6.87 agree well with those given in the literature, 9.83 [7] and 7.0,[8] respectively.

When ethanol is added the pK_a-values increase, at 50 % ethanol concentration the value for *1* is 10.54 and for *2* 7.57.

The increase over the investigated interval seems to be almost linear, and thus added ethanol does not cause any abrupt change in the mode of dissociation of the two phenols, and the difference in acidity between them remains fairly constant.

Acknowledgement. The authors wish to thank Professors O. Mäkitie and H. Saarinen for helpful discussions.

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Investigations of Dithienylglycolic Esters. V. Hydrolytic Stability and Preparation of Some Esters of Glycolic Acids

GUN WALLERBERG and BÖRJE ÖSTMAN

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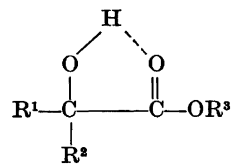
The hydrolytic stability of aryl substituted glycolates has received scant attention. Some data for esters of open-chain alkylamino alcohols have been reported by Kuznetsov and Rozinskaya.[1] They found that the rate of hydrolysis of alkylamino esters in an alkaline medium is higher than that of analogs containing no nitrogen. Knowledge of the hydrolytic stability is important for the elucidation of the structure-activity relationships of these atropin-like anticholinergic compounds. We have therefore synthezised and undertaken a study of the hydrolysis of some cyclic amino alcohol glycolates as well as their corresponding methyl esters [2] with the general structure



	R^1	R^2	R^3
1	Ph	Ph	$-CH_3$
2	3-Th[a]	3-Th	$-CH_3$
3	Ph	2-Th	$-CH_3$
4	3-Th	2-Th	$-CH_3$
5	2-Th	2-Th	$-CH_3$
6	Ph	Ph	Q[b]
7	3-Th	3-Th	Q
8	Ph	2-Th	Q
9	2-Th	2-Th	Q
10	2-Th	2-Th	3-Pip[c]
11	2-Th	2-Th	4-Pip[c]

[a] Th = thienyl. [b] Q = 3-quinuclidinyl. [c] Pip = piperidyl.

In order to determine the extent to which differences in reaction rates depend upon steric interaction from the alcoholic part of the esters, the intramolecular hydrogen bond between the hydroxyl and the carbonyl groups was studied by IR spectroscopy. The strength of the hydrogen bond will also reflect the various inductive effects within the esters.[3] A hydroxyl group hydrogen bonded to a carbonyl group increases the positive charge at the carbonyl

values for *1* 9.59 and *2* 6.87 agree well with those given in the literature, 9.83 [7] and 7.0,[8] respectively.

When ethanol is added the pK_a-values increase, at 50 % ethanol concentration the value for *1* is 10.54 and for *2* 7.57.

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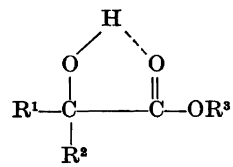

	R^1	R^2	R^3
1	Ph	Ph	$-CH_3$
2	3-Th[a]	3-Th	$-CH_3$
3	Ph	2-Th	$-CH_3$
4	3-Th	2-Th	$-CH_3$
5	2-Th	2-Th	$-CH_3$
6	Ph	Ph	Q[b]
7	3-Th	3-Th	Q
8	Ph	2-Th	Q
9	2-Th	2-Th	Q
10	2-Th	2-Th	3-Pip[c]
11	2-Th	2-Th	4-Pip[c]

[a] Th = thienyl. [b] Q = 3-quinuclidinyl. [c] Pip = piperidyl.

In order to determine the extent to which differences in reaction rates depend upon steric interaction from the alcoholic part of the esters, the intramolecular hydrogen bond between the hydroxyl and the carbonyl groups was studied by IR spectroscopy. The strength of the hydrogen bond will also reflect the various inductive effects within the esters.[3] A hydroxyl group hydrogen bonded to a carbonyl group increases the positive charge at the carbonyl

Table 1. Rate constants for hydrolyses of some aryl substituted glycolates $R^1R^2C(OH)COOR^3$ in glycine-sodium hydroxide buffer system, (50 °C) at pH 9.32, and yields of synthesis *6*, *7*, *9*, *10* and *11*.

Compound	$k_{obs}/10^{-3}$ s^{-1}	Yield/%
1	0.10	
2	0.28	
3	0.57	
4	0.77	
5	1.52	
6	0.17	70
7	0.30	73
8	1.26	
9	1.67	85
10	0.83	50
11	0.43	75

carbon atom which should favour an attack by hydroxyl ion.[4]

Experimental. Compounds *1*–*11* were synthesized according to Kadin and Cannon.[5] By using cyclohexane instead of heptane long periods of reflux time were avoided (7–24 h), and the reactions were completed within 30–60 min. The yields are given in Table 1. The rate constants given in Table 1 were obtained using Hestrin's method.[6,7] Hydrolyses were run at pH 9.32 and 50.0 °C in a glycine-sodium hydroxide buffer system (ionic strength = 0.5) containing 10 % methanol. *1* and *6* were also studied at pH 9.68 and 10.09. The initial concentrations of the esters were in the range 0.003–0.006 M. Aliquots were removed at certain intervals and measured spectrophotometrically. A Beckman Model B Spectrophotometer was used to follow the reaction. Absorbance readings (A_t) were followed for one – two half-lives. It was shown that Lambert-Beer's law was obeyed. The pseudo-first order rate constant (k_{obs}) was obtained by plotting log (A_t) *vs.* time. All the plots were linear and the rate constants from the duplicate runs were in general reproducible within less than ±7 % of the average of the two runs.

Infrared spectra were run on a Perkin-Elmer Model 225 instrument. The solvent used was carbon tetrachloride, dried over activated molecular sieves. The temperature in the cell compartment was 37 °C and the cell path lengths were 3 and 10 mm. Concentrations were 0.006 M and 0.03 M, depending on the cell path length used.

The rate constants of compounds *1*–*5* are given by

$$k_{obs} = k_2[OH^-]$$

and for *6*–*11* by

$$k_{obs} = (k_1K_w + k_2K_E[OH^-])/(K_E + [H^+])$$

where k_1 = second order rate constant for the protonated ester, k_2 = second order rate constant for the basic form of the ester, K_w = thermodynamic ionic product of water, K_E = mixed dissociation constant of the ester.

A plot of log k_{obs} *vs.* pH gives a straight line with a unit slope for 1. The hydrolysis for the other methyl esters is assumed to be first order with respect to ester and hydroxyl ion as well. The second order rate constant of the basic form of the amino alcoholic esters is approximately 1 M^{-1} s^{-1} (and thus of the same magnitude as for the methyl esters). However, the pH range (9.32–10.09) is too narrow for calculations of the second order rate constant of the acid form.

Structure-reactivity relationships may be discussed for the methyl ester series with respect to the aromatic π-systems involved.

A 2-thienyl group is known to display a stronger –I-effect than the phenyl and the 3-thienyl groups.[8] The trend of the k_{obs} values for the alkaline hydrolysis of the methyl esters is therefore in the expected direction (Table 1). A similar trend is noted for the corresponding quinuclidinyl esters with respect to the intramolecular hydrogen bonding ability of the hydroxy group,[3] *i.e.* the bonding becomes stronger as the –I-effect increases.

Ten Thije and Janssen [9] have derived σ^* values for 2-thienyl and 3-thienyl groups to be 0.93 and 0.65, respectively. Using these values and $\sigma^* = 0.60$ for the phenyl group,[10] and assuming additivity of the parameter values, log k_{obs} and log (A''/A') (A' is the intensity of the weak IR band assigned to "free" hydroxyl stretch and A'' is the intensity of the stronger band due to "bonded" hydroxyl stretch) were plotted (Fig. 1) against the sum of the σ^*'s(σ^*) for the methyl esters of Table 1. The plots show reasonable linear dependencies upon the structural changes.

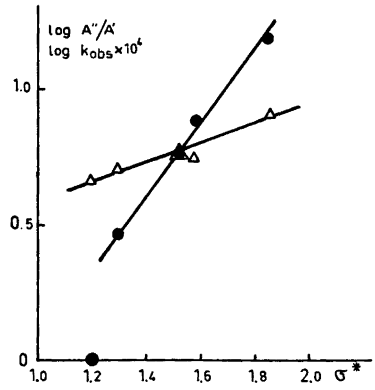

Fig. 1. Plot of log A''/A' (△) and log $k_{obs} \times 10^4$ (●) of the methyl esters of Table 2 *vs.* σ^* (see text).

Thus it appears from Fig. 1 that both the variation in hydrolysis rate and the hydrogen bonding capacity are mainly due to the variation in the inductive effects of the aromatic and heteroaromatic substituents.

It should further be noted that the relative rate ratio of hydrolysis of, *e.g.*, 2,2′-dithienyl/diphenyl for the methyl esters is comparatively large (≈15) being of the same order of magnitude as that (33) of base catalyzed racemization of 2-thienylglycolic acid/phenylglycolic acid.[11] Those data should be compared with the rate factors of alkaline hydrolysis of 2-thenoate/benzoate[12] (1.02) and the *O*-nitration of 2-thenyl alcohol/benzyl alcohol[11] (0.5).

An investigation of 14 glycolates by IR spectroscopy in the OH- and CO-regions suggests that steric interaction from the alcoholic part is likely.[3] Structural variations in this part show that a change in "bulkiness" of the substituents may cause a change in the orientation of the CO-group relative to the α hydroxy group in such a manner that an intramolecular hydrogen bond between these groups is conformationally favoured.

According to Meyerhöffer and Wahlberg[13] the pK_a values of the compounds *6*–*11* decrease in the series *6* > *7* ~ *9* > *8* > *11*.* Although similar data are unavailable for compound *10* it is likely that its pK_a does not exceed that of *6*. For compound *6* the pK_a value corresponds to 3 % protonated ester at 50.0 °C and pH 9.32. Thus for the remaining compounds of this series the contribution of the acid form (k_1) must be even less, and the k_1 term might be disregarded. The hydrolysis data can then be treated as being of the approximately pseudo first order. This suggests that the positive charge on the carbonyl atoms for the 2,2′-dithienylglycolate esters is in the order *9* > *10* > *11*. Since the extent of the intramolecular hydrogen bonding also depends on the charge of the carbonyl group it is interesting to observe that a plot of log (*A*″/*A*′) *vs.* k_{obs} for these esters shows an approximately linear relationship (Fig. 2).

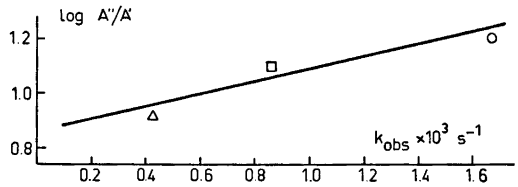

Fig. 2. The measure of intramolecular hydrogen bond strength, log *A*″/*A*′ *vs.* k_{obs}. △, 1-methyl-4-piperidyl 2,2′-dithienylglycolate; □, 1-methyl-3-piperidyl 2.2′-dithienylglycolate; ○, 3-quinuclidinyl 2,2′-dithienylglycolate.

* Estimated from the value of the corresponding diphenyl ester.[13]

In brief, the present work indicates that the hydrolysis rate of the diarylglycolates is influenced inductively by the aryl groups, and sterically by the alcoholic residue. The glycolic link further shows a relatively strong response upon changes of the inductive effects.

Acknowledgements. We wish to express our sincere gratitude to Dr. Johan Santesson, Director of Research, Division of Applied Organic Chemistry, FOA and Dr. Erik Ekedahl for valuable comments and suggestions. Dr. Robert Johnsen has kindly revised the English text.

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Absolute Configuration of 2-*C*-Methylerythritol from *Convolvulus glomeratus*

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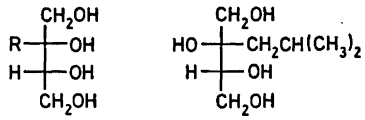
[a] Institute of Chemistry, University of Sind, Pakistan, [b] Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-104 05 Stockholm, Sweden and [c] Organic Chemistry Laboratories, The Norwegian Institute of Technology, University of Trondheim, N-7034 Trondheim-NTH, Norway

In a recent communication [1] we have reported the isolation of a new branched alditol (*1*) from *Convolvulus glomeratus* Choisy. The constitution and relative stereochemistry was deduced by spectroscopic methods and finally by synthesis of racemic *1* from citraconic acid.



1: R = CH_3

2: R = $CH_2CH(CH_3)_2$

3

In order to elucidate the absolute stereochemistry of *1*, a CD study of *1* and the two isobutyltetrols *2* and *3* of known configurations [2] has been performed. As expected, no extrema were reached in the CD measurements on aqueous solutions of *1*–*3*, and only low molecular ellipticities were obtained between 190 and 200 nm. Much more informative spectra were obtained, however, when the tetrols were investigated as their molybdate(VI) complexes (Fig. 1).[3] From these spectra and the known *erythro* configuration of *1*[1] it is obvious that *1* has the 2*S*, 3*R* configuration. Furthermore, since the CD band in the 330 nm region is positive for the erythritols and negative for the threitol this result suggests a method to distinguish between 2-*C*-alkylerythritols and 2-*C*-alkylthreitols.

Experimental. CD spectra were recorded on a Cary 60 instrument using a 1 cm cell for the 5.0 mM solutions of *1*–*3*, and a 0.05 cm cell for the solutions of the molybdate complexes. Following the conditions used by Voelter *et al.*,[3] the latter solutions were 5.0 mM with respect to tetrol and 10.0 mM with respect to sodium molybdate. The pH's were 5.0–5.1, adjusted with hydrochloric acid and measured with a

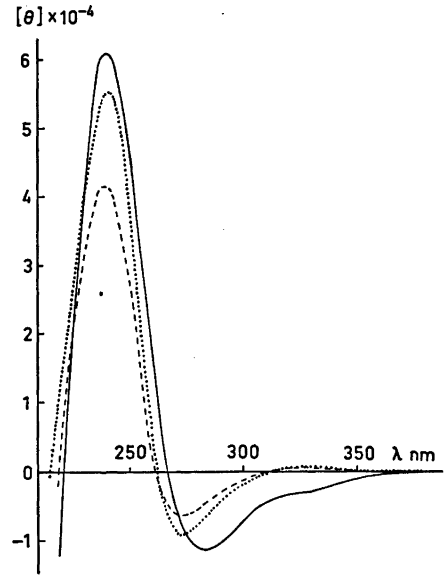


Fig. 1. CD spectra of molybdate(VI) complexes of *1*, ···; *2*, ---; and *3*, —·

Beckman pH meter (accuracy 0.1 unit). The molecular ellipticities are given in degree mol^{-1} cm^2. Obtained for *1*–*3*: $[\theta]_{192.5} = -0.18 \times 10^3$, -0.24×10^3, $+0.10 \times 10^3$, respectively. Obtained for *1*–*3* as molybdate complexes: see Fig. 1.

Acknowledgements. We thank Mr. Jan Glans, Kemicentrum, Lund for measuring the CD spectra, and professor S. Gronowitz for the spectropolarimetry facilities placed at our disposal. This work was supported by the Swedish Natural Science Research Council and Norwegian Agency for International Development.

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Pyridinethiones. I.* Preparation of 3-Formyl-2(1*H*)-pyridinethione and 5,11-Epoxydipyrido[2,3-*b*:2′,3′-*f*]-[1,5]dithiocine

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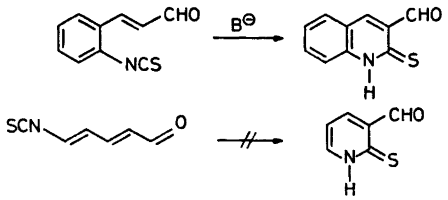
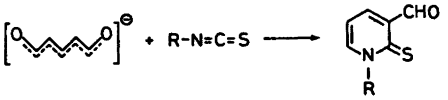
Hull [1a] has reported the formation of 3-formyl-2(1*H*)-quinolinethione by cyclization of *o*-isothiocyanatocinnamaldehyde. However, attempts to prepare 3-formyl-2(1*H*)-pyridinethione (*1a*) from 5-isothiocyanatopenta-2,4-dienal failed.[1b]



We have recently described [2] a general preparation procedure for 1-alkyl- and 1-aryl-3-formyl-2(1*H*)-pyridinethiones (*cf.* Scheme 1). In order to synthesize *1a* we have investigated the thermolytic and hydrolytic behaviour of the 1-*tert*-butyl- and (*R,S*)-1-methylbenzyl-substituted derivatives (*1b* and *1c*, respectively). Previously, Jensen *et al.*[3] have reported the elimination of a 4-*tert*-butyl- and a (*R,S*)-4-methylbenzyl group from thiosemicarbazides by reflux in concentrated hydrochloric acid.

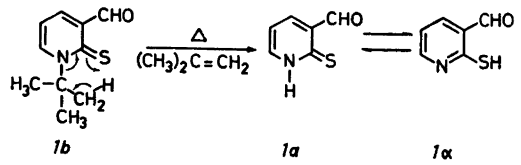


1a R = hydrogen
1b R = *tert*-butyl
1c R = *R,S*-methylbenzyl

Scheme 1.

Results and discussion. Thermolysis. When 1-*tert*-butyl-3-formyl-2(1*H*)-pyridinethione [2] (*1b*) was heated to *ca.* 190 °C, evolution of isobutene took place and 3-formyl-2(1*H*)-pyridinethione (*1a*) was formed (*cf.* Scheme 2). Analogous thermal *syn*-elimination of alkenes from corresponding structural arrangements have been reported (*e.g.* Chugaev reaction).

(*R,S*)-1-Methylbenzyl-3-formyl-2(1*H*)-pyridinethione (*1c*) distilled unchanged at *ca.* 200 °C (1 bar). As the thermolytic elimination of alkenes proceeds through a cyclic transition state, the difference in reactivity of *1b* and *1c* can be expected since in each conformation of the *tert*-butyl group a hydrogen atom fulfils the geometrical requirements of the transition state and elimination can take place, whereas the preferred conformations of the methylbenzyl group do not have a hydrogen atom in the right position.



1b *1a* *1α*

Scheme 2.

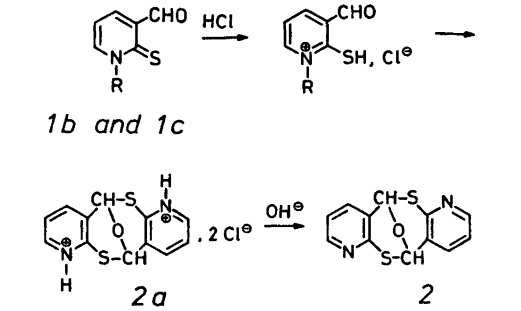
Structure *1a* was assigned to the reaction product on the basis of the way of synthesis and the spectroscopic properties (*cf.* Experimental section and Ref. 2).

3-Formyl-2(1*H*)-pyridinethione may exist in two tautomeric forms, *1a* and *1α* (Scheme 2). By comparing the UV spectrum of the reaction product with the spectrum of 1-methyl-3-formyl-2(1*H*)-pyridinethione [2] it can be concluded that the same chromophore is present in both compounds, and consequently, the thioamide form (*1a*) is the principal one. This conclusion is in accordance with results reported for other α and γ-thionoaza-aromatic compounds.[4]

Hydrolysis. Reflux with concentrated hydrochloric acid converted *1c* to the dihydrochloride of 5,11-epoxydipyrido[2,3-*b*:2′,3′-*f*]-[1,5]dithiocine (*2a*, Scheme 3). The free base (*2*) was obtained in almost quantitative yield by neutralization.

Heating *1b* for *ca.* 0.5 min in concentrated hydrochloric acid gave rise to 3-formyl-2(1*H*)-pyridinethione (*1a*). Prolonged reaction time led to the formation of *2a*.

Structure of 2. In the mass spectrum of *2* a relative abundant molecular ion was observed [*m/e* = 260 (34 %)]. The base peak in the simple spectrum was situated at *m/e* = 154. By ac-

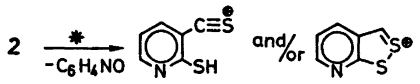


1b and 1c *2a* *2*

Scheme 3.

* This paper is a continuation of the series: Derivatives and Reactions of Glutacondialdehyde, see Ref. 2.

curate mass measurements the elemental composition of the corresponding ion was determined to $C_6H_4NS_2$:



The UV spectrum of the product showed absorptions at 294 and 238 nm. These values are in accordance with results reported for simple 2-alkylthiopyridines; *e.g.* 2-methylthiopyridine absorbs at 292 and 247 nm.[5]

Further evidence for the depicted structure (*2*) of the reaction product was obtained from the ^1H and ^{13}C NMR spectra. The ^1H NMR spectrum showed four resonances with the integrals 1:1:1:1. The H4, H5, and H6 pyridine-ring protons had the expected shifts and couplings.[3] The remaining singlet could be attributed to a CH-group with electronegative substituents. In the ^{13}C NMR spectrum (*cf.* Fig. 1) six signals were observed. The shift values are in agreement with values reported for substituted pyridines.[6]

The geometry of *2* permits the existence of one pair of enantiomers. However, attempts to resolve it have been unsuccessful.

Other 1-substituted-3-formyl-2(1*H*)-pyridinethiones[3] [including the 1-(2′-phenylethyl)-derivative (*cf.* Experimental section)] did not react under the thermolytic and hydrolytic reaction conditions described in this paper.

Experimental. Microanalyses were carried out by the Microanalytical Department of the University of Copenhagen.

Instrumentation. IR: Perkin Elmer 457. UV: Bechmann ACTA III. ^1H NMR: JEOL C-60 HL and Bruker HX-60. MS: AEI-MS 902. ^{13}C NMR: Varian XL-100-15FT. The melting points are uncorrected.

3-Formyl-2(1H)-pyridinethione (*1a*). *Hydrolysis. 1b* (0.3 g) in conc. hydrochloric acid (5 ml) was refluxed for *ca.* 0.5 min. Evaporation (*in vacuo*) of the turbid reaction mixture, addition of water (20 ml) and filtration yielded 0.140 g (66 %) of analytically pure *1a*, yellow crystals, m.p. 214–216 °C.

Thermolysis. When *1b* was heated to *ca.* 190 °C (1 bar) isobutene * was evolved, whereupon the residue crystallized. The resulting orange crystals melted at 214–216 °C and were in all

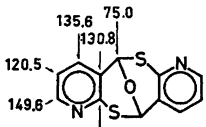


Fig. 1. ^{13}C NMR chemical shifts of 5,11-epoxydipyrido[2,3-*b*:2′,3′-*f*][1,5]dithiocine (*δ*, DMSO-d_6).

* Identified by MS.

respects identical to the product obtained by hydrolysis. *1a* was obtained analytically pure in quantitative yields.

^1H NMR (DMSO-d_6): *δ* 7.85 [dd, H(4)], 6.89 [t, H(5)], 7.96 [dd, H(6)], 10.64 (sCHO), 7.90 (broad, s, NH); $J_{4,5}=6.1$, $J_{5,6}=6.1$, $J_{4,6}=1.5$ Hz. UV [abs. ethanol (log *ε*)]: 375 (3.82), 320 (4.02) 293 sh (3.75), 216 (4.02) nm. IR (KBr): 1678 (CHO) cm^{-1}. MS: $m/e=139$ (44 %, M^+), 67 (100 %). Anal. C_6H_5NOS: C, H, N, S.

5,11-Epoxydipyrido[*2,3*-b:*2′,3′*-f][*1,5*]*dithiocine dihydrochloride* (*2a*). (*R,S*)-1-Methylbenzyl-3-formyl-2(1*H*)-pyridinethione (10 g) was refluxed in concentrated hydrochloric acid (100 ml) for 8 h. The turbid reaction mixture was extracted with carbon tetrachloride and the resulting water phase evaporated *in vacuo*. Washing of the residue with acetone yielded colourless needles of analytically pure *2a* [4.5 g (74 %)], m.p. 248–250 °C d. Titration of *2a* with sodium hydroxide gave the equivalent weight 172 g/mol (calc. 167 g/mol). ^1H NMR (60 MHz, DMSO-d_6): *δ* 7.20 (4, H, s), 7.33 (2 H, dd, *J* 8.3 and 5.3 Hz) 8.03 (2 H, dd, *J* 8.3 and 1.5 Hz) 8.45 (2 H, dd, *J* 5.3 and 1.5 Hz). UV [abs. ethanol (log *ε*)]: 298 (3.97) 242 (4.36) nm. Anal. $C_{12}H_{10}N_2OS_2Cl_2$: C, H, N, S, Cl.

The carbon tetrachloride phase was dried (sodium sulfate) and evaporated *in vacuo*. The resulting oil was distilled (1.0 g, main fraction b.p. 138–141 °C/1.5 mmHg, $n_D^{24}=1.5876$). By refluxing (*R,S*)-1-phenylethyl alcohol and 4 M hydrochloric acid an identical mixture of isomers of 1,3-diphenylbutene was obtained (b.p. 140–145 °C/1 mmHg).[7] ^1H NMR (60 MHz, CCl_4): *δ* 1.44 (3H, d, *J* 9.0 Hz), 3.30–3.70 (1 H, m), 6.25 (1 H, d, *J* 2.3), 7.16 (11 H, m, CH and aryl H). UV [abs. ethanol (log *ε*)]: 252 (3.93) 213 (3.93) nm. MS *m/e* (% rel. int.): M^+ at 208 (100 %).

Bromination of a sample gave white crystals (m.p. 99–112 °C (methanol)). This mixture of stereoisomeric 1,2-dibromo-1,3-diphenylbutanes has previously been identified by Marion.[7]

5,11-Epoxydipyrido[*2,3*-b:*2′,3′*-f][*1,5*]*dithiocine* (*2*). *Method 1. 2a* (4.0 g) was dissolved in water (20 ml) and pH was adjusted to 9 with 2 M sodium hydroxide. The precipitated crystals were collected and dried. Yield: 3.1 g (99 %) analytically pure *2*.

Method 2. (*R,S*)-1-Methylbenzyl-3-formyl-2(1*H*)-pyridinethione (4.5 g) was refluxed in concentrated hydrochloric acid (50 ml) for 10 h. After cooling and extraction, with carbon tetrachloride, pH was adjusted to 7 with 10 M sodium hydroxide. The precipitated crystals were collected and dried [3.9 g (81 %)]. Recrystallization from methanol yielded white needles m.p. = 242–244 °C.

Method. 3. 3-Formyl-2(1*H*)-pyridinethione was refluxed in concentrated hydrochloric acid, pH was adjusted to 7 and the white crystals of analytically pure *2* were isolated. The equivalent weight was found to 260 g/mol (calc.

260 g/mol) by titration with perchloric acid (the monoperchlorate separated as crystals) ^{1}H NMR (60 MHz, DMSO-d_6): δ 7.06 (2 H, s), 7. 25 (2 H, dd, J 8.1 and 5.3 Hz), 7.87 (2 H, dd, J 8.1 and 1.5 Hz), 8.40 (2 H, dd, J 5.3 and 1.5 Hz). UV [abs. ethanol (log ε)]: 298 (3.75) 243 (4.14) nm. IR (KBr): 1095 s (C–O–C) cm^{-1}. Anal. $C_{12}H_6N_2OS_2$: C, H, N, S.

(R,S)-1-Methylbenzyl-3-formyl-2(1H)-pyridinethione (*1c*). Glutacondialdehyde sodium salt (120 g) and (*R,S*)-methylbenzyl isothiocyanate (110 g) in dimethyl sulfoxide (500 ml) were heated to 80 °C for 2 h. The reaction mixture was poured in ice-cold water (4 l). The precipitated orange crystals were collected [119 g (73 %)] and recrystallized from methanol/water (510/88). m.p. 103–105 °C. ^{1}H NMR (60 MHz, DMSO-d_6): δ 1.81 (3 H, d, J 6.2 Hz), 7.40 (5 H, s), 7.46 (1 H, q, J 6.8 Hz), 7.78 (1 H, dd, J 6.2 and 1.5 Hz), 8.30 (1 H, dd, J 6.2 and 1.5 Hz), 10.65 (CHO, s). UV [abs. ethanol (log ε)]: 388 (3.49) 3.18 (4.06) 294 sh (3.77) nm. IR (KBr): 1685 (CHO) cm^{-1}. Anal. $C_{14}H_{13}NOS$: C, H, N, S.

1-(2'-Phenylethyl)-3-formyl-2(1H)-pyridinethione. Glutacondialdehyde potassium salt (2.72 g) and 2-phenylethyl isothiocyanate (3.26 g) in *N,N*-dimethylformamide were heated to 100 °C for 4 h. The reaction mixture was evaporated *in vacuo*, water (200 ml) was added and the dark crystals were collected. Trituration with cyclohexane gave orange crystals [2.8 g (50 %)]. Recrystallization from heptane yielded pale orange crystals with m.p. 132–135 °C. ^{1}H NMR (60 MHz, $CDCl_3$); δ 3.28 (2 H, t, J 7.5 Hz), 4.97 (2 H, t, J 7.5 Hz), 6.59 (1 H, t, J 6.9 Hz), 7.35 (5 H, s), 7.53 (1 H, dd, J 6.9 and 1.5 Hz), 7.88 (1 H, dd, J 6.9 and 1.5 Hz), 11.00 (CHO, s). UV [abs. ethanol (log ε)]: 377 (3.36) 320 (4.01) 294 sh (3.72) nm. IR (KBr): 1682 (CHO) cm^{-1}. Anal. $C_{14}H_{13}NOS$: C, H, N, S.

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Studies on the Kolbe Electrolysis. XII.* Complete Racemization of Optically Active Radicals from (—)-2-Methyloctadecanoate in a Mixed Coupling Reaction

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The hypothesis that the Kolbe anodic coupling of carboxylates proceeds *via* adsorbed radicals would seem to demand at least partial retention of configuration in the coupling product from an initially optically active radical.[1–4] Previous experiments to test this idea have, however, resulted in completely racemized coupling products and hence not proved to be conclusive on this point.[5,6] Only if retention is observed would adsorbed radicals be implicated in the mechanism with any degree of certainty.

The explanation put forward by Muck and Wilson [7] for the remarkably selective Kolbe coupling of long-chain carboxylates, parallel stacking of the long alkyl chains perpendicular to the anode surface with concomitant very low mobility of the alkyl radicals formed, suggests yet another possibility to find a system with a maximal propensity toward retention of configuration, if it is indeed possible to find one at all. We now report a study on the mixed Kolbe coupling between D-(–)-2-methyloctadecanoic acid (*1*) and methyl hydrogen malonate (eqn. 1). Both *1* and the product, methyl 3-methylnonadecanoate (*2*), were known with respect to their maximal optical rotation and configuration.[8,9]

$$\underset{1}{RCH(CH_3)COO^-} + MeOCOCH_2COO^- \xrightarrow[R=C_{16}H_{33}]{\text{Pt anode}} \underset{2}{RCH(CH_3)CH_2COOCH_3} \quad (1)$$

After initial experiments with the (+)-isomer to establish the proper reaction conditions, the crucial experiment was run with (–)-*1* and methyl hydrogen malonate in a 1:8 molar ratio in methanol (total salt concentration ~1 M). Both acids were fully neutralized in order to compensate for the difference in pK between them, and a large Hg cathode was used to avoid alkalinization (by amalgamation of the sodium discharged) during the run.[10] The temperature of the electrolyte solution

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was kept at 60 °C and the current density changed from an initial value of 1.0 to 0.1 A cm^{-2}.

After conventional workup the crude product was distilled and a fraction of b.p. 152–175 °C/1 mmHg was collected. Preparative GLC of this material gave pure *2* in a yield of 4.0 %, $\alpha = 0.000 \pm 0.002°$.

Since (−)-*1* and (+)-*2* have the same configuration, it is essential to establish beyond doubt that the product is not contaminated by starting material which accidentally might give a sample with zero optical rotation. GLC analysis of a sample treated with methanol/sulfuric acid at reflux for 5 h showed that the coupling product contained no detectable amount of *1* (< 0.1 %).

The experiment with (+)-*1* likewise gave a completely racemized product, although the less than perfect conditions gave too small and impure a sample to give a completely reliable estimate.

The optical purity of our sample of (−)-*1* was 60.1 %, so that the maximal obtainable value of $[\alpha]_D^{23}$ for *2* would be +2.28°, corresponding to a measured value of α of 0.325° for the product sample isolated. This permits us to conclude that the reaction has proceeded with at least 99.4 % retention, and that we have added yet another piece of evidence, albeit of negative nature, against the adsorbed radical hypothesis, still tenaciously upheld in some quarters.[11] In view of the apparent lack of more ideal systems to study, we can also add the Kolbe reaction to the growing list of electroorganic reactions for which stereochemistry is most notable by its absence.

Experimental. Starting materials. Partially resolved samples of (+)- and (−)-*1* were prepared according to published procedures,[8] $[\alpha]_D^{23}$ +4.32 and −5.65° (chloroform, *c* 8.2 and 11.7), respectively. The only change was in the resolution procedure, in which one recrystallization of the initially precipitated quinine salt was sufficient for the purpose at hand.

*Coupling reaction between (−)-*1 and *methyl hydrogen malonate.* Methyl hydrogen malonate (11.46 g, 0.097 mol), (−)-*1* (3.62 g, 0.0121 mol) and sodium (2.51 g, 0.109 mol) were dissolved in absolute methanol (100 ml). The solution was electrolyzed between a mercury cathode (580 g, area 28 cm^2) and a platinum anode (wire, 1.5 cm^2) at 60 °C (in order to keep all material in solution) for 185 min. The initial current of 1.5 A eventually decreased to 0.2 A.

The reaction mixture was then evaporated to dryness. Water (20 ml) was added, together with some sodium chloride to avoid the formation of an emulsion. The mixture was extracted with ether (4 × 50 ml) and the extracts were dried with anhydrous sodium carbonate. Distillation afforded a fraction (0.52 g) with b.p. 152–175 °C/1 mmHg which contained the desired product (*2*). This material was separated by preparative GLC (Autoprep Model A-700, 3 m × 9 mm 10 % OV-101 on 45–60 mesh Chromosorb B column at 250 °C, He as carrier gas), which gave a pure sample (157 mg, 4 % yield) of *2*, $\alpha = 0.000 \pm 0.002$ (chloroform, *c* 15.7; Perkin-Elmer model 141 spectropolarimeter).

A small sample of the product (45.6 mg) was refluxed with methanol/sulfuric acid (2.0 ml, molar ratio 25:1) for 5 h. GLC on the isolated product showed that < 0.1 % of the starting material could be present in the original sample.

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Synthesis of the Diastereomeric Mixture (2*RS*)-2-[(1*R*)-3-Cyclohexenyl]propanal

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Bulgarian rose oil contains among other components Δ^1-*p*-menthen-9-al, which is isolated as a partially epimerized diastereomeric mixture of the 4-methyl derivatives of *5a* and *5b* (Chart 1).[1] These compounds are important constituents in perfumes and to obtain them from natural sources is expensive.* Ohloff *et al.*[1] have reported the synthesis of these aldehydes with an optical purity of *ca.* 60 % from (+)-limonene.

3-Cyclohexene-1-carboxylic acid which has been resolved and configurationally assigned [2-4] has been demonstrated to be a useful chiral starting material for the synthesis of optically active substituted fatty alcohols [6] and dicarboxylic acids.[7] The present communication reports the synthesis of (2*RS*)-2-[(1*R*)-3-cyclohexenyl]-propanal, *7a*+*7b*, from (*R*)-3-cyclohexene-1-carboxylic acid, *1*, by the sequence outlined in Chart 1. These aldehydes are lower homologues of Δ^1-*p*-menthen-9-al.

(*R*)-3-Cyclohexene-1-carboxylic acid, *1*, was converted with methyllithium to the methyl ketone, *2*, which with methylenetriphenylphosphorane [8] gave (*R*)-4-isopropenylcyclohexene, *3*. Hydroboration of *3* with 9-borabicyclo[3.3.1]nonane (9-BBN) yielded an optically active diastereomeric mixture of (2*R*)-2-[(1*R*)-3-cyclohexenyl]-1-propanol and (2*S*)-2-[1*R*)-3-cyclohexenyl]-1-propanol, *4a* and *4b*, respectively. Finally, oxidation of this mixture with silver carbonate on Celite,[10,11] gave the diastereomeric mixture of (2*R*)-2-[(1*R*)-3-cyclohexenyl]-propanal, *5a*, and (2*S*)-2-[(1*R*)-3-cyclohexenyl]-propanal, *5b*.

The NMR spectrum of the mixture revealed that the two diastereomers were present in unequal amounts. The chemical shifts of the aldehyde doublets and also of other sets of signals were too close (even at 270 MHz) to allow an accurate estimation of the relative amounts of *5a* and *5b*.

The asymmetric induction apparently occurs in the hydroboration step and to determine the degree of stereoselectivity, we performed the following sequence of reactions. The dextrorotatory diastereomeric mixture *5a*−*5b* was catalytically hydrogenated to the enantiomeric pair *6a*−*6b* (Chart 1). The hydrogenated mixture was optically active which confirmed that asymmetric induction had taken place in the hydroboration step. The aldehyde mixture was then allowed to autoxidize to the enantiomeric acids *7a*−*7b* whose optical properties and absolute configurations have been determined.[12,13] From these values (*cf.* Experimental), assuming that no epimerization or racemization occurs in the oxidation steps, a stereoselectivity of 20 % in the hydroboration step is calculated.

The hydroboration reaction has been demonstrated to proceed *via* a *cis* four-center addition process.[14] Also, considerable asymmetric induction has been found to occur with chiral hydroborating agents [15] and when achiral hydroboranes react with chiral olefins.[16] Our findings are in agreement with these observations.

Experimental. General methods. GLC analyses were carried out on a Perkin-Elmer 900 instrument fitted with flame ionization detectors and 3 mm × 180 cm stainless steel columns packed with 3 % SE-30 on Gas Chrom Q. Preparative LC separations were performed at atmospheric pressure on Merck Kiselgel 60, particle diameter 0.063−0.200 mm. The eluents were con-

* Often structural alterations in olfactory constituents lead to interesting changes in their odours. The *5a*−*5b* mixture has a sweet hyacinth-like odour.

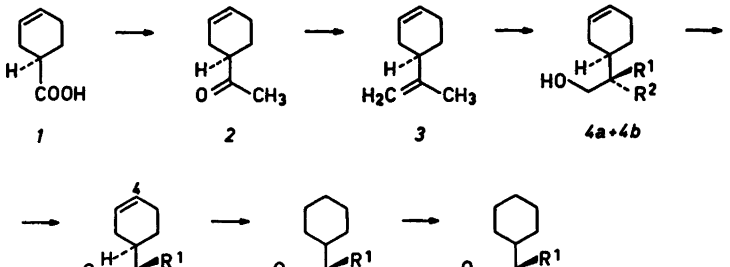

Chart 1. Series *a*: $R^1 = CH_3$, $R^2 = H$; Series *b*: $R^1 = H$, $R^2 = CH_3$.

tinuously analysed with a Pye Unicam LCM2 detector. High pressure LC was performed with a Waters Model 6000 solvent delivery system and R401 RI detector fitted to a 9 mm × 30 cm stainless steel column packed with Porasil. MS were determined on an AEI 902 mass spectrometer or on an LKB 9000 mass spectrometer connected to a gas chromatograph. The 270 MHz NMR spectra were determined in $CDCl_3$ with a Bruker WH270 instrument.

(R)-3-Cyclohexenyl methyl ketone, 2. (*R*)-3-Cyclohexene-1-carboxylic acid, *1*, (2.8 g, 22 mmol), $[\alpha]_D$ +90° (MeOH, $c=5$), which corresponds to 95 % optical purity,[2-4] was dissolved in 60 ml of anhydrous ethyl ether. The solution was kept under dry nitrogen at 0 °C while a 2 M etheral solution of methyllithium (44 mmol) was added dropwise for 30 min. After 1.5 h at room temperature the white suspension was poured into a mixture of ice and 50 ml of 1 M hydrochloric acid. The layers were separated and the water phase was extracted with 4 × 25 ml of ethyl ether. The combined ether extracts were washed once with a saturated solution of sodium carbonate, twice with water, and then dried with anhydrous calcium sulfate. Evaporation of the ethyl ether gave 1.9 g (71 %) of crude product, which was further purified by distillation in a micro distillation apparatus, giving 1.02 g of pure (GC) *2*, b.p. 34 °C/1 Torr (litt.[5] b.p. 78–80 °C/17 Torr), $[\alpha]_D$ +100.2° ($CDCl_3$, $c=4$).

^{1}H NMR (60 MHz): δ 1.2–2.9 (7 H, m), 2.12 (3 H, s) and 5.57 (2 H, s). MS [*m/e* (% rel. int.)]: 125 (5), 124 (47), 109 (13), 95 (6), 91 (8), 83 (5), 82 (6), 81 (81), 80 (28), 79 (51), 78 (8), 77(17), 67 (5), 66 (10), 65 (8), 55 (8), 53 (27), 52 (8), 51 (15), 50 (8), 43 (100), 42 (7), 41 (21), 39 (34), 27 (29), 15 (18). Mol. wt., obs. 124.086 (3), calc. for $C_8H_{12}O$ 124.089.

IR (film): 1710 cm^{-1} (>C=O). UV [abs. ethanol (ε)]: 275 (17) nm.

(R)-4-Isopropenylcyclohexene, 3. Freshly distilled *2* (1.02 g, 8.2 mmol) was added to a solution of methylenetriphenylphosphorane [8] (13.7 mmol) in 20 ml of dimethyl sulfoxide. The solution was stirred for 30 min in a dry nitrogen atmosphere and then distilled giving 607 mg (61 %) of *3* containing traces of dimethyl sulfoxide, $[\alpha]_D$ +67.7° ($CHCl_3$, $c=4$), b.p. *ca.* 50 °C/2 Torr (litt.[17] b.p. 157 °C). The yield of *3* was increased to 70 % when a dry ice trap was used during the distillation and the traces of dimethyl sulfoxide in the distillate were eliminated by chromatography over silica gel (pentane eluent).

^{1}H NMR (60 MHz): δ 1.1–2.5 (7 H, m), 1.70 (3 H, m), 4.62 (2 H, s), 5.58 (2 H, s).

(2RS)-2-[(1R)-3-Cyclohexenyl]-1-propanol, 4a + 4b. 9-BBN (1.02 g, 8 mmol) was dissolved in 12.5 ml of dry tetrahydrofuran. (*R*)-4-Isopropenylcyclohexene (412 mg, 3.4 mmol) in 3 ml of tetrahydrofuran was added to the 9-BBN solution in a dry nitrogen atmosphere.[9] The solution was stirred for 30 min, 5 M sodium hydroxide solution (2.5 ml) was added, followed by 30 % hydrogen peroxide (2 ml). The temperature was kept at 60 °C and the stirring was continued for 1 h. The water phase was saturated with potassium carbonate, separated from the organic phase, and extracted twice with tetrahydrofuran. The combined tetrahydrofuran extracts were dried with potassium carbonate over night. Evaporation and distillation gave 412 mg of product, b.p. *ca.* 70 °C/1 Torr. Liquid chromatography on 40 g silica gel with gradient elution (benzene–ethyl acetate) gave 294 mg (62 % yield) of *4a + 4b*, $[\alpha]_D$ +59.7° ($CDCl_3$, $c=8$). GLC showed the product to have more than 99 % purity.

^{1}H NMR (60 MHz): δ 0.91 (3 H, d), 1.1–2.3 (8 H, m), 2.62 (1 H, s), 3.2–3.9 (2 H, m), 6.70 (2 H, s).

MS [*m/e* (% rel. int.)]: 140 (1.4), 122 (26), 107 (30), 93 (26), 81 (42), 80 (70), 79 (43), 78 (23), 77 (19), 67 (32), 55 (21), 54 (16), 53 (12), 41 (23), 39 (16). Mol. wt., obs. 140.118 (3), calc. for $C_9H_{16}O$ 140.120.

(2RS)-2-[(1R)-3-Cyclohexenyl]propanal, 5a + 5b. Silver carbonate on Celite [10,11] (10 g, 17.5 mmol) was added to a solution of *4a + 4b* (224 mg, 1.6 mmol) in 90 ml of benzene. Benzene-water (5 ml) was azeotropically distilled off and the reaction mixture was refluxed for 12 h. The progress of the oxidation was followed by GLC. The reaction suspension was filtered and the benzene solution was evaporated under reduced pressure giving 172 mg (78 % yield) of *5a + 5b*, $[\alpha]_D$ +62.6° ($CDCl_3$, $c=7$).

^{1}H NMR (60 MHz): δ 1.07 (3 H, d), 1.1–2.6 (8 H, m), 5.52 (2 H, s), 9.55 (1 H, pair of d).

MS [*m/e* (% rel. int.)]: 138 (5), 124 (14), 107 (16), 93 (16), 91 (22), 85 (17), 83 (22), 81 (98), 80 (100), 79 (98), 78 (31), 77 (33), 67 (59), 55 (48), 54 (43), 53 (31), 51 (14), 43 (34), 41 (60), 39 (45). Mol. wt., obs. 138.103 (3), calc. for $C_9H_{14}O$ 138.104.

(RS)-2-Cyclohexylpropanal, 6a + 6b. A solution of *5a + 5b* (63 mg, 0.5 mmol) in 3 ml of ethyl acetate was catalytically hydrogenated at atmospheric pressure with 5 mg of 10 % Pd/C as catalyst. The suspension was filtered and evaporated to give 29 mg of *6a + 6b*, $[\alpha]_D$ −9.5° ($CHCl_3$, $c=4$).* The product was used in the next step without further purification.

(RS)-2-Cyclohexylpropanoic acid, 7a + 7b. Crude *6a + 6b* (29 mg, 0.22 mmol) was allowed to autoxidize for 4 weeks at room temperature to the corresponding acids. The product was purified by high pressure LC (10 % ethyl acetate in hexane) to give *7a + 7b*, $[\alpha]_{578}^{25}$ −3.7° ($CHCl_3$, $c=0.2$), m.p. 53–55 °C.[12,13] GLC showed the product to be more than 98 % pure. ^{1}H NMR (270 MHz): δ 0.93–1.38

* Calculated value from an experiment starting with 27 % optically pure (*S*)-3-cyclohexene-1-carboxylic acid.

(5 H, m), 1.13 (3 H, d), 1.51–1.82 (6 H, m), 2.23–2.34 (1 H, q) and 10.11 (1 H, very broad s).

The sequence of experiments described was also performed with the *S* form of *1* of 27 % optical purity as starting material. The results were in each aspect in agreement with those presented above.

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The Effect of Monovalent Cations on Isoleucyl Transfer-RNA Synthetase Reaction from Baker's Yeast

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The effect of monovalent cations on isoleucyl-tRNA synthetase from baker's yeast has been analyzed. The aminoacylation of $tRNA^{ile}$ is strongly activated by potassium ions. Maximal rate of aminoacylation was obtained in the presence of approximately 140 mM potassium chloride. The rate of the reaction is proportional to $[K^+]^{2.25}$ over the entire range to 140 mM. No activation of the ATP–PPi exchange reaction can be demonstrated but the activity decreased continuously.

The kinetic constants for the aminoacylation reaction show that any activating effect of potassium ions (including those affecting the binding of substrates) must obviously be on the rate of the reaction. Ionic interactions play a major role in the process of tRNA-binding to the enzyme.

In contrast to the specific functions of certain divalent cations, the mode of action of monovalent cations for activating many enzymes has remained relatively obscure.[1] It is generally considered that the active conformation of enzymes activated by monovalent cations is adjusted or stabilized by the ion.

Monovalent cations, usually potassium, are included in the reaction mixtures of almost all work done on aminoacyl-tRNA synthetases. However, very few reports have been published on the action of the monovalent cations. Svensson [2,3] has done some studies of the effect of various salts on the aminoacylation reaction of methionyl-tRNA synthetase. The effect of inhibiting concentrations of salt has been discussed by Loftfield.[4,5] Yarus [6] notes that the major effect of salts is on the association of tRNA and enzyme, not on the rate of aminoacylation. The best explanation seems to be a conformational state of tRNA which is optimal for binding. Pingoud *et al.*[7] estimated the equilibrium and rate constants for the interaction of seryl-tRNA synthetase with $tRNA^{ser}$ at various concentrations of salt.

The present paper reports the activating effect of monovalent cations on isoleucyl-tRNA synthetase (EC 6.1.1.5) from baker's yeast. No activating effect on the ATP–PPi exchange reaction has been found while concentrations above 0.2 M inhibit both the esterification and the ATP–PPi exchange reaction. The site of action of the activating ion is discussed.

MATERIALS AND METHODS

Isoleucyl-tRNA synthetase was prepared from baker's yeast according to a method used to obtain the enzyme from *E. coli*.[8] The yeast cells were broken by sonication and 10 % glycerol was included to the buffer solutions. The specific activity of the enzyme was 53 units/mg protein. One unit of enzyme activity is defined as the aminoacylation of 1 nmol tRNA in 1 min at 30 °C.

Aminoacylation was performed according to Ref. 8. The assay mixture (150 µl) contained, unless otherwise noted, 100 mM Tris–HCl buffer pH 7.5, 2 mM ATP, 4 mM Mg^{2+}, 60 µg tRNA, 60 µM [^{14}C]-isoleucine, 140 mM KCl, 2.5 mM GSH, 0.02 % bovine serum albumin and enzyme.

The pyrophosphate exchange assay was performed according to Ref. 9 except that the anion-exchange paper was soaked in 0.15 M $Na_4P_2O_7$ at pH 8.0 and eluted with a 0.1 M $Na_4P_2O_7$ of the same pH. The enzyme activity is given as the incorporation of 1 µmol [^{32}P]-pyrophosphate into ATP per min at 30 °C.

$tRNA^{ile}$ was prepared from unfractionated baker's yeast tRNA according to the method

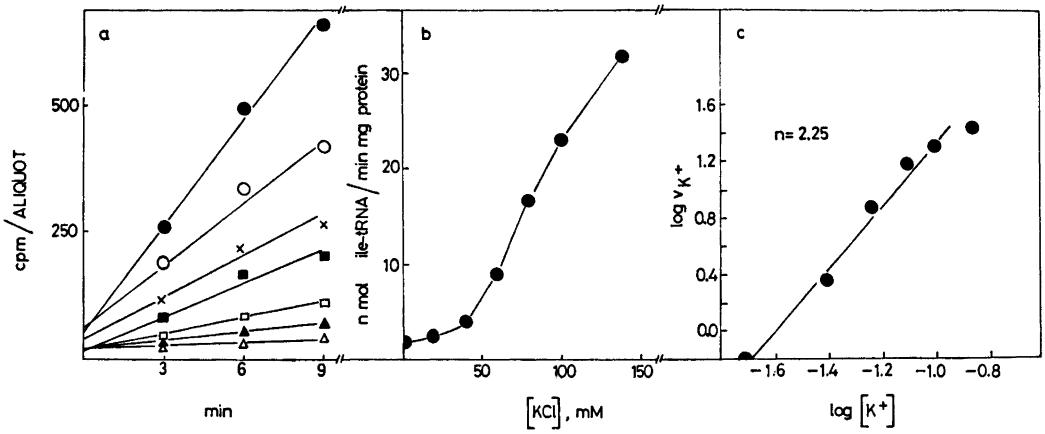

Fig. 1. (a) Dependence of initial rate of isoleucyl-tRNA formation on activating KCl concentrations, (●) 140 mM, (○) 100 mM, (×) 80 mM, (■) 60 mM, (□) 40 mM, (▲) 20 mM and (△) in the absence of added KCl. The concentration of $tRNA^{ile}$ was 3.6×10^{-7} M and the test was carried out as described in Materials and methods. (b) The data of Fig. 1a replotted as the specific activity of isoleucyl-tRNA synthetase *versus* KCl concentration. (c) The data of Fig. 1b replotted as the logarithm of the potassium ion activated rate of reaction *versus* the logarithm of the potassium ion concentration. $v_{K^+} = v - v_0$, v is the activated rate, and v_0 is the rate when no potassium ions were added.

of Gillam *et al.*[10] The purified tRNA had an acceptor activity of 0.9 nmol/A_{260} unit at pH 7.0.

RESULTS

In the presence of 140 mM potassium chloride a 20-fold increase in the rate of aminoacylation is obtained compared to the rate observed in the presence of less than 0.1 mM potassium. A typical experiment for the determination of the effect of potassium chloride on the rate of aminoacylation is shown in Fig. 1a. A similar effect was obtained with potassium sulfate. Sodium ions are also almost as effective as potassium ions. When the rate

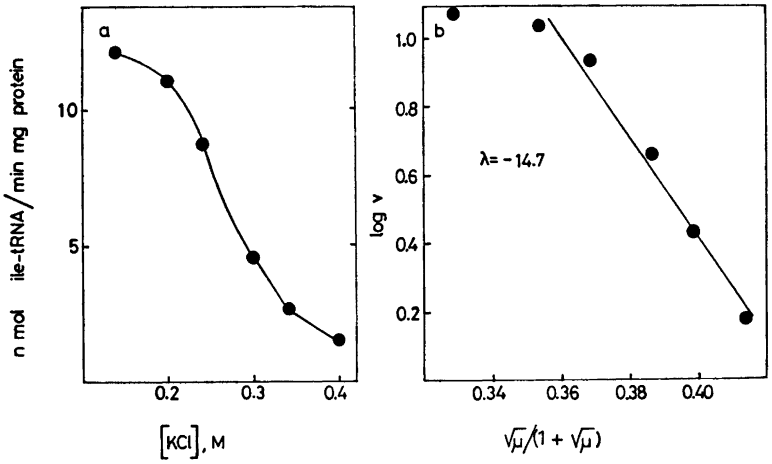

Fig. 2. (a) The dependence of the rate of isoleucyl-tRNA formation of inhibiting KCl concentrations. The concentration of $tRNA^{ile}$ was 1.7×10^{-7} M and the test was carried out as described in Materials and methods. (b) The data of Fig. 2a replotted as the logarithm of rate of reaction *versus* $\sqrt{\mu}/(1+\sqrt{\mu})$. The estimated ionic strength was 0.1 M in the absence of added KCl.

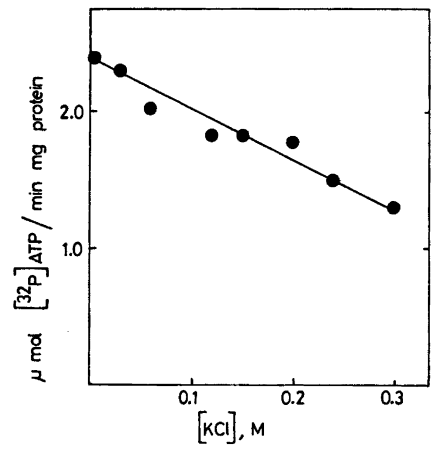

Fig. 3. The dependence of rate of exchange of [^{32}P]-pyrophosphate on KCl concentration. The conditions were as described in Materials and methods.

of aminoacylation is plotted against the concentration of potassium chloride a sigmoidal curve is obtained (Fig. 1b). The rate of aminoacylation is very sensitive to salt concentrations above 140 mM (Fig. 2a). At 0.3 M potassium chloride only about 30 % of the optimal activity is left.

The rate of the ATP – PPi exchange reaction is not sensitive to potassium ions to the same extent as aminoacylation (Fig. 3). No activation whatsoever can be observed. The activity decreases continuously with an increasing potassium chloride concentration. At 0.3 M potassium chloride about 55 % of the original activity is still left.

The effect of potassium ions on the K_m of isoleucine, ATP and tRNA^{ile} was determined by aminoacylation. Fig. 4 shows the effect of potassium ion activation on the estimation of K_m for tRNA. The Eadie plots show the variation of the rate of isoleucyl-tRNA formation with concentrations of tRNA. The data for all three substrates are summarized in Table 1. The K_m's for isoleucine and ATP are substantially the same despite the difference in rate and the concentration of potassium ions. The K_m for tRNA is altered almost exactly as predicted from primary effect of salt on K_m.[4,5]

Table 1. The kinetic constants for isoleucyl-tRNA synthetase determined by aminoacylation in the presence of different amounts of KCl. K_m are in units of mol/l and V_{max} are in units of nmol ile-tRNA/min mg protein.

[KCl]	0	140 mM	240 mM
K_m^{tRNA}	0.58×10^{-7}	3.0×10^{-7}	7.1×10^{-7}
K_m^{ile}	4.2×10^{-6}	9.0×10^{-6}	8.7×10^{-6}
K_m^{ATP}	2.1×10^{-4}	2.4×10^{-4}	1.8×10^{-4}
V_{max}	3	95	65

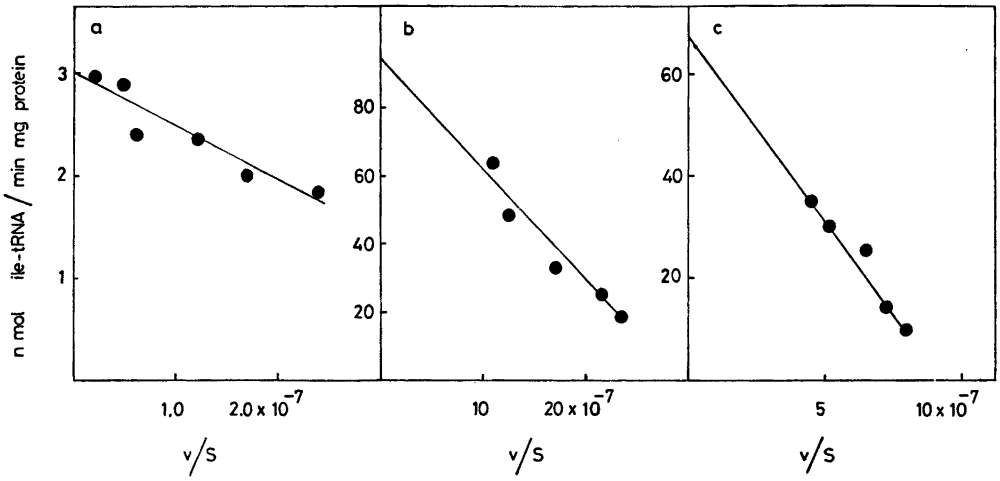

Fig. 4. Eadie plots showing the variation of rate of isoleucyl-tRNA formation with concentrations of tRNA^{ile} in the presence of (a) no added KCl, (b) 140 mM KCl, and (c) 240 mM KCl. Conditions were as described in Materials and methods.

DISCUSSION

According to Suelter [1] the enzymic reactions activated by monovalent cations can be divided into two main classes; phosphoryl transfer and elimination reactions. The usual representation of the aminoacylation of tRNA is through the intermediate formation of an enzyme bound aminoacyladenylate which represents a phosphoryl transfer reaction. Thus the ATP – PPi exchanges would have been expected to be sensitive to potassium ion activation but are not. The transfer of isoleucine from AMP to tRNA should not be sensitive to potassium ions but it is. As the rate of formation of isoleucyl-tRNA increases 20-fold in the presence of 140 mM potassium chloride the rate limiting step of aminoacylation has to be affected in one way or the other. Hence the potassium ions either affect a step of the reaction that they should not activate or then the concerted mechanism,[5] which does not require the formation of enzyme bound aminoacyl-adenylate, has to be considered as an alternative that is sensitive to activating potassium ion concentrations. Based on the fact that the effect on the ATP – PPi exchange reaction is relatively small and inhibiting it seems likely that the enzyme is not substantially altered by potassium ions. On the other hand, in the presence of increasing amounts of potassium ions the K_m is constantly increasing even though the rate of the reaction is first increasing and then decreasing (Table 1 and Fig. 4). Any activating effect (including those affecting the binding of substrates) must obviously be on the rate of the reaction. The rate of the reaction is proportional to $[K^+]^{2.25}$ over the entire range to 140 mM (Fig. 1c). Potassium ions bind apparently to more than one site or one enzymatic species in the mechanism.

Loftfield [4,5] feels that the major force in associating tRNA with ligase is interionic attraction. When the logarithm of the rate of aminoacylation of tRNA is plotted against the Debye-Hückel ionic strength function, $\log v = 0.5z^+z^- \sqrt{\mu}/(\sqrt{\mu}+1)$, usually a linear curve has been obtained with a negative slope.[4,5] If the data for inhibition is plotted according to the Debye-Hückel [11] approximation a negative slope of -1.5 is obtained (Fig. 2b). This observation is consistent with the idea that ionic interactions play a major role in the process of tRNA-binding to the enzyme. The effect of salt on K_m for $tRNA^{ile}$ is as predicted from other studies,[4] but it is more interesting because the K_m is constantly increasing even though the rate of reaction is first increasing then decreasing.

More experimental data are obviously required for identification of the step in the catalytic process that is activated by potassium ions. It has recently been concluded that it is unlikely that enzyme-product dissociation is the rate-limiting step in the synthesis of aminoacyl-tRNA.[12]

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Hydroxylaminolysis of Anilides. III. Hydroxylaminolysis of Formanilide and *p*-Methoxyacetanilide. A Comparison with Acetanilide

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The kinetics of the hydroxylaminolysis of formanilide and *p*-methoxyacetanilide have been studied in the pH range 4.8 – 9.6 at a total hydroxylamine concentration varying from 0.1 to 3.0 M. Under the different conditions the reaction rates are in agreement with a mechanism previously suggested for acetanilide, where the tetrahedral addition intermediate is broken down to products *via* three intermediates which are in protolytic equilibrium. A mechanistically preferable form of the mathematically derived rate expression has now been developed. Using this expression, the kinetic parameters for acetanilide have been recalculated and are compared with the corresponding parameters for formanilide and *p*-methoxyacetanilide. The substituent effects support the suggestion that the three equilibrium intermediates are cyclic.

Previous work [1] from this Laboratory has suggested that the hydroxylaminolysis of acetanilide follows a mechanism involving a complex general acid or general base catalysis of the breakdown of a tetrahedral substrate-hydroxylamine intermediate. In contrast to what was found in the hydroxylaminolysis of thiol esters and thiolactones,[2] the kinetics did not indicate catalysis of the formation of the tetrahedral intermediate. In another paper the hydroxylaminolysis of formamide was studied, but the authors [3] were not able to explain satisfactorily the results obtained at "extreme" pH values. Our recalculation of the data for formamide showed that the mechanism proposed for acetanilide was applicable to formamide over the whole pH range studied, whereas the thiol ester mechanism could not be applied.

The hydroxylaminolysis of trifluoroacetanilide has been reported [4] to be a pure second order reaction in hydroxylamine at pH values higher than approximately 5. This suggests that the strongly activated trifluoroacetanilide might follow the thiol ester mechanism rather than the acetanilide one. It is somewhat unexpected that highly reactive carbonyl compounds such as thiol esters and trifluoroacetanilide should require catalysis of the nucleophilic attack if moderately reactive, nonactivated amide compounds like acetanilide and formamide do not. In order to obtain more detailed information about the nature of the intermediates in the acetanilide mechanism we have now studied the hydroxylaminolysis of formanilide and *p*-methoxyacetanilide.

MATERIALS AND METHODS

Materials

Formanilide. Commercially available formanilide (BDH laboratory reagent) was recrystallized from ligroin + xylene (1 + 9). The resulting white needles were washed with light petroleum, b.p. 30 – 60 °C. The melting point was 47 – 48 °C (lit. 47.5 °C).[5]

p-*Methoxyacetanilide* was prepared from *p*-anisidine and acetic anhydride using the Schotten-Baumann procedure. It was recrystallized, first from water and then twice from benzene, yielding white flakes with a melting point of 130 °C (lit. 129 – 131 °C).[6]

All other chemicals used in the kinetic runs and in the assay were Merck chemicals reagent grade.

Acidity constants

The stoichiometric pK_w at 25 °C in 3.0 M KCl (14.13 ± 0.01) and the pK_a for the hydroxyl-

ammonium ion at 25 °C in 3.0 M KCl (6.32 ± 0.01) had been determined previously.[7,8] The stoichiometric pK_w at 25 °C in 5 % (v/v) dimethyl sulfoxide (DMSO) and 3.0 M KCl and the pK_a for the hydroxylammonium ion in the same medium were determined by potentiometric titration and found to be 14.32 ± 0.01 and 6.40 ± 0.01, respectively.

Kinetic experiments

In the kinetic experiments the medium was water, and the ionic strength was adjusted to 3.0 by addition of KCl. In the case of *p*-methoxyacetanilide the medium contained 5 % DMSO to increase the solubility of the anilide. The concentration of *p*-methoxyacetanilide was about 10^{-2} M, and that of formanilide varied between 4.3×10^{-3} and 4.3×10^{-4} M. The hydroxylaminolysis reaction was studied in the concentration range of 0.1 – 3.0 M hydroxylamine. The H^+ concentration varied from $10^{-5.04}$ to $10^{-9.79}$, corresponding to 5.0 – 99.97 % of the hydroxylamine system as base.

The experiments were performed at 25.00 ± 0.05 °C. The volume of the reaction mixtures was 100.00 ml. The hydroxylamine system was added as hydroxylammonium chloride and the desired percentage of hydroxylamine was obtained by neutralizing the hydroxylammonium ion with a calculated volume of a potassium hydroxide solution of known concentration. At the smallest concentration of the hydroxylamine system in the range of 5 – 80 % free base and for all concentrations at 70 % free base, the obtained pH values agreed very well with the calculated ones. In all other cases, however, medium effects became apparent as the concentration of the hydroxylamine system was increased. At base concentrations below 70 %, the measured pH values were lower than the calculated ones. At 5 % base and 3.0 M total hydroxylamine concentration, the pH was 0.4 units lower than the calculated value. This was the greatest deviation found. At 80 % base and 3.0 M total hydroxylamine concentration the measured pH was 0.1 unit higher than the calculated one. At 90 % base and higher, the pH was adjusted to the calculated values; this was done because of the difficulty in achieving an accurate degree of neutralization by adding measured volumes of the highly concentrated KOH solution.

A Radiometer model pHM 4 equipped with a glass electrode and a saturated calomel electrode was used for the pH control.

At high pH and in the presence of air, hydroxylamine is unstable. However, by excluding air and working under nitrogen,[1] kinetic runs can be performed with a negligible loss of hydroxylamine during the experiment.

Assay

The withdrawn samples were analyzed by means of the so-called aniline method.[7] Since this method was first introduced, it has been successively modified by many authors. The method involves diazotization and coupling of the aniline formed, and will be described in full below.

An aliquot of 1.00 – 5.00 ml was withdrawn and added to a given volume of hydrochloric acid of a given concentration in a volumetric flask. After addition of the aliquot, the volume was 10.00 ml and $[H^+]$ was 0.05 M. The diazotization was started immediately by adding 1.00 ml of 1 M $NaNO_2$ and after 3 min the excess nitrite was destroyed with 5.00 ml of a 5 % ammonium sulfamate solution. After another 3 min 5.00 ml of a 1 % solution of the coupling reagent was added, *N*-(1-naphthyl)-ethylene diammonium dichloride. After 10 min (with aniline as the reaction product) or 2 h (with *p*-methoxyaniline as the reaction product) 1 M HCl was added to give a volume of 25.00 ml and the absorbance was measured at the absorbance maximum; 550 nm for aniline and 580 nm for *p*-methoxyaniline. The molar absorption coefficients were 47 900 for aniline and 48 700 for *p*-methoxyaniline. The spectrophotometer used was a Zeiss spectrophotometer model PMQII.

At least 6 samples were analyzed for each determination of a k_{obs} value. The time during which the reactions were followed varied from 50 min to 48 h for formanilide and from 150 min to 100 h for *p*-methoxyacetanilide.

The k_{obs} values for formanilide were determined from plots of log (remaining anilide) against time, except in a few cases where less than 4 % of the anilide had reacted. Depending on the reaction rate, up to 85 % of the reaction was observed during the kinetic runs. In the case of *p*-methoxyacetanilide the reaction was observed only initially (less than 4 % reacted) and the k_{obs} values were evaluated from plots of concentration of product against time.

Theoretical calculations

The evaluation of k_1 and p values and the preliminary estimation of the constants in eqns. (2a) and (2b) were made by means of a Hewlett-Packard HP 9810A calculator. The final determination of the constants was made on an IBM 370/155 computer. The computer programs used were BMD07R, Biomedical computer programs, University of California and VB01A, Harwell Subroutine Library, AERE, England. Both are nonlinear least squares regression programs for curve fitting.

RESULTS

Observed pseudo first order rate constants, k_{obs}, for the formation of the respective anilines at constant pH and constant total hydroxylamine concentration (C), are given in Figs. 1 and 2 for formanilide and in Figs. 3 and 4 for *p*-methoxyacetanilide. Under most of the experimental conditions, the dependence of k_{obs} on C is close to second order but at high C and low pH the dependence approaches first order.

All the determined k_{obs} values are given in Figs. 1–4 as a function of C at given base %. The figures show that the rate increases with rising base % up to a base content of 60–70 % for formanilide and 50–60 % for *p*-methoxyacetanilide and then decreases continuously until at 99.9 % base no further decrease is apparent.

The curves in Figs. 1–4 have been calculated by means of eqn. (1).

$$k_{obs} = k_1[H_2NOH]pC/(1+pC) \quad (1)$$

In eqn. (1) C is the sum of hydroxylamine (N) and hydroxylammonium ion (NH^+) concentrations, k_1 is a constant and p is a pH-dependent variable, *i.e.* a different p value has been used for each base/acid ratio. The significance of p and its variation with pH has been discussed in a previous paper [1] and will be further commented upon in the discussion below. The values of p and k_1 used to calculate the curves in Figs. 1–4 are given in Table 1.

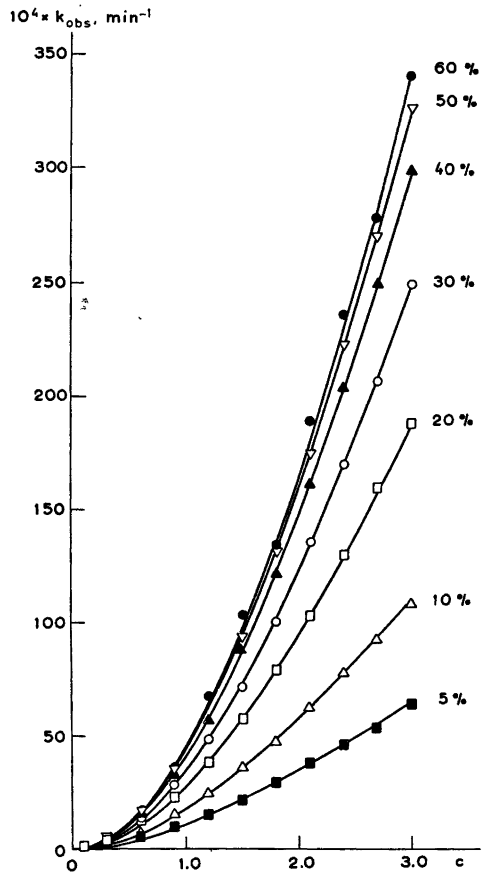


Fig. 1. Formanilide. Plot of k_{obs} *vs.* C at different base percentages. The curves have been calculated from eqn. (1) using the values of k_1 and p listed in Table 1.

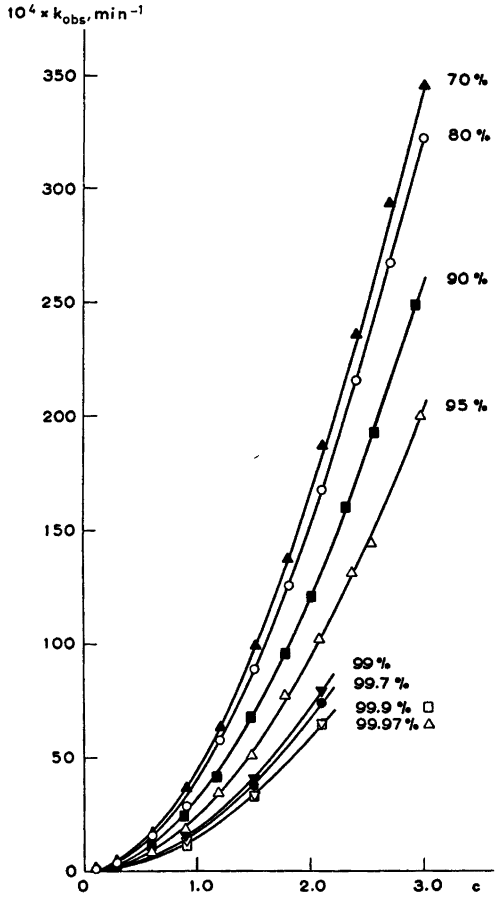


Fig. 2. Formanilide. Plot of k_{obs} *vs.* C at different base percentages. The curves have been calculated from eqn. (1) using the values of k_1 and p listed in Table 1.

Table 1. Values of p used in the calculation of the curves in Figs. 1–4. The values of $k_1 = 8.0 \times 10^{-3}$ M^{-1} min^{-1} for formanilide and $k_1 = 4.5 \times 10^{-4}$ M^{-1} min^{-1} for p-methoxyacetanilide have been used in all calculations. The theoretical p values have been calculated by means of eqn. (2b) using the parameter values given in Table 2.

Formanilide				p-Methoxyacetanilide			
% Base	$-\log [H^+]$	p M^{-1}	p_{theor} M^{-1}	% Base	$-\log [H^+]$	p M^{-1}	p_{theor} M^{-1}
5.00	5.04	0.390	0.390	5.00	5.12	1.55	1.55
10.00	5.37	0.290	0.288	10.00	5.45	0.815	0.833
20.00	5.72	0.210	0.214	20.00	5.80	0.450	0.465
30.00	5.95	0.175	0.176	40.00	6.22	0.244	0.245
40.00	6.14	0.150	0.149	50.00	6.40	0.194	0.190
50.00	6.32	0.125	0.125	60.00	6.58	0.148	0.144
60.00	6.50	0.104	0.104	80.00	7.00	0.079	0.073
70.00	6.69	0.087	0.085	90.00	7.27	0.053	0.047
80.00	6.92	0.068	0.066	99.00	8.27	0.017	0.016
90.00	7.27	0.046	0.046	99.90	9.27	0.012	0.012
95.00	7.60	0.033	0.035	99.97	9.79	0.012	0.012
99.00	8.27	0.024	0.024				
99.70	8.79	0.022	0.021				
99.90	9.27	0.019	0.020				
99.97	9.79	0.019	0.019				

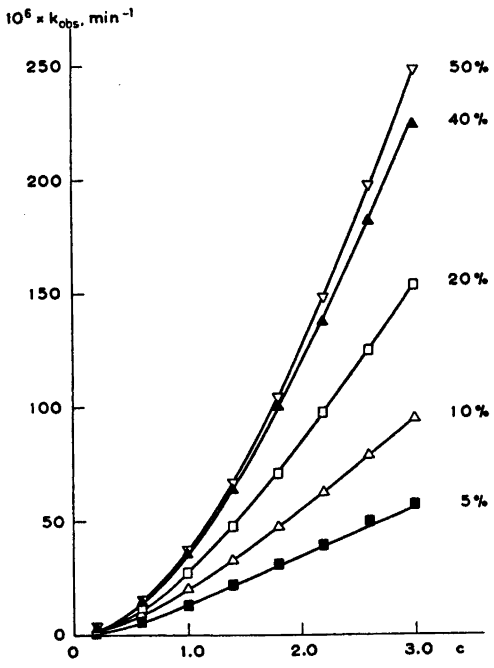


Fig. 3. p-Methoxyacetanilide. Plot of k_{obs} *vs.* C at different base percentages. The curves have been calculated from eqn. (1) using the values of k_1 and p listed in Table 1.

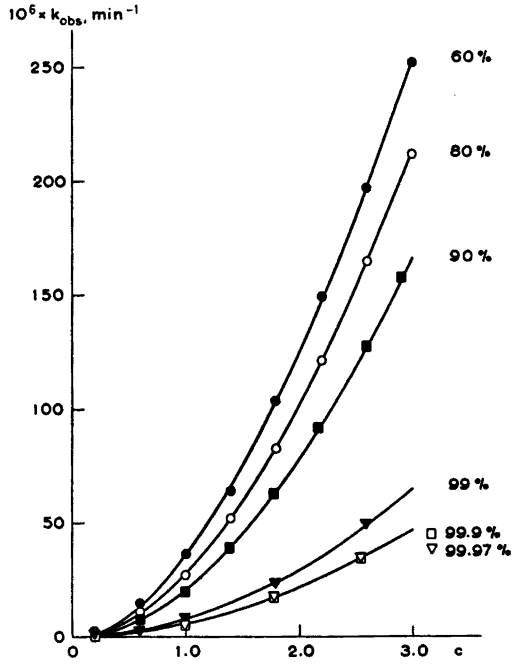


Fig. 4. p-Methoxyacetanilide. Plot of k_{obs} *vs.* C at different base percentages. The curves have been calculated from eqn. (1) using the values of k_1 and p listed in Table 1.

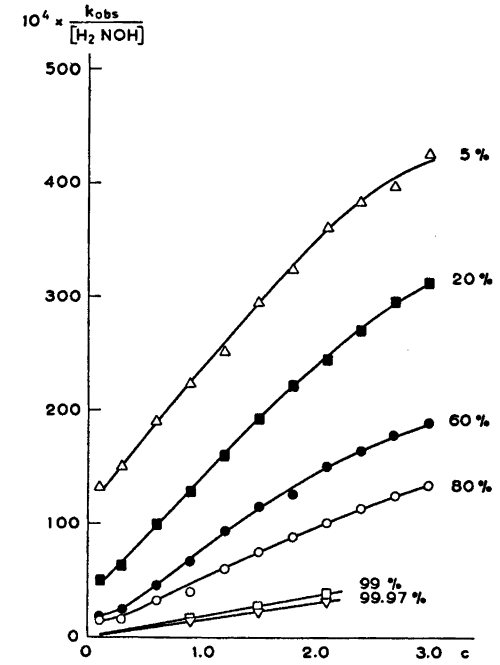


Fig. 5. Formanilide. Plot of $k_{obs}/[H_2NOH]$ *vs.* C at 6 different base percentages. The dots are experimental. The curves have not been calculated.

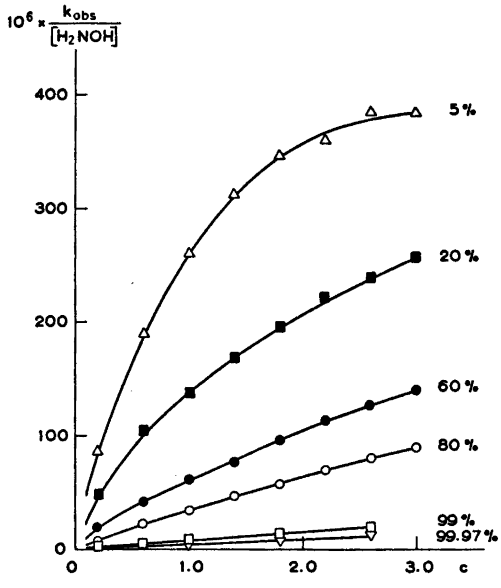


Fig. 6. *p*-Methoxyacetanilide. Plot of $k_{obs}/[H_2NOH]$ *vs.* C at 6 different base percentages. The dots are experimental. The curves have not been calculated.

In the mechanism leading to eqn. (1), k_1 is the rate constant for the nucleophilic attack of hydroxylamine on the carbonylic carbon. The k_{obs} values at high total concentrations at the lowest base percentages are the most useful ones in evaluating k_1. As seen from Figs. 1 and 3 these parts of the plots are almost linear, which indicates that pC in eqn. (1) is considerably larger than 1. A preliminary value of k_1 has been estimated from these plots and then used to calculate the theoretical curves. The value of k_1 first used invariably had to be changed somewhat to give the best possible fit for all base/acid ratios. Too high a k_1 will generally give a bad fit at low base percentages, and too low a k_1 will give a bad fit at high base percentages. These disagreements cannot be compensated for by choosing a suitable value of p. The final k_1 and p values were calculated simultaneously by means of a computer.

The sets of k_1 and p finally chosen were the ones that gave equally good fits to all the plots of k_{obs} *vs.* C, regardless of base percentages.

Generally, values of p deviating from those in Table 1 by approximately 5 % gave a less good fit to the experimental values.

Figs. 5 and 6 show plots of $k_{obs}/[H_2NOH]$ as a function of C; these values have been obtained from some of the curves presented in Figs. 1 – 4. They show how the reaction rate is influenced by a second hydroxylamine molecule or hydroxylammonium ion. The curves in these figures have not been calculated but have been drawn to connect the points as well as possible.

In Fig. 6, which refers to *p*-methoxyacetanilide, the curves have no obvious intercepts. At 5 % base it is quite apparent that the curve approaches a limiting value at the highest C. As the base percentage increases the curves bend less, and at a high pH they show a first order dependency with respect to $[H_2NOH]$.

In Fig. 5, referring to formanilide, the curves have definite intercepts. Owing to smaller p values the curvatures are less marked than in Fig. 6.

In Figs. 7 and 8, k_{obs} values for 4 different C values have been plotted as a function of log $[H^+]$. These figures also show that formanilide reaches its rate maximum at a somewhat smaller $[H^+]$ value than *p*-methoxyacetanilide does, and that a rate limit is reached at small values of $[H^+]$.

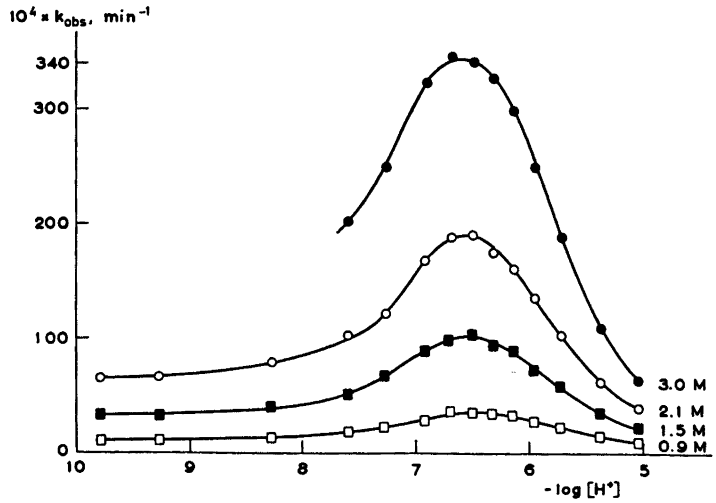


Fig. 7. Formanilide. Plot of k_{obs} *vs.* $-\log [H^+]$ for 4 different C. The dots are experimental. The curves have not been calculated.

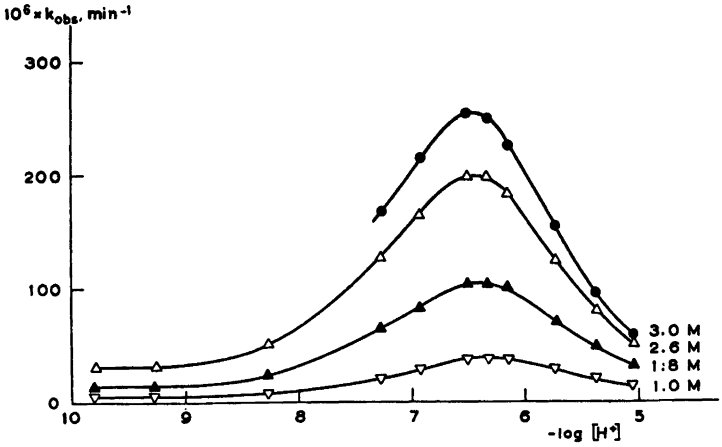


Fig. 8. *p*-Methoxyacetanilide. Plot of k_{obs} *vs.* $-\log [H^+]$ for 4 different C. The dots are experimental. The curves have not been calculated.

The curves in Figs. 7 and 8 have been drawn to connect the points at a given C and have not been calculated.

DISCUSSION

Variation of k_{obs} with C and pH

In the hydroxylaminolysis of acetanilide,[1] plots of k_{obs} *vs.* C were found to be completely described by eqn. (1). This equation may be derived from any mechanism consisting of an uncatalyzed nucleophilic attack on the carbonyl carbon to give an addition intermediate (I, see Scheme 1), followed by a breakdown of the intermediate in which the presence of a second molecule of the nucleophile or its corresponding acid is necessary for the reaction to result in products. By using the steady state approximation, an equation can be derived where all the constants pertaining to the breakdown of the intermediate – be it to starting

materials or products – can be gathered in one complex expression. This expression is here symbolized by p [see eqn. (1)]. A change in the mechanism for the breakdown of the intermediate will give a change in p, but it will not change eqn. (1) as such, as long as the conditions mentioned above are fulfilled.

In the present investigation of the hydroxylaminolysis of formanilide and p-methoxyacetanilide, the plots of k_{obs} *vs.* C are well described by eqn. (1) (see Figs. 1–4).

This is further confirmed for p-methoxyacetanilide in Fig. 6 where k_{obs}/N has been plotted *vs.* C. Eqn. (1) may be rewritten as in eqn. (1a).

$$k_{obs}/N = k_1 pC/(1+pC) \qquad (1a)$$

According to eqn. (1a), the plot of k_{obs}/N *vs.* C should go through the origin. This is indeed the case with p-methoxyacetanilide, as it was with acetanilide.[1] Fig. 5, on the other hand, shows positive intercepts, and a close examination of the plot of k_{obs} *vs.* C in Figs. 1 and 2 shows that the experimental values are indeed higher than the theoretical curve at $C \leq 0.6$ M, whereas at higher concentrations there is good agreement between the theoretical and experimental values.

At 5 % base and the lowest C about 1 % of the total rate can be explained by ordinary acid-catalyzed hydrolysis,[8] but this is far from enough to account for the intercept. At higher C and at a higher pH, the contribution from the acid-catalyzed hydrolysis reaction is negligible.

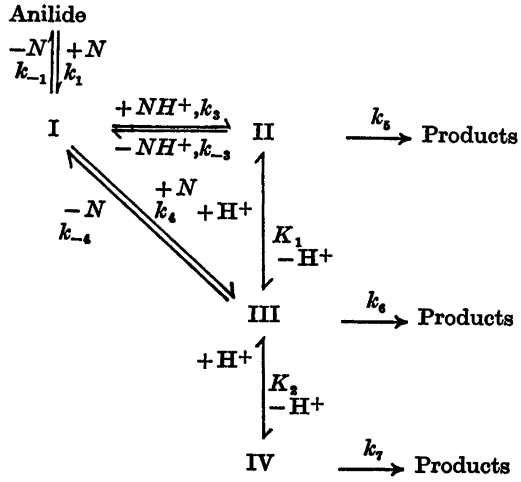
Thus the simplest way to explain the intercepts is by a hydroxylaminolysis reaction where the breakdown of the tetrahedral intermediate to products is spontaneous (catalyzed by water) or catalyzed by H^+ or HO^-. A spontaneous breakdown would give a pH independent intercept. Fig. 5 shows that the intercept increases with rising H^+ concentration, which suggests an acid catalyzed breakdown. No detailed interpretation of the intercepts will, however, be attempted here, since these have been determined by extrapolation and the points at 0.1 M are somewhat uncertain due to experimental difficulties (very low reaction rates).

From the plots of k_{obs} *vs.* $-\log [H^+]$ in Figs. 7 and 8, it is obvious that both N and NH^+ are active in the hydroxylaminolysis reaction, since there is a rate maximum near pK_a for the hydroxylammonium ion. The simplest way to explain this is by a nucleophilic attack by N to form an intermediate, the breakdown of which is assisted by NH^+. Although NH^+ is a potent catalyst, its presence is not absolutely necessary for the breakdown. If it were, the rate should approach zero when the concentration of NH^+ is lowered. Instead, a limiting value is reached at about 99 % base, *i.e.* at a point where an increase of pH will result in a drastic decrease of $[NH^+]$ but in no noticeable increase of $[N]$. Obviously, at high pH, N will act both as nucleophile and catalyst. At low pH no limiting value is to be expected since at least one N must be involved in the nucleophilic reaction.

Interpretation of p

Since in eqn. (1) the second molecule of the nucleophile N and/or its corresponding acid NH^+ is represented by C ($C = N + NH^+$), p will always be dependent on the pH, except in the case where the catalytic effects of the nucleophile and the acid are equal. Thus an investigation of the dependency of p on pH will throw light on the breakdown of the intermediate I to products.

In a previous paper[1] we suggested that the hydroxylaminolysis of acetanilide follows the mechanism presented in Scheme 1.



Scheme 1.

From this mechanism we derived an equation for the dependence of p on [H$^+$] [eqn. (2a)].

$$p = \frac{a + b[H^+] + c[H^+]^2 + d[H^+]^3}{1 + e[H^+] + (fK_a + e/K_a - 1/K_a^2)[H^+]^2 + f[H^+]^3} \quad (2a)$$

Eqn. (2a), where a through f denote big clusters of constants, has two disadvantages. Firstly, a through f have no easily interpretable kinetic meaning. Secondly, they are very complex, and logical errors, such as ascribing two or more different numerical values to the same microconstant (k_3, k_4 *etc.*), may pass unnoticed.

This induced us to rearrange eqn. (2a) into eqn. (2b), see below.

K_I through K_{VI} have distinct kinetic significance. They denote the ratios between the rate constants for the different routes of degradation of the intermediates, as defined in Table 2.

The advantage of eqn. (2a) over (2b) is that it is rather easy to make a first estimate of the parameters a through f. Eqn. (2b) does not involve a larger number of parameters, but it is so complex that it is extremely difficult to make a reasonable guess at where to start the search for the best parameter values. However, from the parameters a through f of eqn. (2a) for acetanilide a fairly good first estimate can be made of the parameters K_I through K_{VI} of eqn. (2b).

The relative simplicity of K_I *etc.* makes it possible to compare the importance of the different paths of decomposition of the intermediates for a certain substrate and to discuss the influence of substituents on the reaction. For this reason, the data for acetanilide have been recalculated using eqn. (2b) instead of eqn. (2a). The result is given in Table 2. By using eqn. (2b) we obtained a slightly better fit to the experimental values of acetanilide than by means of eqn. (2a). Theoretically, the two equations should give the same fit. The difference is probably due to the greater flexibility of eqn. (2b) as well as to a better curve fitting method.

Eqn. (2b) was then used to reproduce the experimental p values of formanilide and p-methoxyacetanilide. The calculated p values are listed in Table 1 and the optimal K_I through K_{VI} values are shown in Table 2.

Formation of intermediate I

On the introduction of a methoxy group into the *para* position of the aniline ring, k_1 decreases from 7.0×10^{-4} to 4.5×10^{-4} M^{-1} min^{-1}. This is in good agreement with the trends of the rate constants for the nucleophilic attack of hydroxide ion on the same substrates: 7.85×10^{-5} M^{-1} s^{-1} for acetanilide [10,11] and 4.20×10^{-5} M^{-1} s^{-1} for p-methoxyacetanilide.[12] The decrease in the rate constants is expected since p-methoxy substitution gives a somewhat higher electron density at the carbonyl carbon.

For formanilide, k_1 of the hydroxylaminolysis increases a hundredfold (see Table 1). This is expected, considering the absence of steric hindrance and the lower electron density at the formyl carbon as compared with the acetyl carbon, and is comparable to the 500-fold increase in k_1 in the hydrolysis of p-nitroacetanilide and p-nitroformanilide [11] and to

Table 2. The parameter values of eqn. (2b) used when calculating the theoretical p values in Table 1.

	$K_I = \frac{k_4}{k_{-1}}$ M^{-1}	$K_{II} = \frac{k_6}{k_7 K_2}$ M^{-1}	$K_{III} = \frac{k_{-4}}{k_7 K_2}$ M^{-1}	$K_{IV} = \frac{k_3}{k_{-1}}$ M^{-1}	$K_V = \frac{k_5}{k_7 K_1 K_2}$ M^{-2}	$K_{VI} = \frac{k_{-3}}{k_7 K_1 K_2}$ M^{-2}
Formanilide	1.89×10^{-2}	1.24×10^{7}	4.48×10^{7}	9.52×10^{-1}	2.34×10^{12}	1×10^{7}
p-Methoxyacetanilide	1.00×10^{-2}	6.30×10^{7}	2.01×10^{10}	9.51×10	3.81×10^{13}	or
Acetanilide	1.30×10^{-2}	2.14×10^{7}	1.40×10^{8}	2.30	8.85×10^{12}	less

$$p = \frac{K_I + [K_{IV}/K_a + K_I K_{II}][H^+] + [K_{II}K_{IV}/K_a + K_I K_V][H^+]^2 + K_{IV}K_V/K_a[H^+]^3}{1 + [1/K_a + K_{II} + K_{III}][H^+] + [(K_{II} + K_{III})/K_a + K_V + K_{VI}][H^+]^2 + (K_V + K_{VI})/K_a[H^+]^3} \quad (2b)$$

the 200-fold difference between acetanilide and formanilide [11] in the total rate of alkaline hydrolysis.

Breakdown of intermediate I

In Table 2 are listed the group constants K_I through K_{VI}. No standard deviations are listed in Table 2, since the two computer programs used in the calculations gave different standard deviations for the same numerical value of a certain constant. In order to test the validity of the constants, we varied them one at a time by ± 1, ± 5, and ± 10 % to see what effect a change in the constants would have on the calculated p values. A variation of K_{III} and K_{IV} by 1 %, of K_{II} and K_V by 5 %, and of K_I by 10 % gave a less good fit to the experimental p values, whereas K_{VI} could be varied by a couple of powers of ten without giving any change at all in the calculated p. K_{VI}, representing the ratio $k_{-3}/k_7K_1K_3$, is evidently too small to be of any significance in the studied pH range; this indicates that the direct breakdown of intermediate II to intermediate I is negligible for all three substances. It seems reasonable to assume that a low variability indicates that a substantial part of the total reaction proceeds via the steps represented in the ratio in question.

At high pH ([H^+] less than 10^{-9}) the experimental p values reach a limiting value. Since at low H^+ concentrations eqn. (2b) is reduced to $p = K_I$, the experimental limiting values were used as the starting values of K_I. These values were not altered in the computerization, but were accepted as the best possible ones.

It has been concluded [12] that the tetrahedral intermediates of the hydrolysis of acetanilide and p-methoxyacetanilide exist long enough for the proton of the $-OH$ group to be exchanged (Fig. 9a). It seems reasonable to assume that the primary tetrahedral intermediate ($I^{\pm}$) in the hydroxylaminolysis reaction, too, should be stable enough to permit proton exchange (Fig. 9b).

Considering the rather large variability of K_I, it is difficult to state positively if this constant, representing k_4/k_{-1}, is significantly altered by the introduction of the p-methoxy group. However, it increases slightly by the abstraction of the methyl group from acetanilide. This suggests that the second hydroxylamine molecule acts as a general base on the hydroxyl group of the acyl carbon or on the amino or hydroxyl group of the nucleophile (Fig. 10). The p-methoxy group is probably too far away to influence significantly the acidity of these protons; the methyl group is sufficiently near to increase the electron densities of the oxygen and the nitrogen at the acyl carbon, thus making the protons less mobile. The hydroxyl group of the original nucleophile is one step further off, but might still be affected.

When [NH^+] = 1.0 M, K_{IV}, representing k_3/k_{-1}, shows that with acetanilide as a substrate, intermediate I will be transformed into intermediate II twice as often as it will decompose into starting materials. This ratio increases fortyfold when a p-methoxy group is introduced and decreases to half the value when the methyl group is abstracted. Since both the p-methoxy group and the methyl group are electron donating, these effects suggest that in the k_3 step a bond is being formed between the hydroxylammonium ion and the free electron pair of the anilide nitrogen.

Combining the substituent effects on K_I and K_{IV} with the mathematical demand from eqns. (2a) and (2b) and Scheme 1 that the intermediates II and III should be interconvertible by the addition or abstraction of a proton only, we come to the conclusion that the hydroxyl-

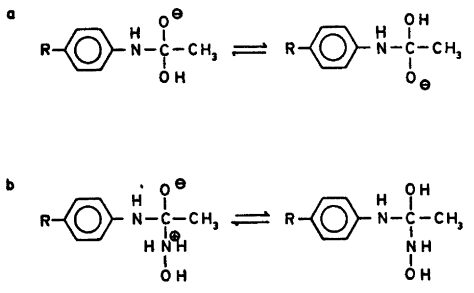


Fig. 9.

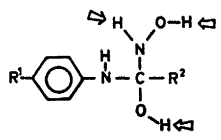


Fig. 10.

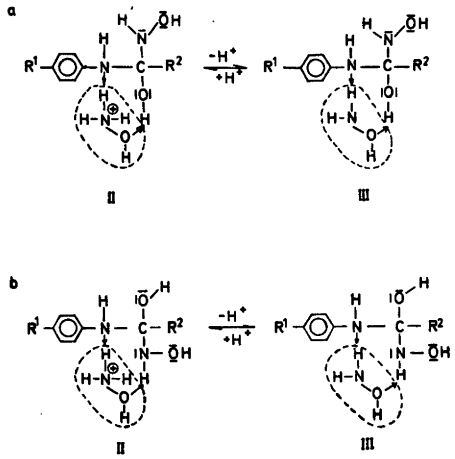


Fig. 11.

amine and the hydroxylammonium ion are bonded to I in a ring structure, the binding sites being the anilide nitrogen and the amino or hydroxyl group (see Fig. 11).

Fig. 11 shows possible structures of the intermediates II and III, the bonding moiety of the acyl part being the hydroxyl group in Fig. 11a and the amino group in Fig. 11b.

It has been suggested [3] that protolytic equilibria between the tetrahedral intermediates in aminolysis reactions will be detectable only when the protolytic constants of the intermediates have the same order of magnitude as that of the nucleophile. From this and from Fig. 10 we may assume the protolytic constants for the conversion of II to III (K_1) to be near to that of the hydroxylammonium ion itself, somewhere around 10^{-6}, but since the K_1 and K_2 values will be influenced by the substituents to an unknown extent, the terms containing these constants, K_{II}, K_{III}, K_V, and K_{VI}, cannot be discussed as they are.

In order to be able to discuss the k_6 and k_{-4} steps, K_{II} is divided by K_{III} to eliminate the k_7K_2 terms. In this way we obtain the following ratios of k_6/k_{-4}: Formanilide 0.28, acetanilide 0.15, *p*-methoxyacetanilide 0.0031. Considering the uncertainty of K_{II} and K_{III}, the slight decrease in the ratio when formyl is replaced by acetyl may not be significant, but there is still a considerable decrease in the ratio when the *p*-methoxy group is introduced. The greater part of the decrease in the k_6/k_{-4} ratio is probably due to a decrease in k_6, since the strength of the amide bond of an anilide is influenced by electron donating or withdrawing substituents in the *para* position of the anilide ring.[11] Electron donating substituents in the acyl part will also strengthen the amide bond but to a smaller extent.

Since all that is known about K_{VI} is that it is too small to be of any significance, there is no possibility of discussing the ratio of k_5/k_{-3} by the same procedure. However, the magnitude of the ratio is worth noticing, since it shows that independent of the substituents the k_5 step is at least a million times larger than the k_{-3} step.

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The Binding of Fluorochromes and Proteins to Cellulose-immobilized Nucleic Acids

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Columns containing DNA immobilized to cellulose were used to determine the quantity of fluorochromes and proteins bound to nucleic acids. The amount of dye bound to the DNA was directly proportional to the AT base content of the polymer. Of the three acridine derivatives tested, acranil had the highest, quinacrine mustard the lowest, and quinacrine intermediate affinity for the nucleic acids. The affinity of the dye Hoechst 33258 varied in relation to the AT base content of the polymer. The higher the AT base content of the DNA, the more poly-L-lysine and the less poly-L-arginine was bound. Binding of polyamino acids or histones to the cellulose-immobilized DNAs partly or wholly prevented the subsequent binding of dyes. Poly-L-lysine was more effective than poly-L-arginine in blocking the dye-binding to DNA. Complete blocking was obtained with protamine. Of the three histone fractions tested, the lysine-rich fraction was twice as effective in blocking dye binding as the intermediate fraction or the arginine-rich fraction.

The past few years have seen many investigations attempting to explain the reactions between fluorochromes and nucleic acid polymers. It has become clear that the reactions between acridine dyes and nucleic acids are base specific. Polyadenylic acid or polynucleotide mixtures with high adenine to guanine ratios increase the fluorescence intensity of the acridines in solution, whereas quenching occurs with polyguanylic acid or polynucleotide mixtures with low adenine to guanine ratios.[1–5] The fluorochrome Hoechst 33258 is an exception to this rule, since both polyadenylic acid and polyguanylic acid enhance its fluorescence intensity in solution. However, the increase in the fluorescence intensity of this dye is higher in mixtures with polyadenylic acid than in mixtures with polyguanylic acid, and hence it, too, is base specific.[6]

The binding affinity of dyes to polymers can be studied in different ways, ultrafiltration, equilibrium dialysis and absorption or fluorescence titration being the methods most commonly used. Such studies have yielded inconclusive or conflicting results (*cf.* Refs. 7 – 12).

The use of immobilized nucleic acids has become wide spread in recent years, especially in the study of nucleic acid enzymes.[13] The application of dyes to immobilized nucleic acids is a suitable technique for dye-binding studies and in this paper we present results from such experiments. Furthermore the successive application of proteins and dyes to cellulose-immobilized nucleic acids allows a quantitation of the blocking effect of proteins on the binding of dyes to nucleic acids. Our data show that the affinity of fluorochromes as well as the binding of certain proteins to nucleic acids are dependent on the base content of the DNA.

MATERIALS AND METHODS

Nucleic acid derivatives. The potassium salt of polyadenylic acid, the sodium salt of polyguanylic acid and the heteropolymerized nucleotide duplex polyadenylic-thymidylic acid were purchased from the Miles Laboratories, U.S.A., and the potassium salt of polycytidylic acid and the potassium salt of polyuridylic acid from Sigma Chemical, Co., U.S.A. DNA from *Clostridium perfringens*, from *Escherichia coli* and from *Micrococcus lysodeikticus* were purchased from Sigma Chemical, Co., U.S.A. DNA from calf thymus or from mouse liver was

extracted according to the method of Marmur.[14]

Proteins. The polyamino acids poly-L-arginine hydrochloride (mol.wt. 65 000) and poly-L-lysine hydrobromide (mol.wt. 180 000) and the calf thymus histone fractions "Type IIA" (containing all histones), "Type III" (lysine-rich fraction) and "Type IV" (arginine-rich fraction) as well as protamine and protamine sulfate were purchased from Sigma Chemical, Co., U.S.A.

Dyes. Acranil and quinacrine were purchased from Bayer Leverkusen, W. Germany and quinacrine mustard from Polysciences, Inc., U.S.A. The dye Hoechst 33258 was a gift from Dr. H. Loewe, Hoechst Ag., Frankfurt, W. Germany.

Labelling of DNA. Mouse L-cells were labelled with ^{3}H-thymidine as described earlier.[15] The L-cell DNA was isolated according to the method of Marmur.[14] The specific activity of the L-cell DNA was approximately 165 counts/min per μg. The radioactivity was assayed by liquid scintillation in 10 ml of Insta-Gel solution from Packard Co., U.S.A.

Preparation of nucleic acid cellulose. Cellulose powder C F 11 was obtained from Whatman, England, and the DNA-cellulose was prepared using irradiation with ultraviolet light according to the method of Litman.[16]

The amount of DNA bound to cellulose was determined as follows: A known amount of nucleic acid cellulose (approximately 100 mg of dry DNA-cellulose) was boiled for 10 min in 5 ml of 5 % perchloric acid. The nucleic acid released into the supernatant was determined spectrophotometrically at 260 nm or by liquid scintillation counting. This method completely removed the DNA from the cellulose (*cf.* Table 1).

The nucleic acid cellulose columns were prepared as follows: The dry DNA-cellulose was suspended in 5 mM NaCl (pH 7.0) and the DNA-cellulose allowed to settle during passage of several milliliters of 5 mM NaCl through the columns (1 cm in length, 2 cm in diameter). An excess of fluorochrome or protein (2 mg/ml in 5 mM NaCl, pH 7.0) was applied to the cellulose columns, and the columns were then washed extensively with 5 mM NaCl to remove unbound fluorochrome or protein. The fluorochrome or protein bound to cellulose-immobilized DNA was removed with 1 M HCl. The recovery of the dye or protein was complete, but no DNA was removed (*cf.* Table 1). The column effluent was collected in 2.5 ml fractions and the amount of fluorochrome or protein in the 5 mM NaCl fractions as well as in the fractions containing 1 M HCl was estimated. The acridine derivatives or poly-L-lysine did not bind to DNA-free acid-washed cellulose, whereas Hoechst 33258 and poly-L-arginine were bound. The values given for Hoechst 33258 or for poly-L-arginine are therefore corrected for this background of binding to cellulose alone.

Table 1. The stability of cellulose-immobilized DNA.

Solution	Released DNA %
Distilled water	0.3
1 M HCl (pH 1.0)	0.5
2 M NaCl (pH 7.0)	1.0
0.25 M KH_2PO_4 – 0.25 M K_2HPO_4 (pH 6.8)	0.5
0.1 M citric acid – 0.2 M Na_2HPO_4 (pH 7.0)	0.4
1 M NaOH (pH 13.0)	40.6
Methanol	14.7
Ethanol (50 %)	13.4
Ethanol (100 %)	9.8
Perchloric acid (5 %, boiling for 10 min)	100.0

Protein assay. L-Arginine and poly-L-arginine were assayed using the Sakaguchi dye reaction.[17] Poly-L-lysine and mixtures of poly-L-arginine plus poly-L-lysine were assayed by precipitation with trichloroacetic acid and by spectrophotometric estimation of the precipitate at 400 nm according to Bonner *et al.*[18] The amino acids and histones were determined by the method described by Lowry *et al.*[19]

Dye assay. The molar extinction coefficients of the fluorochromes at respective absorption maxima were: acranil, 8.3×10^3 at 424 nm; quinacrine, 8.5×10^3 at 424 nm; quinacrine mustard, 14.6×10^3 at 424 nm; Hoechst 33258, 32.7×10^3 at 345 nm.

Abbreviations.

poly(rA), polyadenylic acid
poly(rC), polycytidylic acid
poly(rG), polyguanylic acid
poly(rU), polyuridylic acid
poly(dA-dT), polyadenylic-thymidylic acid
Q, quinacrine
QM, quinacrine mustard

RESULTS

The stability and availability of cellulose-immobilized DNA

To check the stability of the binding between native DNA and cellulose the release of isotopically labelled DNA was estimated after elution of DNA from the cellulose with various solutions (Table 1). It is shown that water or salt solutions at neutral pH-values did not remove DNA from the cellulose. However, at high pH values as much as approximately

40 % of the immobilized DNA is removed (1 M NaOH). Different alcohols removed approximately 10 % of the DNA. If the DNA-cellulose is boiled for 10 min in 5 % perchloric acid the DNA is completely removed.

The capacity of cellulose-immobilized DNA to react with nucleic acids passing through the column was tested as follows: Columns containing a cellulose-immobilized polynucleotide were perfused with complementary and non-complementary polynucleotides and the binding was measured. One mg of cellulose-immobilized poly(rA) bound 0.1 mg of poly(rU) but no poly(rA), poly(rC) or poly(rG). Correspondingly, 1 mg of cellulose-immobilized poly(rG) bound 0.16 mg of poly(rC) but no poly(rA) or poly(rU). These findings indicate that the cellulose-immobilized homopolymers retain their specificity of hybridization to complementary bases, but the binding capacity is not of the same magnitude as it is in solution. This is probably because the bonds between the cellulose and the polynucleotide compete with the pairing of complementary bases.[13]

In contrast, when a mixture of poly(rC) and poly(rG) was applied to poly (dA-dT)-cellulose columns approximately 0.95 mg of poly(rC) plus poly(rG) was bound per mg of poly(dA-dT). Thus, immobilized heteropolymeric nucleotides (poly(dA-dT)) retain their entire availability for hybridization with complementary sequences.

Dimerization

Immobilization induced by UV-light irradiation of nucleic acids probably results in multiple point attachment between the DNA and the cellulose, but the mechanisms are still not completely understood.[13] The nucleic acids form insoluble gels upon UV-light irradiation in solution mostly as a consequence of dimerization between neighboring pyrimidines. The type of dimers formed depends on the base content of the DNA. The formation of thymine dimers (T-T) increases in proportion to the AT base content of the DNA, whereas the formation of cytidine-thymine dimers (C-T) or cytidine dimers (C-C) decreases with increasing AT content. Moreover the total amount of dimers formed is dependent on the base content of the DNA, *i.e.* the higher the AT content of the DNA the more dimers are formed.[21]

Since we used UV light irradiation to immobilize the DNA, it is likely that dimerization occurred in the DNAs during the preparation of the DNA-cellulose. To test the degree of dimerization induced, the following experiments were performed.

The different nucleic acids (approximately 20 μg/ml in 10 mM NaCl) were irradiated with UV-light as in the preparation of nucleic acid-celluloses, and the changes in the absorbance at 260 nm were determined. A slight drop in the absorbance was regularly observed following UV-light irradiation of the DNAs, the hypochromicity being proportional to the base content of the DNA. At low AT content (*M. lysodeikticus* DNA) the decrease in absorbance was approximately 1 %, whereas the greatest hypochromicity (4 %) occurred with DNA from *Cl. perfringens* with high AT content. These figures indicate that dimerization did take place.[21]

The binding of fluorochromes to cellulose-immobilized nucleic acid derivatives

Polynucleotides. Table 2 summarizes the amounts of different fluorochromes bound to cellulose-immobilized polynucleotides. Poly-(rA)-cellulose bound similar amounts of acridines (approximately 300 μg/mg) and the same was true of poly(rG)-cellulose which bound 220 to 240 μg dye/mg. The amount of Hoechst 33258 bound to these two polynucleotide-celluloses was about 30 % lower in each case. Expressed as μg of dye bound per mg of polynucleotide, the binding capacity of poly(rA) was about 20 % higher than that of poly(rG). However, if the values are expressed as P/D (polynucleotide phosphorus divided by the dye concentration) the amount of acridines bound to poly(rA)-cellulose is only 9 % higher than the amount bound to poly(rG)-cellulose (values not given in the table). We therefore conclude that the difference in the dye binding capacity of poly(rA) and poly(rG) is slight or questionable.

Poly(dA-dT)-cellulose binds 1.5 to 3 times more of the acridines than the homopolymeric nucleotide-celluloses. Furthermore, the different dyes had different affinities for cellulose-

Table 2. The binding of fluorochromes to cellulose-immobilized nucleic acid derivatives.

Nucleic acid cellulose	GC %	(μg fluorochrome)/(mg nucleic acid derivative) Acranil	Quinacrine	Quinacrine mustard	Hoechst 33258
Poly(rA)	–	300	290	290	205
Poly(rG)	–	240	220	230	170
Poly(dA-dT)	–	720	640	460	420
M. lysodeikticus	70	140	130	55	170
E. coli	50	390	290	155	270
Cl. perfringens	31	560	540	380	390
Calf thymus	40	640	480	215	450
Mouse liver	40	660	470	230	420

immobilized poly(dA-dT). The relative amounts of acridines bound were as follows: acranil > Q > QM. The amount of Hoechst 33258 bound to poly(dA-dT)-cellulose was of the same order as that of QM.

Nucleic acids. Cellulose-immobilized DNAs with GC contents varying between 31 and 70 % were used to examine the base specificity of the binding between fluorochromes and DNAs. The following conclusions were reached on the basis of the data given in Table 2. The acridines as well as Hoechst 33258 react base specifically with immobilized microbial DNAs. The higher the AT content in the DNA, the greater were the amounts of the fluorochromes bound per mg of DNA. However, the correlation is not rectilinear.

Different proportions of the acridine derivatives were bound to the cellulose-immobilized microbial DNAs, the sequence always being: acranil > Q > QM. The same phenomenon occurred with poly(dA-dT)-cellulose.

The amount of Hoechst 33258 bound to DNAs of different base contents deserves comment. From fluorescence studies in solution it is known that the higher the AT content of the DNA, the stronger is the fluorescence intensity of the solution.[6] At low AT content in DNA-cellulose (*M. lysodeikticus*, 30 % AT) more Hoechst 33258 is bound than any of the acridine derivatives, whereas at high AT content (*Cl. perfringens*, 69 % AT) the reverse is true (Table 2). These findings apparently indicate that the mechanisms by which Hoechst 33258 interacts with nucleic acids are different from those by which the acridines interact.[22,23]

DNA from calf thymus or from mouse liver had similar affinities for the fluorochromes. The greatest amount of acridine bound to mammalian DNAs occurred with the dye acranil and the smallest with QM, whereas the amount of Q bound to mammalian DNAs was intermediate. This is in agreement with the results reported by Modest and Sengupta.[11] They found that the bonds between QM and calf thymus DNA were 25 times as strong as those of Q, but that fewer QM molecules bound to the DNA. The amounts of fluorochromes bound to immobilized mammalian DNAs were generally higher than the amounts bound to immobilized microbial DNAs. The only exception was the very AT rich DNA of *Cl. perfringens*.

The binding of proteins to cellulose-immobilized nucleic acid derivatives

The results are seen in Tables 3 and 4. It has been found that more poly-L-lysine was bound to poly(rA)-cellulose than to poly(rG)-cellulose. Furthermore, more poly-L-arginine was bound to poly(rG)-cellulose than to poly(rA)-cellulose. Thus, our results with immobilized polynucleotides correspond with earlier reports concerning the base specific binding of polyamino acids to DNAs.[24] Poly(dA-dT)-cellulose bound as much poly-L-arginine and poly-L-lysine as did poly(rA)-cellulose (Table 3). The different immobilized polynucleotides bound more poly-L-arginine than poly-L-lysine, *i.e.* poly-L-arginine

Table 3. The binding of polyamino acids to cellulose-immobilized nucleic acid derivatives.

Nucleic acid cellulose	GC %	(μg polyamino acid)/(mg nucleic acid derivative) poly-L-arginine	poly-L-lysine
Poly(rA)	–	260	130
Poly(rG)	–	555	50
Poly(dA-dT)	–	250	150
M. lysodeikticus	70	310	180
E. coli	50	220	230
Cl. perfringens	31	115	450
Calf thymus	40	160	270
Mouse liver	40	160	290

showed higher affinity for polynucleotide-cellulose than did poly-L-lysine.

When polyamino acids reacted with cellulose-immobilized microbial DNAs a clear-cut correlation was observed between the AT content of the DNA and the amount of poly-L-lysine bound, but the relationship was not linear (Table 3). With increasing GC content in the microbial DNAs, the binding of poly-L-arginine also increased; this relationship was linear. DNA from *E. coli* (50 % GC) bound equal amounts of the two polyamino acids.

Differences were also found in the amounts of polyamino acids bound to immobilized mammalian DNAs. The nucleic acids bound more poly-L-lysine than poly-L-arginine. These findings are in marked contrast to the binding of polyamino acids to polynucleotide-celluloses. Both mammalian DNAs tested, each containing about 40 % GC, bound equal amounts of poly-L-arginine, as was also the case with poly-L-lysine.

The amount of poly-L-arginine (160 μg) bound to 1 mg of immobilized mammalian DNAs (approximately 40 % GC) is intermediate between the amount bound to DNA from *E. coli* with 50 % GC (220 μg/mg) and the amount bound to DNA from *Cl. perfringens* with 31 % GC (115 μg/mg). Moreover, the mammalian DNAs bound approximately 280 μg poly-L-

Table 4. The binding of proteins to cellulose-immobilized calf thymus DNA and the effect of proteins on the binding between quinacrine and DNA-cellulose.

Proteins	Protein binding μg protein/mg DNA	Dye binding to DNA-protein complex[a]
Arginine	0	100
Lysine	0	100
Protamine	170	4
Protamine sulfate	0	100
Poly-L-arginine	160	41
Poly-L-lysine	270	7
Poly-L-arginine:Poly-L-lysine		
= 0.33	160	13
= 1.00	180	13
= 3.00	130	14
Histone fractions:		
"Type II A" (intermediate)	270	68
"Type III" (lysine-rich)	310	32
"Type IV" (arginine-rich)	360	68

[a] The inhibition by protein of the capacity of DNA to bind quinacrine is expressed as percent, 100 % being the binding of dye to cellulose-immobilized DNA unexposed to protein.

lysine per mg DNA which is intermediate between the corresponding amounts of poly-L-lysine bound to DNA from *E. coli* (230 μg/mg) and *Cl. perfringens* DNA-cellulose (450 μg/mg). This would seem to indicate that the base content is of importance in determining the binding capacity of polyamino acids to DNAs. The binding of some other proteins to calf thymus DNA was also tested (Table 4). The very arginine-rich protamine was bound to calf thymus DNA. However, immobilized DNA did not bind arginine or lysine. The amounts of different histone fractions bound to DNA-cellulose varied between 270 and 360 μg/mg of DNA. The highest amount bound was found with the arginine-rich histone fraction.

The effect of proteins on the binding between quinacrine and cellulose-immobilized DNA

It is possible partly or wholly to prevent the binding of fluorochromes to DNA-cellulose by saturating the dye-binding sites in the DNA with proteins (Table 4).

The amino acids arginine, lysine or protamine sulfate did not bind to immobilized DNA from calf thymus and hence did not prevent the binding of Q to the DNA-cellulose. However, protamine bound to immobilized DNA was able almost completely to prevent the binding of Q.

As shown above, calf thymus DNA bound more poly-L-lysine than poly-L-arginine (Table 3). Poly-L-lysine was also much more effective in preventing the binding of Q to the immobilized DNA than was poly-L-arginine. Three different mixtures of poly-L-arginine plus poly-L-lysine were also tested. The amounts of the mixtures bound to DNA-cellulose varied between 130 and 180 μg/mg of DNA, but the blocking of Q binding was independent of the poly-L-arginine to poly-L-lysine input ratio. The mixtures were, however, more effective in preventing the binding of the dye than was poly-L-arginine alone. The conclusion is that poly-L-lysine is very effective in blocking the dye-binding sites in cellulose-immobilized DNA. This effect is probably due to competition between poly-L-lysine and the dye for binding sites in the AT-rich regions of the DNA.[24]

Polylysine also has a marked effect on the fluorescence intensity of acridine analogs mixed with DNAs in solution. In all cases the polylysine enhanced the fluorescence intensity of the fluorochrome DNA-complex, *i.e.* the effect of DNA on the dye fluorescence was reversed by polylysine.[25]

Results obtained with microbial DNAs (not shown in Table 4) were similar to those with calf thymus DNA, although the blocking was more complete. Poly-L-arginine as well as poly-L-lysine completely prevented the binding of Q to DNA from *M. lysodeikticus*. Poly-L-lysine was also able wholly to block the binding of the dye to DNA from *E. coli*, whereas the blocking with poly-L-arginine was only partial. It is worth noting that hardly any dye adhered to DNA-cellulose columns saturated with polyamino acids, indicating that the polyamino acids *per se* did not bind the dyes under these conditions.

When different histone fractions were bound to the DNA-cellulose the greatest inhibition of the dye-binding capacity was obtained with the lysine-rich fraction "Type III" (Table 4). The inhibition effects obtained with the two other histone fractions were of the same magnitude (68 %).

DISCUSSION

Although there is now an abundance of data on the effect of nucleic acids on the fluorescence intensity of fluorochromes in solution, less has been written about the quantitative aspects of their binding. Some such studies have been made by ultra-filtration [11] or equilibrium dialysis [23,26–28] and interpreted with the aid of Scatchard plots. O'Brien *et al.*[8] failed to find any DNA base specific fluorescence with quinacrine in solution and this was partly supported by Modest and Sengupta.[11] Similar observations were reported by Müller and Crothers [27] who found that quinacrine shows little base specificity when mixed with DNAs in solution. On the other hand, our previous findings on the base specific effect of DNAs on fluorescence intensity of quinacrine mustard in solution [2,5,29] and similar discoveries by other workers [1–3] indicate base specific interactions between fluorochromes and nucleic acids.

Hence the experimentation needed to be extended.

The use of immobilized nucleic acids seems to give a more direct method, which allows a quantitation of the fluorochromes bound to the DNAs. The experimental procedure involves UV-light irradiation which brings about pyrimidine dimerization of the DNA.[18,21] The UV-light produces chemical and physical changes in the DNA. The absorption maximum of the DNA is reduced and the pyrimidine dimers produce conformational changes in the DNA. These changes have to be taken into consideration when the results in this paper are compared with results obtained by other methods.

Our data reveal that the quantity of the fluorochromes acranil, quinacrine, quinacrine mustard, and Hoechst 33258 bound to DNA is strongly dependent on the base content of the nucleic acid. This finding is different from those presented by others.[8,11,23,27,28] Furthermore, in a recent paper Latt *et al.*[26] reporting Scatchard plots and circular dichroism data, concluded that the DNA base composition has very little effect on quinacrine binding affinity. It is likely that the different results pertaining to the binding affinity presented by others and by us may be due to the differences in methodology.

The AT base specific binding of fluorochromes to cellulose-immobilized DNA shown in this paper may partly be a result of the dimerization of the DNA. The thymine regions in the DNA may bind more or fewer dye molecules than the other regions, due to denaturation of the DNA in the dimerized regions. Earlier studies with fluorochromes and DNAs in solution show that the fluorescence intensity of the fluorochromes is affected by the strandedness of DNA.[5,29,30] The decrease in the absorption maximum of the DNA after irradiation indicates that DNA undergoes conformational changes when exposed to UV-light (*cf.* Results). Although the UV-light irradiation caused damage to the DNA, the cellulose matrix was probably able to partly prevent the conformational changes since even AT-rich cellulose-immobilized DNAs were able to bind fluorochromes. The results in this paper show base specific binding between the fluorochromes and DNAs. This is supported by studies recently reported by Müller *et al.*[23,27,28] They studied the base specific reactions between DNAs and intercalating as well as non-intercalating DNA ligands. Strong AT base specific binding between the dye Hoechst 33258 and DNA was found and the compound seems to need three AT pairs for the binding site.[23]

Our results with proteins bound to cellulose-immobilized DNAs confirm previous studies indicating base specificity of the binding of polyamino acids and histone fractions to DNA.[24] Poly-L-arginine preferentially bound to GC-rich regions, whereas poly-L-lysine bound to AT-rich regions in the DNA. Furthermore the proteins blocked the binding between fluorochromes and DNA, thereby reducing the binding sites of the dyes to DNA. This competition is not surprising since it is known that proteins are bound to the phosphate groups of the DNA backbone.[31] The proteins also occupy either the minor and/or the major grooves of the DNA.[31] Thereby they not only inhibit the ionic bindings between the fluorochromes and the DNA but are also able to compete for the intercalation between dyes and the DNA. The polyamino acids show base specificity when bound to DNA. It is therefore natural that poly-L-lysine and lysine-rich histone fractions have the greatest effect on fluorochromes since they bind preferentially to AT-rich sequences,[32] whose affinity for these fluorochromes is strongest. Our results are consistent with those of Weisblum *et al.*[25] and Latt and Gerald [33] as regards the ability of polylysine to compete for dye-binding to DNA.

It was previously thought that differences in the base content of the DNA could explain the Q-banding of metaphase chromosomes.[34] However, our quantitative data suggest that proteins may have a substantial influence on the *in situ* fluorescence pattern. Similar conclusions could be drawn from studies of the density of nucleoprotein along the metaphase chromosomes.[35]

It may be too early to draw conclusions about the banding of metaphase chromosomes on the basis of binding studies in columns or in solution. Only further work will disclose whether different amounts of fluorochromes bind to different parts of the chromosomes and thereby contribute to banding. However, the present study may open the door to better

understanding of the pattern along metaphase chromosomes since there appears to be a clear-cut interrelation not only between DNA base composition and the intensity of fluorescence, but also between DNA base composition, affinity for different proteins, binding capacity for fluorochromes and finally the intensity of fluorescence.

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Purification and Properties of Two Protease Inhibitors from Rat Skin Inhibiting Papain and Other SH-Proteases

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Two papain inhibitors, I_1 and I_2, from rat skin extract were purified by affinity chromatography on KSCN-modified papain-agarose gel and by gel filtration on Sephadex G-100. I_1 had a molecular weight of 74 000, a pI of 4.6, and it contained 4 % of carbohydrates. I_1 inhibited papain, ficin, bromelain, rat skin benzoylarginine-2-naphthylamide hydrolase, and to a minor extent, rat skin cathepsin C and bovine trypsin. Bovine chymotrypsin or rat skin cathepsin D were not inhibited and benzoylarginine-2-naphthylamide hydrolase was inhibited only at alkaline pH. An inhibitor corresponding to I_1 was present in various rat tissues and also in serum. A similar inhibitor was present in the skin of cat, rabbit, guinea pig, and man. I_2 had a molecular weight of 13 400, a pI of 4.9 and it contained no carbohydrates. I_2 inhibited all thiol proteases tested, but not trypsin, chymotrypsin, or rat skin cathepsin D. I_2 formed an equimolar complex with papain and benzoylarginine-2-naphthylamide hydrolase. I_2 was present in rat skin, muscle, lung, and small intestine, but not in kidney, liver, or serum. A similar inhibitor was found in skin extracts of cat, rabbit, guinea pig, and man.

A number of inhibitors of serine proteases have been characterized from various plant and animal sources,[1] while only a few inhibitors of SH-proteases, such as papain (E.C. 3.4.22.2), cathepsin B1 (cathepsin B, E.C. 3.4.22.1), and cathepsin C (dipeptidyl peptidase, E.C. 3.4.14.1) are known. Finkenstaedt [2] found that the supernatant fraction after differential centrifugation of rat liver homogenate inhibited cathepsins B and C, an observation not confirmed by Bouma and Gruber.[3] A polysaccharide associated with mouse haptoglobin inhibits bovine cathepsin B1, papain, and trypsin.[4,5] However, human haptoglobin does not inhibit human cathepsin B1, while α_2-macroglobulin is inhibitory.[6] Chicken egg white contains an inhibitor with a molecular weight of 12 700 that inhibits the plant proteases papain and ficin (E.C. 3.4.22.3),[7,8] and also the mammalian SH-proteases, cathepsins B1 and C.[9,10] The inhibitor has different active sites for cathepsins B1 and C and it forms complexes with enzymatically inactive Hg^{2+}-derivatives of the enzymes.[9,10] An inhibitor with properties similar to the egg white inhibitor has been demonstrated from Arthus lesions of rabbit [11,12] and guinea pig [13] skin and from rabbit skin burns.[14] Sera of rabbit, guinea pig, and cow also contain an inhibitor that inhibits papain and a neutral inflammatory SH-protease.[14] The serum inhibitor is contained in the α_1-acid glycoprotein fraction and has a molecular weight of about 40 000 – 50 000.

During purification of rat skin benzoylarginine-2-naphthylamide hydrolase (BANA hydrolase), the presence of two inhibitors, I_1 and I_2, of the enzyme in the skin was noticed,[15–17] They also inhibited papain, and in this communication purification of the inhibitors, based on their papain inhibiting capacity, is presented. Some properties of the inhibitors are given and the presence of similar inhibitors in rat tissues and in skin of several mammalian species is demonstrated.

MATERIALS AND METHODS

Reagents. α-*N*-Benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA), α-D-methylmannoside, porcine chymotrypsinogen A, and

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equine cytochrome *c* were from Fluka AG. *N*-Acetyl-L-tyrosine ethylester (ATEE), casein (nach Hammarsten), ethylenediaminetetraacetic acid disodium salt (EDTA), and 4-dimethylaminobenzaldehyde were from E. Merck AG. Bovine hemoglobin (type II), bovine serum albumin, anthrone, indole, and Coomassie Brilliant Blue R-250 were from Sigma Chemical Co. Glycyl-L-arginine-2-naphthylamide (Gly-Arg-NA) was from Fox Chemical Co., dithiothreitol (DTT) from Calbiochem, and cyanogen bromide from Eastman-Kodak. Sepharose® 4B, Concanavalin A-Sepharose® 4B, Sephadex® G-100, Sephadex® G-200, and Blue Dextran were purchased from Pharmacia Fine Chemicals. Goat antiserum against rat serum proteins was obtained from Nordic Immunological Laboratories.

Enzyme preparations. Rat skin BANA hydrolase preparations AI and NII were obtained as described previously.[16] Rat skin cathepsin D was purified as described by Heikkinen *et al.*[18] A cathepsin C preparation was partially purified from rat skin extract by precipitation with acetone, gel filtration on Sephadex G-200, and chromatography on DEAE cellulose. The enzyme hydrolyzed Gly-Arg-NA at pH 5.0 producing glycylarginine and 2-naphthylamine as reaction products, and was activated by DTT. Papain (für biochemische Zwecke, E. Merck AG), ficin (crude, Sigma Chemical Co.), and bromelain (grade II, Sigma Chemical Co.) were dissolved in water (0.1 mg/ml) and used within one day. Bovine trypsin (type III, Sigma Chemical Co.) and chymotrypsin (type II, Sigma Chemical Co.) were dissolved in 1 mM HCl (0.1 mg/ml) and used within four days. Mercuripapain was prepared from Merck's papain by affinity chromatography on an organomercurial-agarose column according to Sluyterman and Wijdenes.[19] The procedure increased the specific activity of papain 3.6-fold.

Inhibition assays. All assays were performed in duplicate. The incubation mixture was composed by pipetting 0.1 ml of an inhibitor solution, 0.1 ml of an enzyme solution, and 0.1 ml of a buffer to a test tube at room temperature. After 10 min, the tube was transferred to a 37 °C water bath, and 0.1 ml of a substrate was added. The mixture was incubated for 10 min, and the reaction products were assayed. The buffers were: 0.2 M Tris-HCl, pH 7.5, 4 mM DTT, 8 mM EDTA for papain, ficin, and bromelain; 0.2 M Tris-HCl, pH 8.0, 10 mM $CaCl_2$ for trypsin and chymotrypsin; Britton-Robinson buffer, pH 5.8,[15] 0.8 mM DTT, 4 mM EDTA, 4 mM KCN for BANA hydrolase; 0.2 M sodium acetate buffer, pH 5.0, 0.2 M NaCl, 4 mM DTT, 4 mM EDTA for cathepsin C, and sodium lactate-acetate buffer, pH 4.3 for cathepsin D.[15] BANA (5 mM) was used as a substrate for papin, ficin, bromelain, BANA hydrolase, and trypsin; Gly-Arg-NA (1 mM) for cathepsin C; hemoglobin (2 %) for cathepsin D, and ATEE (15 mM) or casein (1 %) for chymotrypsin. The reaction products were assayed as described previously.[15–17] The inhibition was expressed as the amount of an enzyme (mg) inhibited by 1 ml of an inhibitor solution (U/ml) or by 1 mg of an inhibitor (U/mg). Linearities of the inhibition assays were always controlled by using serial dilutions of inhibitor solutions.

Other assay methods. Proteins were determined as described previously.[15] Carbohydrates were assayed by indole and anthrone using glucose as a standard.[20]

Tissue extracts. Long Evans rats weighing 150 – 200 g were used. Extracts of rat tissues and of rabbit, guinea pig, cat, and human (a female cadaver) skin were prepared as described previously,[17] but the tissues were minced with scissors and homogenized in 10 mM sodium phosphate buffer, pH 6.0, 1 % KCl, using Ultra-Turrax TP-18 homogenizer. When large quantities of rat skin extract were needed, the skin was minced with a meat mincer and Ultra-Turrax T-45 homogenizer was used.[15] The protein concentration of a typical rat skin extract was 2.1 ± 0.18 mg/ml and the specific papain inhibiting activity was 0.12 ± 0.4 U/mg.

Acetone precipitation. To 50 ml of extract 117 ml of cold (– 18 °C) acetone was slowly added in a cooling bath (– 10 °C), and the precipitated proteins were collected by centrifugation at 8000 *g*, at – 10 °C, for 10 min. The sediment was dissolved in 5 ml of 10 mM sodium phosphate buffer, pH 6.0, 0.1 M NaCl, and undissolved materials were removed by centrifugation at 8000 g, for 10 min, at + 4 °C. The supernatant was filtered by suction through a Millipore® type AP prefilter and AA filter, and applied to a Sephadex G-100 column.

Affinity chromatography on papain-Sepharose. Sepharose 4B was activated by cyanogen bromide and washed according to March *et al.*[21] The washed gel (100 ml) was suspended in 200 ml cold 0.2 M sodium bicarbonate, pH 9.5, and 1 g of papain, dissolved in 100 ml of the bicarbonate buffer, was added. The coupling and washing of the gel was performed as described by March *et al.*[21] To 1 ml of the gel 6.1 mg of papain was coupled as estimated from the protein content of gel washings. The papain-Sepharose gel was suspended in water to obtain a total volume of 200 ml, and 194 g of KSCN was slowly dissolved in the suspension under continuous stirring (the final concentration of KSCN was 5 M). The pH of the suspension was adjusted to 4.0 by 2 M acetic acid, and the suspension was allowed to stand for 2 h at room temperature. This procedure destroyed the BANA hydrolyzing activity of the papain-Sepharose gel. The gel was washed in a glass funnel with 1.0 l of water, 0.5 l of 3 M KSCN in 20 mM trisodium phosphate, and 1.0 l of 10 mM sodium phosphate buffer, pH 6.0, 0.1 M NaCl, and stored as a suspension in 10 mM phosphate buffer, 0.1 M NaCl, 0.02 % NaN_3, at pH 6.0. A column of 2.5×20 cm was packed with the inactivated papain-Sepharose

and equilibrated with 10 mM sodium phosphate buffer, 0.1 M NaCl, pH 6.0. 450 ml of rat skin extract was applied to the column by pumping it two times through the column at a flow rate of 100 ml/h. Unadsorbed material was eluted with 100 ml of the equilibrating buffer, collecting fractions of 10 ml. Nonspecifically adsorbed proteins were eluted with 100 ml of 3 M KCl in the buffer, and KCl was washed out by 100 ml of the equilibrating buffer. Elution of the inhibitors was performed with 200 ml of 20 mM trisodium phosphate, 0.1 M NaCl (pH 12.1). Finally, the column was washed with 100 ml of 3 M KSCN in 20 mM sodium acetate buffer, pH 4.0, and equilibrated with the starting buffer for a subsequent chromatographic run. The pooled inhibitors from papain-Sepharose affinity chromatography were concentrated to 40 ml on Diaflo ® UM-10 (Amicon NV) membrane and applied to a column of Sephadex G-100.

Gel filtration. A column of Sephadex G-100 with dimensions of 5 × 90 cm was used in the purification procedure. The column was equilibrated with 10 mM sodium phosphate buffer, pH 6.0, 0.1 M NaCl, at a flow rate of 60 ml/h, and fractions of 10 ml were collected. A Sephadex G-100 column with dimensions of 2.6 × 32 cm was used to demonstrate inhibitor activities in various tissues and to estimate the molecular weights of purified inhibitors. The column was equilibrated with 10 mM phosphate buffer, pH 6.0, 0.1 M NaCl. A sample of 4 ml was applied to the column at a flow rate of 20 ml/h and fractions of 2 ml were collected. The column was calibrated by using Blue Dextran, bovine serum albumin, chymotrypsinogen A, and cytochrome *c* as standards, and the molecular weights of the inhibitors were estimated according to Andrews.[22]

Chromatography on Concanavalin A-Sepharose. A column of Concanavalin A-Sepharose ® 4 B with dimensions of 1.6 × 6 cm was equilibrated with 10 mM phosphate buffer, pH 6.0, 0.1 M NaCl, and 10 ml of a purified inhibitor preparation was applied to the column at a flow rate of 40 ml/h, collecting fractions of 3 ml. Unadsorbed proteins were eluted with 50 ml of the equilibrating buffer, and adsorbed glycoproteins [23] with 200 ml of 50 mM α-methyl mannoside in the buffer.

Isoelectric focusing. Preparative isoelectric focusings using Ampholine ranges 3.5 – 10 and 3 – 5 were performed as described earlier.[16] Analytical isoelectric focusings were performed according to Wrigley.[24] Dialyzed samples containing 10 μg of protein were entrapped in the gels during photopolymerization. After focusing, the gels were stained for proteins by Coomassie Brilliant Blue R-250 [25] and for polysaccharides by the PAS reaction.[26] To determine the inhibitor activities of the focused bands, unstained gels were sliced longitudinally into halves. One was stained as described above, and the other cut transversely into 1.5 mm pieces. The pieces were extracted for 20 h with 200 μl of water, at 4 °C, and the papain inhibiting activities of the extracts were assayed as usual, but a dilute papain solution (0.02 mg/ml) and a long reaction time (60 min) was used.

Preparation of antisera. 0.5 ml of a purified inhibitor solution, containing 0.05 mg of protein, was emulsified with 0.5 ml of complete Freund's adjuvant and injected intradermally to the dorsal skin of a rabbit in ten aliquots. The injection was repeated three times at three weeks intervals using incomplete Freund's adjuvant. Eight days after the last injection blood was collected and serum prepared. The double diffusion method of Ouchterlony and immunoelectrophoresis on agarose plates, buffered with diethyl barbiturate buffer, pH 8.6, were used to test antigen-antibody reactions.

RESULTS

Evaluation of papain – Sepharose affinity chromatography. The rat skin inhibitors of BANA hydrolase also inhibited papain and were adsorbed on papain-Sepharose in a wide pH-range (pH 3 – 8). The adsorbed inhibitors could not be eluted by 1 mM $HgCl_2$, or by increasing or lowering the pH of the eluting buffer (pH 2 – 12), in the presence of 3 M KCl or 6 M urea. The inhibitors were, however, dissociated from papain-Sepharose by 3 M KSCN in 20 mM acetate buffer, pH 4.0. Repeated elutions with 3 M KSCN, at pH 4.0, destroyed the BANA hydrolyzing activity of the papain-Sepharose adsorbent. It was found

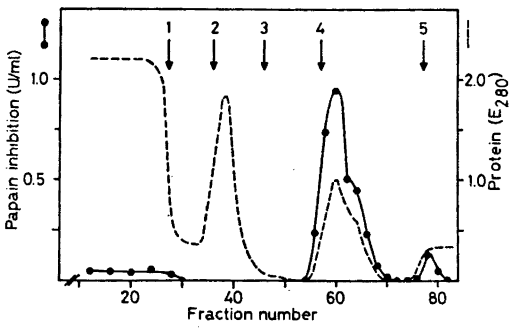

Fig. 1. Papain-Sepharose affinity chromatography. Eluents: 1: 10 mM phosphate buffer, pH 6.0, 0.1 M NaCl. 2: 10 mM phosphate buffer, pH 6.0, 3 M KCl. 3: 10 mM phosphate buffer, pH 6.0, 0.1 M NaCl. 4: 20 mM Na_3PO_4, 0.1 M NaCl. 5: 20 mM Acetate buffer, pH 4.0, 3 M KSCN. Other details are given in "Materials and Methods".

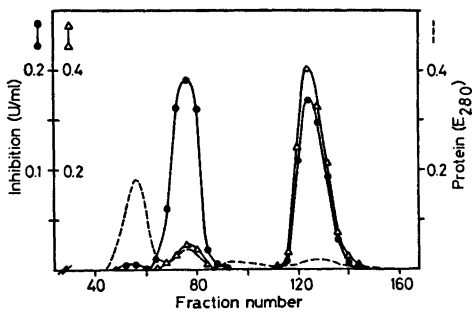

Fig. 2. Sephadex G-100 chromatography of the inhibitors from the papain-Sepharose affinity chromatography. ● papain inhibition. △ BANA hydrolase inhibition. Details are given in "Materials and Methods".

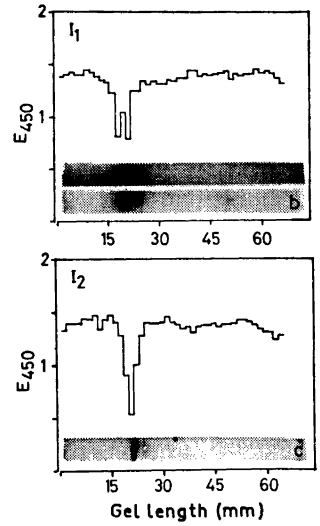

Fig. 3. Analytical isoelectric focusings of I_1 and I_2. The pH range was from 3.5 (left) to 10.0 (right). The curves represent the effects of the focused inhibitors on papain activity (*cf.* "Materials and Methods"). a: PAS staining of I_1. b: Coomassie Brilliant Blue staining of I_1. c: Coomassie Brilliant Blue staining of I_2. PAS staining of I_2 was negative and is not shown.

that the inhibitors were adsorbed to a KSCN-inactivated papain-Sepharose, and that from the inactivated adsorbent they could also be eluted without KSCN by increasing the pH of the elution buffer to 8.9, or higher. The milder elution of the inhibitors from the inactivated adsorbent at alkaline pH resulted in an increased recovery of the inhibitors. Thus the papain-Sepharose gel was inactivated before use and the inhibitors were eluted at alkaline pH, as described in "Materials and Methods".

Purification of inhibitors. The papain-Sepharose affinity chromatography is depicted in Fig. 1. The inhibitors eluted with the alkaline buffer were pooled (fractions Nos. 56–65). The pooled inhibitors had a specific papain inhibiting activity of 1.23 U/mg. The yield was 42 % and the purification factor 11, as compared to the activity of the extract.

Concentrated inhibitors from papain-Sepharose affinity chromatography were separated into two fractions I_1 and I_2 on Sephadex G-100 (Fig. 2). Pooled I_1 (fractions Nos. 71–82) had a specific papain inhibiting activity of 2.6 U/mg and pooled I_2 (fractions Nos. 120–132) 5.8 U/mg. The purification factors, as compared to the activity of the extract, were 24 for I_1 and 53 for I_2. The total inhibitor yield (I_1+I_2) was 27 % of the activity in the extract. A typical purification procedure is summarized in Table 1.

Properties of the inhibitors. The approximate molecular weights of 74 000 and 13 400 were obtained for I_1 and I_2 respectively.

Table 1. Summary of a typical purification procedure of the rat skin papain inhibitors.

Purification step	Volume ml	Protein mg/ml	Activity U/ml	Total activity U	Specific activity U/mg	Yield %	Purification factor
Extract	450	2.0	0.22	99	0.11	100	1
Papain-Sepharose	90	0.38	0.47	42	1.2	42	11
Sephadex G-100							
I_1	116	0.046	0.12	14	2.6	14	24
I_2	130	0.017	0.099	13	5.8	13	53

The carbohydrate content of I_1 was 4 %, while I_2 did not contain carbohydrates. As expected for a glycoprotein, I_1 was adsorbed to a column of concanavalin A-Sepharose and could be eluted with α-methyl mannoside in the buffer. The procedure did not increase specific activity of I_1. I_2 was not adsorbed to concanavalin A – Sepharose.

The isoelectric point of I_1 was 4.6 and that of I_2 4.9, as measured after preparative isoelectric focusing. Analytical isoelectric focusing of I_1 separated 4 – 5 protein bands, which were also stained by the PAS reaction for carbohydrates (Fig. 3). The papain inhibiting activity of I_1 was focused in two peaks, which coincided with the two main protein bands of I_1. I_2 was focused in one major band and two minor bands, which were stained by the protein dye but not by the PAS reaction. The inhibitor activity of I_2 coincided with the main protein band.

At −18 °C the inhibitors were stable at least 6 months, and at +4 °C, in the presence of NaN_3 (0.2 g/l), at least 30 days. I_2 retained all its inhibitor activity after an incubation period of 20 min in a boiling water bath. I_1 lost 2 % of its activity at 65 °C, 32 % at 70 °C, and 78 % at 75 °C, during on incubation period of 20 min. The stability of the inhibitors was similar in 10 mM acetate buffer, pH 4.0, in 10 mM phosphate buffer, pH 6.0, and in 10 mM Tris buffer, pH 8.0, in the presence of 0.1 M NaCl.

Antiserum raised against I_1 was polyvalent. After immunoelectrophoresis anti-I_1 serum precipitated 6 bands from rat serum, although only one band was formed with I_1. The precipitate of I_1 and the major band of serum were located in the α_1-globulin fraction. In immunodiffusion according to Ouchterlony the precipitation band of I_1 fused with a heavy band of serum, which was a sum of several precipitates. Anti-I_1 serum did not form precipitates with I_2.

The antiserum raised against I_2 formed a sharp precipitation band with I_2 and in addition, faint bands with I_1 and rat serum. After immunoelectrophoresis the precipitate of I_2 was located in the α_2-fraction, while the precipitates of I_1 and serum were found in the α_1-fraction. The results suggest that rat serum may contain a protein antigenically and electrophoretically similar to I_1, while I_2 is not present in serum.

Rat skin BANA hydrolase (preparation NII) and cathepsin C did not form precipitates with anti-I_1 and anti-I_2 serums, indicating that during purification of the enzymes they are dissociated and separated from I_1 and I_2. A commercial goat antiserum against rat serum proteins did not precipitate I_1 or I_2.

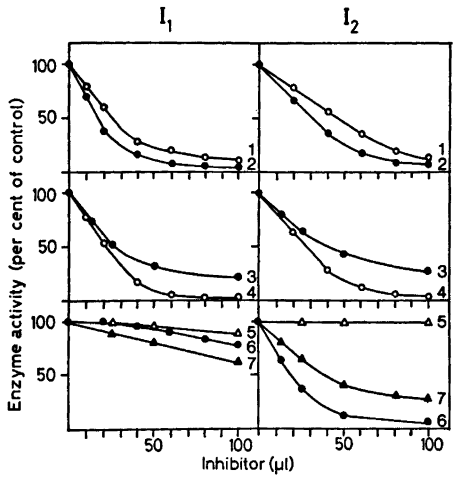

Fig. 4. Inhibition of several proteases by I_1 and I_2. 1: papain (10 µg). 2: mercuripapain (1.8 µg protein). 3: bromelain (10 µg). 4: ficin (10 µg). 5: trypsin (10 µg). 6: BANA hydrolase (1.2 µg). 7: cathepsin C (1.2 µg). 100 µl of I_1 solution contained 9 µg and I_2 solution 1.7 µg of protein.

Table 2. Inhibition of several proteolytic enzymes by I_1 and I_2 as calculated from the data in Fig. 4.

Enzyme	Inhibition (U/mg) I_1	I_2
Papain	2.2	6.4
Mercuripapain	0.64	1.7
Ficin	2.5	10.6
Bromelain	2.2	8.4
BANA hydrolase	< 0.05	1.9
Cathepsin C	< 0.05	0.5
Cathepsin D[a]	0	0
Trypsin	0.1	0
Chymotrypsin[a]	0	0

[a] Not shown in Fig. 4.

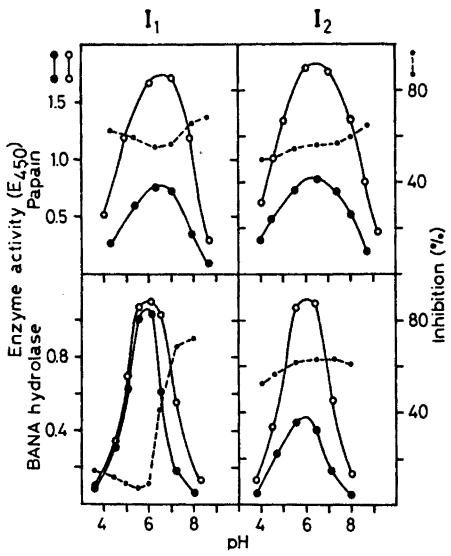

Fig. 5. Effect of pH on inhibition of papain and BANA hydrolase by I_1 and I_2. 9 μg and 27 μg of I_1 was used in inhibition assays of papain and BANA hydrolase, respectively. 1.7 μg of I_2, 10 μg of papain, and 1.2 μg of BANA hydrolase were used. The buffer was Britton-Robinson universal buffer,[17] containing 4 mM dithiothreitol and 8 mM EDTA. ○ No inhibitor, ● with inhibitor.

Inhibition of several proteolytic enzymes by I_1 and I_2 is presented in Fig. 4 and Table 2. I_2 inhibited all SH-proteases tested, but not at all trypsin, chymotrypsin, or cathepsin D. I_1 inhibited strongly the plant SH-proteases papain, ficin, and bromelain, while only a slight inhibition of cathepsin C and BANA hydrolase (at pH 5.8) was noticed. In addition, slight inhibition of trypsin was noticed, while chymotrypsin or cathepsin D were not inhibited. Only mercuripapain and BANA hydrolase (preparation AI) were pure enough to permit the estimation of molar ratios of enzyme-inhibitor complexes. One mg of I_1 inhibited 0.64 mg of mercuripapain and one mg of I_2 1.7 mg of mercuripapain, or 1.9 mg of BANA hydrolase (the amount of mercuripapain was determined by the Lowry-method and is given as milligrams of protein). Using the molecular weights of the inhibitors and enzymes (23 000 for papain and 27 000 for BANA hydrolase) it can be calculated that one mol of I_1 inhibits 2.1 mol of mercuripapain, while one mol of I_2 inhibits

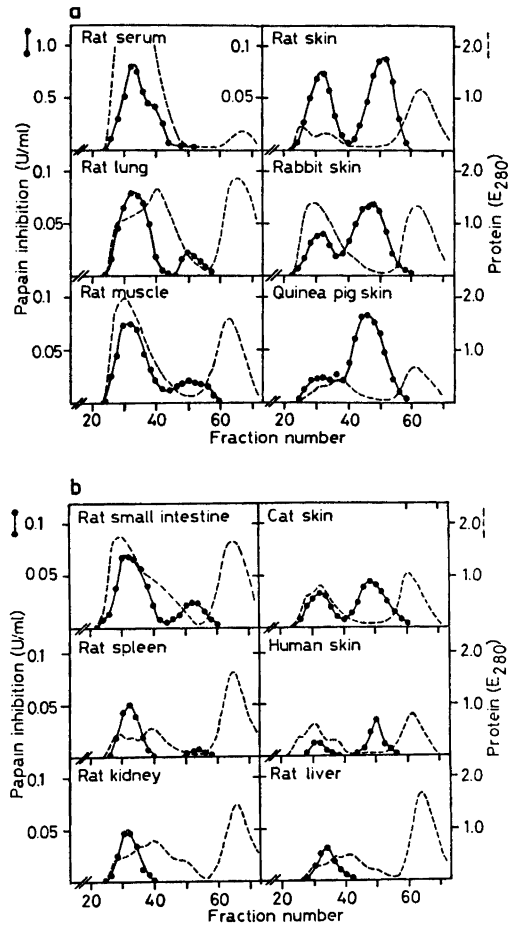

Fig. 6. a. Sephadex G-100 chromatograms of rat serum and acetone precipitated tissue extracts. *b.* Sephadex G-100 chromatograms of acetone precipitated tissue extracts.

1.0 mol of mercuripapain or 0.94 mol of BANA hydrolase. The results suggest the molar ratio of 2:1 for papain-I_1 complex and 1:1 for papain-I_2 and BANA hydrolase-I_2 complexes. The inhibitors did not hydrolyze BANA, Gly-Arg-NA, or hemoglobin.

Inhibition of papain by I_1 and I_2 and inhibition of BANA hydrolase by I_2 was only slightly affected by pH of the assay solution (Fig. 5). In contrast, inhibition of BANA hydrolase by I_1 increased 7-fold, when pH of the assay mixture was changed from 6 to 8.

Presence of inhibitors in tissues. In Fig. 6 a and b fractionation of papain inhibitors of

various rat tissues and of rabbit, guinea pig, cat, and human skin is presented. An inhibitor corresponding to the elution volume of I_1 (fraction No. 32) was present in rat serum and in all tissue extracts studied, and also in human serum (not shown in Fig. 6). An inhibitor corresponding to I_2 was present in all skin extracts studied and in rat lung, muscle, small intestine, and slightly in spleen, but was not found in rat serum, kidney, or liver.

DISCUSSION

The fact that the rat skin inhibitors of cathepsin B_1-like benzoylarginine-2-naphthylamide hydrolase also inhibited papain [18] suggested that papain immobilized on agarose beads could be used as an affinity chromatography material in the purification of the inhibitors. Indeed, the inhibitors were bound to papain-Sepharose and the problem was how to elute them in active form. The inhibitors were successfully eluted with 3 M KSCN, at pH 4.0. SCN^- is known to be a chaotropic ion that causes dissociation of multienzyme complexes,[27] antigen-antibody complexes,[28] and collagenase – α_2-macroglobulin complexes.[29,30]

The inhibitors could not be eluted from papain-Sepharose with Hg^{2+}-ions, 3 M KCl, or 6 M urea, at pH 2 – 12 suggesting that free SH-group of papain, ionic forces, or hydrogen bonds are of minor importance for binding of the inhibitors. Similar results have been obtained by Sen and Whitaker,[8] who failed to purify the egg white papain-ficin inhibitor on a ficin – CM-cellulose adsorbent.

Irreversible inactivation of papain-Sepharose by 5 M KSCN, at pH 4.0 weakened complexes of the inhibitors with the adsorbent, and after inactivation of the gel, the inhibitors were dissociated at alkaline pH, but not by 3 M KCl, at pH 6. The changes in Sepharose-bound papain caused by KSCN are not known. It is possible that papain is modified by thiocarbamylation or by denaturation. Denaturation of Sepharose-bound papain may be restricted by the numerous covalent linkages of papain with Sepharose in such a manner that inhibitors binding capacity of the gel is retained. Binding of protease inhibitors to inactive enzymes is known to occur in several cases. The egg white papain inhibitor forms complexes with inactive papain and cathepsin B1,[7–9] and enzymatically inactive anhydrotrypsin, anhydrochymotrypsin and methyl-chymotrypsin form complexes with protease inhibitors.[31,32]

KSCN-inactivated papain-Sepharose did not adsorb all papain inhibitors from rat skin extract. A possible reason is the presence of competing enzymes in the extract. Inactive proteins are also adsorbed to the papain-Sepharose column. This is understandable, because papain is known as a potent proteolytic enzyme that has an affinity for numerous proteins. Most, but not all of the inactive proteins that are adsorbed to papain-Sepharose, are eluted with 3 M KCl in the equilibrating buffer.

I_1 inhibited effectively plant SH-proteases, while a high excess of I_1 was needed to inhibit rat skin cathepsin C and BANA hydrolase. BANA hydrolase was more effectively inhibited at alkaline than at neutral or acidic pH, which may explain the presence of a high-molecular-weight BANA hydrolyzing compound in gel chromatograms of rat skin extract, when elution was performed at pH 8.0.[18] At pH 7.5 such a high-molecular-weight aggregate was not noticed.[16]

An inhibitor corresponding to I_1 seems to be quite common in rat tissues and in skin of various species. The similar molecular size and immunological reactivity of I_1 and the rat serum papain inhibitor suggest that I_1 in rat tissues might be derived from serum. I_1 is an acid glycoprotein and may be similar to the papain and neutral SH-protease inhibiting substance isolated by Tokaji from sera of cow, rabbit, and guinea pig.[14] Trypsin is also slightly inhibited by I_1, but it is not known, whether this inhibition is caused by I_1 itself, or by a contaminating rat serum trypsin inhibitor.[33] Mouse haptoglobin is associated with a polysaccharide that inhibits papain, cathepsin B1, and also trypsin.[5] It is not known, if I_1 is related to the haptoglobin-polysaccharide complex.

I_2 inhibited all SH-proteases tested, of both plant and animal origin. An inhibitor corresponding to I_2 was present in several rat tissues and abundantly in skin of various species. Its absence from rat serum suggests its intracellular nature.

I_2 seems to be similar to the inhibitor purified from rabbit skin with healing Arthus inflamma-

tion and from rabbit skin burns.[14] I_2 and the Arthus inhibitor[11,12] have much in common with the chicken egg white papain inhibitor:[7–10] they all inhibit thiol proteases, but not serine proteases, they have a molecular weight of about 13 000, contain no carbohydrates, and are resistant to high temperatures. In addition, both I_2 and the egg white inhibitor form equimolar complexes with papain, and also form complexes with enzymatically inactive papain.[7–8] It is not known if I_2 is, like the egg white inhibitor, a multihead inhibitor with different active sites for cathepsin B1 (or papain) and cathepsin C.[10]

The specific activity of a rat skin BANA hydrolase preparation NII, which had been purified 250-fold, increases markedly when the enzyme is highly diluted, or preincubated at pH 4, at 55 °C, for 20 min, before enzyme assay.[16,17] The presence of a dissociable inhibitor in the enzyme preparation has been suggested.[17] The antisera raised against purified rat skin inhibitors I_1 and I_2 did not form immunoprecipitates with BANA hydrolase preparation NII, suggesting that the effect of dilution on BANA hydrolase activity is not caused by I_1 or I_2.

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2-Halogen Substituted Selenides and their Reactions with Nucleophilic Reagents

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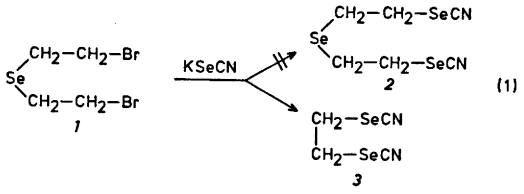
The reactions between bis(2-bromoethyl) selenide (*1*) and a variety of nucleophiles have been studied. Normal substitution products as well as different types of abnormal products have been obtained. A mechanism for these reactions has been proposed which accounts for the formation of all the various products. The reactions of bis(2-haloethyl) selenide dihalides and bis(2-haloethyl) selenoxides with potassium selenocyanate have also been studied. In all cases 1-halo-2-selenocyanatoethanes were isolated as products.

The selenium containing compounds corresponding to mustard gas and its bromine analogue (*1*) were first synthesized by Gibson *et al.*[1,2] These substances were later studied by Smedslund in efforts to elucidate their chemical properties.[3] This investigation was initiated by an unsuccessful attempt to prepare bis(2-selenocyanatoethyl) selenide (*2*). Instead of *2*, diselenocyanatoethane (*3*) was isolated from the reaction mixture (eqn. 1).



1 2 3 (1)

This behaviour justified a closer study of the action of various nucleophiles on 2-halogen substituted selenides.[4]

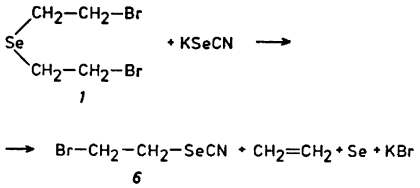
RESULTS

When reacting bis(2-bromoethyl) selenide (*1*) with potassium selenocyanate, ethylene and selenium were produced together with diselenocyanatoethane (*3*). The reaction was easily followed with ^{1}H NMR spectroscopy using acetone-d_6 as solvent. In the spectrum of *1*, which was of the A_2B_2 type (δ 3.4), peaks at δ 5.35 and 3.6 corresponding to ethylene and *3* were obtained on addition of potassium selenocyanate. The formation of ethylene was also qualitatively demonstrated by passing the gaseous products through a solution of selenium tetrabromide in benzene. Bis(2-bromoethyl) selenide dibromide (*4*) was isolated from the mixture.

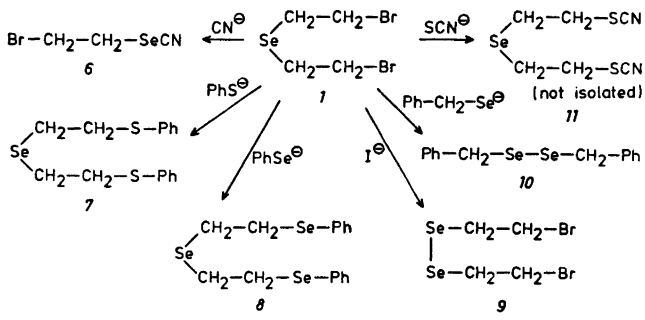
A number of solvents, such as *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetone, ethyl acetate, and ethanol were used as media for the substitution reaction between *1* and potassium selenocyanate. No change of reaction path was, however, observed when using the different solvents. When the reaction was performed in ethanol a competing solvolysis giving bis(2-ethoxyethyl) selenide was noted, in agreement with Smedslund's observations.[5]

When bis(2-chloroethyl) selenide (*5*) was treated with potassium selenocyanate the same reaction as with *1* was observed.

The molar ratio of starting materials in the reaction between potassium selenocyanate and *1* was 2:1. However, when equimolar amounts of the starting materials were used it was possible to isolate 1-bromo-2-selenocyanatoethane (*6*) (eqn. 2). Formation of ethylene was also observed.



1 6 (2)



Scheme 1.

A number of different nucleophilic reagents were allowed to react with *1* (Scheme 1). When using benzenethiolate as well as benzeneselenolate ion as nucleophiles the normal substitution products, *7* and *8* respectively, were obtained. The reaction between *1* and thiocyanate ion gave no isolable product. On following the reaction by ^{1}H NMR spectroscopy an A_2B_2 spectrum was obtained consisting of two groups of peaks centred around a δ value of 3.3. The product formed is most probably the normal substitution product (*11*) since only a minute amount of ethylene was produced during the reaction.

When *1* was allowed to react with potassium cyanide, sodium iodide or sodium phenylmethaneselenolate, the expected substitution products could not be isolated. Cyanide ion reacted with *1* giving *6* as the product when DMF was used as solvent. No reaction was obtained in acetone solution. The reaction between iodide ion and *1* gave bis(2-bromoethyl) diselenide (*9*), ethylene and iodine. Dibenzyl diselenide (*10*) and ethylene were formed in the reaction between phenylmethaneselenolate ion and *1*.

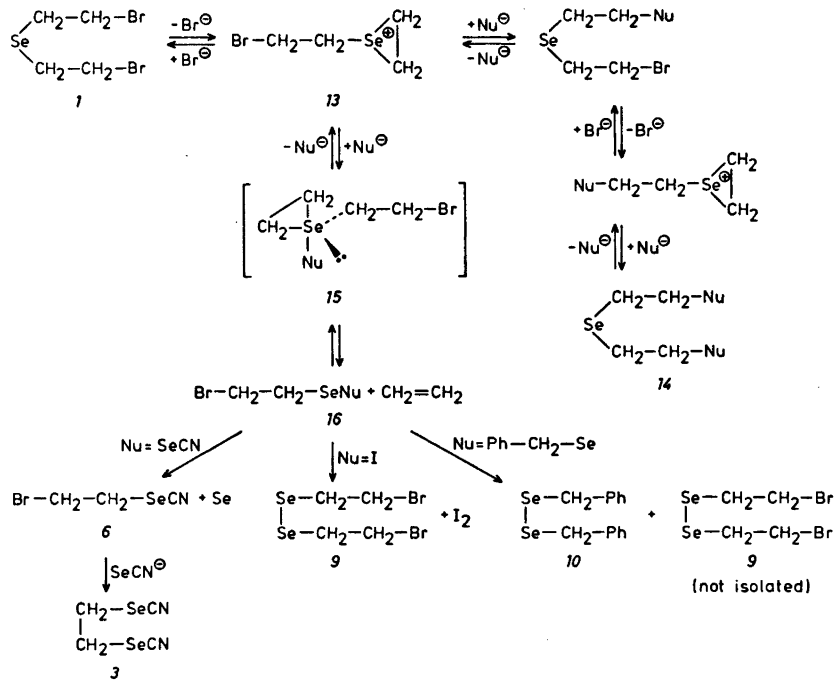
To substantiate the above results the reactions between 2-bromoethyl phenyl selenide (*17*) [6] and iodide and phenylmethaneselenolate ion were studied. When iodide ion was used as the nucleophile diphenyl diselenide (*18*), ethylene and iodine were produced. The reaction between *17* and phenylmethaneselenolate ion gave a mixture of products, which was shown by mass and ^{1}H NMR spectroscopy to consist of four compounds, *18*, benzyl phenyl diselenide (*20*), *10*, and 1,5-diphenyl-1,4-diselenapentane (*19*).

For comparison, thiocyanate [7] and selenocyanate ion were allowed to react with bis(2-bromoethyl) sulfide (*12*); normal substitution products were obtained in both cases.

DISCUSSION

The question arose as to whether it is possible to explain the reaction between bis(2-bromoethyl) selenide (*1*) and different nucleophiles by a common mechanism. The action of various nucleophiles on mustard gas, which is the sulfur analogue to *5*, has been kinetically studied and the formation of an episulfonium ion intermediate in the rate determining step has been proposed.[8,9] A number of relatively stable episulfonium salts have been generated in non-nucleophilic solvents and these when subjected to nucleophilic reactions give varying amounts of Markownikov and *anti*-Markownikov products.[10] It has also been possible to isolate some stable episulfonium salts.[11,12] One of these has been allowed to react with nucleophiles and a competitive attack on sulfur and on carbon has been obtained.[11] Thus in studies on reactions of 2-halogen substituted sulfides it has been possible to confirm the intermediacy of episulfonium ions.

Cyclic selenonium ions as intermediates have until recently been proposed only a few times.[13–15] However, a number of episelenonium salts have now been prepared.[16] In one of the methods of preparation, 2-halogen substituted selenides were used as the starting material. The products obtained have been subjected to nucleophilic substitution by chloride, bromide, and acetate ion. In all cases nucleophilic attack was at the carbon atom and competitive attack at selenium was not observed.



Scheme 2.

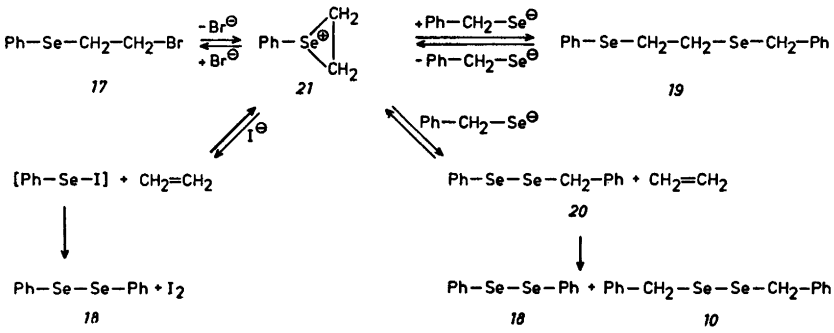
The formation of an episelenonium ion (*13*) as a common intermediate in the reactions between *1* and various nucleophiles is thus very probable [4] (Scheme 2). The cyclic selenonium ion formed can be attacked at carbon or selenium. Attack on carbon gives rise to the normal substitution product (*14*). When the positive selenium atom of the selenonium ion is attacked by a nucleophile, the intermediate formation of an episelenurane (*15*), in analogy with episulfonium compounds,[17] can be proposed. The episelenurane decomposes to give ethylene and a selenenyl compound (*16*). A stable episelenurane has recently been isolated in the reaction between an arylselenenyl halide and ethylene.[18] The identity of this compound has, however, been questioned.[19]

The selenenyl compound *16* can undergo further reaction depending on the nature of the nucleophile. When the nucleophile is the selenocyanate ion, *16* corresponds to 1-bromo-2-(selenenylselenocyanato)ethane. Stable aliphatic selenenyl selenocyanates have not been isolated. The aromatic representatives of this type of compound decompose at higher temperature to give the corresponding selenocyanate and selenium.[20] In the reaction between *1* and potassium selenocyanate, selenium is formed and 1-bromo-2-selenocyanatoethane (*6*) is trapped as an intermediate. Intermediate *6* then reacts with another molecule of potassium selenocyanate to give *3*.

The nucleophilic attack of cyanide ion on *13* gives rise to compound *16* which corresponds to *6*. (Scheme 2).

The isolation of bis(2-bromoethyl) diselenide (*9*), iodine and ethylene when sodium iodide and *1* are allowed to react is explained by the formation of an intermediate *16* corresponding to a selenenyl iodide, which decomposes to give *9* and iodine (Scheme 2).

In the reaction between *1* and phenylmethaneselenolate ion, the formation of ethylene and dibenzyl diselenide (*10*) is explained in accordance with the mechanism in Scheme 2. The intermediate selenenyl compound *16* corresponds to an unsymmetrical diselenide, benzyl 2-bromoethyl diselenide. The isolation of *10* from the reaction mixture can be explained by a disproportionation of the unsymmetrical



Scheme 3.

diselenide to give *10* and *9*. Bis(2-bromoethyl) diselenide (*9*) has, however, not been isolated from the reaction mixture. The analogous reactions between *17* and iodide and phenylmethaneselenolate ion were mechanistically explained in analogy with the results obtained above for *1* (Scheme 3). An intermediate episelenonium ion *21* is formed from *17* and then attacked by iodide ion to give a selenenyl iodide, which decomposes to give *18* and iodine. When phenylmethaneselenolate ion is used as nucleophile *21* is attacked on carbon as well as on selenium giving the normal substitution product *19* and the unsymmetrical diselenide *20*, respectively. The unsymmetrical diselenide then partially disproportionates to give *10* and *18* (Scheme 3).

The formation of a cyclic selenonium ion as the first step in the reaction between 2-halogen substituted selenides and different nucleophiles has been discussed above. The question arises as to whether it is possible, by blocking the selenium atom by certain substituents, to prevent the formation of an episelenonium ion and thereby obtain exclusive formation of normal substitution product. Selenide dihalides and selenoxides obtained from the corresponding bis(2-haloethyl) selenides were therefore allowed to react with potassium selenocyanate. The reaction between *4* and potassium selenocyanate gave *6* as product. Potassium selenocyanate and bis(2-chloroethyl) selenide dichloride gave 1-chloro-2-selenocyanatoethane (*22*) as product. When bis-(2-bromoethyl) selenoxide and bis(2-chloroethyl) selenoxide, respectively, were allowed to react with potassium selenocyanate, *6* and *22* were isolated. Normal substitution products were not observed.

EXPERIMENTAL

The selenium analyses were performed by a microanalytical method developed by Bengtsson [21] and very similar to that of Gould.[22] The melting points are uncorrected.

The reaction between bis(2-bromoethyl) selenide (1) and potassium selenocyanate (molar ratio 1:2). To a solution of 8.85 g (0.03 mol) of *1* [3] in 150 ml of acetone were added 9.50 g (0.066 mol) of potassium selenocyanate [23] dissolved in 75 ml of acetone. The mixture was refluxed for 2 h, filtered, the residue washed with acetone and the filtrate evaporated. The product was recrystallized from benzene. Yield: 5.3 g (74 %) of *3* [24] m.p. 136–137.5 °C. Elemental selenium [2.0 g (84 %)] was regained. During the reaction ethylene is evolved. This can qualitatively be shown by letting all volatile fumes formed pass through a trap containing selenium tetrabromide in benzene. Theoretically 0.03 mol of ethylene is evolved. Thus, by adding 1.65 ml [2.93 g/cm³; 4.8 g (0.03 mol)] of bromine to a suspension of 1.2 g (0.015 mol) of selenium in 25 ml of benzene, a solution containing 0.015 mol of selenium tetrabromide was obtained. When the reaction is complete 1.1 g of the trapping product *4* is obtained, m.p. 124–125 °C. (Analysis for *3*: Found: C 20.23; H 1.76; Se 66.35. Calc. for $C_4H_4N_2Se_2$: C 20.18; H 1.70; Se 66.35).

The reaction between 1 *and potassium selenocyanate (molar ratio 1:1).* To a solution of 5.9 g (0.02 mol) of *1* in 100 ml of acetone were added 3.2 g (0.022 mol) of potassium selenocyanate dissolved in 50 ml of acetone. The reaction mixture was allowed to stand under stirring at room temperature for 2 h, the solvent was evaporated and a yellow oil obtained. Elemental selenium [1.3 g (82 %)] was regained. The oil was distilled giving 0.6 g (14 %) of product, b.p. 77–78 °C/0.02 mmHg. IR and ^{1}H NMR spectra of the compound showed it to be *6*, though slightly impure.

The reaction between 1 *and potassium cyanide.* To a solution of 5.9 g (0.02 mol) of *1* in 100 ml of DMF a suspension of 2.9 g (0.044 mol) of potassium cyanide in 50 ml of DMF were added. The reaction mixture was allowed to stand at room temperature for 5 h, then filtered, poured into water and the mixture extracted with ether. From the mother liquor 0.1 g of diselenocyanatoethane was obtained. Evaporation of the dried ether gave an oil which was distilled, b.p. 80–88 °C/0.1 mmHg. Yield: 1.3 g (31 %). Redistillation gave 0.35 g of product, b.p. 79–80 °C/0.05 mmHg. The product was shown by IR and ^{1}H NMR spectroscopy to consist of *6*.

The reaction between 1 *and sodium benzenethiolate.* To a sodium methoxide solution, prepared by reacting 1.01 g (0.044 mol) of sodium with 20 ml of absolute methanol, were added 4.85 g (0.044 mol) of freshly distilled benzenethiol. The solution was evaporated to dryness under stirring at reduced pressure. The residue was dissolved in 25 ml of acetone and a filtered solution of 5.9 g (0.02 mol) of *1* in 50 ml of acetone was added. The mixture was allowed to stand for 5 h with stirring at room temperature and in a nitrogen atmosphere. The reaction mixture was filtered and the filtrate evaporated. The product was recrystallized from ethanol. Yield: 4.3 g (61 %). To obtain the analytically pure compound another recrystallization from cyclohexane and treatment with active carbon was necessary, m.p. 66.5–67 °C. (Found: C 54.52; H 5.06; Se 22.38. Calc. for $C_{16}H_{18}S_2Se$: C 54.37; H 5.14; Se 22.34).

The reaction between 1 *and sodium benzeneselenolate.* To a sodium methoxide solution prepared by reacting 1.01 g (0.044 mol) of sodium with 20 ml of absolute methanol, was added 6.91 g (0.044 mol) of newly distilled benzeneselenol. The solution was evaporated to dryness under stirring at reduced pressure. The residue was dissolved in 25 ml of acetone and a filtered solution of 5.9 g (0.02 mol) of *1* in 50 ml of acetone was added. The mixture was allowed to stand for 2 h at room temperature in a nitrogen atmosphere. The reaction mixture was filtered and the filtrate evaporated. The product was recrystallized from ethanol. Yield: 5.1 g (57 %). The analytical specimen was obtained by repeated recrystallization from ethanol and cyclohexane (active carbon), m.p. 78–79 °C. (Found: C 42.92; H 4.06; Se 52.97. Calc. for $C_{16}H_{18}Se_3$: C 42.97; H 4.07; Se 52.97).

The reaction between 1 *and sodium iodide.* To a solution of 2.9 g (0.01 mol) of *1* in 50 ml of acetone was added 1.65 g (0.011 mol) of sodium iodide dissolved in 25 ml of acetone. Immediate formation of iodine was observed. The reaction was performed at room temperature and was considered complete after 15 min. Ethylene was shown to be evolved by isolating *4*, m.p. 122–124 °C as mentioned before. The reaction mixture was filtered and the filtrate was evaporated to give a solid residue which was dissolved in ether. The ether solution was washed with aqueous sodium pyrosulfite and dried with calcium chloride. The residue after evaporation was recrystallized from a mixture of light petroleum and ethyl ether (19/1) at −60 °C. From the reaction mixture 0.1 g of elemental selenium was recovered. The product was unstable and decomposed at room temperature. ^{1}H NMR and mass spectra of the compound were consistent with the spectra of *9*.

Unsuccessful efforts were made to trap the intermediate selenenyl iodide as 2-bromoethyl 2′-iodocyclohexyl selenide by adding an excess of cyclohexene to the reaction mixture.

Bis(2-bromoethyl) diselenide (9). A solution of 4.0 g (0.016 mol) of bis(2-hydroxyethyl) diselenide [6] in dry chloroform was cooled in ice while dry hydrogen bromide gas was passed through the solution. After a few minutes a heavy oil [8] separated and after some further time formation of red selenium started. The introduction of gas was stopped, the reaction mixture filtered through a bed of calcium chloride and the solvent evaporated. The residue was extracted with ether, the ether extract washed with an aqueous sodium hydrogen carbonate solution and dried. After evaporation, the product was recrystallized from a mixture of light petroleum and ethyl ether (19/1) at −60 °C. The yellow product, which melted at about 25–30 °C, was unstable and decomposed rapidly even below room temperature. When recording the ^{1}H NMR spectrum in deuteriochloroform and acetone-d_6, the product decomposed with formation of free selenium. The ^{1}H NMR spectrum changed gradually to that of *1*. Presumably *9*, when dissolved in the above solvents, decomposes to give elemental selenium and *1*.

The reaction between 2-bromoethyl phenyl selenide (17) and sodium iodide. To a solution of 2.65 g (0.01 mol) of *17* in 50 ml of acetone was added 1.65 g (0.011 mol) of sodium iodide dissolved in 25 ml of acetone. On addition the reaction mixture became brownish owing to the formation of free iodine. The reaction mixture was allowed to stand at room temperature for 3 h. A small amount of *4* was isolated as described earlier. After filtering the reaction mixture, the solvent was evaporated and the residue dissolved in ether. The ether solution was washed with an aqueous sodium pyrosulfite solution and dried. After evaporation, a solid residue was obtained which was recrystallized from ethanol to give 0.5 g (32 %), m.p. 60.5–61.5 °C, of *18*.

The reaction between bis(2-bromoethyl) sulfide (12) and potassium selenocyanate. To a solution of 15 g (0.06 mol) of *12* in 25 ml of acetone 19.0 g (0.132 mol) of potassium selenocyanate dissolved in 50 ml of acetone was added. The mixture was refluxed for 5 h. After the stipulated time the reaction mixture was filtered and the filtrate evaporated, giving an oily residue. By shaking the oil with ethyl ether

a crystalline product was obtained. Recrystallization from ethanol gave 10.3 g (58 %) of product. Repeated recrystallization from ethyl acetate-light petroleum gave the pure bis(2-selenocyanatoethyl) sulfide m.p. 38–39 °C. (Found: C 24.18; H 2.70; S 11.30; Se 53.15. Calc. for $C_6H_8N_2SSe_2$: C 24.17; H 2.71; S 10.75; Se 52.97).

The reaction between 1 *and sodium phenylmethaneselenolate.* A sodium methoxide solution was prepared by reacting 1.01 g (0.044 mol) of sodium with 20 ml of absolute methanol. To the solution obtained, 7.55 g (0.044 mol) of phenylmethaneselenol was added. The red-brown solution was evaporated under stirring at reduced pressure. The residue was dissolved in 25 ml of DMF and added to a filtered solution of 5.8 g (0.02 mol) of *1* in 50 ml of DMF. The reaction was performed in a nitrogen atmosphere at room temperature with stirring for 3 h. Evolution of ethylene was detected as described earlier by letting the gaseous products pass through a solution of selenium tetrabromide; 2.5 g of *4* was isolated. When the reaction was complete, the reaction mixture was poured into water and the solution extracted with ether. The dried ether solution was evaporated and the residue recrystallized from a mixture of ethanol and water giving 5.0 g (74 %) of *10*. The structure of the product was verified using ^{1}H NMR and IR spectroscopy. The reaction could also be performed in acetone.

The reaction between 17 *and sodium phenylmethaneselenolate.* To a sodium methoxide solution prepared by reacting 0.3 g (0.013 mol) of sodium with 10 ml of absolute methanol, was added 2.0 g (0.012 mol) of phenylmethaneselenol. The red-brown solution was evaporated under stirring at reduced pressure. The residue was dissolved in 25 ml of acetone and the solution obtained added to a filtered solution of 2.65 g (0.01 mol) of *17* in 50 ml of acetone. The reaction mixture was allowed to stand at room temperature with stirring in a nitrogen atmosphere for 3 h. After filtration of the reaction mixture, the filtrate was evaporated giving an oil as residue, which was investigated by ^{1}H NMR and mass spectroscopy. The ^{1}H NMR spectrum consisted of three singlets at δ 3.73, 3.65 and 3.03 and an A_2B_2 spectrum centred around a δ value of 2.79. The mass spectrum suggested that the oily product was a mixture of four different compounds. A molecular peak at *m/e* 356, corresponded to the normal substitution product *19*. Peaks were also obtained at *m/e* 342, 328 and 314, which must be due to the molecular peaks of the diselenides *10*, *20* and *18*, respectively. The assumption, that the product of the reaction between *17* and phenylmethaneselenolate ion consists of four separate substances, is based on the following reasoning. It is very improbable that the three peaks at *m/e* 342, 328 and 314 are formed by fragmentation of *19*; as has been described earlier *10* does not fragment to give peaks at *m/e* 328 and 314.[25] It is also unlikely that the peak at *m/e* 314 is caused by fragmentation of *20*, with its molecular peak at *m/e* 328. The most probable fragmentation of an unsymmetrical diselenide should be breakage of the diselenide bond. Assignment of the resonances in the ^{1}H NMR spectrum to various compounds was made as follows. When integrating the spectrum it was possible to relate the singlet at δ 3.65 to the A_2B_2 spectrum at δ 2.79, as the integrals were in the proportion 1:2. These resonances are consequently due to *19*. To confirm that the A_2B_2 spectrum was not due to starting material, a little *17* was added to the NMR tube and another spectrum was run. A new A_2B_2 spectrum was obtained centred around a δ value of 3.29 corresponding to *17*. On addition of *10* to the NMR tube and running a spectrum, a distinct increase of the singlet at δ 3.73 was obtained. All resonances are now identified except that at δ 3.03, which is tentatively assigned to the methylene group of the unsymmetrical diselenide *20*. Efforts were made to separate the four components by preparative TLC using a mixture of methylene chloride and pentane (3/7) as migrating phase. Two slightly impure fractions were obtained, one containing the three diselenides and the other the normal substitution product.

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The Crystal Structure of Methyl α-D-Galactofuranoside

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The crystal structure of methyl α-D-galactofuranoside has been determined from three-dimensional single-crystal X-ray diffraction data. The compound crystallizes in the monoclinic space group $P2_1$ with two molecules in the unit cell of dimensions $a = 6.217(2)$ Å, $b = 14.806(5)$ Å, $c = 4.811(2)$ Å, and $\beta = 105.62(1)°$. The structure was solved by direct methods and refined by full-matrix least-squares technique to an R_w value of 0.037 ($R = 0.048$) for 1394 observed reflections.

The furanose ring has the twist conformation ${}^2_1T$ with the anomeric carbon atom 0.28 Å below and its neighbouring carbon atom 0.33 Å above the plane of the remaining ring atoms. The exocyclic anomeric C—O bond is short [1.395(3) Å]. All oxygen atoms except for the glycosidic one are engaged in intermolecular hydrogen bonds forming a three-dimensional network.

The furanose ring occurs in a variety of important biological molecules, particularly in the nucleic acids. During the last years crystal structures of several complex carbohydrate compounds in which the carbohydrate component is in the furanose form have been determined. Due to their biological interest attention has been concentrated on D-ribofuranose and 2-deoxy-D-ribofuranose derivatives. However, little structure information has appeared in the literature of compounds where the furanose residue constitutes the major part of the molecule.

In several simple carbohydrates containing a pyranose ring the exocyclic anomeric C—O bond is short except in the case of an axial glycosidic group where there is evidence of a distinction between the two ring C—O bonds.[1] The C—O—C—N system of glycofuranosides shows unequal C—O bond lengths, the cyclic anomeric C—O bond being shorter than the other ring C—O bond.[1] Few data, on the other hand, are available for the C—O—C—O system of glycofuranosides.[2-4]

The structure analysis of methyl α-D-galactofuranoside (Me α-D-Gal*f*) has been undertaken in order to supplement our knowledge of the conformation of furanose carbohydrates and to provide data for study of carbon to oxygen distances in such compounds.

EXPERIMENTAL

Me α-D-Gal*f*, m.p. 89 °C, $[\alpha]_D^{20}$ +104 (*c* 0.25, water) was prepared as described by Augestad and Berner.[5] Crystals suitable for X-ray studies were obtained by slow crystallization from ethyl acetate.

Oscillation, Weissenberg, and precession photographs showed the space group to be $P2_1$. Setting angles for 15 reflections (Cu$K\beta$, $\lambda = 1.3922$ Å) determined on a Picker manual four-circle diffractometer were used for calculation of the unit cell dimensions. Intensity data were collected on a Picker automatic four-circle diffractometer using Mo$K\alpha$ radiation (graphite monochromator). A crystal of dimensions $0.15 \times 0.15 \times 0.30$ mm was mounted with the plane $(11\bar{2})$ normal to the ϕ axis of the goniostat. Intensities were measured using the $\omega - 2\theta$ scanning technique with a 2θ scan speed of 1° min^{-1} through the scan range from 0.7° below $2\theta(\alpha_1)$ to 0.7° 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, measured at regular intervals throughout the data collection showed no significant fluctuations and were accordingly not used for scaling. Estimated standard deviations $\sigma(I)$ of the intensities were taken as $[\sigma_s^2 + (0.02\ C_N)^2]^{\frac{1}{2}}$ (σ_s is the standard deviation due to counting statistics and C_N is the net count). Of 2171 unique reflections measured with $2\theta < 75°$ 1374 (529 with $2\theta > 55°$) had intensities greater than $2.5\sigma(I)$ and were regarded as observed. The intensities were corrected for Lorentz and polarization effects.

CRYSTAL DATA

Methyl α-D-galactofuranoside, $C_7H_{14}O_6$, M = 194.19 amu. Space group $P2_1$ (No. 4).
$a = 6.217(2)$ Å, $b = 14.806(5)$ Å, $c = 4.811(2)$ Å, $\beta = 105.62(1)°$, $Z = 2$, $D_m = 1.51(1)$ g cm^{-3}, $D_x = 1.51$ g cm^{-3}.

STRUCTURE DETERMINATION

The phase problem was solved by direct methods.[6] The hydrogen atoms were located in difference Fourier maps. Least-squares refinement including all observed reflections and with anisotropic thermal parameters for non-hydrogen atoms gave a final R_w of 0.037 (R = 0.048). The intensity data showed no secondary extinction effects. A final difference Fourier synthesis showed electron density fluctuations not exceeding 0.3 e Å^{-3}.

The atomic scattering factors used were those of Doyle and Turner [7] for carbon and oxygen and of Stewart *et al.*[8] for hydrogen. Except for ORTEP [9] and those for phase determination all programs applied are described in Ref. 10.

RESULTS

Lists of observed and calculated structure factors are available from the authors on request. The final fractional coordinates and thermal parameters with their estimated standard deviations for the non-hydrogen atoms are given in Table 1 and for the hydrogen atoms in Table 2. The eigenvalues of the vibration tensors for the non-hydrogen atoms are also included in Table 1. A stereoscopic illustration of the molecule is shown in Fig. 1 where also the numbering of the atoms is indicated.

Rigid-body analysis of translational, librational, and screw motion [11] gave an r.m.s. difference between observed and calculated U_{ij}'s of 0.0041 Å^2 when all the non-hydrogen atoms were included. The whole molecule can therefore not be regarded as a rigid body. By excluding the terminal atoms except for substituents of the ring an r.m.s. value of 0.0025 Å^2 is obtained, thus indicating that this part of the molecule may be regarded as an oscillating rigid body. The latter description was adopted when correcting the coordinates for libration.

Table 3a contains the bond lengths. For

Table 1. Fractional coordinates ($\times 10^4$), anisotropic thermal parameters U_{ij} ($\times 10^4$), and r.m.s. amplitudes of vibration ($\times 10^3$) for the non-hydrogen atoms. The parameters U_{ij} are terms in the temperature factor expression $\exp[-2\pi^2(U_{11}h^2a^{*2}+\cdots+2U_{23}klb^*c^*)]$. The e.s.d.'s given in parentheses refer to the last figure of respective values.

Atom	x	y	z	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}	$w1$	$w2$	$w3$
O1	4124(3)	7504(3)	5876(4)	290(8)	323(9)	392(11)	84(7)	136(8)	68(8)	21	18	15
O2	8383(3)	7189(3)	8655(5)	216(8)	285(9)	787(16)	8(8)	147(9)	99(10)	29	16	14
O3	7530(3)	5090(3)	9778(5)	237(8)	348(10)	413(12)	97(8)	68(8)	68(9)	21	19	13
O4	2936(3)	6528(3)	8990(4)	241(7)	253(8)	376(10)	3(6)	154(7)	−53(7)	20	16	13
O5	−6(3)	5279(3)	5675(5)	179(7)	475(12)	340(11)	15(8)	74(8)	−38(10)	22	18	13
O6	1692(3)	4070(3)	2171(4)	417(11)	235(9)	363(11)	−18(8)	73(8)	−46(8)	21	19	15
C1	4491(4)	7205(3)	8716(6)	243(10)	271(11)	331(14)	−3(9)	98(9)	−21(11)	18	16	15
C2	6724(4)	6719(3)	9598(6)	232(10)	281(12)	343(15)	−23(9)	59(10)	14(11)	19	17	15
C3	6141(3)	5792(3)	8263(6)	172(9)	276(11)	271(12)	32(8)	72(9)	41(10)	18	15	13
C4	3717(3)	5649(3)	8328(5)	220(10)	225(11)	246(12)	8(8)	99(9)	4(9)	16	15	13
C5	2253(3)	5287(3)	5507(5)	172(9)	227(10)	261(12)	20(8)	75(8)	10(9)	16	15	13
C6	2973(4)	4341(3)	4934(6)	275(12)	265(12)	367(16)	41(9)	50(11)	−30(12)	20	17	15
C7	2089(5)	8000( )	4889(8)	299(14)	454(19)	536(22)	98(13)	122(14)	122(17)	25	20	16

Table 2. Fractional coordinates ($\times 10^3$) and isotropic thermal parameters U ($\times 10^3$) for the hydrogen atoms. The temperature factor expression is exp $(-8\pi^2 U \sin^2\theta/\lambda^2)$. The e.s.d.'s given in parentheses refer to the last figure of respective values.

Atom	x	y	z	U
HO2	953(6)	690(2)	880(7)	48(10)
HO3	838(6)	502(3)	905(8)	45(11)
HO5	−62(6)	517(3)	417(8)	43(10)
HO6	178(5)	356(2)	206(7)	40(10)
H1	423(4)	770(2)	996(5)	18(6)
H2	712(4)	670(2)	1168(6)	28(7)
H3	618(4)	581(2)	634(5)	16(6)
H4	364(4)	522(2)	986(6)	24(6)
H5	240(4)	567(2)	401(5)	13(6)
H6A	457(5)	437(2)	510(6)	33(7)
H6B	275(5)	390(2)	635(6)	35(8)
H7A	200(6)	847(2)	620(8)	54(11)
H7B	203(5)	826(3)	303(8)	53(10)
H7C	86(6)	766(3)	491(8)	63(11)

bonds not involving hydrogen atoms corrected bond lengths are also listed. Bond angles and torsion angles involving non-hydrogen atoms are listed in Table 3b and Table 3c, respectively. The hydrogen bonding scheme is shown in Fig. 2 and the corresponding hydrogen bonding data are given in Table 3d. The estimated standard deviations were calculated from the least-squares correlation matrix.

DISCUSSION

The furanose ring in Me α-D-Gal*f* is non-planar. The displacements of C1 and C2 from the plane of C3, C4, and O4 are −0.285 and 0.327 Å, respectively, showing that the ring adopts a twist conformation. Alternatively, the conformation of the furanose ring is conveniently described by the torsion angles τ_j ($j=0,4$) about the five ring bonds.[12,13] Starting

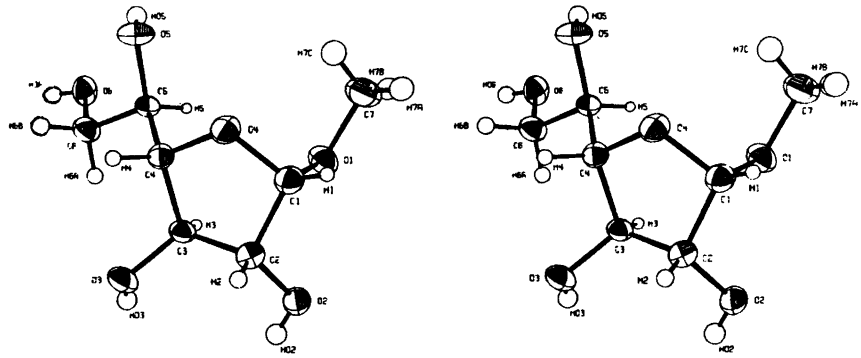

Fig. 1. Stereoscopic drawing of the methyl α-D-galactofuranoside molecule with atom numbering. The hydrogen atoms are assigned isotropic thermal parameters corresponding to one third of the values listed in Table 2. The thermal ellipsoids are scaled to 50 % probability.

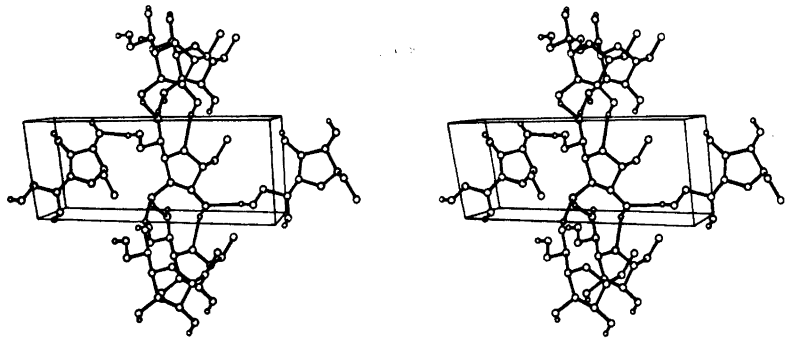

Fig. 2. Stereoscopic drawing showing the hydrogen bonding scheme. Hydrogen atoms not involved in hydrogen bonds are omitted for clarity.

Table 3. (a) Bond lengths. For bonds between non-hydrogen atoms bond lengths corrected for libration are also listed. (b) Bond angles involving non-hydrogen atoms. (c) Torsion angles involving non-hydrogen atoms. (d) Hydrogen bond parameters. The e.s.d.'s given in parentheses refer to the last figure of respective values.

(a) Bond lengths (Å)

O1–C1	1.395(3)	1.401	O6–C6	1.412(3)	1.414	O2–HO2	0.81(3)	C4–H4	0.99(3)
O1–C7	1.429(3)	1.431	C1–C2	1.520(3)	1.524	O3–HO3	0.72(3)	C5–H5	0.94(2)
O2–C2	1.416(3)	1.420	C2–C3	1.517(4)	1.520	O5–HO5	0.74(4)	C6–H6A	0.98(3)
O3–C3	1.420(3)	1.423	C3–C4	1.530(3)	1.536	O6–HO6	0.75(3)	C6–H6B	0.98(3)
O4–C1	1.424(3)	1.428	C4–C5	1.514(3)	1.519	C1–H1	0.98(3)	C7–H7A	0.95(4)
O4–C4	1.454(3)	1.457	C5–C6	1.518(3)	1.520	C2–H2	0.96(3)	C7–H7B	0.97(4)
O5–C5	1.428(3)	1.431				C3–H3	0.93(2)	C7–H7C	0.92(4)

(b) Bond angles (°)

C1–O1–C7	112.6(2)	O2–C2–C1	111.5(2)	C2–C3–C4	103.9(2)	O5–C5–C4	108.4(2)
C1–O4–C4	109.6(2)	O2–C2–C3	114.9(2)	O4–C4–C3	105.7(2)	O5–C5–C6	110.1(2)
O1–C1–O4	112.2(2)	C1–C2–C3	102.4(2)	O4–C4–C5	110.9(2)	C4–C5–C6	110.7(2)
O1–C1–C2	108.7(2)	O3–C3–C2	113.7(2)	C3–C4–C5	112.9(2)	O6–C6–C5	108.3(2)
O4–C1–C2	103.4(2)	O3–C3–C4	110.8(2)				

(c) Torsion angles (°)

C7–O1–C1–O4	68.3(3)	O1–C1–C2–O2	42.9(3)	C1–C2–C3–O3	−151.7(2)	O4–C4–C5–O5	−54.9(2)
C7–O1–C1–C2	−177.9(2)	O1–C1–C2–C3	−80.4(2)	C1–C2–C3–C4	−31.2(2)	O4–C4–C5–C6	−175.9(2)
C4–O4–C1–O1	84.7(2)	O4–C1–C2–O2	162.3(2)	O3–C3–C4–O4	135.2(2)	C3–C4–C5–O5	−173.3(2)
C4–O4–C1–C2	−32.2(3)	O4–C1–C2–C3	39.0(2)	O3–C3–C4–C5	−103.4(2)	C3–C4–C5–C6	65.8(3)
C1–O4–C4–C3	12.3(3)	O2–C2–C3–O3	87.3(3)	C2–C3–C4–O4	12.8(3)	O5–C5–C6–O6	65.3(3)
C1–O4–C4–C5	−110.4(2)	O2–C2–C3–C4	−152.2(2)	C2–C3–C4–C5	134.2(2)	C4–C5–C6–O6	−174.8(2)

(d) Hydrogen bond distances (Å) and angles (°)

O2···O4^{i}	2.958(2)	HO2···O4^{i}	2.17(3)	O2–HO2···O4^{i}	163(3)
O3···O5^{i}	2.819(3)	HO3···O5^{i}	2.16(4)	O3–HO3···O5^{i}	153(4)
O5···O3^{iii}	2.852(3)	HO5···O3^{iii}	2.12(4)	O5–HO5···O3^{iii}	171(4)
O5···O2^{ii}	2.812(3)	HO6···O2^{ii}	2.06(4)	O6–HO6···O2^{ii}	172(3)

(i) $1+x,y,z$
(ii) $1-x,-\frac{1}{2}+y,1-z$
(iii) $-1+x,y,-1+z$

at the O4–C1 bond ($j=0$), the values along the ring are −32.2, 39.0, −31.2, 12.8, and 12.3°. These torsion angles correspond to a phase angle of pseudorotation P and an amplitude of pucker τ_m of 143.6 and 38.7°, respectively.[14] In terms of the formalism suggested by Altona and Sundaralingam[14] the Me α-D-Gal*f* ring has approximately the twist conformation ${}^{1}_{2}T$. This conformation is different from those of a variety of other furanose rings in which either C2 or C3 (or both) have been shown to be the out-of-plane ring atoms.[15] The puckering in Me α-D-Gal*f* may be related to eclipsed interaction between the substituents at C1 and C2. For the five-membered ring in methyl β-D-arabinofuranoside, which is configurationally related to Me α-D-Gal*f*, a ${}_{2}E$ conformation has been proposed from a consideration of non-bonded interaction.[16]

In view of the puckering observed in the furanose ring the substituents cannot exhibit a "true" *cis* or *trans* relation. This affects mainly the C1 and C2 substituents, a fact which is reflected in the torsion angle about the C1–C2 bond between O1 and O2 of 42.9°. The orientation of the methyl group relative to the furanose ring oxygen is $+sc$[12] (*gauche*). When relating O4 to O5 about the C4–C5 bond and O5 to O6 about C5–C6, the conformations are $-sc$ (*gauche*) and $+sc$, respectively.

The C–C bond lengths in Me α-D-Gal*f* average to 1.520 Å. None of the C–C bond lengths in the ring are significantly different from this mean or from the value of 1.523 Å reported by Sundaralingam as an average value for furanose rings.[15] The C–O bonds lengths in the molecule average to 1.422 Å. The values for O1–C1 (1.395 Å) and O4–C4 (1.454 Å) both deviate significantly from this mean. It may be pointed out that a short exocyclic anomeric bond [1.382(11) Å] is also found in methyl α-D-lyxofuranoside.[4] The two ring C–O bond lengths in Me α-D-Gal*f* (1.428 and 1.454 Å) agree well with those reported as average values for such bonds (1.427 and 1.450 Å).[15]

Except for the bond angle at O4 (109.6°) all the internal angles in the furanose ring are significantly less than the tetrahedral angle. The internal C–C–C bond angle (102.4°) at the out-of-plane carbon atom C2 is less than the C–C–C bond angle (103.9°) at the in-plane carbon atom C3. Other characteristic features of bond angles in furanosides described by Sundaralingam[15] do not fit well for this structure. This may be related to the different mode of puckering in the present structure.

The Me α-D-Gal*f* molecules are linked through hydrogen bonding and except for O1 all oxygen atoms participate in the three-dimensional hydrogen bond network (Fig. 2 and Table 3d). Each of the oxygen atoms O2, O3, and O5 donate as well as accept protons, O6 acts as a donor only, and O4 as an acceptor. The O2···O4^{i} separation of 2.958 Å is somewhat longer than the other O···O distances of Table 3c. However, the position of the hydrogen atom HO2 (O–H···O angle of 163°) indicates the presence of a hydrogen bond. The molecules pack with the ring planes (five-atom least-squares planes) nearly parallel to the *ab* plane.

The present structure shows two short intermolecular contacts involving non-hydrogen atoms, one oxygen oxygen contact (O6···O3^{iii}) of 2.949(3) Å and one carbon oxygen contact (C3···O5^{i}) of 3.077(3) Å.

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Chlorinated Long-chain Fatty Acids. Their Properties and Reactions. IX. The Kinetics and Stereochemical Course of the Neutral and the Base-promoted Dehydrochlorination of Sodium *erythro*- and *threo*-9(10)-Chloro-10(9)-hydroxyoctadecanoates

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The base-promoted and neutral dehydrochlorination of sodium *erythro*- and *threo*-9(10)-chloro-10(9)-hydroxyoctadecanoates in water has been studied. The appreciable contribution of the neutral reaction was taken into account by determining the rate coefficients for this and for the base-catalysed reaction simultaneously. The observation that the different reaction rates of the diastereoisomers are mainly due to the differences in the ground state energies of their *anti* conformations [$k_{erythro}/k_{threo} = 4.3 \pm 0.6$ (k_{OH}) and 2.3 ± 0.7 (k_0) and 4.5 (est.) at 298 K] confirmed the postulation that the reaction is stereochemically a *trans* process.

Ketola pointed out recently [1] that in alkaline water solution sodium *erythro*- and *threo*-9(10)-hydroxyoctadecanoates undergo a dehydrochlorination reaction which is promoted both by water and the base. The rate equation in his report was very approximate and hence both rate coefficients (k_0 and k_{OH}) may be estimated more accurately using the method described by Salomaa [2] and Euranto.[3] Moreover, Ketola's earlier results for the *erythro* isomer were not accurate enough [1] that we ought to redetermine them whereas his original results [1] were used to recalculate the rate coefficients for the *threo* isomer.

The different reaction rates in the base-catalyzed removal of the first chlorine atom of sodium *erythro*- and *threo*-9,10-dichlorooctadecanoates were mainly due to the differences in the ground state energies of their reactive conformations.[4] Since the present reactions are also *trans* processes [1] it was worth trying to correlate the reaction rates of the isomers with their ground state energies.

EXPERIMENTAL

erythro-9(10)-Chloro-10(9)-hydroxyoctadecanoic acid was synthesized from elaidic acid by the method of Swern.[1,5] The product which was a mixture of the two positional isomers melted at 326 – 331 K (lit.[1,5,7] 326 – 327, 331.0 – 331.8, 325 – 330 K) and had a chlorine content of 10.39 ± 0.13 % (calc. 10.59 %). The *threo* isomer was not prepared but the original experimental results of Ketola [1] were used to recalculate the values of the rate constants.

Kinetic experiments. The alkaline dehydrochlorination of *erythro*-9(10)-chloro-10(9)-hydroxyoctadecanoic acid was studied at four temperatures at 4 to 5 different base concentrations (Table 1). The reaction solutions were prepared by pouring a weighed amount of the standard base (NaOH) solution into a mixture of weighed amounts of substrate and distilled water. The acid dissolved at once and the initial base concentration had to be corrected accordingly. Before mixing both components were thermostated for half an hour. The removal of the chlorine atom was followed by titrating the chloride ions formed with 0.01 N $Hg(NO_3)_2$ in 80 % (v/v) aqueous methanol. Otherwise the kinetic measurements were accomplished as reported earlier. [1,7,8]

Calculation of the rate coefficients. In alkaline water solution the dehydrochlorination reactions of sodium *erythro*- and *threo*-9(10)-chloro-

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Table 1. The values of the rate coefficients for the neutral (k_0) and base-catalyzed (k_{OH}) dehydrochlorination of sodium *erythro*-9(10)-chloro-10(9)-hydroxyoctadecanoate at different temperatures. For the definition of [S], [OH^-], $\bar{k}_t$ and $\bar{c}_t$ see the text.

Temp. K	10^3[S]/ mol kg^{-1}	10^3[OH^-]/ mol kg^{-1}	$10^3\bar{k}_t$/ min^{-1}	$10^3\bar{c}_t$/ mol kg^{-1}	10^4k_0/ s^{-1}	10^3k_{OH}/ kg mol^{-1} s^{-1}
288	10.08	19.31	5.35	14.87		
	9.82	24.96	6.52	21.04		
	10.13	29.79	7.51	25.48	4.24 ± 0.24[a]	31.80 ± 0.95[a]
	10.04	39.66	9.37	36.02		
293	9.93	15.73	6.60	11.71		
	9.86	20.59	8.30	16.78		
	9.96	30.40	10.84	26.83	5.69 ± 0.34	46.78 ± 1.34
	10.32	41.86	14.10	37.99		
298	9.96	15.90	9.63	11.86		
	9.99	17.92	10.98	14.40		
	9.89	23.48	13.21	19.62	7.70 ± 0.44	72.69 ± 2.19
	10.09	25.25	14.09	21.27		
	9.78	32.59	16.99	28.66		
303	9.94	12.76	10.30	8.51		
	10.19	14.27	12.17	10.07		
	10.14	16.97	13.79	12.82	9.39 ± 1.61	101.77 ± 12.55
	10.35	22.17	16.40	18.01		

[a] Standard deviation.

Table 2. The values of the rate coefficients for the neutral (k_0) and base-catalyzed (k_{OH}) dehydrochlorination of sodium *threo*-9(10)-chloro-10(9)-hydroxyoctadecanoate at different temperatures. For the definition of [S], [OH^-], $\bar{k}_t$ and $\bar{c}_t$ see the text.

Temp. K	10^3[S]/ mol kg^{-1}	10^3[OH^-]/ mol kg^{-1}	$10^3\bar{k}_t$/ min^{-1}	$10^3\bar{c}_t$/ mol kg^{-1}	10^4k_0/ s^{-1}	10^3k_{OH}/ kg mol^{-1} s^{-1}
293	20.72	20.52	2.51	14.84		
	20.71	40.40	3.27	33.89	2.32 ± 0.60[a]	10.50 ± 1.21[a]
	20.00	82.41	6.32	76.89		
298	20.68	21.68	3.87	15.96		
	20.71	40.25	4.99	33.21	3.32 ± 0.85	16.87 ± 1.74
	20.00	82.00	9.84	76.48		
303	20.95	38.33	8.27	32.42		
	20.93	59.06	12.70	53.02	5.24 ± 2.30	28.08 ± 4.03
	19.98	82.28	15.84	77.07		

[a] Standard deviation

10(9)-hydroxyoctadecanoates follow the rate law:[1,9]

$$\frac{dx}{dt} = (k_0 + k_{OH}[OH^-])[S] \quad (1)$$

where k_0 is the rate coefficient for the neutral and k_{OH} for the base-promoted reaction and [OH^-] and [S] the base and substrate concentrations. The reaction was carried out at several base concentrations (Tables 1 and 2) to obtain the values of k_0 and k_{OH} from the equation

$$\bar{k}_t = k_0 + k_{OH}\bar{c}_t \quad (2)$$

The average base concentrations $\bar{c}_t$ for each run were calculated using a program written

Table 3. Values of thermodynamic functions of activation at 298 K for the neutral (A) and alkaline (B) dehydrochlorination of the *erythro* (I) and *threo* (II) acids.

Compound	Reaction	$\Delta H^{\ddagger}$/ kJ mol^{-1}	$\Delta S^{\ddagger}$/ J mol^{-1}K^{-1}	$\Delta G^{\ddagger}$/ kJ mol^{-1}
I	A	36.6±2.2[a]	−182.2±7.5[a]	90.9±0.04[a]
I	B	54.6±1.7	−83.7±5.8	79.6±0.04
II	A	57.6±4.8	−118.1±16.1	92.8±0.07
II	B	70.2±2.2	−43.4±7.4	83.1±0.03

[a] Standard deviation

for an IBM 1130 computer with the aid of the trapezoidal rule [2,3]

$$\bar{c}_t = c_0 - \frac{1}{2t}\sum_{j=1}^{i}(x_j + x_{j-1})(t_j - t_{j-1}) \quad (1)$$

where x_j is the amount of the chloride ion formed in time t_j. The average rate coefficients for each run were obtained from the normal first-order rate -equation involved in the above program.

The values of k_0 and k_{OH} obtained from the above equation by the method of least squares ($r = 0.995 - 0.999$) are shown in Tables 1 and 2. The values for sodium *threo*-9(10)-chloro-10(9)-hydroxyoctadecanoate have been recalculated from the experimental results of Ketola.[1] The values of thermodynamic functions of activation (Table 3) for the neutral and alkaline dehydrochlorination of sodium *erythro*- and *threo*-9(10)-chloro-10(9)-hydroxyoctadecanoates were obtained from the values of k_0 and k_{OH} (Tables 1 and 2) by the method of least squares.

RESULTS AND DISCUSSION

The values obtained in this work for the rate coefficients (k_0) of the neutral reaction of sodium *erythro*-9(10)-chloro-10(9)-hydroxyoctadecanoate do not differ appreciably from those of Ketola [1] whereas our values for k_{OH} are 2−3 times higher than his original ones. This is due to the different computational methods and the improved quality of the sample used for kinetic experiments. In the case of the *threo* isomer the recalculated values of k_0 remained practically equal to the earlier results [1] whereas those of k_{OH} increased by 16 to 48 %.

If we compare the rate coefficients determined for the neutral and base-catalysed dehydrochlorination of the studied octadecanoates with those of 2-chloroethanol[1,8,10] we observe that the substitution increases the rate of both reactions but more that of the neutral reaction.

Kinetics and mechanisms of dehydrochlorination. The product analyses [1] and a stereochemical treatment (see below) confirm that the dehydrochlorination reaction is principally a *trans* process. The rate-determining step in the base-catalysed reaction is an intramolecular substitution reaction (S_Ni) [1,10] where an inversion occurs at the α-carbon during the ring closure. In the neutral reaction the primary product may well be a diol [11] though in the case of some reactive chlorohydrins the epoxide may still be an intermediate.[12]

The values of thermodynamic functions of activation for the neutral and base-catalysed reactions are close to those for the reactions of 2-chloroethanol.[1,10] The values let us suppose that the mechanisms are the same for the compounds studied here and for 2-chloroethanol though the results for the neutral reaction reveal the need of a thorough study to clarify the detailed course of this reaction.[12]

Stereochemistry of the dehydrochlorination

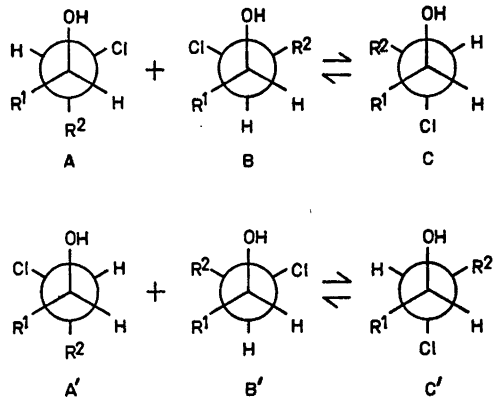

Fig. 1. The minimum energy conformations of sodium *threo* (A−C) and *erythro*-9(10)-chloro-10(9)-hydroxyoctadecanoates (A′−C′).

reaction. It has been pointed out[1] that *threo* and *erythro* isomers produce *cis*- and *trans*-9,10-epoxyoctadecanoic acids, respectively. A stereochemical requirement for these products is that the reacting groups (OH and Cl) have an *anti*-arrangement.[5]

Both sodium *threo*- and *erythro*-9(10)-chloro-10(9)-hydroxyoctadecanoates have three minimum energy conformations (Fig. 1) from which only C and C′ can undergo a *trans* process. The total rate coefficient of a given reaction of a conformationally heterogeneous system may be presented by the relation

$$k = \sum x_i k_i \quad (4)$$

where x_i is the mol fraction and k_i the rate coefficient of the reaction studied for the ith conformation. Since the studied reaction[1] has been postulated to be a *trans* process the total rates for the dehydrochlorination of sodium *threo*- (k_t) and *erythro*-9(10)-chloro-10(9)-hydroxyoctadecanoates (k_e) may be written

$$k_t = x_C k_C \quad (5)$$

$$k_e = x_{C'} k_{C'} \quad (6)$$

Consequently, if we assume that $k_C \sim k_{C'}$

$$k_e/k_t = x_{C'}/x_C \quad (7)$$

In other words if the reaction really occurs almost exclusively through the *anti* conformations C and C′ (Fig. 1) the relative rates should be determined by the ground state energies and hence by the relative populations of the reactive conformations. If we use the following values (in kJ mol^{-1}) for the various *gauche* interactions[4,13] at 298 K: R^1-R^2 +2.7, R^1-Cl or R^2-Cl −0.2, R^1-OH or R^2-OH 0.8, and $Cl-OH$ −4.0 the estimated interaction energies for the different conformations (Fig. 1) are then

threo	A	−1.3	B	−3.4	C +3.3
erythro	A′	−1.5	B′	−0.5	C′ +0.6

from which we obtain $x_C = 0.045$ and $x_{C'} = 0.204$. Accordingly, $k_e/k_t = 0.204/0.045 = 4.5$ in fair agreements with the experimental findings 4.3 ± 0.6 for the base-promoted reaction and 2.3 ± 0.7 for the neutral reaction at 298 K.

Finally, we like to emphasize that no attention has been paid to the possible difference in the dehydrochlorination rates of, *e.g.*, sodium *erythro*-9-chloro-10-hydroxy- and *erythro*-10-chloro-9-hydroxyoctadecanoates. The influence of the rather remote carboxylate group is likely to be very small[14] and sometimes even an α-carboxylate group has practically no neighbouring group effect on the halide ion release.[15]

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Determination of the Conformation of Substituted 4,6-Dioxo-1,3-dioxanes. Part III.* ^{13}C NMR Chemical Shifts, $^{13}C,H$ Coupling Constants and Spin-lattice Relaxation Times of 2-, 2,2-, 2,5-, and 2,2,5-Substituted Derivatives

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^{13}C NMR chemical shifts, methyl substituent effects, $^{13}C,H$ coupling constants and spin-lattice relaxation times (T_1) have been measured for a number of substituted 4,6-dioxo-1,3-dioxanes. The parameters are discussed on the basis of the known conformational behaviour of the compounds.

In recent time increasing attention has been paid to ^{13}C NMR spectra of various ring systems, including those of cyclohexane,[1] cyclohexanone,[2] 1,3-dioxane,[3] and glutaric anhydride.[4] It has been shown that in general the parameters derived from ^{13}C NMR spectra are highly dependent on the configuration of the substituents and on the conformation of the ring. In this paper some ^{13}C NMR parameters for 2-, 2,5-, 2,2-, and 2,2,5-substituted 4,6-dioxo-1,3-dioxanes are reported. These compounds have been shown to exist predominantly in a boat conformation[5,6] and the ^{13}C NMR spectra were expected to give supporting evidence. Furthermore, no previous ^{13}C NMR studies have been reported on the compounds under consideration.

EXPERIMENTAL

The synthesis of the compounds studied is reported elsewhere.[5,6] The ^{13}C NMR spectra were recorded on a JEOL FX-60 NMR spectrometer equipped with the dual probe for ^1H and ^{13}C resonance observation. The compounds were examined as *ca.* 20 % w/v solutions in acetone-d_6 and/or in deuteriochloroform. Chemical shifts are given relative to internal TMS. Their accuracy is believed to be within 0.1 ppm. A spectral width of 4 kHz and a data memory size of 8K were used. The routine spectra were taken under conditions of proton-noise decoupling. Selective proton decoupling, off-resonance decoupling and T_1 measurements were performed in certain cases for signal identification purposes. Inversion-recovery method was used in the T_1 measurements with a pulse repetition time of 150 s. Some of the spectra were also recorded without noise-decoupling to find out the various types of ^{13}C, H couplings. The accuracy of the reported coupling constants is believed to be within 1 Hz (spectral resolution due to limited data capacity was *ca.* 1 Hz).

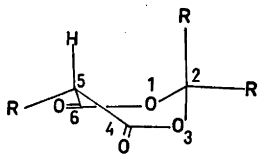


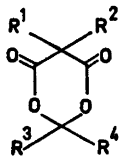
Fig. 1. The preferred ring conformation of 2-, 2,5-, 2,2-, and 2,2,5-substituted 4,6-dioxo-1,3-dioxanes.

RESULTS AND DISCUSSION

1. Spectral assignments. The ^{13}C NMR chemical shift data of the ring carbon atoms together with the shifts of the methyl and aryl side

* Parts I and II: *Finn. Chem. Lett.* (1976) 110, 115.

Table 1. The ^{13}C NMR chemical shifts (δ). Solvent: acetone-d_6.



Compound	R^1	R^2	R^3	R^4	C-2	C=O	C-5	2-Me	5-Me
1	H	H	H	Ph	98.4	164.9	39.8	—	—
2	H	Me	H	Ph	97.2	167.8	44.2	—	9.6
3	H	H	Me	Me	106.4	164.0	36.8	27.5	—
4	H	H	Me	i-Bu	108.1	164.0	36.9	26.0	—
5	H	H	Me	*t*-Bu	111.2	164.3	36.7	21.6(eq)	—
6	H	Me	Me	Me	105.2	166.8	41.9	26.1(ax) 28.7(eq)	10.7
7	H	Me	Me	Et	106.6	167.0	42.1	24.3(ax)	10.7
7B	H	Me	Et	Me	107.6	167.0	41.9	25.4(eq)	11.0
8	H	Me	Me	i-Pr	108.1	167.0	42.2	22.2(ax)	10.7
8B	H	Me	i-Pr	Me	108.1	167.0	41.8	21.1(eq)	11.4
9	H	Me	Me	*t*-Bu	109.5	166.8	41.9	20.4(eq)	10.7
10	H	Me	Ph	Me	105.1	166.8	44.2	30.8(ax)	10.1
					Aryl C-1′	C-2′,6′	C-3′,5′	C-4′	
1					133.4	127.2	129.4	131.2	
2					134.0	127.6	129.6	131.4	
10					140.6	125.3	130.1	130.4	

chain carbons are collected in Table 1. The assignment of the ring carbon signals is straightforward: the carbonyl carbon is found on the lowest field around 165 ppm and is easily recognized. Next to the higher field, at about 100 ppm, is the signal of the C-2 atoms. The C-5 signal is found at around 40 ppm. The aliphatic side chains produce signals in the high field region of the spectrum sometimes obscuring the signal of C-5. They could, however, be identified unequivocally by means of off-resonance, selective decoupling and T_1-measurements. The signals of the aromatic substituent appear at 120 – 140 ppm and are thus clearly separated from the rest of the signals. The signal of C-1′ of the aromatic ring is easily recognized due to its weak intensity in the routine spectrum. The signal of C-4′ is identified as the second weakest signal in this part of the spectrum while the carbons 2′,6′ and 3′,5′ give about twice as strong signals. The order of these two last-mentioned signals given in Table 1 could be reversed but the given order is the same as in Ref. 7 for aryl-substituted glutaric anhydrides.

2. Chemical shifts; substituent effects on the ^{13}C chemical shifts of the ring carbons. It is well known that the substituent effects are indicative of the geometry of the compound under examination though there seem not to be any simple rules. Additivity and similarity of substituent effects in a series of substituted derivatives can be used as a criterion of conformational homogeneity and the absolute values of substituent effects give information about the configuration of the substituent and other geometrical relationships. In the case of 4,6-dioxo-1,3-dioxanes, which are considered in this context, the only useful substituent effect is that of 5-methyl substituent which can be elucidated from the compound pairs (*1* – *2*), (*3* – *6*) and (*5* – *9*) and these are given in Table 2. The corresponding effects in cyclohexane,[1] cyclohexanone,[2] 1,3-dioxane,[3] and glutaric anhydride [4] are given in the same table for comparison.

The similarity of the calculated substituent effects show that these six 4,6-dioxo-1,3-dioxane derivatives have a similar ring conformation, *i.e.* a boat, as reported earlier,[5,6] The

Table 2. The calculated 5-methyl substituent effects (ppm). Positive values mean shifts to higher fields. Solvent is acetone-d_6. Values found in some other cyclic systems are listed for comparison.

Compound	α(C-5)	β(C=O)	δ(C-2)
1−*2*	−4.4	−2.9	1.2
3−*6*	−5.1	−2.8	1.2
5−*9*	−5.2	−2.5	1.7
Cyclohexane [1]	−6.0		0.3
Cyclohexanone (Me at C-2) [2]	−3.6	−1.5	0.5
1,3-Dioxane (Me at C-5) [3]	−3.1		0.2
Glutaric anhydride (Me at C-2) [4]	−5.7	−1.8	

α-effect of the 5-Me is quite "normal" if compared to cyclohexane but is clearly more negative than in 1,3-dioxane and in cyclohexanone but more positive than in glutaric anhydride. The β-effect of the 5-Me on the carbonyl carbon shielding (on average −2.7 ppm) is significantly more negative than in cyclohexanone (−1.5 ppm) or in glutaric anhydride (−1.8 ppm). This may be a reflection of the more flattened region (O−CO−C(5)−CO−) in 4,6-dioxo-1,3-dioxane than the corresponding regions in cyclohexanone (−C−CO−C−C) or in glutaric anhydride (−O−CO−C−C). The observed δ-effect is anomalously large if compared to the effects of equatorial methyl groups found in other cyclic systems (Table 2). Interestingly, a δ-effect of about the same size (1.1 to 1.4 ppm) can be observed in a series of 1-methyl-substituted norbornanes,[8] where the six-membered ring is forced to a boat conformation. Obviously this large δ-effect is an indication of the boat conformation.

3. Chemical shifts of the methyl and aryl substituents. The chemical shifts of the methyl groups are in the expected range [9,10] and are collected in Table 1. It is easily seen that the shifts of the 5-methyl groups in stereoisomer pairs (*7,7B*) and (*8,8B*) are not markedly different. The same is true for the 2-methyl groups; the 5-Me group is equatorial in all compounds but the 2-Me substituent can be axial (as in *7* and *8*) or equatorial (as in *7B* and *8B*). When there are two methyl substituents at C-2 (as in *6*), the signal of the axial methyl is at a slightly higher field but if the other of the substituents is changed to another alkyl group the order may be reversed, depending on the kind of the alkyl group: in the epimer pair (*7,7B*) the axial 2-methyl is at higher field but in the isopropyl derivatives (*8,8B*) the axial methyl signal is at a lower field.

In the aryl-substituted derivatives *1* and *2* the corresponding chemical shifts of the aryl carbons (Table 1) are very similar but the shifts of the third aryl-derivative (*10*) are distinctly different. This is in agreement with the earlier observation [6] that in *1* and *2* the aromatic side chain is equatorial but is axial in *10*.

4. $^{13}C,H$ coupling constants. The ^{13}C,H couplings of some representative compounds were measured from non-decoupled spectra and are collected in Table 3. The one-bond couplings are of the expected order of magnitude.[9,10]

An interesting phenomenon occurs in the case of 2-methyl-−2-*tert*-butyl derivative (*5*): the C-5 signal appears in the coupled spectrum as a quartet indicating two different $^1J_{5C5H}$ coupling constants. To analyze this X-part of an ABX-system the ^{13}C-H satellite signals of the C-5 protons in the ^{1}H spectrum were located. The ABX-analysis yielded the following values: $^2J_{AB}=21.0$ Hz (main quartet from the ^{12}C-H spectrum: 21.4 Hz), $\delta_{AB}=36.4$ Hz (main quartet: 36.6 Hz), $J_{AX}=129.0$ Hz and $J_{BX}=138.3$ Hz (A=axial proton at C-5, B=equatorial proton at C-5). As δ_{AB} is relatively large, this is a nearly first order spectrum: the difference of the inner peaks of the C-5 quartet is 8.5 Hz in acetone-d_6, close to the value $J_{BX}-J_{AX}=9.3$ Hz. In deuteriochloroform, however, the corresponding difference is only 3.9 Hz as δ_{AB} in this solvent is much smaller (*ca.* 11 Hz) than in acetone-d_6 (36.4 Hz). This is a demonstration of a case where first

Table 3. The J_{CH} coupling constants of the compounds studied. Solvent $CDCl_3$ if not otherwise indicated. Mutually coupled nuclei in italics.

Compound	*2*[a]	*5*	*6*	*7*	*7B*	*9*	*10*
$^1J_{2C2H}$	173.4	—	—	—	—	—	—
$^1J_{5C5Heq}$	—	138.3*a*	—	—	—	—	—
$^1J_{5C5Hax}$	124.5	129.0*a*	124.5	124.5	124.5	120.1	123.0
$^1J_{2CH_3eq}$	—	—	129.2	—	129.9	—	130.5
$^1J_{2CH_3ax}$	—	128.6	128.6	128.9	—	128.4	—
$^1J_{5CH_3}$	130.5	—	131.6	131.8	131.8	131.8	131.8
$^1J_{5CH_35H}$	5.4	—	5.9	6.0	6.0	6.1	5.3
$^2J_{CCH_3eq}$	—	—	5	—	[b]	—	[b]
$^2J_{CCH_3ax}$	—	5	5	[b]	—	5	—
$^2J_{CO5Heq}$	—	6.8[c]	—	—	—	—	—
$^2J_{CO5Hax}$	[b]	6.8[c]	9.3	9.5	[b]	9.3	9.3
$^2J_{5CCH_3}$	[b]	—	4.2	4.4	4.4	4.9	4.9
$^3J_{CO5CH_3}$	[b]	—	4.6	4.5	[b]	4.6	4.6
$^3J_{2CH_32CH_3}$	—	—	3.3	2.4[d]	2.9[d]	—	—

[a] Solvent $(CD_3)_2CO$. [b] Not resolved. [c] The X-part of the ABX_2-system of $5H_a5H_e(CO)_2$ is a triplet with spacings 6.8 Hz. See text. [d] Actually a coupling $^3J_{2CH_32CH_3CH_3}$.

order treatment of the ^{13}C spectrum would lead to an erroneous result.[11] It is also noted that in all 5-methyl derivatives the $^1J_{C,H}$ at C-5 is in the range 120 – 130 Hz indicating an axial 5-proton, in agreement with the previous result.[5,6]

The other $^1J_{C,H}$ in this ring system is that for C-2. A value of 173.4 Hz was measured from the coupled spectrum of 2-phenyl-5-methyl-4,6-dioxo-1,3-dioxane (*2*). This value is large if compared to ^1J at C-5 and is a result of the neighbourhood of the two electronegative oxygen atoms.[9,10]

Several ^2J's and ^3J's could be detected. For example, the carbonyl carbon signal of *5* is a triplet indicating a ^2J of 6.8 Hz to the methylene protons at C-5. This value was determined in $CDCl_3$ and actually the couplings can differ slightly due to the close chemical shifts of the 5-protons; however, the sum of these couplings is 13.6 Hz. In the 5-methyl derivatives the C=O signal appears as a doublet of quartets with couplings of *ca.* 9 and 4.5 Hz. The first coupling is the above-mentioned ^2J of the carbonyl carbon with the axial proton at C-5 and the second is the ^3J between the carbonyl carbon and the protons of the equatorial methyl group at C-5. This is a typical, large long range coupling of properly substituted esters and other carbonyl compounds.[12]

Other long range couplings include the typical ^3J's of the 5-methyl carbon with the methine proton at C-5 and the ^3J coupling of the C-2 with the methyl or methylene protons of the C-2 substituents. A ^3J of *ca.* 3 Hz could be resolved in the spectrum of *6* in both 2-methyl carbon signals: these carbons are coupled to the protons of the second methyl substituent attached at C-2. Couplings of same type were detected also in the case of compounds *7* and *7B*.

Slight variations are noted in values of the ^{1}J coupling of the C-2 methyl carbon and the methyl protons: in equatorial configuration the coupling is about 1 to 2 Hz larger than in axial configuration of this substituent (compounds *6*, *7* and *7B* in Table 3).

5. T_1 *measurements.* To get more information about the structural parameters in these compounds, T_1 measurements of some representative compounds were performed. The Auto-T_1 program of the JEOL FX-60 computer system with the pulse sequence $(180\text{-}\tau\text{-}90t_r)_n$ was used. A value of 150 s was used for t_r (*ca.* 4 times the longest T_1) and τ was varied from 0.1 to 150 s. Concentrated samples in $CDCl_3$ were used and the cycle for each τ was repeated 25 – 50 (n) times to achieve a proper S/N ratio. Degassing of the samples was found to be necessary due to the slow relaxation of the

Table 4. The measured T_1 values (s) of the compounds studied (mean values of two independent measurements). Solvent $CDCl_3$. For experimental details, see text.

Compound	C=O	C-2	C-5	2-CH_3eq	2-CH_3ax	5-CH_3	Substituents CH_2-CH_3		$C\equiv(CH_3)_3$	
3	49.7	40.1	2.7	1.7						
5	37.0	41.1	2.7		3.0				23.6	2.3
6	40.4	28.1	2.7	1.2	2.2	1.7				
7[a]	46.5	25.9	2.2		2.1	1.5	1.8	1.8		
7B[a]	46.5	29.3	1.9	1.0		1.3	2.0	2.6		
9	41.3	38.1	2.5		3.4	1.8			21.3	1.4
10	43.2	32.7	2.7	0.8		1.9				
Mean	43.0	33.6	2.5	1.0	2.7	1.6				

[a] These values were determined from a mixture of *7* and *7B*. The chemical shifts of the C=O carbons are the same for both compounds.

Table 5. The measured NOE's for compounds *3* and *9*. Solvent $CDCl_3$. For experimental details, see text.

Compound	C=O	C-2	C-5	2-CH_3	5-CH_3	2-*t*-Bu
3	1.1	2.2	2.1	2.1		
9	1.9	1.7	2.0	2.2	1.9	1.8[a]

[a] Both $C(CH_3)_3$ and $C(CH_3)_3$.

nonprotonated carbons, C=O and C-2. The results are collected in Table 4.

The computer program did not yield standard deviations of the least squares fits but results from parallel independent measurements indicate a probable error of *ca.* 20 %.

It is known that several mechanisms may be responsible for the observable spin-lattice relaxation process. In general, the dipole-dipole mechanism is the most effective for protonated carbons in medium-sized compounds and is prevailing also for nonprotonated carbons.[9,10,13] A measure for the preference of the dipole-dipole mechanism can be derived by measuring the Nuclear Overhauser Effects (NOE) by integration of the fully relaxed spectrum and assuming complete NOE for protonated carbons or by internally comparing the intensities in normal and gated (proton decoupling off under FID sampling) spectra. The latter method was used (pulse interval 200 s) to find out the NOE's of compounds *3* and *9* (these represent the "smallest" and "largest" of the compounds under examination) and the results are collected in Table 5. The probable error is *ca.* 10 %. As the theoretical maximum of NOE in proton noise-decoupled spectra is 1.998, the results indicate that dipole-dipole relaxation is prevailing for all carbon atoms in compound *9*. In compound *3* the same is true for all but the C=O carbons, for which the observed NOE is only 1.1 indicating two or more competing relaxation mechanisms. This compound (*3*), moreover, is a conformational mixture of two interconverting identical boat conformations, and also hereby differing from the rest of the compounds in Table 4.

The differentiation between the protonated and nonprotonated carbon atoms is an easy task as the nonprotonated carbons relax distinctlty slower. However, there are slight variations from compound to compound indicating that also other factors are involved. It is known that in case of a uniform relaxation mechanism (here dipole-dipole mechanism) the molecular motion is reflected in T_1 values.[9,10,13] Thus in medium-sized compounds methyl carbons usually relax slower than secondary or tertiary carbons of a rigid skeleton, this being related to the short correlation time, *i.e.* fast rotation of the substituent (in small symmetrical molecules spin-rotation mechanism will also contribute). Similarly, in substituted benzenes and biphenyls, the *para* carbons

relax faster (long correlation time) than *ortho* and *meta* carbons (short correlation time) due to preferred rotation of the molecule around the C_1-C_{para} axic.[14,15]

Some conclusions from the T_1 values in Table 4 can be drawn. The longer relaxation of the C-2 in compounds *5* and *9* if compared to this relaxation time in the rest of the compounds, obviously reflects the lack of nearby hydrogens. The relaxation of C-2 of *3* is also slow but here it can be explained by the conformational mobility of this compound. The relaxation of the carbonyl carbon is also very slow and the fastest relaxation is observed in the case of *5* where there are two hydrogens at position 5. As mentioned above, other relaxation mechanisms may contribute to some extent for these carbons. The relaxation of the C-5 atom does not vary much from compound to compound. The fastest relaxation would be expected in the case of *5*. Actually the relaxation time is about the same as in the methyl derivative *9*.

The T_1 values of the methyl carbons show a significant dependence on the location and configuration of the substituent. Slowest relaxation is found in the case of axial 2-methyl (mean 2.5 s) while the equatorial 2-methyl (mean 1.0) and equatorial 5-methyl (mean 1.6) relax distinctly faster. An explanation to this phenomenon may be given in terms of anisotropic tumbling of the molecule around a certain preferred axis. The equatorial methyl groups lie close to the average plane of the ring the axial methyl group being more displaced from this plane. Rotation of the molecule about an axis in the symmetry plane of the molecule and close to the average plane of the ring would lead to a situation where the axial 2-methyl group has a shorter correlation time and, consequently, a slower relaxation (Fig. 2). A similar trend can be noted in the T_1 values of the carbons of the ethyl chain at C-2 (compounds *7* and *7B*: in axial configuration these carbons relax slower (compound *7*) than in equatorial configuration (compound *7B*).

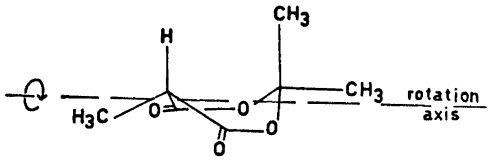


Fig. 2. An explanation to the observed differences in the T_1 values of the methyl groups. The molecules have a preferred axis of rotation and the carbons more displaced from this axis have a shorter relative correlation time and hence a longer relative relaxation time than the carbons close to the axis.

Summarizing, the current ^{13}C NMR data are found to be consistent with conclusions obtained from the earlier ^1H NMR data of 4,6-dioxo-1,3-dioxans.[5,6]

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Chemistry of *gem*-Dihalocyclopropanes. X. Configuration Determination of 2,2,2′,2′-Tetrahalobicyclopropyl Derivatives from Dipole Moment Measurements; Crystal Structure of *meso*-2,2,2′,2′-Tetrachloro-3,3,3′,3′-tetramethylbicyclopropyl

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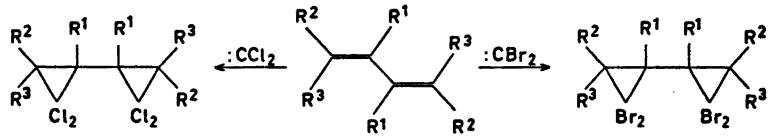
The dipole moments of several alkylated 2,2,2′,2′-tetrahalobicyclopropyl derivatives (*2* and *3*) and 1,1-dihalocyclopropanes (*4*—*9*) have been measured in benzene solution. These results are analysed to determine the configurations of the former compounds and it turns out that the isomers with the lower dipole moments (< 1.2 D) have a *meso* configuration.

In order to distinguish the *meso*-form with certainty from the racemate an X-ray structure determination of the lower-melting form of 2,2,2′,2′-tetrachloro-3,3,3′,3′-tetramethylbicyclopropyl at −160 °C was carried out. The crystals are triclinic, space group $P\bar{1}$. Using 2244 reflections recorded by counter methods the structure was refined to an R-value of 0.05. The analysis proved the compound to be the *meso*-form; bond lengths and angles are discussed.

The addition of dihalocarbene to a diene may yield the corresponding bis-adduct as one of the products and when properly substituted this product may be a mixture of a *meso*-form and a racemate.[1,2] In some cases the stereoisomers have been separated.[1,3–6]

The molecular dipole moment has proved to be a useful tool to determine the configurations of molecules containing polar substituents.[7] In the field of cyclopropanes Kumler *et al.*[8] have established the configurations of several compounds containing the *gem*-dibromocyclopropane system by comparing the measured dipole moments with those calculated for different configurations and conformations of the molecules. In the bis-adducts studied here only the rotation around the inter-ring C–C bond will affect the magnitude of the dipole moment; consequently it is in principle possible to calculate the dipole moment of any conformation of both the *meso*- and the racemic form. The measured dipole moments will correspond to a number of calculated conformations and by



3 1 2

a R^1 = Me, R^2 = R^3 = H
b R^1 = H, R^2 = R^3 = Me
c R^1 = H, R^2 = t-Bu, R^3 = Me

Scheme 1.

analyses of these with regard to interatomic interactions it should be possible to reject some of the solutions and draw a conclusion at least with respect to the configurations of the bis-adducts.

RESULTS

The addition of dibromocarbene, generated from bromoform and potassium *tert*-butoxide,[9] to the dienes *1a*, *1b* and *1c* gave the corresponding bis-adducts in varying yields (Scheme 1). The dienes *1b* and *1c* were also reacted with dichlorocarbene and in the former case 2,2,2′,2′-tetrachloro-3,3,3′,3′-tetramethylbicyclopropyl (*3b*) was isolated in good yield while in the latter case no bis-adduct was detected; the only isolable product was a liquid that consisted mainly of two isomeric chlorides of so far unknown constitutions in a ratio of approximately 3:2.

The conjugated dienes *1a*, *1b* and *1c* are all symmetrically substituted and the corresponding bis-adducts can therefore be mixtures of *meso*- and racemic forms. As shown by thin-layer chromatography (TLC) bicyclopropyl *2b* was obtained as an isomeric mixture; this result is supported by the NMR spectrum of the bis-adduct according to which the compound is a mixture of 70 % of one of the stereoisomers and 30 % of the other [1,2] (see Experimental). On the other hand adduct *2a* appeared homogeneous by TLC as was observed also with *2c* which is formed only in the *meso* configuration.[10]

The product *3b* could not be analyzed by TLC due to volatility; however, it was shown by gas chromatography to be a mixture of two compounds in a ratio of 3:1. Separation by preparative GLC afforded pure samples of the two isomers, m.p. 90 °C (predominant) and 102 °C. The *meso*-form and the racemate of 2,2,2′,2′-tetrabromo-3,3,3′,3′-tetramethylbicyclopropyl (*2b*) were separated by column chromatography and after recrystallization the isomers were isolated as colourless needles, the predominant isomer melting at 121 – 122 °C and the other at 135 – 136 °C.

The dipole moments of the bis-adducts and of six *gem*-dihalocyclopropanes, measured to get the relevant CCl_2 and CBr_2 group moments,[11] were measured in benzene at 25 °C; these results are summarized in Table 1. It is worth noting that replacing a cyclopropyl substituent with a methyl group hardly influences the dipole moment of a substituted *gem*-dihalocyclopropane.

Table 1. The dipole moments of bis-adducts and *gem*-dihalocyclopropanes measured in benzene at 25 °C.

Compound (m.p.)	Dipole moment (D)
2a	0.37
2b (121 – 122 °C)	1.15
2b (135 – 136 °C)	3.44
2c	0.75
3b (89.5 – 90 °C)	1.08
3b (102 – 102.5 °C)	3.74
1,1-Dibromo-2,2-dimethylcyclopropane (*4*)	2.20
1,1-Dibromo-2,2,3-trimethylcyclopropane (*5*)	2.31
1,1-Dibromo-2,2,3,3-tetramethylcyclopropane (*6*)	2.49
2,2-Dibromo-1,1′-dimethylbicyclopropyl (*7*)	2.30
1,1-Dichloro-2,2,3-trimethylcyclopropane (*8*)	2.34
1,1-Dichloro-2,2,3,3-tetramethylcyclopropane (*9*)	2.42

DISCUSSION

The tetrahalides *2a*, *2b*, *2c* and *3b* studied here can be considered as dimers of *gem*-dihalo-

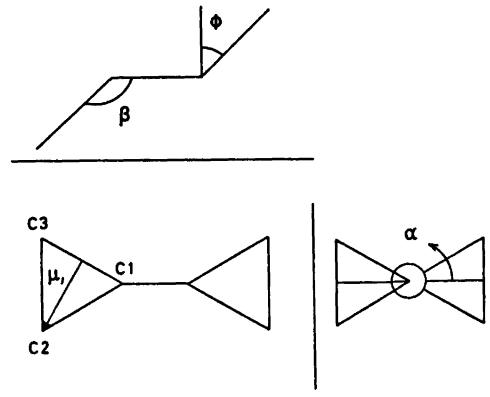


Fig. 1. Projections of the bicyclopropyl molecule showing quantities used for calculation of dipole moments.

cyclopropanes; hence the dipole moment μ can be expressed approximately as a function of the torsional angle α about the central C–C bond (taken here as 0° for a *trans* configuration) and the dipole moment μ_1 of a *gem*-dihalocyclopropane (Fig. 1). If the cyclopropane rings are assumed to be equilateral triangles,[12,13] an assumption that fits experimental results well,[14,15] and the dipole moment of the *gem*-dihalocyclopropane is directed along the normal from C2 to the C3-C1 bond as depicted (Fig. 1) simple vector addition gives the eqns. I and II for the dipole moment of a *meso* and a racemic bis-adduct, respectively.

$$\mu = (2^{\frac{1}{2}}/2)[(3+\cos^2\phi)(1-\cos\alpha)]^{\frac{1}{2}}\mu_1 \qquad \text{I}$$

$$\mu = (2^{\frac{1}{2}}/2)\{(3+\cos^2\phi)[1+\cos(\alpha+53.14°)]\}^{\frac{1}{2}}\mu_1 \qquad \text{II}$$

Among the bicyclic compounds studied the value of β (see Fig. 1) is known only for one of the isomers of tetrachloride *3b* (127.3°, crystal structure determination, see below). From the works of others, however, it seems as if the substituents have only a slight influence on the magnitude of this angle [12,14,15] and an angle of 127.3° was therefore taken between the inter-ring C–C bond axis and the plane of each ring, *i.e.* $\phi = 37.3°$.

As a good approximation, the exact value of μ_1 that is not known for any of the bis-adducts can be replaced by the dipole moment of an appropriate *gem*-dihalocyclopropane derivative. Among the measured dipole moments of compounds *4* – *9* (see Table 1) the moment of compound *7*, *viz.* 2.30 D, was chosen as μ_1 primarily because the structure best resembles each *gem*-dihalocyclopropyl group of the bis-adducts, but also because the magnitude of the moment is close to the average of the dipole moments of the six *gem*-dihalocyclopropanes recorded in Table 1. The variations in the dipole moment for *meso* and racemic bis-adducts as a function of α are shown in Fig. 2.

Agreement between calculated and measured dipole moments for the lower-melting form of bis-adduct *3b* is achieved when $\alpha = 29$ and 331° (eqn. I) and $\alpha = 98$ and 155° (eqn. II). Examination of molecular models indicates that the latter values result in sterically impossible conformations whereas the first two are free of substantial steric strain; consequently the lower-melting isomer of *3b* should be the *meso*-

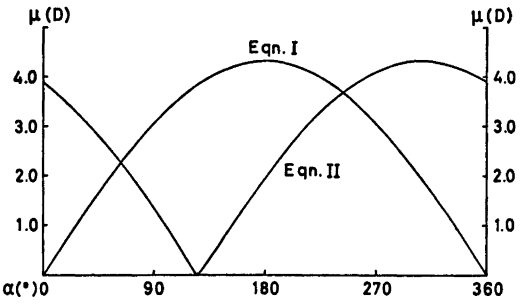


Fig. 2. The variations of the dipole moment μ for *meso* (eqn. I) and racemic (eqn. II) bis-adducts as a function of α.

form. A complete X-ray analysis of this isomer proved this conclusion (see below). Hence, the higher-melting isomer of *3b* must be a racemate and this is also borne out by the calculations; the conformations corresponding to both solutions of eqn. I ($\alpha = 116$ and 244°) and one of the solutions of eqn. II ($\alpha = 243°$) appear impossible because of considerable steric interactions whereas the second solution of eqn. II, $\alpha = 10°$, leads to a conformation that is sterically acceptable. It is worth noting that for this bis-adduct (and also for the tetrabromo analog *2b*) the correspondence between configurations and melting points are opposite to that usually observed, *i.e.* the high-melting isomer is the *meso* form.[1]

The values of α giving coincidence between calculated and observed dipole moments for the isomers of adducts *2a*, *2b* and *2c* are compiled in Table 2. As for the stereoisomers of adduct *3b* examination of scale molecular models of *2c* and the isomers of *2b* indicates their configurations. The lower-melting isomer of bicyclopropyl *2b* must have a *meso* configuration with either $\alpha = 31$ or 329°, whereas for the higher-melting isomer a racemate with

Table 2. The solutions of eqns. I and II for the tetrabromobicyclopropyl derivatives.

Bis-adduct (m.p.)	α (°)			
	Eqn. I		Eqn. II	
2a	9	351	117	136
2b (121 – 122 °C)	31	329	96	157
2b (135 – 136 °C)	103	257	23	230
2c	20	340	107	146

$\alpha = 23°$ is the only acceptable solution of eqns. I and II. Finally, bis-adduct *2c* is a *meso*-form with either $\alpha = 20$ or 340°, a result that is confirmed by the observed crystallographic symmetry.[2,10] For the adduct *2a*, however, the analyses of molecular models with respect to steric interactions are less conclusive because all four values of α correspond to sterically acceptable conformations. Since a deviation of 9° from the *s-trans* position is much more likely than more than 115°, the only isolated isomer of 2,2,2′,2′-tetrabromo-1,1′-dimethylbicyclopropyl is probably a *meso*-form.

Crystal structure of 2,2,2′,2′-tetrachloro-3,3,3′,3′-tetramethylbicyclopropyl, lower-melting form. The X-ray crystal structure analysis showed

Table 3. Selected interatomic distances, bond angles, and dihedral angles. For numbering of atoms, see Fig. 3.

Bond distances (Å)			
C1 – C1′	1.481(4)	C2 – Cl1	1.763(2)
C1 – C2	1.504(2)	C2 – Cl2	1.756(2)
C2 – C3	1.508(2)	C3 – C4	1.510(3)
C1 – C3	1.538(2)	C3 – C5	1.508(3)
Bond angles (°)			
C1′ – C1 – C2	120.9(2)	Cl2 – C2 – C3	120.3(1)
C1′ – C1 – C3	122.6(2)	C1 – C3 – C2	59.2(1)
C2 – C1 – C3	59.4(1)	C2 – C3 – C4	118.4(2)
C1 – C2 – Cl1	118.2(1)	C2 – C3 – C5	118.9(2)
C1 – C2 – Cl2	119.5(1)	C1 – C3 – C4	117.4(2)
C1 – C2 – C3	61.4(1)	C1 – C3 – C5	119.9(2)
Cl1 – C2 – Cl2	110.2(1)	C4 – C3 – C5	113.1(2)
Cl1 – C2 – C3	119.9(1)		
Dihedral angles (°) (positive for a right-hand screw)			
C1′ – C1 – C2 – Cl1	−137.4(2)	Cl1 – C2 – C3 – C4	−1.3(2)
C1′ – C1 – C2 – Cl2	1.5(3)	Cl1 – C2 – C3 – C5	142.6(1)
C1′ – C1 – C3 – C4	142.4(2)	Cl2 – C2 – C3 – C4	−144.1(1)
C1′ – C1 – C3 – C5	−1.4(3)	Cl2 – C2 – C3 – C5	−0.1(1)

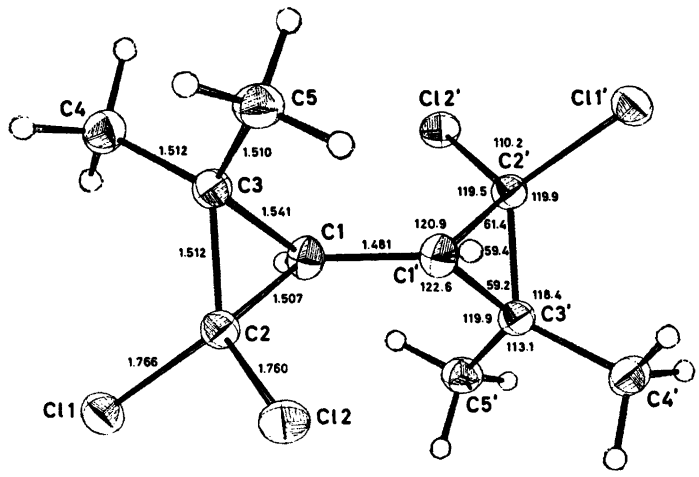


Fig. 3. Perspective view of the molecule showing bond lengths (A) corrected for thermal libration and angles (°). Nonhydrogen atoms are represented by thermal ellipsoids defined by the principal axes of thermal vibration and scaled to include 50 % probability.

the compound to be the *meso*-form, the molecular centra of symmetry being required by the crystallographic symmetry. Selected interatomic distances, bond angles and dihedral angles are listed in Table 3; bond lengths corrected for thermal libration are given in Fig. 3 which also shows the conformation, thermal ellipsoids and the numbering of the atoms. The C–H distances were found in the range 0.94(3) to 1.00(3) Å, mean value 0.96 Å.

The central C–C bond is found to be of normal length for a bicyclopropyl derivative, 1.481(4) Å, as compared to 1.487(4) Å in bicyclopropyl[14] and 1.49(2) Å in 2,2'-dibromobicyclopropyl;[15] this value corresponds to that given for a single bond between sp^2 hybridized carbon atoms (1.478 Å). The central C–C bond forms angles (β) of 127.3° with the planes of the cyclopropane rings.

The $C_{methyl}-C_{ring}$ distance (mean value 1.511 Å) corresponds to the sum of single bond radii for sp^2 and sp^3 hybridized carbon atoms (1.505 Å). The $Cl-C_{ring}$ bond length (mean value 1.763 Å) is found to be slightly longer than the C–Cl bond found in 1,1-dichlorocyclopropane (1.734 Å),[16] but within the accuracy of the determinations the same as in 1,1-dichloro-2,2-diphenylcyclopropane (1.754 Å).[17] The $C_{methyl}-C_{ring}-C_{methyl}$ angle is 113.1(2)° and the Cl–C–Cl angle 110.2(1)°; the corresponding $C_{phenyl}-C_{ring}-C_{phenyl}$ and Cl–C–Cl angles in 1,1-dichloro-2,2-diphenyl-cyclopropane are 113.9 and 110.9°, respectively.

A survey of ring carbon-carbon bond lengths observed in various cyclopropane derivatives is given in Ref. 17. The bond lengths seem to be influenced by the type of substituents, the observations being in the range 1.44–1.56 Å with an average of 1.511 Å; standard deviation from the mean is 0.024 Å. In the present investigation two of the C–C bonds are of the same length (1.512 and 1.507 Å) as in cyclopropane itself (1.510 Å),[18] whereas the C–C bond across the ring relative to the carbon atom with chlorine attached was found as long as 1.541 Å.

Intermolecular distances were found to be as expected for a crystal in which the packing forces are of the van der Waals type.

EXPERIMENTAL

General. NMR spectra were measured on Varian Associates A-60A and HA-100-15D spectrometers. Mass spectral data were obtained using an A.E.I. MS902 mass spectrometer. The IR spectra were recorded on a Perkin-Elmer model 457 spectrophotometer. Gas chromatographic analyses were performed with Varian Aerograph Models 90P and 711. All reactions were carried out under pure nitrogen.

2,3-Dimethyl-1,3-butadiene (*1a*) and 2,5-dimethyl-2,4-hexadiene (*1b*) are commercial products and were used without further purification, whereas *E,E*-2,2,3,6,7,7-hexamethyl-3,5-octadiene (*1c*) was prepared as previously described.[2] The purity as determined by GLC was in no case less than 98 %.

Preparation of bis-adducts. The bis-adducts were prepared from the dienes, potassium *tert*-butoxide and haloform following the procedure of Kleveland and Skattebøl.[2]

2,2,2',2'-Tetrabromo-1,1'-dimethylbicyclopropyl (2a). Bis-adduct *2a* was isolated in 31 % yield, m.p. 99 °C from ethanol (lit.[1] m.p. 96–99 °C). The product was shown to be homogeneous by TLC (silica gel; light petroleum (40–65 °C), carbon tetrachloride, benzene, ether, ethyl acetate and methanol), a conclusion supported by its NMR spectra in different solvents.[1]

2,2,2',2'-Tetrabromo-3,3,3',3'-tetramethylbicyclopropyl (2b). From the reaction of diene *1b* and dibromocarbene adduct *2b* was isolated in 65 % yield, m.p. 109 °C from ethanol (lit.[1] m.p. 109 °C). This product was shown to be a mixture of isomers by TLC [silicagel, light petroleum (40–65 °C); R_F-values 0.39 and 0.55 (predominant)] and these were separated by column chromatography [silica gel (40–60 mesh), light petroleum (40–65 °C)]. After recrystallization from benzene the compounds gave sharp melting points, 121–122 °C for the predominant form and 135–136 °C for the other. ^{1}H NMR (60 MHz, CCl_4; the lower-melting compound): δ 1.19 (2 H, s), 1.43 (6 H, s), 1.52 (6 H, s). ^{1}H NMR (60 MHz, CCl_4; the higher-melting compound): δ 1.23 (2 H, s), 1.32 (6 H, s), 1.48 (6 H, s). The IR spectra of the two compounds as solids are almost identical but different in carbon tetrachloride. Their mass spectra did not show any significant differences; in no case was the molecular ion observed and the highest observable mass was a result of extensive fragmentation.

According to its NMR spectrum (100 MHz, CS_2) bis-adduct *2b* is composed of 70 % of the lower-melting and 30 % of the higher-melting form.

E,E-2,2,2',2'-Tetrabromo-3,3'-di-tert-butyl-3,3'-dimethylbicyclopropyl (2c). Bicyclopropyl *2c* was isolated in a yield of 25 %, m.p. 142 °C from ethanol (lit.[2] m.p. 143 °C).

2,2,2',2'-Tetrachloro-3,3,3',3'-tetramethylbicyclopropyl (3b). From the reaction of diene *1b* with dichlorocarbene adduct *3b* was isolated

in 80 % yield, m.p. 79 – 80 °C from light petroleum (40 – 65 °C) (lit.[19] m.p. 78 °C). The product could not be analyzed by TLC because no compound was visible by any means even after a short distance of migration. However, GLC analysis (30 % Apiezon L, 170 °C) of an acetone solution of the bis-adduct disclosed that the compound was an isomeric mixture; the isomer with the shorter retention time amounts to 75 % and the other to 25 % of the mixture. The isomers were separated by preparative GLC (30 % Apiezon L, 170 °C) and after recrystallization from acetone, the isomer with the shorter retention time melted at 89.5 – 90 °C, whereas the other melted at 102 – 102.5 °C. The IR spectra of the isomers as solids are different as well as those in carbon tetrachloride. ^{1}H NMR (60 MHz, CS_2; the lower-melting form): δ 1.03 (2 H, s), 1.32 (6 H, s), 1.41 (6 H, s). ^{1}H NMR (60 MHz, CS_2; the higher-melting form): δ 1.11 (2 H, s), 1.19 (6 H, s), 1.37 (6 H, s). The NMR spectrum (100 MHz, CS_2) of bis-adduct *3b* confirms the ratio between the stereoisomers (3:1).

Reaction of diene 1c *with dichlorocarbene.* Reaction of potassium *tert*-butoxide [from 4.3 g (0.11 mol) of potassium], 14.3 g (0.12 mol) of chloroform and 7.7 g (0.04 mol) of diene *1c* in 100 ml of dry pentane gave, except 3.5 g (46 %) of unreacted starting material, 3.1 g of a liquid, b.p. 64 – 68 °C (0.4 mmHg), which consisted essentially of two isomers with composition $C_{15}H_{25}Cl$ in a ratio of 3:2 as shown by mass spectrometry and GLC; the structures of these isomers have not yet been elucidated.

Dipole moments. Dielectric constants were measured at 25 °C in a Weilheim Dipolmeter DM01 on four different solutions in benzene. Refractive indices were measured on the same solutions in a Brice-Phoenix Differential Refractometer. Calculation of dipole moments was performed according to Hedestrand,[20] using no correction for atomic polarization.

X-Ray structure determination of 3b. Single crystals were prepared by recrystallization from pentane; a specimen of approximate dimensions $0.25 \times 0.25 \times 0.05$ mm^3 was used for the X-ray experiments.

Determination of unit cell dimensions and collection of intensity data were carried out on a SYNTEX $P\bar{1}$ four-circle diffractometer at – 160 °C using graphite crystal monochromated Mo$K\alpha$-radiation. The $\theta/2\theta$ scanning mode was employed with scan speeds (2θ) of 2 – 8° min^{-1} depending on the peak intensity. The scan range was from 1.1° below $2\theta(\alpha_1)$ to 1.1° above $2\theta(\alpha_2)$ and background counts were taken for 0.35 times the scan time at each of the scan limits. Three standard reflections were measured for every 40 reflections; they showed a systematic fluctuation and the data were accordingly adjusted. All unique reflections with $2\theta < 45°$ were recorded; for reflections with $45° < 2\theta < 75°$ the intensities were measured only if larger than a preset value according to a quick scan. The standard deviations were taken as $\sigma(I) = [C_T + (0.05C_N)^2]^{\frac{1}{2}}$, where C_T is the total number of counts and C_N is the net count.

2244 reflections with $\sin\theta/\lambda < 0.85$ Å^{-1} and intensities larger than $2\sigma(I)$ were used in the structure determination. They were corrected for Lorentz and polarization effects and for absorption.

The atomic form factors were those of Doyle and Turner [21] for chlorine and carbon atoms and of Stewart, Davidson and Simpson [22] for hydrogen. Computer programs used in the structure determination are described in Ref. 23. The full-matrix least-squares program employed minimizes the quantity $\sum w\Delta F^2$ where w is the inverse of the variance of the observed

Table 4. Fractional atomic coordinates for the heavy atoms (× 10^4) and for hydrogen atoms (× 10^3). The thermal parameters (× 10^4) are given according to the expression exp $-(B11h^2 + B22k^2 + B33l^2 + B12hk + B13hl + B23kl)$; for hydrogen atoms the common B-value is 2.7 Å^2.

Atom	x	y	z	$B11$	$B22$	$B33$	$B12$	$B13$	$B23$
Cl1	9091(1)	2732(1)	1754(1)	164(2)	123(1)	85(1)	152(2)	79(2)	45(1)
Cl2	4555(1)	1821(1)	2187(1)	159(2)	88(1)	100(1)	60(2)	99(2)	73(1)
C1	6262(3)	5135(3)	478(2)	132(5)	122(4)	54(2)	90(7)	59(6)	65(4)
C2	6869(3)	3745(3)	1770(2)	126(5)	88(3)	60(2)	79(6)	60(5)	42(4)
C3	7572(3)	6094(3)	2439(2)	133(5)	89(3)	59(2)	75(7)	47(5)	40(4)
C4	10188(4)	7529(3)	3046(3)	146(6)	105(4)	95(3)	53(8)	39(7)	43(5)
C5	6262(4)	6755(3)	3471(3)	187(7)	116(4)	78(3)	124(8)	80(7)	19(5)

Atom	x	y	z	Atom	x	y	z
HC1	723(4)	544(4)	– 20(3)	H1C5	453(5)	583(4)	311(3)
H1C4	1092(5)	710(4)	230(4)	H2C5	630(5)	819(5)	335(4)
H2C4	1109(5)	742(4)	420(4)	H3C5	698(5)	682(4)	467(4)
H3C4	1033(5)	901(5)	309(4)				

structure factors.

Crystal data: 2,2,2′,2′-tetrachloro-3,3,3′,3′-tetramethylbicyclopropyl, $C_{10}H_{14}Cl_4$, triclinic. $a = 6.352(3)$ Å; $b = 6.743(4)$ Å; $c = 8.249(4)$ Å; $\alpha = 93.63(4)°$; $\beta = 108.97(4)°$; $\gamma = 109.34(4)°$; $V = 309.4$ Å^3. ($T = 110$ K) Mol.wt. 276.03. $Z = 2$. $D_{calc} = 1.48$ gcm^{-3}, $\mu(MoK\alpha) = 9.1$ cm^{-1}. Space group $P\bar{1}$ (No. 2).

The structure was solved by Patterson methods and refined by Fourier and least-squares methods. Final refinements including all positional parameters, anisotropic thermal parameters for the heavy atoms and a common isotropic thermal parameter for hydrogen atoms yielded a conventional R-factor of 0.051, $R_w = 0.066$ and goodness of fit $(S = (\sum w \Delta F^2/(n-m)^{\frac{1}{2}}) = 1.92$. The parameters with estimated standard deviations are given in Table 4; the structure factor listing is available from the authors. An analysis of the thermal parameters in terms of rigid-body motion of one half of the molecule (r.m.s. $\Delta U = 5 \times 10^{-4}$ Å^2) served as the basis for correction of bond lengths for libration.

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Studies of Pig Kidney Diamine Oxidase

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Preparations of pig kidney histaminase were analyzed for total compositions, *N*-terminal residues, patterns of tryptic peptides and for subunits under dissociating conditions. The crystalline enzyme is pure by several criteria but not completely homogeneous. It is rich in acidic or amidated residues and has a low content of cysteine/halfcystine. Preparation analyzed consistently contained a few percent of carbohydrate. Subunits are concluded to be identical or highly similar with a molecular weight of about 90 000, which is consistent with other analyses.

Pig kidney histaminase or diamine oxidase (diamine: oxygen oxidoreductase, EC 1.4.3.6) has been crystallized [1] and is known to contain pyridoxal phosphate and copper.[1–5] From the metal content, a minimum molecular weight of about 87 000 was determined [1–3] but the whole enzyme is larger,[3] probably twice this size.[1] Various amine oxidases differ in molecular weights, in subunit arrangements and in cofactor contents (flavin, copper, pyridoxal phosphate) but few other structural characteristics are known. Similarities in monomer sizes (80 000 – 100 000) between different amine oxidases have been suggested [3] but the subunits of some other flavin-linked oxidases are smaller.[6]

In the present work, crystalline enzyme preparations were analyzed for total compositions, *N*-terminal residues and subunit sizes. The result give further characteristics of the enzyme, and conclusions from these chemical analyses are compatible with those obtained from physicochemical and metal analyses.

MATERIALS AND METHODS

Enzyme protein. The enzyme was purified from fresh pig kidney and crystallized as previously described.[1] The crystalline preparation migrated as a single band on agarose gel electrophoresis, pH 8.6, but in some preparations a minor, more acidic component was also present. Histaminase activity was determined by incubation of the sample with [ring-2-^{14}C]labelled histamine for 15 min at 37 °C in 0.04 M sodium phosphate buffer, pH 7.5, and subsequent separation of substrate and reaction products on phosphocellulose columns,[7] from which eluted fractions were measured in a Packard Tri-Carb liquid scintillation spectrometer. Total protein was determined by weights of samples dried at 105 °C. The crystalline enzyme preparations converted 0.33 μmol of histamine per min and mg protein.

Analyses. Samples for amino acid analysis were hydrolyzed in evacuated tubes with 6 M HCl containing 1 % phenol, at 110 °C for 20, 48 and 72 h, and analyzed on a Beckman Unichrom amino acid analyzer. Cysteic acid was determined after oxidation with performic acid [8] and tryptophan after hydrolysis with 4 M methanesulfonic acid containing 0.2 % 3-(2-aminoethyl)indole.[9,10] Neutral monosaccharides were determined with the orcinol-H_2SO_4 method [11] and identified by thin-layer chromatography on silica gel (20 × 20 cm, Merck AG) in 1-propanol:water:2-butanone (2:1:1, v./v.) after hydrolysis in 0.5 M H_2SO_4 at 100 °C for 8 h. Glucosamine was determined with the amino acid analyzer and sialic acid with the thiobarbituric acid method.[12]

Reduction of histaminase was performed in 8 M urea, 0.1 M Tris, 2 mM EDTA, pH 8.1 (10 mg protein/ml) with dithiothreitol (0.08 μg/mg protein) for 2 h at 37 °C, and subsequent carboxymethylation with iodoacetate (1.5 μmol/mg protein) under identical conditions. Reagents were removed by dialysis against distilled water. *N*-Terminal residues were determined by the dansyl method after coupling in 8 M urea.[13] Electrophoresis in 10 % polyacrylamide slab gels containing 0.1 % sodium dodecylsulfate,[14] and chromatography on Sepharose 4B in buffered 5 M guanidine.HCl[15] were performed as previously described. Tryptic peptides were mapped by different steps of

Table 1. Composition of a crystalline histaminase preparation analyzed by different methods as given in the text.

Component	%[a]	Component	%[a]
Aspartic acid/Asparagine	8.4	Phenylalanine	5.1
Threonine	5.5	Lysine	4.2
Serine	5.8	Histidine	2.9
Glutamic acid/Glutamine	12.2	Arginine	5.1
Proline	6.7	Cysteine/Half-cystine	1.5
Glycine	7.8	Tryptophan	1.2
Alanine	7.2	Galactose	0.4
Valine	7.0	Mannose	1.0
Methionine	1.8	Fucose	0.2
Isoleucine	4.0	*N*-Acetylglucosamine	1.4
Leucine	9.5	*N*-Acetylneuraminic acid	0.1
Tyrosine	4.1		

[a] Content per 100 amino acid residues.

electrophoresis and chromatography on paper [16] or by two-dimensional thin-layer separation on cellulose.[14]

RESULTS AND DISCUSSION

Purity and dissociation experiments. The enzyme is crystalline and pink in colour. The best preparations moved as a single band on agarose gel electrophoresis at pH 8.6, revealed only one *N*-terminus (below) and are previously known to be homogeneous in the ultracentrifuge.[1] These results suggest that the histaminase preparations are reasonably pure. The subunit composition was analyzed on the carboxymethylated protein under dissociating conditions by sodium dodecylsulfate polyacrylamide gel electrophoresis and by Sepharose chromatography in guanidine.HCl. In both cases, one major component was obtained, with a molecular weight against marker proteins (β-galactosidase, adenovirus hexon, bovine serum albumin, and horse liver alcohol dehydrogenase) of about 85 000-90 000. The protein was not completely homogeneous and there was some variation between different preparations. Apart from trace amounts of different components, a minor component with an apparent molecular weight of 40 000 was present. The origin of this fragment is unclear. It may be unrelated to the enzyme or, due to the variability, proteolytically obtained.

Analytical results. The total compositions of the enzyme preparation calculated from different hydrolysates is shown in Table 1. Values for serine and threonine were extrapolated to zero time (addition of 4 and 3 %, respectively, to the 20 h values), those for valine and isoleucine are the maximum yields, whereas those for remaining residues are average values. Different preparations showed highly similar compositions, including carbohydrate and the less abundant residues. The results show that the histaminase preparation is rich in acidic or amidated residues and contains small amounts of carbohydrate.

End-group analysis of the carboxymethylated protein in 8 M urea with the dansyl method [13] revealed the presence of *N*-terminal threonine apart from trace amounts in variable yield of a few other residues (Gly, Ser, Leu). If the possibility of blocked polypeptide chains is disregarded, this result is compatible with an essentially pure protein preparation containing *N*-terminal threonine.

Mapping of tryptic peptides was performed on cellulose thin-layer and on paper. All spots, especially the neutral ones, were not resolved on thin-layer but on paper at least 55 spots (26 basic, 9 acidic, 20 neutral) were revealed. With the ^{14}C-carboxymethylated protein, 11 of these were radioactively labelled. Due to partial cleavages, overlapping of peptide positions, and presence of weakly staining peptides,

correlations between peptide mapping and total compositions are extremely difficult with large proteins [15,17] and all fragments are usually not detected. Nevertheless, the number of total and labelled tryptic peptides detected, compared with the content of lysine, arginine and halfcystine/cysteine in the enzyme (Table 1) is compatible with the presence of one unique monomer with a size similar to that of the 90 000 dalton component shown in the dissociation experiments.

Structural characteristics. The present results show that crystalline histaminase is pure by several criteria but not completely homogeneous. The enzyme is rich in dicarboxylic residues and has a low content of cysteine/half cystine. Carbohydrate was consistently found in the preparations, and if histaminase-bound would correspond to a glycoprotein with a few percent carbohydrate. A protein subunit with a molecular weight of 90 000 is demonstrated by electrophoresis and chromatography under dissociating conditions. This is compatible with the results of peptide mapping and of analysis for total composition, which combined further suggest that all subunits are identical or highly similar. These data support conclusions from previous determinations of only copper and pyridoxal phosphate in the protein [1-5] and are consistent with analyses of another diamine oxidase.[18]

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Thermal Reactions of *erythro*- and *threo*-1-(1-Acetoxyethyl)-indene.[1] Competing Epimerisation, Rearrangement and Acetic Acid Elimination

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Thermally induced hydrogen migrations competing with 1,2-elimination of acetic acid have been studied. Thus, when one of the diastereomeric 1-(1-acetoxyethyl)indenes (*1*) was pyrolysed (neat) at 182 °C, not only the olefin *trans*-1-ethylideneindene (*4a*) was produced, but even the epimer (*2*) and the rearranged ester 3-(1-acetoxyethyl)indene (*3*) were obtained along with starting material. The other diasteromer (*2*) gave both *trans*- and *cis*-olefin (*4a* and *4b*) together with the three isomeric acetates. Compound *3* also produced the olefins and the epimers, but the rearrangement of this compound was slow. By deuterium-labeling experiments, a common dipolar intermediate for the reactions can be ruled out. The results indicate that the epimerisations and the 1,3-proton transfer rearrangements proceed with suprafacial [1,5]-sigmatropic shifts. The kinetic deuterium isotope effect of the disappearance of *1* was determined to be 2.8 ± 0.6.

Thermal suprafacial [1,5]-sigmatropic shifts of hydrogen have been shown in the indene system by deuterium-labeling experiments,[2,3] by isolation of the indene-maleic anhydride adduct,[3,4] and by studying isomerisation and racemisation of an optically active indene derivative.[5] These experiments strongly support the intervention of an isoindene intermediate in the hydrogen migrations. Studies have also been made of other migrating groups. Thus, hydrogen has been shown to migrate much faster than phenyl which in turn migrates faster than methyl.[6]

Pyrolysis of alkyl acetates has been reviewed by Saunders and Cockerill.[7] The elimination of acetic acid proceeds *syn*-stereospecificially *via* a cyclic six-membered transition state.

Bordwell and Landis [8] have explained the non-stereospecific Chugaev reactions of *erythro*- and *threo*-3-*p*-tolylsulfonyl-2-butyl-*S*-methyl xanthate which produce the *trans*- and *cis*-olefins in different proportions by a mechanism involving a dipolar ion intermediate. Internal rotation leads to an interconversion of the *cis*- and *trans*-form of the dipolar intermediate and formation of the more stable olefin as the main product.

Taylor *et al.*[9] have shown that some charge separation occurs in the transition state of acetate pyrolysis. They studied the elimination of acetic acid from 1-arylethyl acetates in the gas phase at 600 K.

Rearrangement seldom accompanies elimination in acetate pyrolysis. However, when the substrate lacks a β-hydrogen rearrangement has been observed.[7] Isomerisation prior to elimination has been proposed to explain that the same product mixture of 1,3- and 2,4-heptadiene was obtained from 2-acetoxy-3-heptene and 4-acetoxy-2-heptene.[10]

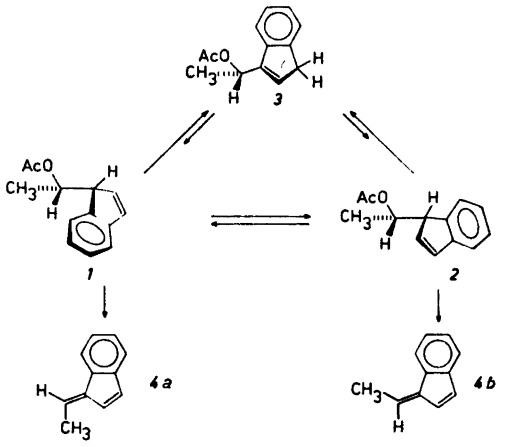
Few isotope effects of pyrolysis of esters have been reported.[11–13] Depuy, King and Froemsdorf [11] found a kinetic deuterium isotope effect of 1.9 at 400 °C and 1.7 at 500 °C for the pyrolysis of 2,2,4,4-d_4-1-methylcyclohexyl acetate.

Recently, Bock has shown that thermal 1,4-elimination of acetic acid from some 3-acetoxy-1,4-cyclohexadien derivatives proceeds stereochemically *syn*.[14] However, multistep reactions could not be excluded.

The present study was initiated in order to establish the correlation between the two diastereomeric 1-(1-acetoxyethyl)indenes and the structures *1* and *2* in Scheme 1. However, we found that thermally induced hydrogen migrations to epimerised and rearranged material compete with elimination of acetic acid. Therefore we decided to test the hypothesis that the reactions proceed *via* a common (dipolar) intermediate.

RESULTS AND DISCUSSION

When one of the diastereomeric 1-(1-acetoxyethyl)indenes (*1*) was pyrolysed (neat) at 182 °C, the olefin *trans*-1-ethylideneindene (*4a*) was formed. Along with the olefin and starting material, the epimer (*2*) and the rearranged ester 3-(1-acetoxyethyl)indene (*3*) were also obtained. The other diastereomer (*2*) gave both *trans*- and *cis*-olefin (*4a* and *4b*) together with the two isomeric acetates (Table 1). Compound *3* was also shown to produce a mixture of the diastereomers and the olefins.



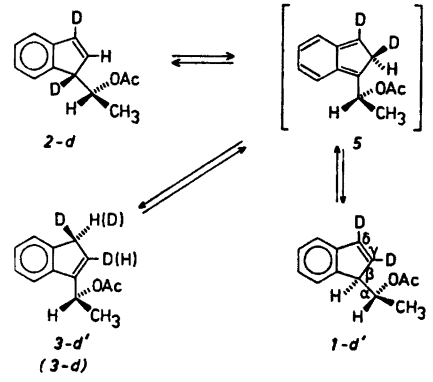
Scheme 1.

The above experiments made it possible to assign the structure *1* in Scheme 1 to the diastereomer *1* and the structure *2* to diastereomer *2*. These assignments were also suggested by studies of methoxide-promoted eliminations, because *anti*-elimination from *2* was favoured over *syn*-elimination (92 % *anti* and 8 % *syn*).[15]

By using specifically deuterated substrate (*2-d*) in the reactions, we found that the epimerisation reaction does not proceed *via* an intermediate internal ion pair. Instead, the results indicate that the epimerised material is formed mainly by suprafacial [1,5]-sigmatropic shifts *via* the isoindene intermediate *5* (Scheme 2). This was established by isolating the *1-d'* (by HPLC) formed from *2-d* after about 50 % reaction and determining the content of protium in the β-, γ- and δ-positions of *1-d'*. The result was (as determined by ^{1}H NMR spectroscopy with the α-proton as a reference): 91 ± 10 atom-% H in the β-position and 0 ± 10 atom-% H in the γ- and δ-positions, respectively. The corresponding result for isolated starting material *2-d* was 0 ± 10 atom-% H in the β-position. This experiment indicates that the hydrogen migrations have a common intermediate of isoindene-type formed by [1,5]-sigmatropic shifts.

Table 1. Product compositions obtained from thermal reactions with *1*, *2* and *3* at 181.6 ± 0.4 °C (^{1}H NMR).

Run No.	Substrate	Time/min	Result/mol-% *1*	*2*	*3*	*4a*	*4b*
1	*1*	75	80.1	2.4	14.5	2.9	–
2	*1*	93	77.1	3.3	16.4	3.2	–
3	*1*	185	60.6	5.8	30.1	3.5	–
4	*1*	226	50.4	10.5	34.9	4.2	–
5	*2*	226	10.2	41.2	45.4	1.6	1.6
6	*2*	226	8.6	43.6	44.0	1.6	2.2
7	*3*	125	2.1	2.2	95.0	0.3	0.3
8	*3*	226	3.0	3.4	92.8	0.5	0.3
9	*3*	375	4.8	5.3	89.1	0.5	0.3
10	*1-d*	216	79.8	2.7	14.8	2.7	–
11	*1-d*	228	79.1	3.1	14.6	3.2	–
12	*2-d*	420	8.8	51.5	37.7	1.0	1.0
13	*2-d*	462	9.5	49.9	39.0	0.8	0.8



Scheme 2.

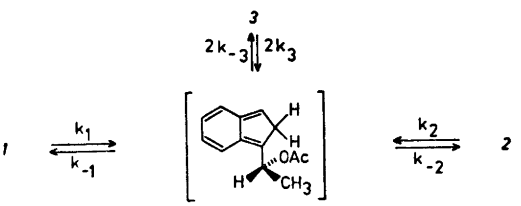
The rate constant of the disappearance of *1* in the thermolysis was determined to be $(4.80 \pm 0.48) \times 10^{-5}$ s^{-1}, and of *1-d* to be $(1.73 \pm 0.17) \times 10^{-5}$ s^{-1} from the data in the table (run Nos. 1, 2, 10, and 11). Accordingly, the kinetic isotope effect is 2.8 ± 0.6, which can be considered to be the isotope effect of the isomerisation of *1*, since elimination is a much slower process. The theoretical maximal isotope effect at 182 °C is ≈ 4.5.[16] An effect smaller than this can be explained with a transition state which is highly asymmetric and/or non-linear with respect to hydrogen migration. A mechanism involving an intermediate which returns to starting material faster than it goes on to product can be ruled out, because analysis of the reaction rates, which can be calculated from the data in Table 1, shows that the isotope effect of the intermediate-forming step is only slightly decreased due to reversibility. The transition states of 1,5-sigmatropic shifts in the indene system must be non-linear due to the rigidity of the ring system. The [1,5]-sigmatropic shift in 1,3-pentadiene has a much larger deuterium isotope effect,[17] 5.4 at 185 °C which includes secondary isotope effects, but in this molecule the transition state has the possibility of being somewhat more linear.

The isotope effect which we have reported above is of similar magnitude to the isotope effect (≈ 3 at 140 °C) determined by Almy and Cram for the rearrangement of 1-methyl-3-*tert*-butylindene.[5] They also found an isotope effect of 2.7 – 2.9 for the reaction of the isoindene intermediate to products by studying the deuterium content of the formed materials.

The equilibrium constants at the reaction temperature were determined to be $[3]_{eq}/[1]_{eq} = 9.6 \pm 2.0$ and $[3]_{eq}/[2]_{eq} = 9.6 \pm 2.0$. These values are considerably lower than those obtained in methanol at 30 °C.[15]

The collapse ratios of the isomerisation reactions could be calculated by assuming a common isoindene intermediate: $k_{-2}/k_{-1} \approx 1$ and $k_{-3}/k_{-1} \approx 2.5$ (Scheme 3). These figures can be compared with Almy and Crams collapse ratio of ≈ 1.[5]

The elimination of acetic acid from *1* is a stereospecific *syn*-elimination (Table 1 and Scheme 1). Only the presumably more stable olefin *4a* is formed. The other epimer *2* produces both of the olefins. Isolated olefin mixture (from run No. 6) was obtained by HPLC and analysed by ^{1}H NMR spectroscopy, which showed about 40 % *4a* and 60 % *4b*. However, at least the main part of the olefin *4a* must have been formed *via* epimerised material, and there is no experimental indication that *2* gives *4a* by a route not involving *1*. This is also supported by an experiment in which *2-d* was reacted about 25 % and the olefins separated from the reaction mixture. Analysis by mass spectroscopy showed a ratio of the mol peaks *m/e* 143 and 144 of 2, *i.e.* about 30 % of the olefins must have been formed from *1-d'*.



Scheme 3.

No interconversion of *4a* and *4b* during the reactions could be detected.

The compound *3* produced the olefins at a low rate (Table 1). Multicenter, 1,4-elimination reactions are thermally allowed.[14] However, the distance between the single-bonded oxygen and the δ-protons in *3* is large, and the rate of a reaction following this mechanism can therefore be expected to be very low. A mechanism in which the acetoxy group picks up a δ-proton and gives a dipolar intermediate is also improbable due to the large distance. The olefins have probably been formed instead *via* the rearranged materials *1* and *2*.

It is interesting to note that a common intermediate has been found also for other reactions with similar compounds. Thus we have found strong indications for the intermediacy of a common ion pair in base-catalysed 1,3-proton transfer competing with 1,2- and 1,4-elimination reactions in protic solvents with tertiary amines as bases.[18] The substrates were 1- and 3-(2-acetoxy-2-propyl)indene.

EXPERIMENTAL

General

The ^{1}H NMR spectra were obtained with Varian A 60D and JEOL FX 60 NMR spectrometers. The HPLC was performed with a Waters

6000A solvent delivery system and 440 absorbance detector. The purity of the acetates was checked with HPLC using a silica column (Waters μPorasil 0.30 m).

Syntheses

1-(1-Hydroxyethyl)indene. A diastereomeric mixture of the alcohols was prepared according to the method given by Courtot.[19] The alcohol (*1-OH*) corresponding to the acetate *1* was formed predominantly (≈70 % of the mixture). Pure *1-OH* was obtained by recrystallization of the distilled product twice from light petroleum. The other diastereomer (*2-OH*) was separated from the remaining oil (containing 35 % *1-OH* and 65 % *2-OH*) by liquid chromatography on a Sephadex LH-20 column with 80 % 1,2-dichloroethane and 20 % cyclohexane as solvent. The pure *2-OH* crystallized in the refrigerator.

3-(1-Hydroxyethyl)indene. In 110 g of dry pyridine 55.0 g (0.343 mol) of 1-(1-hydroxyethyl)indene (diastereomeric mixture) was dissolved. The reaction mixture was heated under dry nitrogen, and after 20 h at 65 °C, an equilibrium mixture was obtained containing about 4 % of each of the diastereomer along with the rearranged alcohol and traces of eliminated material. The product mixture was poured onto ice, and after addition of 200 ml of water, extraction was performed with 3 × 200 ml of ether. The combined ether extracts were washed, first with 100 ml of water, then with 2 M HCl until acidic, and finally with four further portions of HCl. The organic phase was washed with water until neutral and then with saturated sodium chloride solution. After drying over sodium sulfate the ether was evaporated and the residue flash-distilled in vacuum. The final purification of the rearranged alcohol was performed on a split-tube column (Fischer HMS 300) at 13 Pa. The high-boiling fraction was pure and free from isomers and eliminated material, as shown by ^{1}H NMR spectroscopy.

1-(1-Acetoxyethyl)indene (1). In 23.9 g (0.234 mol) of acetic anhydride 8.26 g (0.0516 mol) of diastereomerically pure 1-(1-hydroxyethyl)-indene (*1-OH*) and 0.25 g of anhydrous zinc(II) chloride were dissolved. The reaction mixture, which was stirred under dry nitrogen, became warm, and after 2 h no trace of unreacted starting material could be detected with ^{1}H NMR spectroscopy. The reaction solution was diluted with 50 ml of benzene, washed with 3 × 5 ml of water and dried over magnesium sulfate. After evaporation of the benzene and unreacted acetic anhydride, the residue was distilled in vacuum through a short Vigreux column. Yield 7.2 g (69 %), b.p. 90.5–91.5 °C/7 Pa, m.p. 31–32.5 °C. ^{1}H NMR spectrum and HPLC showed the compound to be pure and free from isomerised and eliminated material. MS (IP 12 eV): *m/e* 202 (M). ^{1}H NMR (60 MHz, CCl_4): δ 0.87 (3 H, d, *J* 6.2 Hz), 1.97 (3 H, s), 3.67 (1 H, d, *J* 5.7 Hz and further small coupling), 5.23 (1 H, p, *J* 6.2 Hz), 6.33 (1 H, dd, *J* 5.6 Hz and 1.9 Hz), 6.74 (1 H, dd, *J* 5.6 Hz and 1.9 Hz), 6.88–7.35 (4 H, complex).

1-(1-Acetoxyethyl)indene (2) was prepared from *2-OH* according to the method above. The product, which was a liquid at room temperature, had the same b.p. as its diastereomeric isomer, and was pure and free from isomerised and eliminated material as shown by ^{1}H NMR spectroscopy and HPLC. MS (IP 12 eV): *m/e* 202 (M). ^{1}H NMR (60 MHz, CCl_4): 0.90 (3 H, d, *J* 6.2 Hz), 1.91 (3 H, s), 3.64 (1 H, p, *J* 4.6 Hz and 1.9 Hz), 5.28 (1 H, qd, *J* 6.2 Hz and 4.6 Hz), 6.22 (1 H, dd, *J* 5.5 Hz and 1.9 Hz), 7.49 (1 H, dd, *J* 5.5 Hz and 1.9 Hz), 6.81–7.41 (4 H, complex).

3-(1-Acetoxyethyl)indene (3). In 60 ml of acetic anhydride 36.2 g (0.226 mol) of 3-(1-hydroxyethyl)indene and 0.5 g of anhydrous zinc(II) chloride were dissolved. The reaction mixture was stirred under dry nitrogen for 4 h, 100 ml of benzene was added and the solution washed with 3 × 10 ml of water and finally dried over magnesium sulfate. The benzene and the excess of acetic anhydride were removed in vacuum and the residue distilled through a short Vigreux column. Yield 32.9 g (72 %), b.p. 90–92 °C/13 Pa. ^{1}H NMR showed that the compound was pure and free from isomerised and eliminated material. MS (IP 12 eV): *m/e* 202 (M). ^{1}H NMR (60 MHz, CCl_4): δ 1.51 (3 H, d, *J* 6.5 Hz), 1.92 (3 H, s), 3.19 (2 H, t, *J* very small), 5.84 (1 H, qd, *J* 6.5 Hz and 1.1 Hz), 6.23 (1 H, q, *J* 1.1 Hz), 6.90–7.30 (4 H, complex).

Mixture of trans- *and* cis-*ethylideneindene (4a and 4b).* Synthesis of this compound has been reported previously by Courtot.[20]

In this work the olefins were prepared for calibration purposes from *1* by elimination with potassium *tert*-butoxide in *tert*-butyl alcohol. After one distillation, b.p. 66–67 °C/13 Pa, a pure mixture of the *trans*- and *cis*-olefins was obtained (88 % *4a* and 12 % *4b*). MS (IP 19 eV): *m/e* 142 (M).

1,3-d$_2$-1-(Hydroxyethyl)indene. The preparation of 1,1,3-*d*$_3$-indene has been described previously.[18c]

To 0.36 mol BuLi in hexane/ether 41.0 g (0.344 mol) of 1,1,3-*d*$_3$-indene (containing 98.7 ± 1.0 atom-% D in the 1 and 3 positions) was added dropwise under dry nitrogen. The temperature was kept below −50 °C for 1.5 h, and then 45.0 g (1.02 mol) of acetaldehyde was added dropwise to the cooled solution. After the addition the reaction mixture was stirred for 5 min and then poured slowly into a stirred mixture of 2 M HCl (excess) and ice. The water phase was extracted with 2 × 100 ml of ether. The combined organic phases were washed first with water until neutral and then with saturated sodium chloride solution, and

were finally dried over magnesium sulfate. Evaporation of the ether and distillation of the products through a short Vigreux column gave a viscous liquid containing 56 % of the alcohol corresponding to *1-d* and 44 % of the other diastereomer. The liquid was dissolved in light petroleum, and *1-d-OH* crystallized from the solution. The crystalline product, which was recrystallized once, was pure and free from rearranged, epimerised and eliminated material as shown by ^{1}H NMR spectroscopy.

The other diastereomer (*2-d-OH*) was separated from the remaining oil by LC, and a product free from rearranged, epimerised and eliminated material and any other impurity was obtained. Yield (before separation of the diastereomers) 35.2 g (63 %), b.p. 98 – 102 °C/27 Pa.

1,1-d_2*-3-(1-Hydroxyethyl)indene* was prepared from the previous compound and purified according to the method for the corresponding protium compound, but the reaction time was increased to 120 h at 60 °C. The isomeric purity of the distilled product was > 99.5 %, and the compound was free from other impurities detectable with ^{1}H NMR.

1,3-d_2*-1-(1-Acetoxyethyl)indene (*1-d*)*. In 25 ml acetic anhydride 7.0 g (0.0432 mol) of diastereomerically pure 1,3-d_2-1-(hydroxyethyl)-indene (*1-d-OH*) was dissolved and 0.25 g of anhydrous zinc(II) chloride was added. The mixture was stirred under dry nitrogen for 3 h and then 50 ml of benzene was added. The solution was washed with 3 × 5 ml of water and dried over magnesium sulfate. The benzene and unreacted acetic anhydride were removed in vacuum and the residue was distilled through a short Vigreux column. Yield 6.4 g (73 %), b.p. 83 – 84 °C/9 Pa. The deuterium content was 98.2 ± 1.0 atom-% (in the 1 and 3 positions, ^{1}H NMR). HPLC and ^{1}H NMR showed the compound to be pure and free from isomerised and eliminated material.

1,3-d_2*-1-(1-Acetoxyethyl)indene (*2-d*)* was prepared from *2-d-OH* according to the method above. Also this acetate was pure and free from isomerised and eliminated material as shown by ^{1}H NMR spectroscopy and HPLC. The deuterium content was the same as in *1-d*.

1,1-d_2*-3-(1-Acetoxyethyl)indene (*3-d*)*. In 20 ml of acetic anhydride 5.0 g (0.0308 mol) of 1,1-d_2-3-(1-hydroxyethyl)indene was dissolved, and 0.20 g of anhydrous zinc(II) chloride was added. The mixture was stirred under dry nitrogen for 12 h, and then 50 ml of benzene was added. The solution was washed with 3 × 5 ml of water and dried over magnesium sulfate. The benzene and the unreacted acetic anhydride were removed in vacuum and the residue was distilled through a short Vigreux column. Yield 4.5 g (71 %), b.p. 90 – 92 °C/13 Pa. The deuterium content was 98.2 ± 1.0 atom-% in the 1 position (^{1}H NMR). No trace of isomerised or eliminated material or any other impurity could be detected.

Thermal reactions

Base-catalysed 1,3-proton transfer rearrangements of the substrates together with base-promoted elimination of acetic acid occur at much lower temperatures.[15] Therefore, the equipment was thoroughly washed to remove traces of base.[5] Furthermore, the acetic acid formed in the thermal eliminations suppresses the base concentration. Polymer was formed after long reaction times. However, when the reaction from *1* was stopped after about 50 % reaction, less than 3 mol-% of the material had been polymerised.

Pyrex tubes (3 mm i.d.) were sealed at one end, cleaned with chromic acid, and then rinsed with water, dilute ammonium hydroxide, distilled water, dilute acetic acid and again with distilled water before drying at 130 °C at least over night. The samples were added to the tubes, which were then degassed four times under 13 Pa. Dry nitrogen was introduced between the evacuations. The tubes were sealed under vacuum and placed in a silicone oil bath at 181.6 ± 0.4 °C. After the appropiate time the reactions were quenched by dipping the tubes in light petroleum. The reaction mixtures were dissolved in CCl_4 and analysed by ^{1}H NMR spectroscopy. The temperature was measured with a calibrated mercury thermometer.

The isolation of *1-d'* was performed with reversed phase HPLC (recycling on a Waters 610 × 7 mm C_{18}/Porasil B column, 40 % by weight ethanol-water solution, 280 nm). The purity of the chromatographic fractions was checked on a Waters μBondapak C_{18} analytical column (0.30 m).

The rate constants were obtained by one-point kinetics (run Nos. 1, 2, 10, and 11 in Table 1). The estimated errors are maximal errors including random errors and maximal systematic errors calculated considering the maximal errors in the concentration determinations of *1* and *1-d* to be ± 2 mol-%.

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N-Terminal Amino Acid Sequences of Pepsinogens from Dogfish and Seal and of Bovine Pepsinogen B

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The *N*-terminal amino acid sequences of dogfish pepsinogen (8 residues), seal pepsinogen (26 residues) and bovine pepsinogen B (25 residues) have been determined. The primary structures of all three pepsinogens show considerable homology with those of zymogens of gastric proteases sequenced previously.

At acidic pH the zymogens of the gastric proteases are converted into active enzymes by limited proteolysis during which *N*-terminal segments of about 45 amino acid residues are removed. It has been suggested that at neutral pH these zymogens are stabilized in inactive conformation through electrostatic interaction between positive charges of basic amino acids in the *N*-terminal end of the peptide chain and negative charges of aspartyl and glutamyl residues in the enzyme moiety of the zymogens.[1,2]

Previous determinations of the primary structures of porcine pepsinogen, bovine pepsinogen A and bovine prochymosin have shown a high degree of homology in the *N*-terminal amino acid sequences of these zymogens.[2-4] In order to investigate the general validity of the observations, especially with respect to distribution of basic amino acid residues, we have isolated and analysed pepsinogens from two more distantly related species (dogfish and ring seal) and one more of the bovine pepsinogens.

EXPERIMENTAL

Enzyme assay. To monitor the potential proteolytic activity during the purification of pepsinogen, modifications of the radial diffusion method[5] were used: For assay of seal pepsinogen gels were made of 1.1 % agarose and 0.3 % acid precipitated casein in 0.1 M sodium acetate buffer (pH 4.0). The gels were spread on glass plates to a thickness of 1.5 mm. Wells of 2 mm in diameter were punched in the gels. Before the assay, samples of pepsinogen solutions were converted into pepsin by standing at room temperature for 10 min at pH 2. Five μl of pepsin solutions were placed in the wells, and digestion took place for 20 h at 37 °C. After staining with Coomassie Brilliant Blue R-250 clear zones were observed around the pepsin containing wells. Calibration experiments carried out with porcine pepsin showed that the diameters of the clear zones were proportional to the logarithm of the pepsin concentrations, in these experiments 125 ng of porcine pepsin produced a clear zone of 5.0 mm in diameter.

A similar method was used for estimation of the potential proteolytic activity of dogfish pepsinogen, but in this case the most well defined zones were obtained with a gel layer containing 0.05 % acid denatured hemoglobin in formate buffer (0.1 M, pH 2.8), under such conditions both activation and digestion took place for 20 h at 37 °C. In this system 50 ng of porcine pepsin produced a clear zone of 6.6 mm in diameter.

The potential proteolytic activity is expressed as equivalents of μg of porcine pepsin/ml (ppa).

During the preparation of bovine pepsinogen B the potential enzymatic activity was determined by the milk clotting test.[1]

Purification of pepsinogens. Pepsinogen from ring seal (*Phoca hispida*). The seals were shot at Upernavik (Greenland) and the stomachs shipped in frozen state to the laboratory. Mucosa from 5 stomachs (500 g) was extracted with 2 l of 0.05 M Tris-HCl (pH 7.0) containing 0.1 M NaCl. The crude extract was clarified by formation of aluminium phosphate gel *in situ*.[1] All following operations were carried out at 5 °C. The precipitate was removed by centrifugation, and the supernatant dialysed against

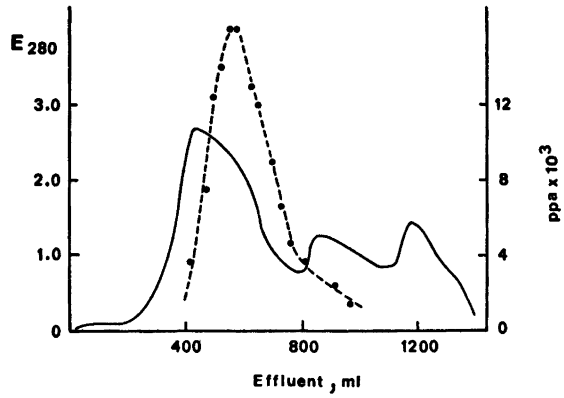

Fig. 1. Ion exchange chromatography of seal pepsinogen. Column: DEAE cellulose (2.5 × 43 cm). Elution: Linear gradient of 500 ml of 0.05 M Tris-HCl (pH 7.0) and 500 ml of 0.5 M NaCl in the same buffer. Flow rate 39 ml/h. Full line: E_{280}. Dashed line: Potential proteolytic activity (ppa).

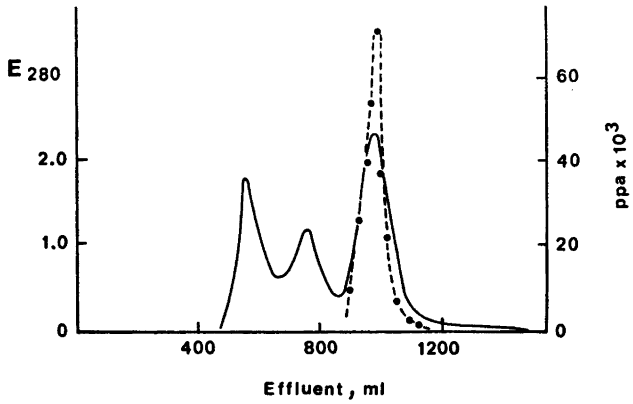

Fig. 2. Gel filtration of seal pepsinogen. Column: Sephadex G-100 (5 × 90 cm). Eluent: 0.05 M Tris-HCl (pH 7.0). Flow rate 70 ml/h. Full line: E_{280}. Dashed line: Potential proteolytic activity.

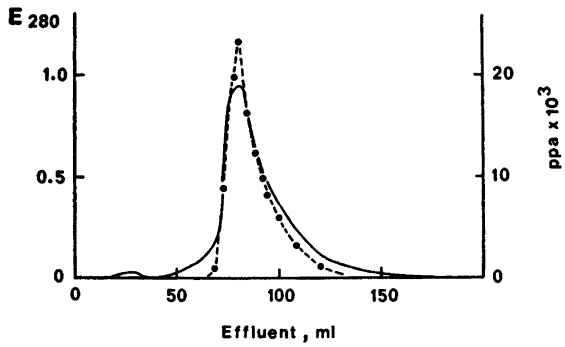

Fig. 3. Ion exchange chromatography of seal pepsinogen. Column: DEAE cellulose (0.9 × 15 cm). Elution: Linear gradient of 75 ml of 0.05 M piperazine-HCl (pH 6.0) and 75 ml 0.2 M NaCl in the same buffer. Flow rate 4 ml/h. Full line: E_{280}. Dashed line: Potential proteolytic activity.

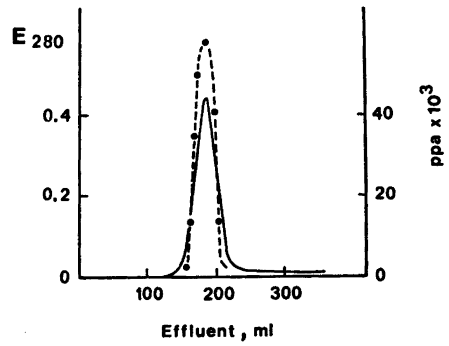

Fig. 4. Gel filtration of seal pepsinogen. Column: Sephadex G-75 (2.5 × 100 cm). Eluent: 0.02 M piperazine-HCl (pH 6.0). Flow rate 18 ml/h. Full line: E_{280}. Dashed line: Potential proteolytic activity.

0.05 M Tris-HCl (pH 7.0) and applied to a column of DEAE cellulose. Inert material was washed from the column with 500 ml of 0.05 M Tris-HCl (pH 7.0) before elution with a linear gradient from the starting buffer to 0.5 M NaCl in the starting buffer. The elution was completed with 350 ml of 0.5 M NaCl in the starting buffer. The results are illustrated in Fig. 1. The effluent from 364 ml to 780 ml was concentrated to 35 ml by ultrafiltration.

After gel filtration on Sephadex G-100 as shown in Fig. 2, effluent from 896 to 1064 ml was concentrated to 10 ml by ultrafiltration and applied to a column of DEAE cellulose (0.9 × 15 cm) equilibrated with 0.05 M piperazin-HCl (pH 6.0). Elution took place with a linear gradient from the equilibration buffer to 0.2 M NaCl in the same buffer (Fig. 3). Effluent from 76 to 110 ml was concentrated to 10 ml by ultrafiltration and gel filtration was carried out on a column of Sephadex G-75 as illustrated in Fig. 4. Two aliquots of the peak fraction, each containing *ca.* 15 nmol of pepsinogen, were freeze-dried and sequenced in parallel.

Pepsinogen from dogfish (*Squalus acanthias*). The dogfish was caught in the North Sea, stomachs were frozen immediately after landing and shipped frozen to the laboratory. Mucosa from 10 stomachs (180 g) was extracted with 600 ml of 0.05 M Tris-HCl (pH 7.5). Clarification with aluminium phosphate followed by centrifugation and dialysis was performed as described above. The following purification was carried out by a procedure similar to that published.[6] The zymogen was adsorbed to a column of DEAE cellulose (2.5 × 29 cm). Inert material was washed from the column with 150 ml 0.03 M of Tris-HCl (pH 7.5), and elution took place with a linear gradient (500 ml of 0.03 M Tris-HCl (pH 7.5) + 500 ml of 0.5 M NaCl in the same buffer). The major pepsinogen component was subjected to gel filtration on Sephadex G-100 (0.3 M Tris-HCl, pH 7.5) and finally purified by chromatography on a column of DEAE cellulose (0.9 × 13 cm), eluted with a linear gradient of 75 ml of 0.01 M sodium phosphate (pH 5.0) and 75 ml of 0.02 M NaCl in the same buffer. Of the peak fraction 0.9 ml (containing 16 nmol of pepsinogen) was freeze-dried and used for sequencing.

Bovine pepsinogen B is one of the minor components in bovine gastric mucosa. The starting material was extract of cow's stomachs obtained from Chr. Hansen's Laboratory Ltd., Copenhagen. The zymogen was prepared as described by Antonini and Ribadeau Dumas[7] except for the use of an additional step of chromatography on polylysine Sepharose 4B.[3] Bovine pepsinogen B was converted into pepsin B by activation at pH 2 for 30 min in an ice-bath. The activation peptides were purified by chromatography on DEAE cellulose and paper electrophoresis as previously described.[3] Activation peptides are denoted Act and numbered from the *N*-terminus of pepsinogen.

Sequencing. For Edman degradation of proteins the method of Weiner *et al.*[8] was modified to allow identification of the liberated thiazolinone derivatives by conversion into free amino acids.[9] The reaction was carried out in test tubes of 12 × 70 mm by the following procedure:

Coupling: dissolve 15 – 25 nmol of protein in 150 µl of 0.5 M $NaHCO_3$ (pH 9.8), add 20 µl of 10 % sodium dodecyl sulfate. Flush with nitrogen for 1 min, add 10 µl of phenylisothiocyanate, flush again with nitrogen for 1 min, and seal with parafilm. Place the test tube in a water bath at 50 °C and shake thoroughly at intervals of 5 min. After 60 min place the test tube in a freezer (– 20 °C) for 4 to 5 min and add 1 ml of acetone (stored in the freezer). Shake thoroughly and collect precipitate by centrifugation, withdraw and discard the yellowish supernatant. Wash precipitate once more with 1 ml of cold acetone, after centrifugation and withdrawal of supernatant, evaporate the last amount of acetone by flushing with nitrogen while vortexing the tube to distribute the precipitate over the lower part of the tube. Place the tube horizontally in a desiccator, and dry under vacuum for 20 min at 60 °C.

Cyclization and cleavage: cool to room temperature and add 200 µl of trifluoroacetic acid. Flush with nitrogen for 30 s and seal with double layer of parafilm. Leave the tube for 5 min at 50 °C. Evaporate trifluoroacetic acid by flushing with nitrogen in the hood while vortexing the tube to distribute the precipitate (NB, use gloves). Dry the precipitate under vacuum in a desiccator with solid NaOH for 10 min at 60 °C.

Extraction and identification: cool the tube to 0 °C in an ice-bath. Add 100 µl of ice-cold water and extract three times with 500 µl

of ice-cold butyl acetate saturated with water. Dry the water-phase and the protein is now ready for the next cycle of Edman degradation. To remove any contaminating peptides or protein from the combined extracts of butyl acetate, extract this with 100 µl of water. Evaporate the butyl acetate in a desiccator at 60 °C. Add 200 µl of hydriodic acid and seal the tube under vacuum. The thiazolinone is now converted into free amino acid by reaction for 24 h at 127 °C.

Edman degradation of peptides was carried out by coupling and cyclization as described in Ref. 10. After evaporation of trifluoroacetic acid, the dried residue was dissolved in 50 µl of ice-cold water; extraction was performed with 200 µl of butyl acetate and the further treatment was as described above.

The amino acids were analysed quantitatively on a Durrum D-500 amino acid analyzer. To recover methionine as metsulfone, Act-3 was oxidized with performic acid, otherwise the known artifacts of the conversion [9] were taken into account.

The *N*-termini were also analysed by dansylation.[10]

Cyanogen bromide cleavage of bovine pepsinogen B was carried out in 70 % formic acid. Fragments were separated by gel filtration (Sephadex G-50, eluent: 0.05 M acetic acid). The purified fragments are denoted CB and numbered from the *N*-terminus of pepsinogen.

RESULTS AND DISCUSSION

The *N*-terminal amino acid sequences of dogfish and ring seal pepsinogen were obtained by direct Edman degradation of the proteins, while bovine pepsinogen B was analysed by Edman degradation of the protein up to residue No. 9.

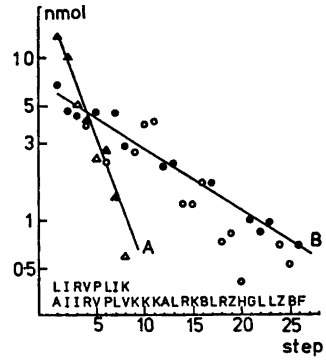

Fig. 5. Recoveries in nmol of amino acids obtained after conversion with hydriodic acid, semilogarithmic plots against steps of Edman degradation. A: Dogfish pepsinogen. B: Ring seal pepsinogen. Solid marks illustrate amino acids of high recovery [9] (Ala, Gly, Ile, Leu, Phe or Val), straight lines are drawn on basis of these. Letters above steps are amino acids in single letter code.[11]

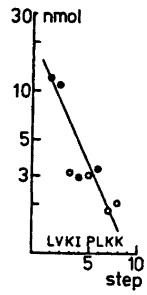

Fig. 6. Recoveries of amino acids after Edman degradation of bovine pepsinogen B. Notation as in Fig. 5.

Table 1. Analyses of activation peptides from bovine pepsinogen B. Amino acid compositions are expressed in stoichiometric ratios. Recoveries after Edman degradation are given directly in nmol.

Step of Edman degradation	Act-1 Amino acid composition		Recovery nmol	Act-2 Amino acid composition		Recovery nmol	Act-3 Amino acid composition		Recovery nmol
1	Leu	2.0	11.4	Phe	0.9	9.2	Ile	1.0	14.8
2	Val	0.9	9.4	Lys	1.0	9.0	Met	0.7	14.8
3	Lys	3.1	7.6	Ser	0.9	2.1	Lys	1.9	13.6
4	Ile	1.0	6.3	Ile	0.9	5.2	Glu	1.1	10.6
5	Pro	0.8	5.8	Arg	1.0	2.9	Lys		11.3
6	Leu		5.0	Glu	1.0	7.2	Gly	1.0	14.2
7	Lys		3.9				Leu	1.1	13.8
8	Lys		2.5						

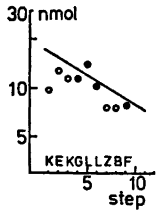

Fig. 7. Recoveries of amino acids after Edman degradation of CB-2 from bovine pepsinogen B. Notation as in Fig. 5. This fragment continues in another *ca.* 25 residues.

The recoveries from the sequencing are illustrated as semilogarithmic plots:[9] Figs. 5 and 6 show the results from entire proteins, and Fig. 7 shows the *N*-terminal sequence of CB-2 from bovine pepsinogen B, the sequences being given in single letter code.[11] The analyses of activation peptides from bovine pepsinogen B are shown in Table 1. CB-1 was identified only from amino acid composition and by *N*-terminal Dns-Leu. The final sequences are summarized in Table 2.

Quantitative results from sequencing experiments are presented without correction for "background", due to "out of step" reactions and other side reactions. It is a general experience that such reactions will lead to decreasing yield during the sequencing, it is further well known that such difficulties may vary much from protein to protein, and in addition to this a large and uncontrolled loss often occurs in the first step of Edman degradation.[9,12] Our results reflect the same difficulties. The repetitive yields were calculated to be 70 and 92 % by sequencing dogfish and seal pepsinogen; this allowed an unambiguous interpretation of 8 and 26 steps, respectively. The repetitive yield of bovine pepsinogen B was 74 %, in this case samples after step 9 were lost due to failure of the amino acid analyzer.

By conversion of thiazolinone derivatives into free amino acids serine represents a special problem, since it is converted into low yields of alanine. After conversion, high yield of alanine was found in the first step of Edman degradation of seal pepsinogen. The *N*-terminus of another sample was therefore investigated by the dansylation method; alanine was found as predominant *N*-terminal amino acid, but minor amounts of serine were also observed.

Table 2. *N*-Terminal amino acid sequences of zymogens of gastric proteases.

	5	10	15	20	25
Dogfish pepsinogen	Leu-Ile-Arg-Val-Pro-Leu-Ile-Lys/				
Ring seal pepsinogen	Ala-Ile-Ile-Arg-Val-Pro-Leu-Val-Lys-Lys-Lys-Ala-Leu-Arg-Lys-Asx-Leu-Arg-Glx-His-Gly-Leu-Leu-Glx-Asx-Phe/				
Bovine pepsinogen B	Leu-Val-Lys-Ile-Pro-Leu-Lys-Lys-Phe-Lys-Ser-Ile-Arg-Glu-Ile-Met-Lys-Glu-Lys-Gly-Leu-Leu-Glx-Asx-Phe/				
	Act-1	Act-2	Act-3		
	CB-1		CB-2		
Porcine pepsinogen A[2]	Leu-Val-Lys-Val-Pro-Leu-Val-Arg-Lys-Lys-Ser-Leu-Arg-Gln-Asn-Leu-Ile-Lys-Asp-Gly-Lys-Leu-Lys-Asp-Phe/				
Bovine pepsinogen A[3]	Ser-Val-Val-Lys-Ile-Pro-Leu-Val-Lys-Lys-Lys-Ser-Leu-Arg-Gln-Asn-Leu-Ile-Glu-Asn-Gly-Lys-Leu-Lys-Glu-Phe/				
Bovine prochymosin[4]	Ala-Glu-Ile-Thr-Arg-Ile-Pro-Leu-Tyr-Lys-Gly-Lys-Ser-Leu-Arg-Lys-Ala-Leu-Lys-Glu-His-Gly-Leu-Leu-Glu-Asp-Phe/				
Common	Pro Leu	Lys	Arg	Gly	Leu Phe

In seal pepsinogen residue No. 13 (step No. 12) was only identified after conversion to free amino acid (good yield of alanine, *cf.* Fig. 5B). The corresponding residue in bovine pepsinogen B was unambiguously identified since only serine was found by amino acid analysis of Act-2. The electrophoretic mobilities of Act-2 and Act-3 show the presence of glutamyl residues.[13] In seal pepsinogen information about amides at positions 17, 20, 25, and 26 is lost in the present way of sequencing. For position 25 and 26 this is also the case in bovine pepsinogen B, but these minor ambiguities do not interfere with the pattern of common structure that emerges from the sequences.

To facilitate comparison among the *N*-terminal sequences of the gastric zymogens sequenced up till now, Table 2 also includes the previously published sequences.[2-4] In five of the zymogens we find basic amino acids at positions 5, 10, 12, and 15. Dogfish pepsinogen has only been sequenced up to residue No. 10, but again with basic amino acids at positions Nos. 5 and 10. In this part of the sequence only Pro No. 7 and Leu No. 8 are identical in all the zymogens; however, the majority of the remaining residues show substitutions of very conservative character like Ile/Val. This means that we may predict that these sequences will fit very similar tertiary structures in all the zymogens.

In this context it is noteworthy that among the peptides liberated during activation of bovine pepsinogen A, a peptide consisting of 17 residues (No. 2 to No. 18 in Table 2) was identified as an inhibitor of the milk clotting action of pepsin.[3]

The information is still scattered, but it comprises sequences of three zymogens from one species (cow) and three more from other vertebrate species of which two (ring seal and dogfish) are distantly related to cow. Thus we may tentatively conclude that in the *N*-terminal sequence of the gastric zymogens a pattern of basic amino acid residues with spacings mainly of apolar amino acid residues are necessary for stabilizing the zymogen molecules in inactive conformation at neutral pH. This pattern appears to be common from the cartilaginous fishes (dogfish) to mammalia whether these are carnivorous (seal) or herbivorous (cow).

Acknowledgement. The work was supported by the Danish Natural Science Research Council and by the Carlsberg Foundation.

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Mechanism of the Grignard Addition Reaction. XII. The Reversibility of the Addition of Allylic Grignard Reagents to Di-*t*-butyl Ketone

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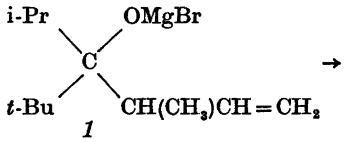
The reaction between di-*t*-butyl ketone with α, γ-dimethylallylmagnesium bromide has been studied by chemical, thermochemical and kinetic methods. Derivatives of both the ketone and the Grignard reagent have been obtained starting with the tertiary alcohol. The enthalpy of activation of the reverse reaction equals the sum of $\Delta H^{\neq}$ (forward) and ΔH_r (reverse). The *cis*/*trans* isomerisation of the tertiary alcohol occurs by retro addition-readdition.

In a recent study by Benkeser and Broxterman [1] on the reaction of *t*-butyl isopropyl ketone with crotylmagnesium bromide the observed slow conversion of the reaction product *1* to a mixture of *cis* and *trans* *2* was interpreted as taking place through a reversal of the Grignard addition reaction.



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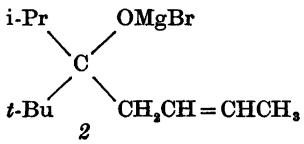
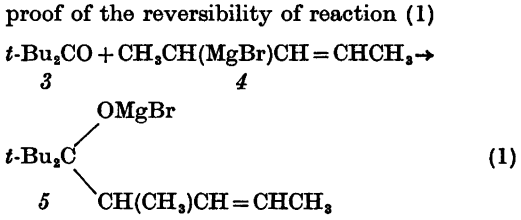


2

The theory of a retro Grignard addition was based solely on this observed rearrangement. It would seem, however, that the isomerisation might equally well be explained as, *e.g.*, a 1,3-radical type rearrangement of the allylic system and that more direct evidence was needed to prove, whether the Grignard addition is, in fact, truly reversible.

In the present investigation the most convenient system for the study of the problem was found to be di-*t*-butyl ketone (*3*) and α,γ-dimethylallylmagnesium bromide (*4*). Definitive proof of the reversibility of reaction (1)



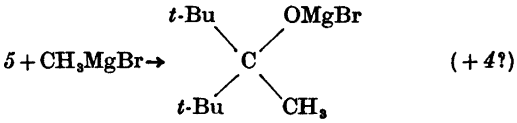
(1)

would require the isolation of both starting materials *3* and *4* after thermally induced decomposition of *5*. Indirect evidence would be obtained by isolating derivatives of each starting material, *e.g.* by treating *5* with a reagent which might displace the hypothetical equilibrium (1) by scavenging either *3* or *4* as fast as it is formed. The problem in this case would be whether the derivatives were formed after spontaneous dissociation of *5* into *3* and *4* or whether the splitting was induced by attack on *5* of the reagent used for the scavenging.

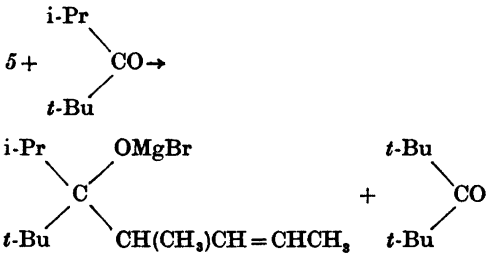
In the present work derivatization of *3* and *4*, when starting from *5*, has been combined with kinetic and thermochemical investigations of the reactions concerned. The accordance in the observations leaves no doubt that eqn. 1 represents a true equilibrium.

A derivative of *3* was obtained when *5* was heated with an excess of a Grignard reagent, *e.g.* methyl- or allylmagnesium bromide. After work-up a tertiary alcohol was obtained in

which the dimethylallyl radical was displaced by the radical of the "foreign" Grignard reagent:



To get a derivative from *4* the alcoholate *5* was heated with isopropyl-*t*-butyl ketone, and after work-up pentenyl-isopropyl-*t*-butyl carbinol was obtained together with di-*t*-butyl ketone, evidently as the result of reversal of eqn. 1 followed by addition of *4* to the "foreign" ketone:



Heating of *5* alone was found to induce *cis/trans* isomerisation from an initial ratio of *ca.* 1:1 to a thermodynamically determined equilibrium of *ca.* 1:3.

The thermochemistry and kinetics of reaction (1) and related reactions were investigated by means of the flow stream thermographic procedure [2] or, for slow reactions, by the use of a steady state heat flow calorimeter.[3]

From the measurements shown in Table 1 it is seen that the enthalpy of reaction of allylic Grignard reagents with ketones is numerically low compared with values obtained with methylmagnesium bromide. Furthermore the enthalpy of reaction of allylic reagents with the highly hindered di-*t*-butyl ketone is very much less negative than the values obtained with acetone. The reaction of *3* and *4* in (1) then shows only an enthalpy of reaction of −106 kJ/mol which is little more than half the value obtained with acetone and methylmagnesium bromide.

The reaction rates between allylic Grignard reagents and various substrates are given in Table 2. With an unhindered ketone like acetone the rates are exceedingly high even with highly hindered reagents, and with an unhindered reagent like allylmagnesium bromide even di-*t*-butyl ketone reacts momentarily. Hindrance in both ketone and the reagent, however, leads to measurable rates, and the rate of *3* reacting with *4* for example is remarkably low.

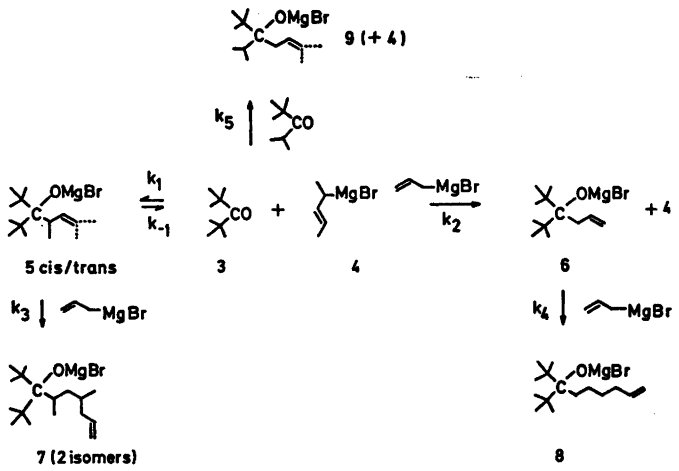
For a reversible reaction the enthalpy of reaction should equal the difference between the enthalpies of activation for the forward and the reverse reaction (eqn. (2)].

Table 1. Enthalpy of reaction in kJ/mol of ketones reacting with Grignard reagents in diethyl ether.

	Me_2CO	i-PrCO-*t*-Bu	*t*-Bu_2CO	Ph_2CO
$CH_2=CHCH_2MgBr$	−178		−108	−157
$CH_3CH=CHCH_2MgBr$		−105		
$CH_3CH=CHCH(MgBr)CH_3$			−106	
CH_3MgBr	−204		−150	−171

Table 2. Pseudo first order rate constants in s^{-1} for the reaction of 0.05 M ketone in diethyl ether at 20 °C with allylic Grignard reagents at the concentrations stated. Rates of addition to azobenzene showed for comparison.

	Me_2CO	i-PrCO-*t*-Bu	*t*-Bu_2CO	PhN=NPh
$CH_2=CHCH_2MgBr$ 0.35 M	$>10^4$	$>10^4$	$>10^4$	120
$CH_3CH=CHCH_2MgBr$ 0.22 M	$>10^4$	64	19	13
$CH_3CH=CHCH(MgBr)CH_3$ 0.21 M	$>10^4$	100	2.3	50



Scheme 1.

$$\Delta H_r(1) + \Delta H^{\ddagger} \text{ (forward)} = \Delta H^{\ddagger} \text{ (reverse)} \quad (2)$$

The enthalpy of activation for reaction (1) was determined on the basis of rate measurements at 20 and 40 °C and was found to be 31 kJ/mol. For the less hindered reactants isopropyl-*t*-butyl ketone and crotylmagnesium bromide the value was 27 kJ/mol.

In order to obtain the kinetics of the reverse reaction it was necessary to use a scavenging agent which was much more reactive toward *3* than is *4*. This condition is fulfilled by allylmagnesium bromide (see Table 2). A problem using this reagent is that it adds to the double bond of the magnesium salt of the allyl carbinol, a reaction analogous to the addition of allylmagnesium bromide to the magnesium salt of 4,4-diphenylbut-3-en-1-ol. This means that two reactions are competing in a mixture of *5* and allylmagnesium bromide leading to a reaction mixture (Scheme 1) containing the products *7* and *8*. Since the product *6* was never observed it was concluded that k_4 was large.

The occurrence of the side reaction was a complication in determining the enthalpy of activation for the reverse reaction (1). The procedure adopted was to run the reaction between *5* and a large excess allylmagnesium bromide at various temperatures and to find the time necessary to obtain, after work up, equal heights in the gas chromatogram of the peaks corresponding to *5* and *7*. Over the temperature range 52 °C to 109 °C the reaction varied from *ca.* 4000 to *ca.* 5 min, Table 3. The ratio of 7/8 in the reaction mixture decreased over the temperature range by approximately a factor of 2, and *7* was not considered in the calculations.

An Arrhenius treatment of the data yielded an enthalpy of activation of *ca.* 125 kJ/mol for the formation of *7* from *5* and since k_{-1} is assumed to be rate limiting this represents $\Delta H^{\ddagger}$ (reverse). Calculation of $\Delta H^{\ddagger}$ (reverse) from eqn. (2) leads to $105 + 31 = 136$ kJ/mol. The apparent discrepancy between the two values probably does not exceed the experimental uncertainty. If the difference was significant it would suggest that the retro addition is not following exactly the same path as the addition, but that a short-cut allows formation

Table 3. Reaction time for approximately 30 % conversion of di-*t*-butyl-2-pent-3-enyl carbinol (*5*) (*ca.* 0.1 M) in 0.7 M allylmagnesium bromide (54 % excess Br^+) in diethyl ether at various temperatures.

Temperature, °C	51.7	70.5	82.3	100	109
Time, min	3930	360	77	16	5
Ratio *7/8*	1.3	1.3	2.0	3.3	3.0

Table 4. Time for 80 % conversion of *ca.* 0.1 M bromomagnesium di-*t*-butyl-2-pent-3-enyl carbinolate (*5*) in diethyl ether in the reaction with isopropyl *t*-butyl ketone *ca.* 0.2 M.

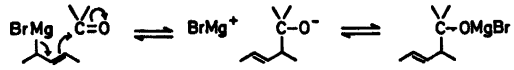
Temperature, °C	55.5	80.1	100
Time, min	1122	37	3

of *6* without the intermediacy of entirely free ketone.

Derivatives of the Grignard reagent *4* were obtained by heating the alcoholate *5* with an excess of a "foreign" ketone. Since isopropyl-*t*-butyl ketone is a much more reactive reagent toward α,γ-dimethylallylmagnesium bromide than is di-*t*-butyl ketone the conversion of *5* into *9* (see Scheme 1) should have the rate limiting step in common with the above mentioned conversion of *5* into *8*. This step in both cases should be the thermal dissociation of the alcoholate into ketone and Grignard reagent with the rate constant k_{-1}.

The rate of formation of *9* was followed at various temperatures and determination of the enthalpy of activation was attempted. The rate was found to be 8 – 10 times faster than the rates observed for formation of *8* (see Table 4). The enthalpy of activation was approximately 130 kJ/mol, not inconsistent with the value obtained by "Grignard exchange". That the "ketone exchange" rate is higher may be a solvent effect, since it may be concluded from Benkeser and Broxtermans work that the exchange of ether by THF is also causing a rate increase. This effect of the better ionizing solvents may indicate that the first step in the retro addition is ionization of the alcoholate. In accordance with this assumption is the observation that the presence of a proton donor like *t*-butyl alcohol is prohibitive for the fission of the alcoholate *5* since no ketone was formed even after prolonged heating at 100 °C. Treatment of the potassium salt of *5* with-butyl alcohol in ether produced the ketone in a smooth reaction at 100 °C.

The values calculated for the entropy of activation for the retro Grignard addition are approximately 25 J/°C when Grignard reagent is in excess and 42 J/°C when ketone is in excess. For the forward reaction the value is – 146 J/°C. The six-centered cyclic transition state, which was proposed by Benkeser *et al.* for both reactions is not consistent with these values, which indicate an associative addition mechanism and a dissociative retro addition. It has been shown by Felkin *et al.*[5] that addition of allylic Grignard reagents are S_E2' and not S_Ei' and the mechanism shown in Scheme 2 is therefore indicated.



Scheme 2.

The suggested mechanism is purely ionic. The relative reactivities of Grignard reagents seem to be easier to explain if the first step is divided into a rate limiting transfer of a single electron (SET) and a fast combination of radical ions. That methylmagnesium bromide has much less reactivity than either benzyl- or ethylmagnesium bromide is not consistent with a purely ionic mechanism since the effect of substitution with the electronegative phenyl should be opposite the effect of the electropositive methyl.[6,7] The relative reactivities would, however, be reasonably well explained by the operation of SET. The intense yellow colour which appears during the reaction of *3* and *4* might also indicate the formation of coloured ion radicals by SET.

With inclusion of SET the complete mechanism for the Grignard addition and for the reverse reaction would have three steps:

$$R'-MgX+R_2C=O \rightleftharpoons R'.\overset{+}{M}gX+R_2\dot{C}-O^- \rightleftharpoons$$
$$R_2R'C-O^-+MgX^+ \rightleftharpoons R_2R'C-OMgX$$

Felkin *et al.* have published observation of the *cis/trans* ratios obtained in reactions of α, γ-dimethylallylmagnesium bromide with various electrophilic substrates.[8] They obtained ratios near 6 with strongly electrophilic substrates like CO_2 and with electrophiles with decreasing strengths the ratios decreased, reaching with the weakest electrophiles a minimum of 0.3.

Table 5. The *cis/trans* ratio of di-*t*-butyl-2-pent-3-enyl carbinol determined by GLC after heating at 100 °C as a 0.2 M solution of the bromomagnesium salt in diethyl ether in the presence of 0.2 M di-*t*-butyl ketone. Also shown is the ratio of di-*t*-butyl carbinol/pentenyl-di-*t*-butyl carbinol in the reaction mixture.

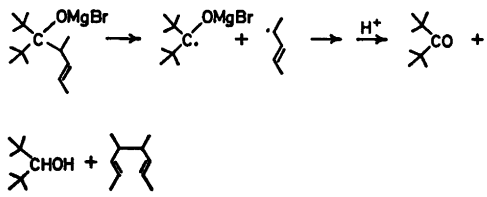
100 °C	Start	1 min	3 min	6 min	12 min
cis/trans carbinol %	1.0	0.76	0.57	0.46	0.42
sec/tert carbinol %	3.3	4.2	6.1	9.2	14.6
54.4 °C	Start	1600 min			
cis/trans carbinol %	1.0	0.46			
sec/tert carbinol %	3.3	4.6			

The results were explained by assuming transition states with varying degree of anionic character.

Assuming the stepwise mechanism outlined above the results of Felkin *et al.* might be interpreted by the difference in life time of different ion radical pairs, since the radical ion formed by SET might be "born" in the *cis* configuration, but would tend to isomerise during its life as radical.

When the alcoholate *5* was heated the initial *cis/trans* ratio of 1:1 changed to the equilibrium value *ca.* 1:3. It seemed a possibility that the isomerisation could take place in an intermediate ion pair and that the rate and activation energy for the isomerisation reaction might therefore be different from the parameters for the retro addition. Determination of the rate of isomeration at 50 and at 100 °C, however, revealed (see Table 5) that the activation energy for the process was *ca.* 120 kJ/mol which indicates that the isomerisation occurs by retro addition-readdition and that the life time of an eventual radical ion pair is at least not long enough to allow rotation from *cis* to *trans* configuration.

The gas chromatograms revealed that during the heating of the alcoholate *5* increasing amounts of di-*t*-butyl carbinol were produced. The most reasonable explanation for this is that a homolytic cleavage (2) of the carbon-carbon bond is competing with the heterolytic (1) as shown in Scheme 3. A lower temperature coefficient for the homolytic cleavage seems to indicate a lower activation enthalpy.



Scheme 3.

EXPERIMENTAL

Materials. Ketones were prepared by alkylation of diisopropyl ketone [9] and were purified to 99 % (GLC) by fractional distillation. Allylic bromides were prepared from the alcohols by addition of gaseous hydrogen bromide. The allylic Grignard reagents were prepared by extremely slow addition of the bromide to excess sublimed magnesium (Specpure, Johnson, Matthey Chemicals) in diethyl ether distilled from lithium aluminum hydride with stirring using evacuated equipment. From allylic Grignard reagents and hindered ketones were obtained tertiary alcohols as reported.[10] The products *5 cis*, *5 trans*, *7*, *8*, and *9* were not isolated, but were identified by the combination of gas chromatography and mass spectroscopy, in which the tertiary alcohols produced strong peaks corresponding to the various oxonium ions arising by the loss of either a *t*-butyl or an alkenyl group from the molecular ions, which were not visible.

Kinetics. Reaction mixtures were prepared at room temperature and distributed in sealed ampoules. The ampoules were heated in a thermostated oil bath for the specified time. The reaction mixtures were treated with saturated ammonium chloride, washed with water and base and dried with potassium carbonate. The product distributions were evaluated by GLC.

Fast reactions were measured in a flow stream reactor by thermography [2] using a liquid speed in the reaction tube of 300 cm/s.

Thermochemistry. Enthalpies of reaction of fast reactions were obtained from the thermographic data. The reaction between methyl magnesium bromide and di-*t*-butyl ketone is slow and the enthalpy of reaction was obtained by the use of the steady state heat flow calorimeter [2] adding the ketone at a constant rate to an excess of the Grignard reagent over a period of 1 – 2 h.

Because of the low reactivity of methylmagnesium bromide compared with allylic reagents its reaction as a 1 M ethereal solution with *5* (0.1 M) required several hours at 124 °C. The yield of methyl di-*t*-butyl carbinol increased with time since α,γ-dimethylallylmagnesium bromide was slowly removed from the equilibrium by thermal decomposition. After 48 h the conversion was quantitative as determined by GLC.

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Short Communications

Monitoring of Solid-phase Peptide Synthesis by Potentiometric Titration of Samples

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Potentiometric titration with perchloric acid on the whole batch of resin has been used for monitoring solid-phase peptide synthesis.[1–5] However, titration on withdrawn samples is in some cases to be considered as advantageous, as the main portion of resin bound product is not subjected to the washing procedure preceding the titration or to the perchloric acid titration itself. Also in large scale preparations titration of the entire amount of resin bound peptide may be disadvantageous. A prerequisite for quantitation of the results, however, is that throughout the synthesis it is possible to account for withdrawn material. In laboratory scale syntheses this can be done by weighing, and as example is presented the monitoring of the chain elongation in the synthesis of the cyclic decapeptide antamanide.

*Experimental. Synthetic procedure.** Boc-Phe-O-resin (0.46 mmol Phe/g) was prepared by esterification of the tetramethylammonium salt of Boc-Phe to a chloromethylated (0.81 mmol Cl/g) polystyrene, crosslinked with 2 % divinyl benzene, Bio Beads S-X2, 200–400 mesh from BIO-RAD.[6] Boc-Phe-Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe-O-resin was synthesized using 25 g of Boc-Phe-O-resin. Each coupling was performed with a threefold excess of Boc-amino acid and DCC and allowed to proceed for 17–19 h. After coupling the whole amount of resin was dried, weighed and an aliquot withdrawn. Boc-groups were cleaved with 1 N HCl/acetic acid.

Titration. Potentiometric titrations were carried out on samples of approx. 1 g in acetic acid/dichloromethane (1:1 v/v), using a Radiometer titration equipment. Due to the increasing proportion peptide/resin, 0.5 to 0.3 meq was titrated. The titrant was 0.0296 N perchloric acid in acetic acid. The accuracy of the titration was 0.475 ± 0.0015 meq/g, *i.e.* ± 0.3 %, an improvement compared to earlier experiments,[2,4] in which the entire amount of resin bound product was titrated.

Amino acid analysis. 10–15 mg of the samples withdrawn after each coupling step was hydrolyzed for 4 h with 6 N hydrochloric acid–propionic acid 1:1 v/v at 130 °C in sealed ampoules. The cyclic product was hydrolyzed for 24 h with 6 N hydrochloric acid at 110 °C in a sealed ampoule. A Beckman model 121 amino acid analyzer was used for analyzing the hydrolysates.

Cleavage and cyclization. The end product was cleaved from the resin with HBr in trifluoroacetic acid (5 × 20 min). The crude peptide was subjected to high voltage electrophoresis at pH 2 on Whatman No. 1 and to thin-layer chromatography in chloroform–methanol–acetic acid 18:1:1, 2-butanol–formic acid–water 75:15:10, 2-butanol–aqueous ammonia 85:15, and 1-butanol–pyridine–acetic acid–water 30:20:24:6. In all systems a high degree of purity of the product was indicated. Cyclization was performed according to Ref. 7 on the crude peptide. The peptide (6.9 g, 5.5 mmol) was dissolved in dimethylformamide (150 ml), *N*-hydroxysuccinimide (2.76 g. 24.0 mmol) added and the mixture diluted with dichloromethane (1.35 l). The solution was cooled to 0 °C and DCC (2.7 g, 13.1 mmol) in dichloromethane (50 ml) and triethylamine (1 ml, 7.1 mmol) were added. The reaction mixture was stirred at 0 °C for 1.5 h and allowed to attain room temperature and left for 70 h. The solvents were evaporated and the product dissolved in tetrahydrofuran. The precipitated dicyclohexylurea was filtered off and the filtrate concentrated to an oil. 1.7 g of antamanide (27 %) was isolated by chromatography on aluminium oxide and silica gel. M.p. 170–172 °C, Ref. 7, 170–174 °C. Amino acid analysis in μmol/mg: Phe 3.53, Val 0.846, Pro 3.60, Ala 0.844. Theoretical, respectively: 3.38, 0.845, 3.38, and 0.845. $[\alpha]_D^{20}$ −163° (*c*, 1, methanol), Ref. 7, −148°. The product was homogeneous by thin-layer chromatography. Molecular weight determined by mass spectrometry was 1146.

Results. The course of the chain elongation is demonstrated in Fig. 1. The values are corrected for withdrawal of the samples. As the chain elongation proceeds, an increase in the titration values of the Boc-protected product is seen to occur after step 5, due to *N*-alkylation.[8] Furthermore, a slight decrease (8 %)

* Abbreviations: Boc = *tert*-butoxycarbonyl; DCC = *N*,*N*′-dicyclohexylcarbodiimide.

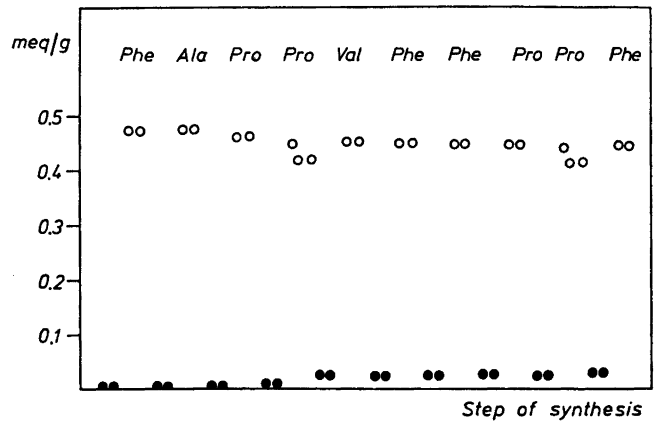

Fig. 1. Titration values obtained during the chain elongation in the synthesis of antamanide carried out from the *C*-terminal Phe (left) to the *N*-terminal Phe (right). The ordinate values are the numbers of meq calculated per g of Boc-Phe-O-resin. Filled circles before, and open circles after Boc-group cleavage.

Table 1. Amino acid content of the ten samples from the synthesis of antamanide as determined by titration and amino acid analysis. Values shown in μmol/mg.

Sample No.	Phe Titr.	A [a]	Ala Titr.	A	Pro Titr.	A	Val Titr.	A
1	0.47	0.46	–	–	–	–	–	–
2	0.46	0.44	0.46	0.44	–	–	–	–
3	0.44	0.42	0.44	0.42	0.43	0.42	–	–
4	0.42	0.41	0.42	0.41	0.80	0.81	–	–
5	0.41	0.39	0.41	0.39	0.78	0.78	0.37	0.39
6	0.73	0.74	0.38	0.37	0.74	0.74	0.35	0.37
7	1.01	1.05	0.36	0.35	0.69	0.70	0.33	0.35
8	1.01	1.02	0.36	0.34	1.00	1.02	0.33	0.34
9	0.97	0.97	0.34	0.33	1.24	1.31	0.31	0.33
10	1.21	1.26	0.33	0.32	1.20	1.26	0.30	0.31

[a] A = Amino acid analysis.

is demonstrated in the titration values after deprotection, most likely due to cleavage of peptide from the resin. The evident irregularities by the titration of the proline residues coupled as Nos. 4 and 9 cannot be explained, but the subsequent coupling yields correspond to the first obtained titration value. The yield in single steps and the overall yield are to be calculated by subtracting the respective values obtained by titration of the Boc-protected product from the deprotected.[5] Thus an overall yield of 89 % was achieved.

In Table 1 is presented the content of the amino acid residues calculated from the titration values in the single steps of chain elongation, and determined by amino acid analysis. A good agreement is shown to exist between the two methods of determination of the amino acid content.

Discussion. The results obtained demonstrate that a quantitative determination of the yields in the single steps during the synthesis of a peptide can be performed by titration on withdrawn samples. A prerequisite is, however, that the proportion between the amount of the sample and the total amount can be determined with sufficient accuracy. Regarding the calculation of the content of the amino acids in a sample, the reliability of the results is somewhat dependent on the course of the synthesis. Thus, if much peptide is lost during the synthesis, this must be taken into consideration by the calculation of the content of the amino acid residues preceding the *N*-terminal

amino acid in the sample. If a decrease during the synthesis is due to blocking, the titration results will show the actual content of the amino acids. In the present experiment it has not been possible to explain the reason for the gradual slight decrease of the titration values during the synthesis.

In the synthesis of still larger amounts of resin bound peptides, determination of the proportion of the sample to the entire amount by weighing is not possible in practice. In such cases an internal standard has to be used for determination of the amount of derivatized polystyrene in the actual sample, allowing the results to be calculated per weight unit of the polymer. Whether a radioactive labelling of the methylene group in the benzyl ester linkage insures a sufficient accuracy will have to be evaluated by experiments.

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Resolution and Absolute Configuration of 1-Ethyl-1-methyl-2-propynylamine

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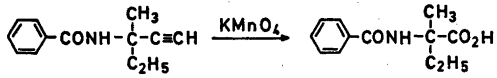
The enantiomers of some *N*-(4-*tert*-amino-1-methyl-2-butynyl)-substituted succinimides and 2-pyrrolidones show strong stereospecificity in blocking the motor effects of the muscarinic agent oxotremorine, *N*-(4-pyrrolidino-2-butynyl)-2-pyrrolidone. The (*R*)-(+)-isomers are about twice as active as their corresponding racemates in this respect while the (*S*)-(−)-isomers are practically inactive.[1,2] In order to investigate the influence of further alkyl substitution on stereospecificity we decided to prepare optical isomers of compounds containing both a methyl and an ethyl group in the 1-position of the butynyl chain. For this purpose we needed the enantiomers of 1-ethyl-1-methyl-2-propynylamine (*1*) as starting material and this communication deals with the resolution of this amine and the determination of its absolute configuration.

The amine was resolved into its (+)- and (−)-enantiomers by fractional crystallization of its (+)- and (−)-hydrogen tartrates, respectively. In order to estimate the optical purity of the enantiomers we used ^{1}H NMR spectroscopic analyses of the diastereomeric amides formed when optically impure amine is acylated with optically pure (−)-*O*-methylmandelyl chloride.[2,3]

The absolute configuration of the amine was established by transforming its benzoyl derivative to the corresponding isovaline derivative.



The benzoyl derivative obtained from the (+)-enantiomer of the amine yielded upon oxidation with potassium permanganate the benzoyl derivative of (+)-isovaline. Thus (+)-*1* and (+)-isovaline must be configurationally identical. The latter compound has been correlated into the natural amino acid series (L- or *S*-configuration) both by chemical[4] and enzymatic methods.[5] Dextrorotatory *1* can therefore be assigned the *S* configuration.

Experimental. Melting points were determined in a metal block using open capillary tubes and calibrated Anschütz thermometers. Microanalyses were carried out at the Microanalytical Laboratory, Royal Agricultural College, Uppsala. IR spectra were run on a Perkin-Elmer 157 G spectrophotometer and ^{1}H NMR spectra

amino acid in the sample. If a decrease during the synthesis is due to blocking, the titration results will show the actual content of the amino acids. In the present experiment it has not been possible to explain the reason for the gradual slight decrease of the titration values during the synthesis.

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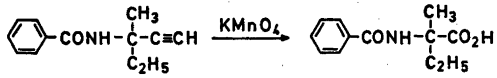
The amine was resolved into its (+)- and (−)-enantiomers by fractional crystallization of its (+)- and (−)-hydrogen tartrates, respectively. In order to estimate the optical purity of the enantiomers we used ^{1}H NMR spectroscopic analyses of the diastereomeric amides formed when optically impure amine is acylated with optically pure (−)-*O*-methylmandelyl chloride.[2,3]

The absolute configuration of the amine was established by transforming its benzoyl derivative to the corresponding isovaline derivative.



The benzoyl derivative obtained from the (+)-enantiomer of the amine yielded upon oxidation with potassium permanganate the benzoyl derivative of (+)-isovaline. Thus (+)-*1* and (+)-isovaline must be configurationally identical. The latter compound has been correlated into the natural amino acid series (L- or *S*-configuration) both by chemical[4] and enzymatic methods.[5] Dextrorotatory *1* can therefore be assigned the *S* configuration.

Experimental. Melting points were determined in a metal block using open capillary tubes and calibrated Anschütz thermometers. Microanalyses were carried out at the Microanalytical Laboratory, Royal Agricultural College, Uppsala. IR spectra were run on a Perkin-Elmer 157 G spectrophotometer and ^{1}H NMR spectra

on a Perkin-Elmer R 12 B spectrometer. Optical rotations were measured with a Perkin-Elmer 141 spectropolarimeter.

Resolution of 1-ethyl-1-methyl-2-propynylamine. Racemic amine (60 g, 0.62 mol), prepared as previously described,[6] was added to a solution of (+)-tartaric acid (92.5 g, 0.62 mol) in 300 ml of absolute ethanol. The solution was left overnight at room temperature. The salt obtained (60.5 g) required several recrystallizations from about 10 % solutions in 95 % ethanol before constant physical properties of the salt, of the hydrochloride, and of the (−)-*O*-methylmandelyl derivative of the liberated amine were obtained. Yield 18.0 g (24 %) of resolved (+)-hydrogen tartrate, m.p. 164.5–166 °C, $[\alpha]_D^{22}$ +17.8° (*c* 1.0, water). Anal. $C_{10}H_{17}NO_6 \cdot \frac{1}{2}H_2O$: C, H, N.

The first filtrate from the above resolution was concentrated *in vacuo* and the residue dissolved in saturated K_2CO_3-solution. After extracting the amine with ether and drying the extract (K_2CO_3), the solution was fractionated through a helix-packed column. The amine fraction was added to a solution of (−)-tartaric acid in 95 % ethanol and the salt formed was purified as described above for the enantiomeric salt. The yield, based on recovered amine, of resolved (−)-hydrogen tartrate was 26 %, m.p. 165–166 °C, $[\alpha]_D^{22}$ −18.5 (*c* 1.0, water). Anal. $C_{10}H_{17}NO_6 \cdot \frac{1}{2}H_2O$: C, H, N.

(S)-(+)-*1-Ethyl-1-methyl-2-propynylamine.* Since the amine liberated from the resolved (+)-hydrogen tartrate was found to contain traces of impurities (as shown by ^{1}H NMR and GC, 20 % Carbowax 20 m), the (+)-hydrogen tartrate was dissolved in saturated K_2CO_3-solution and the amine extracted with ether. The hydrochloride of the amine was precipitated from the ethereal extract, dried, and dissolved in saturated K_2CO_3-solution. The pure amine was then obtained through the procedure described above, b.p. 104–105 °C, n_D^{22} 1.435, $[\alpha]_D^{22}$ +6.6° (*c* 1.0, ethanol), yield 58 % (from the hydrochloride). ^{1}H NMR ($CDCl_3$, 37 °C): δ 1.03 (3 H, t, *J* 7.0 Hz, CH_2CH_3), 1.37 (3 H, s, $C-CH_3$), 1.3–1.8 (2 H, m, CH_2) 2.28 (1 H, s, $\equiv CH$). *Hydrochloride:* m.p. > 250 °C (from ethanol-ether), $[\alpha]_D^{22}$ +6.9° (*c* 0.8, ethanol). *Benzamide:* m.p. 104–105 °C (from ligroin), $[\alpha]_D^{22}$ +3.4° (*c* 1.0, ethanol). Anal. $C_{13}H_{15}NO$: C, H, N.

(R)-(−)-*1-Ethyl-1-methyl-2-propynylamine* was obtained similarly from the (−)-hydrogen tartrate *via* the hydrochloride salt, b.p. 104–106 °C, n_D^{22} 1.435, $[\alpha]_D^{22}$ −6.2° (*c* 1.0, ethanol), yield 57 %. *Hydrochloride:* m.p. > 250 °C, $[\alpha]_D^{22}$ −6.4°. (*c* 1.4, ethanol), Anal. $C_6H_{11}N.HCl$: C, H, N, *Benzamide:* m.p. 104–105 °C, $[\alpha]_D^{22}$ −3.4° (*c* 1.0, ethanol). Anal. $C_{13}H_{15}NO$: C, H, N.

N-[(S)-*1-Ethyl-1-methyl-2-propynyl*]-(R)-O-*methylmandelamide.* (*R*)-(−)-*O*-methylmandelic acid,[7] $[\alpha]_D^{22}$ −148.7° (*c* 0.6, ethanol), was converted to its acid chloride with which (*S*)-(+)-1-ethyl-1-methyl-2-propynylamine was acylated according to a method described in the literature,[8] m.p. 53.5–54.5 °C (from light petroleum), $[\alpha]_D^{22}$ −58.5° (*c* 0.7, ethanol). ^{1}H NMR (C_6H_6, 37 °C): δ 0.95 (3 H, t, *J* 7.2 Hz, CH_2CH_3), 1.58 (3 H, s, $C-CH_3$), 2.06 (1 H, s, $\equiv CH$), 1.55–2.45 (2 H, m, CH_2), 2.96 (3 H, s, OCH_3), 4.43 (1 H, s, CH). Anal. $C_{15}H_{19}NO_2$: C, H, N.

N-[(R)-*1-Ethyl-1-methyl-2-propynyl*]-(R)-O-*methylmandelamide* was prepared similarly from (*R*)-(−)-1-ethyl-1-methyl-2-propynylamine, m.p. 31–33 °C (from light petroleum), $[\alpha]_D^{22}$ −68.4° (*c* 1.2, ethanol). ^{1}H NMR (C_6H_6, 37 °C): δ 0.87 (3 H, t, *J* 7.2 Hz, CH_2CH_3), 1.71 (3 H, s, $C-CH_3$), 2.05 (1 H, s, $\equiv CH$), 1.40–2.30 (2 H, m., CH_2), 2.95 (3 H, s, OCH_3) 4.42 (1 H, s, CH). Anal. $C_{15}H_{19}NO_2$: C, H, N.

(S)-(+)-N-*Benzoylisovaline. A. Acylation of* (S)-(+)-*isovaline.* (*S*)-(+)-Isovaline, $[\alpha]_D^{22}$ +10.9° (*c* 1.1, water), obtained by resolution and hydrolysis of (+)-*N*-formylisovaline,[9] was benzoylated under customary Schotten-Baumann conditions. Yield 30 %, m.p. 175–177 °C (from 40 % methanol), $[\alpha]_D^{22}$ +11.2° (*c* 0.7, methanol), lit.[10] m.p. 176–178 °C, $[\alpha]_D^{15}$ +10.3° (*c* 1.4, methanol).

B. Oxidation of (S)-(+)-N-*(1-ethyl-1-methyl-2-propynyl)-benzamide.* A saturated solution of $KMnO_4$ was added dropwise to a stirred suspension of the finely pulverized benzamide (0.85 g, 0.04 mol) in water (25 ml) at 0–5 °C. When no more $KMnO_4$ was consumed the solution was filtered and acidified with 5 M H_2SO_4. Sodium pyrosulfite was added to remove excess of permanganate. The product crystallized from the clear solution. Yield 59 %, m.p. 176–177 °C, $[\alpha]_D^{22}$ +10.8° (*c* 1.2, methanol). Anal. $C_{12}H_{15}NO_3$: C, H, N.

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N-Isocyanoimines. Preparation and Characterisation

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Aromatic *N*-isocyanoimines have been reported as intermediates in the synthesis of formhydrazonoesters.[1-3] In a few cases the *N*-isocyanoimines have been isolated, but comments on yield and reactivity are sparse. Aliphatic *N*-isocyanoimines have not been described.

This paper reports the synthesis of aliphatic *N*-isocyanoimines. The hitherto unknown compounds were characterized by their α-addition products from reactions with piperidine and with ethanethiol.

N-Isocyanoimines (*1*) were synthesized by two different methods: dehydration of *N'*-alkylidene formohydrazides by means of $POCl_3$ and triethylamine in CH_2Cl_2-solution (method A), and reaction between hydrazones and $CHCl_3$ in NaOH-solution, with use of benzyltriethylammonium chloride as phase transfer catalyst (Method B).

$$\begin{array}{l} RR'C{=}N{-}NHCHO + POCl_3 + Et_3N \xrightarrow{(A)} \\ RR'C{=}N{-}NH_2 + CHCl_3 + OH^- \xrightarrow{(B)} \end{array} \quad \underset{1a-e}{RR'C{=}N{-}N{=}C}$$

Scheme 1. (Compound, R, R'): (a, Me, Me), (b, Et, Et), (c, Pr^i, Pr^i), (d, Me, Ph), (e, Ph, Ph).

Attempts to purify *N*-isocyanoimines by distillation *in vacuo* resulted in products contaminated with triethylamine. The distillate was collected in an acetone/dry ice trap. The compounds (*1*) decomposed rapidly at room temperature, whereas they were stable for some days in solution. All formed *N*-isocyanoimines showed strong, sharp IR absorptions around 2100 cm^{-1}, and had the characteristic isocyanide smell. The use of pyridine in the dehydrating step instead of triethylamine gave no *N*-isocyanoimine.

For further characterisation of *1a–e* the CH_2Cl_2-phase from the reaction mixture was used. The reaction with piperidine, catalysed by CuCl, was found to be convenient for trapping the *N*-isocyanoimines, as it took about 2 h at room temperature, giving formamidrazones in overall yields from 11 to 38 %. The analogous reaction for *t*-butyl isocyanide proceeds in 10 h at room temperature.[4]

$$\underset{1a-d}{RR'C{=}N{-}N{\equiv}C} + HN(CH_2)_5 \rightarrow \underset{2a-d}{RR'C{=}N{-}N{=}CH{-}N(CH_2)_5}$$

Scheme 2.

N-Isocyano-3-pentanimine (*1b*) was used for investigations of the reactivity in other α-addition reactions. The reaction with aniline, which is known to give good yields of formamidine on boiling with normal isocyanide [4] gave no α-addition product. This is probably due to the elevated temperature which causes rapid decomposition of the *N*-isocyanoimine. Reaction with ethanethiol [5] catalysed by CuCl resulted in formation of the α-addition product (*3b*) in low yield. No isothiocyanate was detected.

$$\underset{1b}{(Et)_2C{=}N{-}N{\equiv}C} + EtSH \rightarrow \underset{3b}{(Et)_2C{=}N{-}N{=}CH{-}SEt}$$

Scheme 3.

Experimental. Microanalyses were carried out in the Microanalysis Department of Chemical Laboratory II, The H. C. Ørsted Institute. ^1H NMR spectra were obtained on a JEOL JNM MH 60/II instrument. IR spectra were recorded on a Perkin-Elmer model 225 grating spectrograph or model 157 NaCl spectrophotometer. Mass spectra were taken on an AEI-902 instrument operating at 70 eV. Melting points are uncorrected.

N'-(3-Pentylidene) formohydrazide and N'-(1-phenylethylidene) formohydrazide were prepared as described for *N'*-(2-propylidene) formohydrazide.[6] *N'-(3-Pentylidene) formohydrazide.*[7] Yield 89 %, m.p. 73–74 °C. Anal. $C_6H_{12}N_2O$: C, H, N. *N'-(1-Phenylethylidene) formohydrazide.* Yield 60 %, m.p. 157–158 °C. Anal. $C_9H_{10}N_2O$: C, H, N. *N'-[2,4-Dimethyl-(3-pentylidene)] formohydrazide* was prepared by refluxing 2,4-dimethyl-3-pentanone hydrazone [8] (0.19 mol) in ethyl formate (60 ml) for 6 h. After subsequent stirring at room temperature for 10 days, the mixture was evaporated to dryness and the resulting product was recrystallized from ethanol. M.p. 113 °C, yield 55 %. Anal. $C_8H_{16}N_2$: C, H, N.

N-Isocyano-2-propanimine (1a). *N'*-(2-Propylidene) formohydrazide (0.1 mol) and triethylamine (60 ml) were dissolved in CH_2Cl_2 (50 ml). $POCl_3$ (9.3 g) was added dropwise under cooling with ice. The temperature was 42–45 °C. The resulting mixture was stirred at room temperature for 3 h. Saturated Na_2CO_3-solution (70 ml) was added under cooling, the CH_2Cl_2-layer separated, dried over K_2CO_3, and used for further reactions.

An attempt to isolate the *N*-isocyanoimine by distillation of a CH_2Cl_2-layer evaporated previously to *ca.* 30 ml gave 1.5 g of a liquid, b.p. 15–20 °C/1 mmHg. The distillate was cooled in dry ice/acetone. IR absorption (CH_2Cl_2): NC 2100 cm^{-1}. On heating to room temperature the liquid decomposed rapidly, with a colour change from light yellow to dark brown. In CH_2Cl_2 or CCl_4-solution, the decomposition proceeded slower; there was still an NC IR absorption in CCl_4-solution after 7 h at room temperature. ^1H NMR spectra showed that the distillate was a mixture of triethylamine and *N*-isocyanoimine.

N¹-Pentamethylene-N³-(2-propylidene) formamide hydrazone (2a). The CH_2Cl_2-phase described above was mixed with piperidine (0.1 mol) and CuCl (100 mg). After stirring for 2 h at room temperature the mixture was filtered and the filtrate distilled *in vacuo*. B.p. 63–64 °C/0.1 mmHg, yield 22 %. Anal. $C_9H_{17}N_3$: C, H, N. ^1H NMR ($CDCl_3$): 7.86 (1 H, s), 3.1–3.5 (4 H, m), 2.02 (3 H, s), 1.95 (3 H, s), 1.33–1.75 (6 H, m). MS *m/e* (% of base peak): 167(57)M^+, 152(5), 111(11), 84(74), 83(100), 58(13), 56(24), 55(40), 42(35), 41(30).

N-Isocyano-3-pentanimine (1b) was prepared in CH_2Cl_2/Et_3N-solution as described for (*1a*). Evaporation *in vacuo* to a volume of *ca.* 30 ml followed by distillation *in vacuo*, gave 2.4 g of a mixture of *N*-isocyanoimine (*1b*) and triethylamine b.p. 14–18 °C/0.5 mmHg. IR absorption (CH_2Cl_2): NC 2100 cm^{-1}.

N¹-Pentamethylene-N³-(3-pentylidene) formamide hydrazone (2b) was prepared analogous to *2a*. B.p. 75 °C/0.05 mmHg, yield 38 %. Anal. $C_{11}H_{21}N_3$: C, H, N. ^1H NMR ($CDCl_3$): δ 7.82 (1 H, s), 3.2–3.5 (4 H, m), 2.51 (2 H, q), 2.25 (2 H, q), 1.5–1.7 (6 H, m) 1.11 (3 H, t), 1.07 (3 H, t). MS *m/e* (% of base peak): 196(14), 195(64)M^+, 166(13), 111(53), 86(27), 84(100), 83(91), 69(11), 56(34), 55(34), 42(10), 41(27).

N³-(2,4-Dimethyl-3-pentylidene) N¹-pentamethyleneformamide hydrazone (2c) was prepared from *N*-isocyano-2,4-dimethyl-3-pentanimine (Method A) as described for *2a*, yield 36 %, b.p. 78–80 °C/0.02 mmHg. Anal. $C_{13}H_{25}N_3$: C, H, N. ^1H NMR ($CDCl_3$): δ 7.76 (1 H, s), 3.51 (1 H, sep.), 3.48–3.19 (4 H, m), 2.58 (1 H, sep.), 1.55–1.65 (6 H, m), 1.17 (6 H, s), 1.06 (6 H, s). MS *m/e* (% of base peak): 224(11), 223(50)M^+, 180(16), 139(22), 114(11), 113(11), 112(15), 111(27), 85(9), 84(100), 83(42), 70(13), 69(16), 56(13), 55(31), 42(23), 42(27), 41(37).

N-Isocyano-2,4-dimethyl-3-pentanimine (1c). (Method B). A mixture of diisopropyl ketone hydrazone [8] (0.1 mol), chloroform (0.1 mol), aqueous NaOH-solution (50 ml, 50 %), benzyltriethylammonium chloride (0.5 g) and CH_2Cl_2 (50 ml) was stirred at room temperature for 2.5 h (slightly exothermic reaction). The CH_2Cl_2-layer was separated and dried over K_2CO_3. IR (CH_2Cl_2): NC 2095 cm^{-1}. The *N*-isocyanoimine could be stored for a few days in solution, with slight decomposition. Subsequent treatment with piperidine and CuCl as described for *1a* gave 11 % of *2c*.

N-Isocyano-1-phenylethanimine (1d). Preparation by Method B gave mainly acetophenone azine. Method A gave the *N*-isocyanoimine in solution, IR (CH_2Cl_2): NC 2090 cm^{-1}.

N¹-Pentamethylene-N³-(1-phenylethylidene) formamide hydrazone (2d). Prepared analogous to *2a*. B.p. 138–140 °C/0.05 mmHg, m.p. 43 °C (EtOH), yield 30 %. Anal. $C_{14}H_{19}N_3$: C, H, N. ^1H NMR ($CDCl_3$): δ 8.05 (1 H, s), 7.2–7.8 (5 H, m), 3.2–3.6 (4 H, m), 2.42 (3 H, s), 1.5–1.7 (6 H, m). MS *m/e* (% of base peak): (azine contaminated) 230(15), 229(70)M^+, 217(62), 145(15), 120(85), 119(15), 118(17), 111(23), 110(13), 104(32), 103(36), 99(21), 97(25), 85(10), 84(77), 83(100), 77(60), 72(51), 71(32), 58(13), 57(13), 56(47), 55(38), 51(27), 50(12), 44(40), 43(15), 42(38), 41(34), 40(9).

N-Isocyanodiphenylmethanimine (1e). Benzophenonehydrazone (0.1 mol), $CHCl_3$ (0.1 mol), NaOH-solution (50 ml, 50 %) and benzyltriethylammonium chloride (0.5 g) were stirred in 50 ml CH_2Cl_2 for 5 days at room temperature. The CH_2Cl_2-layer was separated and dried over K_2CO_3. IR (CH_2Cl_2): NC 2060 cm^{-1}. After treatment with piperidine no formamide hydrazone was isolated.

S-Ethyl-N-(3-pentylidene) thioformhydrazonate (3b). *N*-Isocyano-3-pentanimine in CH_2Cl_2/Et_3N-solution, ethanethiol (0.5 mol) and CuCl (0.5 mmol) were stirred at room temperature for 1 h. The solvent was evaporated and the residue distilled *in vacuo*, b.p. 46 °C/0.1 mmHg, yield 19 %. ^1H NMR ($CDCl_3$): δ 8.25 and 7.65 (1 H, singlets, intensity 1/3), 3.1–2.1 (6 H, 3 quartets), 1.5–0.8 (9 H, 3 triplets). MS *m/e* (% of base peak): 172(32)M^+, 143(14), 139(13), 111(25), 88(42), 86(21), 84(10), 61(25), 60(13), 56(100), 55(10), 54(19), 45(11), 41(17).

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On the Reaction between Azide Ion and Thiobenzophenone or Thiobenzophenone S-Oxides. Thiatriazolines as Possible Intermediates

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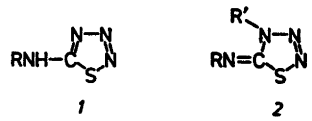
Examples of Δ^2-1,2,3,4-thiatriazolines (*2*) have been described recently. 5-Arylamino- and 5-arylsulfonylaminothiatriazoles (*1b* and *1a*) are alkylated in position 4 by diazomethane to give *2b* and *2a*, respectively [1] and trialkyloxonium tetrafluoroborates similarly alkylate *1b* and *1c* in position 4.[2] 4-Alkyl-5-arylsulfonyliminothiatriazolines (*2a*) are obtained from alkyl azides and the reactive arylsulfonyl isothiocyanates [3] and thiatriazolines (*2b*) are probably also formed from alkyl azides and aryl isothiocyanates but the primary products cannot be isolated.[4,5]

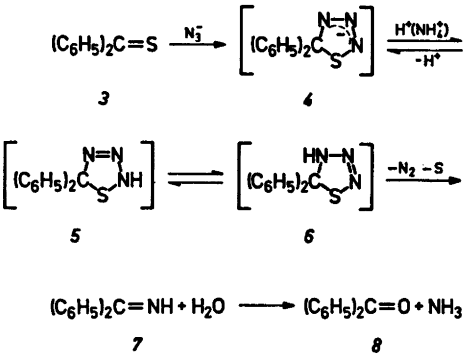


1 *2*

a, R = arylsulfonyl
b, R = aryl
c, R = alkyl
R' = alkyl

We report on the apparent transient formation of thiatriazolines *5* and/or *6* and their anion *4* (Scheme 1) and the corresponding *S*-oxides.

When a blue ethanolic solution of thiobenzophenone (*3*) is left together with ammonium azide at room temperature decolorization is observed. After 2½ h all colour has vanished, and the resulting solution contains diphenylmethaneimine (*7*) and benzophenone (*8*) in almost equal amounts as well as elemental sulfur. In aqueous ethanol, only benzophenone (100 %) is formed in agreement with the facile hydrolysis of the imine [6] while in non aqueous solution diphenylmethaneimine is formed in better than 90 % yield. The reaction between hydrogen azide and *3* in aqueous ethanol proceeds with formation of benzophenone only but the reaction is much slower than with azide ion. No reaction takes place between hydrogen azide and thiobenzophenone in ether/methylene chloride unless triethylamine is added, which suggests that the azide ion is the reactive species. A general scheme explaining the observed products is depicted below (Scheme 1).

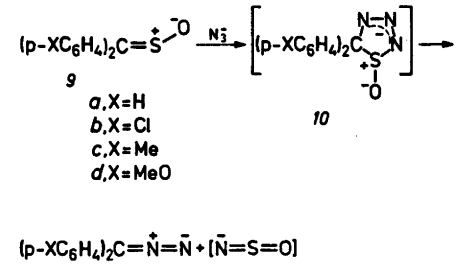


3 *4* *5* *6* *7* *8*

Scheme 1.

We have not been able to observe directly the formation of the assumed thiatriazoline intermediates either by TLC or through monitoring the reactions with UV spectroscopy.

The almost colourless thiobenzophenone *S*-oxide (*9a*) also reacts with azide ion in ethanol. Under these conditions a red color is slowly developed, identified as due to diphenyldiazomethane (88 %). Benzophenone is observed in small amounts, probably owing to reaction of diphenyldiazomethane with oxygen. The formation of diphenyldiazomethane in this reaction may be formulated as shown in Scheme 2.



9 *10* *11*

a, X = H
b, X = Cl
c, X = Me
d, X = MeO

Scheme 2.

In agreement with nucleophilic attack on the thiocarbonyl group it has qualitatively been found that formation of diaryldiazomethane is accelerated by substitution with electron-attracting groups in the benzene rings. Thus 4,4'-dichlorothiobenzophenone *S*-oxide (*9b*) reacts faster than thiobenzophenone *S*-oxide (*9a*), which again reacts faster than 4,4'-dimethylthiobenzophenone *S*-oxide (*9c*), while no reaction is observed over a long period of time with 4,4'dimethoxythiobenzophenone *S*-oxide (*9d*).

Reaction with hydrogen azide in aqueous ethanolic solution instead of azide ion gives

rise to the same products, but as in the thiobenzophenone case it proceeds at a much lower rate, resulting in increased amounts of benzophenone at the expense of diphenyldiazomethane. Only a very slow reaction takes place with hydrogen azide in ether/methylene chloride but the red colour of diaryldiazomethane is immediately produced on addition of triethylamine. As in the thiobenzophenone case this strongly suggests that the azide ion is the reactive species.

The formation of diphenyldiazomethane is remarkable since this is the first example of a thiatriazole or thiatriazoline which does not fragment by loss of nitrogen.[7] The anion ($\bar{N}=S=O$, *11*) of thiocarbonylimide ($HN=S=O$)[8] which apparently is extruded in these processes could not be isolated. From the solution a salt was obtained, but it contained approximately 50 % oxygen and was not identified.

Experimental. Reaction of thiobenzophenone (3) with ammonium azide and with hydrogen azide. All reactions involving *3* were carried out under oxygen-free conditions.

Thiobenzophenone (1 mmol) and ammonium azide (1 mmol) were dissolved in ethanol (20 ml). After 2½ h the blue colour of *3* had disappeared, and the reaction mixture was evaporated to dryness. The residue consisted of almost equal amounts of benzophenone and diphenylmethaneimine according to TLC, IR and UV spectroscopy.

Thiobenzophenone (1 mmol) in ethanol (25 ml) was added to an aqueous solution of hydrogen azide (1 mmol). After 72 h the colour of *3* had disappeared. Analysis as above revealed the formation of benzophenone in better than 90 % yield.

Triethylamine (1 mmol) in methylene chloride (20 ml) was added to a mixture of thiobenzophenone (1 mmol) and hydrogen azide (1 mmol) in ether (10 ml). After 2½ h the colour of *3* had disappeared. Analysis as above revealed the formation of diphenylmethaneimine in better than 90 % yield.

Reaction of thiobenzophenone S-*oxide (9a) with ammonium azide and with hydrogen azide.* Thiobenzophenone *S*-oxide (18.7 mmol) and ammonium azide (20 mmol) were dissolved in ethanol (200 ml). After 18 h a deep red colour had developed. Addition of water (10 ml) caused precipitation of inorganic material, which was removed by filtration. After addition of more water (500 ml) the aqueous phase was extracted with ether (3 × 75 ml), and the combined ether extracts were dried over $MgSO_4$ and evaporated to dryness. The residue was dissolved in light petroleum (b.p. 40 – 60 °C), filtered, and evaporated to dryness, affording deep red crystals of diphenyldiazomethane with m.p. 26 – 27 °C (lit. 29 – 32 °C),[9] yield 88 % based on *9a*. The infrared spectrum was identical with that of an authentic sample.

The reaction of an ethanolic solution of *9a* (10^{-4} M) with excess ammonium azide was monitored by UV spectroscopy. The absorption maximum of *9a* at 329 nm[10] disappeared simultaneously with the appearance of a new maximum at 287 nm (diphenyldiazomethane). An isosbestic point was observed at 312 nm.

Thiobenzophenone *S*-oxide (*9a*) (1 mmol) in ethanol (25 ml) was added to an aqueous solution of hydrogen azide. After 24 h the red colour of diphenyldiazomethane had developed, but according to TLC and UV spectroscopy more than 85 % of *9a* was still present. After 1 week the now colourless reaction mixture was evaporated to dryness and analyzed by means of TLC and UV spectroscopy. Benzophenone (*ca.* 50 %) was found together with traces of tetraphenylethylene, while *ca.* 40 % of sulfine *9a* remained. Diphenyldiazomethane was not detected.

Thiobenzophenone *S*-oxide (*9a*) (1 mmol) in ethanol (25 ml) was added to an aqueous solution of acetic acid (1 mmol). After 1 week the reaction mixture was analyzed by means of UV spectroscopy. Benzophenone (15 %) was detected together with unreacted sulfine *9a* (85 %). This shows that hydrolytic formation of benzophenone directly from *9a* is less important.

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Tobacco Chemistry 31. (1*S*,4*S*,8*R*,11*S*,12*R*)-8,12-Epoxy-2*E*,6*E*-thunbergadiene-4,11-diol, a New Constituent of Greek Tobacco

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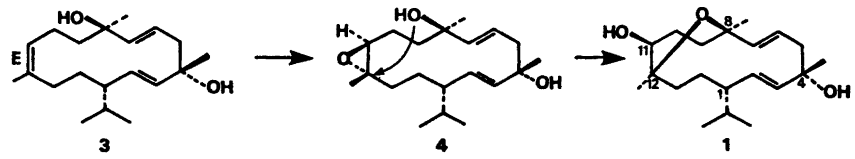
As part of a general study of tobacco isoprenoids and their degradation products, we have recently determined the absolute configuration of the tobacco thunberganoids and shown the chirality at the carbon carrying the isopropyl group, C(1), to be *S* in these compounds as well as in several tobacco nor-thunberganoids.[1]

Further examination of Greek tobacco has now resulted in the isolation of a new thunberganoid (*1*), obtained in minute quantities as the corresponding mono-acetate (*2*) (3.5 mg, 12 ppb) after acetylation and chromatography of a medium-volatile fraction of an ether extract.[2] Its composition $C_{20}H_{34}O_3$ (MS), and the presence of an isopropyl group [δ 0.85 (d) and 0.88 (d); ν_{max} 1370 and 1390 cm^{-1}] and three methyl groups [δ 1.09 (s), 1.18 (s) and 1.34 (s)], probably linked to oxygenated carbons, implied a diterpenoid structure. A tertiary (ν_{max} 3480 cm^{-1} in *2*) and a secondary hydroxy group [δ 3.52 (1 H, m) in *1* shifted to δ 4.85 in *2*], accommodated two of the three oxygens. This left an ether moiety extending from two fully substituted carbons to account for the third oxygen since there were no further $-CHO-$ resonances in the ^{1}H NMR spectrum. ^{1}H NMR resonances corresponding to four olefinic protons indicated the presence of two *trans* disubstituted double bonds, one of which was flanked by a methine group and a fully substituted carbon atom, $-\overset{|}{C}H-CH=CH-\overset{|}{C}-$ (AB-part of an ABX-system at δ 5.13 and 5.34, J_{AB} 15.5, J_{AX} 7.5 Hz; ν_{max} 979 cm^{-1}). The new compound should thus be carbo-monocyclic and the presence of the isopropyl and three methyl substituents suggests that the remaining fourteen carbon atoms are joined in one ring, incorporating the two double bonds and an ether bridge extending between two of the methyl substituted carbon atoms. Although these results implied a thunberganoid structure, the scarce amount of the new compound excluded ^{13}C NMR and correlative chemical studies and its structure and relative stereochemistry was determined by X-ray diffraction analysis of the monoacetate.

Intensity data for the acetate, which crystallizes in the orthorhombic space group $P2_12_12$ with $a = 18.190$ (7), $b = 12.199$ (2) and $c = 9.839$ (2) Å, were collected on the Philips computer-controlled PW 1100 diffractometer. An *E* map with structure-factor phases determined by direct methods displayed all non-hydrogen



Scheme 1. Probable biogenesis of compound *1*.

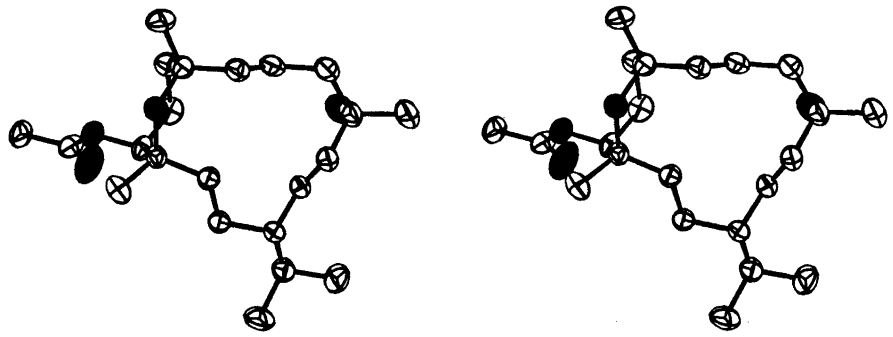


Fig. 1. Stereoscopic view of (1*S*,4*S*,8*R*,11*S*,12*R*)-8,12-epoxy-11-acetoxy-2*E*,6*E*-thunbergadien-4-ol (*2*).

atoms, while all hydrogen atoms were located from a difference Fourier synthesis. The structure was refined to an R value of 0.040 with anisotropic thermal parameters assigned to the carbon and oxygen atoms, and fixed isotropic temperature factors assigned to the hydrogen atoms. A stereoscopic view of the acetate (oxygen atoms shaded) summarising the X-ray results to be discussed in detail elsewhere,[3] is given in Fig. 1.

The absolute configuration of the new thunberganoid (1*S*,4*S*,8*R*,11*S*,12*R*) was inferred from the CD-curves of the corresponding 11-*O*-benzoate and 11-*O*-*p*-nitrobenzoate. These displayed positive ^1L_a bands at 227 (MeOH, $\Delta\varepsilon_{227} \approx 0.1$) and 260 nm (MeOH, $\Delta\varepsilon_{260} \approx 0.2$), respectively, in compliance with the fact that C(12), which is vicinal to the benzoxylated C(11) and carries the ether oxygen, occurs in a positive sector when a Dreiding model of the benzoate possessing the stereostructure and conformation shown in Fig. 1 is aligned according to the benzoate sector rule.[4,5]

Since the new compound (*1*) possesses the expected 1*S*-configuration and the chirality at C(4) is *S*, it is likely to be derived from the known tobacco diol (*3*) (*cf.* Scheme 1). Similar to other ether bridged tobacco thunberganoids of established absolute configuration, whose formation can be viewed as a result of an oxidative attack on the 11,12-double bond from the α-side of the appropriate 4,8-diols and anchimeric assistance by the 8-hydroxy-group in the formation of the 8,11-ether bridge, it can be inferred from the 11-*S*,12*R*-configuration of *1* that it is formed in a corresponding manner involving the 11*S*,12*S*-epoxide (*4*) or related species as intermediate.

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Alkylation of Enamines. A Convenient Route to 1,4-Dicarbonyl Compounds

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Methods for preparation of 1,4-dicarbonyl compounds are of current interest, and have been subject to a recent review.[1] Alkylation of enamines with α-halocarbonyl compounds have been reported previously, although these reports deal with enamines derived from cyclic ketones and aldehydes.[2–5]

In the present investigation we have studied the reactions of enamines derived from acyclic aliphatic ketones towards several α-bromocarbonyl compounds, as a possible route to acyclic 1,4-dicarbonyl compounds. The enamines used were the morpholino, dimethylamino and pyrrolidino derivatives of methyl isopropyl ketone and methyl *tert*-butyl ketone (pinacolone), respectively. The α-bromocarbonyl compounds used were bromomethyl isopropyl ketone,[6] bromomethyl *tert*-butyl ketone,[7] bromomethyl phenyl ketone,[8] and ethyl bromoacetate. The reactions were carried out using equimolar amounts or excess of enamine, without any solvent present.

Results and discussion. The yields of the 1,4-dicarbonyl compounds obtained in the reactions of α-bromocarbonyl compounds with the enamines are summarized in Table 1 (calculated from ^{1}H NMR spectra). Physical data are summarized in Table 2. The reaction mechanism is under consideration and will be reported later.

Experimental. The IR spectra were obtained on neat samples using a Perkin Elmer 257 spectrometer, the ^{1}H NMR spectra were recorded on a JEOL C60-HL spectrometer and the ^{13}C NMR spectra were recorded on a JEOL PFT-60HL spectrometer. TMS was used as internal standard. Deuteriochloroform (^{1}H NMR) was used as solvent, and the sample concentrations were *ca.* 1 M. ^{13}C NMR spectra were obtained using neat samples and a D_2O capillary. Probe temperature 23 °C.

Alkylation of enamines. A typical procedure was: Bromomethyl *tert*-butyl ketone, 1.90 g (10.6 mmol), was placed in an Erlenmeyer flask equipped with a magnetic stirrer, dropping funnel and reflux condenser fitted with a $CaCl_2$ drying tube. 2-Dimethylamino-3-methyl-1-butene, 6.3 g (56 mmol, excess), was added in portions to the stirred bromoketone, the stirring being continued for 0.5 h to ensure complete reaction. The precipitated ketoimmonium salt was hydrolyzed with 20 ml of acidulated (HCl) water and stirred for an additional 0.5 h. The aqueous phase was extracted with 2 × 50 ml of ether, and the combined ethereal layers were treated with 10 ml

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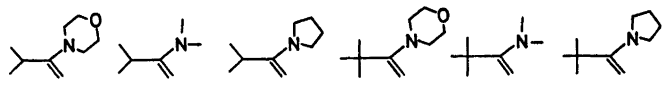
Table 1. Yields (%) of 1,4-dicarbonyl compounds. Without asterisk: excess enamine; with asterisk: equimolar amounts of bromoketone and enamine.



1 *2* *3* *4* *5* *6*

α-Bromocarbonyl compound	*1*	*2*	*3*	*4*	*5*	*6*
Bromoethyl isopropyl ketone	58	85	75	trace	29*	59*
Bromomethyl *t*-butyl ketone	trace	87	40	–*	trace	51*
Bromomethyl phenyl ketone	73	59*	–*	–*	35*	72*
Ethyl bromoacetate	–	40	44	–*	63	88

Table 2. Physical data of 1,4-dicarbonyl compounds formed.

Compound	IR (cm^{-1})	^{13}C NMR (ppm)	^{1}H NMR (δ)
$(CH_3)_2CHCOCH_2CH_2COCH(CH_3)_2$	1709 (C=O)	211.1 (C=O)	1.1 (d, 12 H, J=7 Hz), 2.8 (s, 4 H) 2.7 (sept, 2 H, J=7 Hz, part. overlapped)
$(CH_3)_2CHCOCH_2CH_2COC(CH_3)_3$	1707 (C=O)	212.4 (*t*-BuCO) 211.1 (i-PrCO)	1.1 (d, 6 H, J=7 Hz), 2.8 (s, 4 H) 2.7 (sept, 1 H, J=7 Hz, part. overlapped) 1.2 (s, 9 H)
$(CH_3)_3CCOCH_2CH_2COC(CH_3)_3$	1706 (C=O)	212.7 (C=O)	1.2 (s, 18 H), 2.8 (s, 4 H)
$C_6H_5COCH_2CH_2COCH(CH_3)_2$	1710 (i-PrCO) 1687 (PhCO)	211.9 (i-PrCO) 198.0 (PhCO)	1.1 (d, 6 H, J=7 Hz), 3.0 (m, 4 H) 2.7 (sept, 1 H, J=7 Hz, part. overlapped) 8.1–7.9 (m, 2 H) 7.6–7.3 (m, 3 H)
$C_6H_5COCH_2CH_2COC(CH_3)_3$	1702 (*t*-BuCO) 1686 (PhCO)	213.0 (*t*-BuCO) 197.8 (PhCO)	1.2 (s, 9 H), 3.1 (m, 4 H) 8.1–7.9 (m, 2 H), 7.6–7.3 (m, 3 H)
$CH_3CH_2O_2CCH_2CH_2COCH(CH_3)_2$	1714 (i-PrCO) 1737 (R-COO)	211.3 (i-PrCO) 172.4 (R-COO)	1.2 (d, 6 H, J=6.5 Hz), 2.7 (m, 4 H) 1.3 (t, 3 H, J=7 Hz) 2.5 (sept, 1 H, J=6.5 Hz, part. overlapped) 4.2 (quart, 2 H, J=7 Hz)
$CH_3CH_2O_2CCH_2CH_2COC(CH_3)_3$	1708 (*t*-BuCO) 1736 (R-COO)	212.5 (*t*-BuCO) 172.3 (R-COO)	1.2 (s, 9 H), 2.7 (m, 4 H) 1.3 (t, 3 H, J=6.5 Hz) 4.1 (quart, 2 H, J=6.5 Hz)

of 2 % $NaHCO_3$ solution and washed with 3 x 10 ml of water. The organic layer was dried ($MgSO_4$) and the ether evaporated *in vacuo*, yielding 1.70 g (87 %) of 2,2,7-trimethyl-3,6-octanedione (calculated from ^{1}H NMR spectra).

For preparative scale: 2-Dimethylamino-3-methyl-1-butene, 13.0 g (0.12 mol), was added in portions to bromomethyl phenyl ketone 19.9 g (0.10 mol) with stirring followed by the procedure given above. The yield obtained after distillation (119–120 °C/0.3 mmHg) was 12.0 g (59 %) of 1-phenyl-5-methyl-1,4-hexanedione.

The haloketones were prepared according to methods described in the literature.[6–8] Commercially available ethyl bromoacetate was used. The enamines were prepared according to White and Weingarten.[9]

Acknowledgement. Assistance by Jan Forsberg in the preparation of starting materials is gratefully acknowledged.

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The Crystal Structure of Succinylcholine Chloride Dihydrate

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The crystal structures of three different succinylcholine salts have earlier been determined.[1–4] Succinylcholine chloride is the only choline ester salt known to crystallize as a hydrate. It was therefore found worth-while to examine the crystal structure as part of solid state studies of choline ester salts, in which hydrogen bonding of the choline ester ion cannot *a priori* be excluded.

Bond lengths and angles calculated from the final parameters (Table 1) are shown in Fig. 1 and are in general agreement with accepted values. The conformations of the succinylcholine ions in the present crystal structure (the torsion angles are given in the legend to Fig. 1) and in the crystals of succinylcholine perchlorate[3] are approximately the same. A stereo view of the crystal packing is given in Fig. 2. The water oxygen atom is donor for two hydrogen bonds $O11-H111\cdots Cl^-_{-x,-y,1-z}$ and $O11-H112\cdots Cl^-$. The $O\cdots Cl^-$ distances are 3.300(4) and 3.255(4) Å, respectively, and the $O-H\cdots Cl^-$ angles are 170(3) and 172°(5), respectively. No hydrogen bonding involves the ester oxygen atoms O3 and O4. The ability of choline ester ions to be acceptors for hydrogen bonds seems to be low, as the same situation now has been found in several choline ester salts: Acetylcholine β-resorcylate,[5] acetylcholine (+)-bitartrate[6] (both of which have two formula units in the asymmetric unit), γ-aminobutyric acid choline ester diiodide and (±)-tartrate,[7] lactoylcholine iodide,[8] and the present structure.

Experimental. Succinylcholine chloride dihydrate appears as a white microcrystalline material. Several attempts to grow single crystals from a variety of solvents using different techniques have been unsuccessful. Finally a few transparent crystals were found among the microcrystalline material obtained by slow evaporation of an aqueous solution.

Crystal data. Succinylcholine chloride dihydrate, $C_{14}H_{34}N_2O_6Cl_2$, $M=397.34$, m.p. 190–191 °C. Space group $P\bar{1}$, $a=8.941(4)$, $b=$

Table 1. Final positional and thermal parameters. The estimated standard deviations, referring to the last figure, are given in parentheses. Thermal parameters are $\times 10^3$. The temperature factor is defined by:

$$\exp\left[-2\pi^2(U_{11}h^2a^{*2}+\cdots+2U_{12}hka^*b^*+\cdots)\right]$$

ATOM	X/A	Y/B	Z/C	U11	U22	U33	U12	U13	U23
C 1	.0895(4)	.5084(5)	.5768(3)	2.4(1)	4.5(2)	2.4(1)	0.6(1)	1.0(1)	1.1(1)
C 2	.2318(4)	.4385(4)	.5416(3)	2.5(1)	3.8(2)	2.6(2)	0.5(1)	0.8(1)	1.3(1)
O 3	.2157(3)	.3876(4)	.4108(3)	3.3(1)	6.3(1)	3.0(1)	1.2(1)	1.5(1)	1.5(1)
O 4	.3865(3)	.4383(4)	.6777(3)	2.4(1)	6.0(1)	2.9(1)	1.1(1)	1.0(1)	1.8(1)
C 5	.5245(4)	.3509(6)	.6498(4)	3.2(2)	7.1(2)	3.8(2)	1.4(2)	1.9(1)	2.6(2)
C 6	.6963(4)	.3760(5)	.8088(4)	2.6(2)	5.2(2)	4.0(2)	0.5(1)	1.5(1)	1.9(1)
N 7	.7170(3)	.2245(4)	.8963(3)	2.3(1)	3.5(1)	2.5(1)	0.6(1)	1.1(1)	0.8(1)
C 8	.5963(4)	.2570(6)	.9590(4)	3.1(2)	6.0(2)	4.1(2)	1.3(1)	2.2(1)	2.2(2)
C 9	.6908(5)	.0041(6)	.7949(5)	4.0(2)	4.2(2)	3.8(2)	0.5(1)	1.2(2)	0.0(1)
C10	.9047(4)	.2669(5)	1.0390(4)	2.4(1)	4.7(2)	3.3(2)	0.3(1)	1.0(1)	0.7(1)
O11	-.2375(4)	.0587(5)	.4630(4)	4.0(2)	9.6(2)	4.8(2)	-0.7(1)	1.9(1)	0.3(2)
CL-	.1331(1)	.1470(1)	.8119(1)	4.35(5)	5.12(5)	3.72(5)	-0.04(3)	2.03(4)	1.20(3)

ATOM	X/A	Y/B	Z/C	ATOM	X/A	Y/B	Z/C
H 11	.121(5)	.647(6)	.643(5)	H 91	.571(5)	-.026(5)	.717(5)
H 12	.085(5)	.427(5)	.638(5)	H 92	.767(5)	-.009(6)	.757(5)
H 51	.492(5)	.188(6)	.587(4)	H 93	.714(5)	-.082(6)	.860(5)
H 52	.548(5)	.439(5)	.600(5)	H101	.977(5)	.242(5)	.994(4)
H 61	.792(5)	.359(5)	.787(4)	H102	.916(5)	.414(6)	1.102(5)
H 62	.711(5)	.525(6)	.887(4)	H103	.914(5)	.176(6)	1.097(5)
H 81	.623(5)	.405(6)	1.030(5)	H111	-.200(5)	.015(6)	.407(5)
H 82	.472(5)	.224(5)	.864(5)	H112	-.149(6)	.070(6)	.538(5)
H 83	.614(5)	.170(6)	1.020(5)				

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The Crystal Structure of Succinylcholine Chloride Dihydrate

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The crystal structures of three different succinylcholine salts have earlier been determined.[1–4] Succinylcholine chloride is the only choline ester salt known to crystallize as a hydrate. It was therefore found worth-while to examine the crystal structure as part of solid state studies of choline ester salts, in which hydrogen bonding of the choline ester ion cannot *a priori* be excluded.

Bond lengths and angles calculated from the final parameters (Table 1) are shown in Fig. 1 and are in general agreement with accepted values. The conformations of the succinylcholine ions in the present crystal structure (the torsion angles are given in the legend to Fig. 1) and in the crystals of succinylcholine perchlorate[3] are approximately the same. A stereo view of the crystal packing is given in Fig. 2. The water oxygen atom is donor for two hydrogen bonds $O11-H111\cdots Cl^-_{-x,-y,1-z}$ and $O11-H112\cdots Cl^-$. The $O\cdots Cl^-$ distances are 3.300(4) and 3.255(4) Å, respectively, and the $O-H\cdots Cl^-$ angles are 170(3) and 172°(5), respectively. No hydrogen bonding involves the ester oxygen atoms O3 and O4. The ability of choline ester ions to be acceptors for hydrogen bonds seems to be low, as the same situation now has been found in several choline ester salts: Acetylcholine β-resorcylate,[5] acetylcholine (+)-bitartrate[6] (both of which have two formula units in the asymmetric unit), γ-aminobutyric acid choline ester diiodide and (±)-tartrate,[7] lactoylcholine iodide,[8] and the present structure.

Experimental. Succinylcholine chloride dihydrate appears as a white microcrystalline material. Several attempts to grow single crystals from a variety of solvents using different techniques have been unsuccessful. Finally a few transparent crystals were found among the microcrystalline material obtained by slow evaporation of an aqueous solution.

Crystal data. Succinylcholine chloride dihydrate, $C_{14}H_{34}N_2O_6Cl_2$, $M=397.34$, m.p. 190–191 °C. Space group $P\bar{1}$, $a=8.941(4)$, $b=$

Table 1. Final positional and thermal parameters. The estimated standard deviations, referring to the last figure, are given in parentheses. Thermal parameters are $\times 10^3$. The temperature factor is defined by:

$$\exp\left[-2\pi^2(U_{11}h^2a^{*2}+\cdots+2U_{12}hka^*b^*+\cdots)\right]$$

ATOM	X/A	Y/B	Z/C	U11	U22	U33	U12	U13	U23
C 1	.0895(4)	.5084(5)	.5768(3)	2.4(1)	4.5(2)	2.4(1)	0.6(1)	1.0(1)	1.1(1)
C 2	.2318(4)	.4385(4)	.5416(3)	2.5(1)	3.8(2)	2.6(2)	0.5(1)	0.8(1)	1.3(1)
O 3	.2157(3)	.3876(4)	.4108(3)	3.3(1)	6.3(1)	3.0(1)	1.2(1)	1.5(1)	1.5(1)
O 4	.3865(3)	.4383(4)	.6777(3)	2.4(1)	6.0(1)	2.9(1)	1.1(1)	1.0(1)	1.8(1)
C 5	.5245(4)	.3509(6)	.6498(4)	3.2(2)	7.1(2)	3.8(2)	1.4(2)	1.9(1)	2.6(2)
C 6	.6963(4)	.3760(5)	.8088(4)	2.6(2)	5.2(2)	4.0(2)	0.5(1)	1.5(1)	1.9(1)
N 7	.7170(3)	.2245(4)	.8963(3)	2.3(1)	3.5(1)	2.5(1)	0.6(1)	1.1(1)	0.8(1)
C 8	.5963(4)	.2570(6)	.9590(4)	3.1(2)	6.0(2)	4.1(2)	1.3(1)	2.2(1)	2.2(2)
C 9	.6908(5)	.0041(6)	.7949(5)	4.0(2)	4.2(2)	3.8(2)	0.5(1)	1.2(2)	0.0(1)
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ATOM	X/A	Y/B	Z/C	ATOM	X/A	Y/B	Z/C
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H 82	.472(5)	.224(5)	.864(5)	H112	-.149(6)	.070(6)	.538(5)
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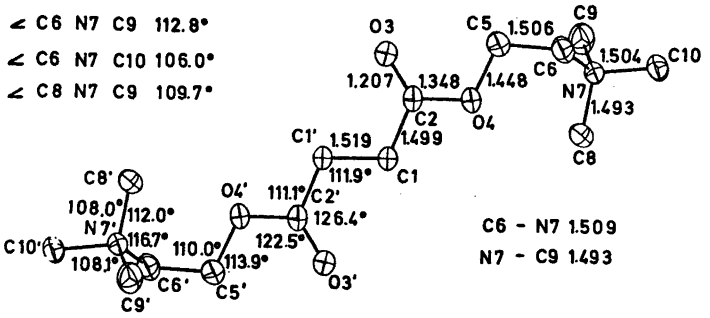

Fig. 1. The dimensions of the succinylcholine ion. The torsion angles are C1′ C1 C2 O4∓175.0°; C1 C2 O4 C5±173.6°; C2 O4 C5 C6±174.6°; O4 C5 C6 N7±78.8°; C5 C6 N7 C8∓68.8°. The estimated standard deviations on bond lengths and angles are about 0.005 Å and 0.3°, respectively. The drawings were produced by ORTEP.[12]

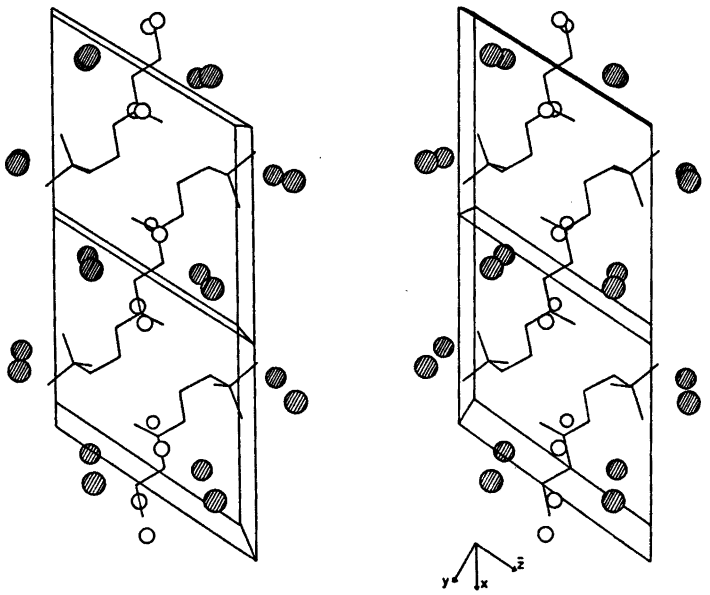

Fig. 2. A stereo view of the packing of succinylcholine chloride dihydrate. The chloride ions are shaded.

6.825(3), $c = 10.260(5)$ Å, $\alpha = 108.13(3)$, $\beta = 122.00(3)$, $\gamma = 84.91(3)°$. $V = 502.3$ Å^3. $D_m = 1.31$ g cm^{-3}, $Z = 1$, $D_c = 1.31$ g cm^{-3}. Linear absorption coefficient for X-rays [λ(Mo$K\alpha$ = 0.7107 Å)], $\mu = 3.5$ cm^{-1}. $F(000) = 214$. The unit-cell parameters were refined by least-squares techniques from the θ angles measured for 45 reflections on a NONIUS three-circle automatic diffractometer. The density was measured by flotation. The melting point was determined on a Leitz hot stage microscope.

Intensity data were collected on the diffractometer from a slightly imperfect single crystal of irregular shape (*ca.* 0.25 × 0.30 × 0.45 mm) using Mo$K\alpha$ radiation and omega scan. Out of the 1578 independent reflections in the range $2.5 \leq \theta \leq 25.0°$, 1382 had $I_{net} \geq 3.0\ \sigma(I)$, where σ is the standard deviation from counting statistics. No absorption corrections have been made.

The structure was solved by the heavy atom method and refined by full matrix least-squares techniques to a final R value of 0.055, using the X-RAY-system.[9] The final cycles of refinement included positional parameters for all atoms and anisotropic thermal parameters for

all non-hydrogen atoms while a fixed common thermal parameter ($B=3.5$) was assigned to all hydrogen atoms. The quantity minimized was $\sum w(|F_o|-|F_c|)^2$ where $w=1/\{1+[(F_o-B)/A]^2\}$, $A=8.0$ and $B=6.0$. The X-ray atomic scattering factors used for hydrogen were those of Stewart, Davidson and Simpson [10] and for all other atoms those listed in International Tables for X-Ray Crystallography.[11] All atoms but Cl^- were treated as uncharged. The final list of structure factors is available from the author on request.

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